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Dhinaut Julien

Ecologie évolutive du priming immunitaire chez le ténébrion meunier, *Tenebrio molitor*

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Titre : Ecologie évolutive du priming immunitaire chez le ténébrion meunier, *Tenebrio molitor*

Mots clés : *Tenebrio molitor*, génétique quantitative, transfert trans-générationnel d'immunité, mémoire immunitaire

Résumé : Il est maintenant connu que de nombreux invertébrés peuvent moduler leur réponse immunitaire en fonction de leur expérience immunologique. Ce phénomène est appelé priming immunitaire. Si les mécanismes du priming immunitaire restent encore assez méconnus, il a pour conséquence d'apporter un bénéfice aux individus lors d'une seconde rencontre avec un agent pathogène, via une élévation de leur immunocompétence. Une caractéristique assez étonnante du priming immunitaire est qu'il peut se manifester chez la descendance. Ce transfert trans-générationnel d'immunité (TTGI), ainsi que le priming immunitaire, doivent avoir évolués à la suite de challenges répétitifs par les mêmes agents pathogènes durant la vie des individus et au fil des générations. Ainsi, le priming et le TTGI doivent être plus efficaces et moins coûteux vis-à-vis des parasites exposant l'hôte à la plus grande probabilité de réinfection. De plus, il est maintenant prouvé que la réponse immunitaire chez les insectes est génétiquement variable. Pour comprendre l'évolution du TTGI et de son potentiel de réponse à la sélection, il convient d'étudier la composante génétique de sa variabilité. Au cours de cette thèse, j'ai associé l'expression du priming et du TTGI chez un insecte à un type de bactéries, qui a dû agir comme la principale pression de sélection sur le système immunitaire de cette espèce hôte. Cela s'est fait via l'identification de différents coûts et bénéfices, qui ont également mis en exergue certains mécanismes possibles dans la réalisation de ces phénomènes immunitaires. Pour ce faire, j'ai utilisé comme organisme modèle le ténébrion meunier, *Tenebrio molitor*.

Title : Evolutionary ecology of immune priming in the mealworm beetle, *Tenebrio molitor*

Keywords : *Tenebrio molitor*, trans-generational immune priming, quantitative genetics, immunology

Abstract: Many organisms can improve their immune response as a function of their immunological experience, a phenomenon called immune priming. While the mechanisms through which immune priming is achieved remain unknown, individuals that survived to a given parasite are better protected against subsequent exposures. This immune priming can cross generations (trans-generational immune priming – TGIP), preparing offspring for prevailing parasite environment. Both individual and trans-generational immune priming might be adaptive and may have evolved from repeated challenges by the same pathogens during the host lifetime or across generation. While protection could be cross-reactive, a certain level of specificity may exist in response to the range of pathogens from which immue priming may have evolved. Thus, immune priming and TGIP should be more efficient and less costly with respect to pathogens exposing the host to the greatest probability of re-infection. Moreover, it is now known that insect immune response is genetically variable. To understand the evolution of TGIP and its impact on life history evolution, we need to explore its quantitative genetics. During my thesis, I found that the expression of individual immune priming and TGIP in the mealworm beetle, *Tenebrio molitor*, is dependent of a range of pathogens that might have been a major selective pressure on the immune system of this insect species. This was done through the characterisation of costs and benefits of the expression of immune priming in response to challenges with a large range of bacterial pathogens. This work also highlighted potential mechanisms through which these immune phenomena could be achieved.

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Université Bourgogne Franche-Comté
32, avenue de l'Observatoire
25000 Besancon

“The biggest obstacle to creativity is breaking through the barrier of disbelief.”

Rodney Mullen

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Résumé

Il est maintenant connu que de nombreux invertébrés peuvent moduler leur réponse immunitaire en fonction de leur expérience immunologique. Ce phénomène est appelé priming immunitaire. Si les mécanismes du priming immunitaire restent encore assez méconnus, il a pour conséquence d'apporter un bénéfice aux individus lors d'une seconde rencontre avec un agent pathogène, via une élévation de leur immunocompétence. Une caractéristique assez étonnante du priming immunitaire est qu'il peut se manifester chez la descendance. Ce transfert trans-générationnel d'immunité (TTGI), ainsi que le priming immunitaire, doivent avoir évolués à la suite de challenges répétitifs par les mêmes agents pathogènes durant la vie des individus et au fil des générations. Ainsi, le priming et le TTGI doivent être plus efficaces et moins coûteux vis à vis des parasites exposant l'hôte à la plus grande probabilité de réinfection. De plus, il est maintenant prouvé que la réponse immunitaire chez les insectes est génétiquement variable. Pour comprendre l'évolution du TTGI et de son potentiel de réponse à la sélection, il convient d'étudier la composante génétique de sa variabilité. Au cours de cette thèse, j'ai associé l'expression du priming et du TTGI chez un insecte à un type de bactéries, qui a du agir comme la principale pression de sélection sur le système immunitaire de cette espèce hôte. Cela s'est fait via l'identification de différents coûts et bénéfices, qui ont également mis en exergue certains mécanismes possibles dans la réalisation de ces phénomènes immunitaires. Pour ce faire, j'ai utilisé comme organisme modèle le ténébrion meunier, *Tenebrio molitor*.

Dans le premier chapitre, nous avons étudié la survie d'individus adultes de *T. molitor* face à une infection bactérienne, en fonction de leur propre expérience immunitaire ou de celle de leur mère. Nous avons constaté que le priming et le TTGI étaient plus efficaces et moins coûteux vis à vis des bactéries à Gram-positif. Cette étude a également révélé que, contrairement à ce que de précédentes recherches suggérent, les hémocytes ne jouent pas nécessairement un rôle majeur dans le priming immunitaire et le TTGI.

Dans le deuxième chapitre, nous avons stimulé le système immunitaire de femelles adultes de *T. molitor* avec deux bactéries Gram-positives. Nous avons mis en évidence que la protection transmise aux oeufs pouvait résulter d'un transfert maternel de peptides antibactériens, ou que ces peptides pouvaient être produits par l'œuf lui-même, en fonction de la bactérie utilisé pour stimuler la mère. Il s'avère que quel que soit le mécanisme, le TTGI améliore le taux d'éclosion des œufs et peut même s'accompagner d'un bénéfice en survie pour les jeunes larves.

Dans le troisième chapitre, nous avons stimulé le système immunitaire de femelles de lignées consanguines afin de quantifier la variation génétique de l'investissement maternel à la protection des œufs et mesuré d'autres traits associés à la valeur sélective des mères et de la descendance. Malheureusement, du fait d'un nombre trop faible de lignées et d'individus utilisés au sein de nos lignées, il nous a été impossible de conclure quant à l'existence de bases génétiques associées au TTGI.

Dans le quatrième chapitre, nous avons passé en revue l'ensemble des études concernant le TTGI. Cela nous a permis de mettre en exergue les principales caractéristiques et les mécanismes identifiés, en fonction de l'écologie et de l'évolution du phénomène.

Les bénéfices et les coûts associés au priming ainsi qu'au TTGI suggèrent que les bactéries à Gram-positif ont été la principale pression de sélection ayant contraint l'évolution du système immunitaire de *T. molitor*. En ce qui concerne le TTGI, de plus amples recherches sont nécessaires afin de trancher quant à l'existence de bases génétiques associées au phénomène.

Abstract

Many organisms can improve their immune response as a function of their immunological experience, a phenomenon called immune priming. While the mechanisms through which immune priming is achieved remain unknown, individuals that survived to a given parasite are better protected against subsequent exposures. This immune priming can cross generations (trans-generational immune priming – TGIP), preparing offspring for prevailing parasite environment. Both individual and trans-generational immune priming might be adaptive and may have evolved from repeated challenges by the same pathogens during the host lifetime or across generation. While protection could be cross-reactive, a certain level of specificity may exist in response to the range of pathogens from which immue priming may have evolved. Thus, immune priming and TGIP should be more efficient and less costly with respect to pathogens exposing the host to the greatest probability of re-infection. Moreover, it is now known that insect immune response is genetically variable. To understand the evolution of TGIP and its impact on life history evolution, we need to explore its quantitative genetics. During my thesis, I found that the expression of individual immune priming and TGIP in the mealworm beetle, *Tenebrio molitor*, is dependent of a range of pathogens that might have been a major selective pressure on the immune system of this insect species. This was done through the characterisation of costs and benefits of the expression of immune priming in response to challenges with a large range of bacterial pathogens. This work also highlighted potential mechanisms through which these immune phenomena could be achieved.

In a first chapter of this thesis, we examined the survival of individuals to infection with different bacteria according to their own immunological experience or that of their mother with these bacteria. We found that priming response to Gram-positive bacteria was particularly more efficient and less costly than priming response to Gram-negative bacteria. This study also shows that, contrary to what is currently believed, the cellular component of the *T. molitor* immune system does not necessarly play a major role in providing immune protection through individual immune priming or TGIP.

In a second chapter, we have stimulated the immune system of adult females with two Gram-positive bacteria to study maternal transfer of immunity to the eggs. We found that the process throght which eggs are protected is dependent on the bacterial pathogen used to immune challenge the mother. Indeed, depending of the bacterial pathogen that immune challenged the mother, antibacterial activity in the eggs are either transfeered by the mother or produced by the egg itself, Furthermore, whatever the mechanism through which egg protection was achieved, primed eggs exhibited enhanced hatching rate and the resulting larvae even showed improved early survival to food privation.

In a third chapter, we used inbred lines of T. molitor to study the quantitative genetics of TGIP. The aim of this work was to test whether TGIP could be heritable and whether its expression is genetically associated to other fintness traits of mothers and offspring. Unfortunately, due to a low number of inbred lines available and a low number of samples within some of these lines, it was impossible to conclude about the genetic basis associated to TGIP.

In a fourth chapter, we produced a review on TGIP. This allowed us to highlight the main characteristics and mechanisms curently identified, and the ecology and the evolution of the phenomenon.

Costs and benefits associated to immune priming and TGIP suggest that Gram-positive bacteria might have been a major selective pressure at the origin of these phenomena in *T. molitor*. Whether TGIP has genetic basis still required further research.

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Introduction générale

Introduction générale

L'immunité chez les invertébrés

Les études sur le système immunitaire ont longtemps opposé ses composantes innées et adaptatives. L'immunité de type adaptative est généralement la plus connue car c'est au sein de ce type de réponse que l'on retrouve le phénomène de mémoire immunitaire, qui entraine une réaction rapide et spécifique, dépendante de l'expérience des individus (Pancer et Cooper 2006). Ce type d'immunité a longtemps été exclusivement attribué aux vertébrés, car il repose sur l'action des anticorps, produits par les lymphocytes B et T, inexistant chez les invertébrés (Theopold et al. 1996). Le système immunitaire de ces derniers était donc connu pour n'être que de type inné. Quelle que soit l'expérience immunitaire des individus, cette composante innée est non spécifique et n'est pas modulable.

Chez les insectes qui font partie de ces organismes n'ayant qu'une immunité de type innée, celle-ci est activée à la suite de la reconnaissance d'un important registre de motifs du non-soi. Ces derniers, communément appelés PAMPs (Pathogen Associated Molecular Patterns) proviennent de molécules de surface des cellules pathogènes, tels que les peptidoglycanes, les LPS (lipopolysaccharides), les β-1,3 glucanes ou d'autres fragments de sucres, qui sont des éléments extrêmement conservés et essentiels pour les microbes (Theopold et al. 1999). Une fois ces PAMPs identifiés par des protéines spécialisées dans leur reconnaissance : les PRRs (Pathogen Recognition Receptors), différentes voies immunitaires sont activées, afin de recruter le ou les effecteur(s) immunitaire(s) approprié(s).

L'un des effecteurs immunitaires le plus important chez les insectes est une tyrosinase : la phénoloxydase (PO ; Chase et al. 2000). Cette enzyme catalyse les premières étapes de la synthèse de la mélanine et de la sclérotine (Sugumaran 2002). La production de PO à partir de son précurseur inactif, la pro-phénoloxydase (PPO), est déclenchée par l'activation en cascade de sérines protéases, elles-mêmes initiées par la détection des PAMPs (Söderhäll & Cerenius 1998 ; passé en revue dans Cerenius & Söderhäll 2004). L'action de la PO produit de la mélanine qui peut encapsuler les agents pathogènes, comme c'est le cas chez de nombreux insectes tels que les bourdons (Allander & Schmid-Hempel 2000) ou les moustiques (Gorman & Paskewitz 2001). Un autre type de capsule peut être formé lorsque des cellules, appelés les hémocytes, viennent englober le corps étranger et que la PO mélanise l'ensemble (Lackie et al. 1985). Quel que soit le type de capsule formé, le corps étranger est ensuite isolé dans l'hémocœle. L'ensemble du phénomène est appelé encapsulation.

Les hémocytes font également partie des principaux effecteurs immunitaires des insectes et sont responsables de la phagocytose. Cet évènement a lieu lorsqu'un hémocyte rencontre un agent pathogène plus petit que lui. Après l'avoir identifié, il l'absorbe et l'élimine. Cette élimination se fait via l'utilisation d'oxyde nitrique (Nathan & Hibbs 1991) et des dérivés réactifs de l'oxygène (Robinson & Badwey 1994) chez les mammifères. Ces deux composés ayant été identifiés chez les insectes (Weiske & Wiesner 1999 ; Luckhart et al. 1998 ; Hao et al. 2003 ; Whitten & Ratcliffe 1999 ; Dettloff et al. 2001 ; Glupov et al. 2001), il est probable qu'ils aient une fonction similaire. Lorsqu'un élément tel qu'un regroupement de bactéries, est trop important pour être phagocyté par un seul hémocyte, plusieurs couches d'hémocytes qui seront ensuite mélanisées vont venir l'englober. On parlera alors de nodulation.

Il existe un dernier grand type d'effecteur immunitaire classiquement étudié chez les insectes. Il s'agit des peptides antibactériens. Ces derniers sont capables de pénétrer et de perturber les membranes des agents pathogènes. Contrairement aux autres effecteurs humoraux, ils sont hautement spécifiques, ce qui se traduit d'ailleurs par une vitesse de réaction plus lente. En effet, l'expression des gènes associés aux peptides antimicrobiens ne débute qu'une à trois heures après une stimulation immunitaire (Lemaitre et al. 1997) et le pic de cette activité n'intervient que dans les trois à douze heures suivant cette stimulation. Cette activité peut être maintenue à des niveaux assez élevés en fonction de l'agent pathogène utilisé pour la stimulation et des peptides considérés (Lemaitre et al. 1997). Par exemple chez *Drosophila melanogaster*, l'expression des Drosomycines reste très importante 72 heures après une stimulation avec *Beauveria bassiana* (Lemaitre et al. 1997). Chez la mite *Pseudoplusia includes*, l'expression de gènes associés aux peptides antimicrobiens est détectable dans l'hémolymphe 2 heures après une stimulation immunitaire et le pic d'activité est observé entre 8 et 24 heures après cette même stimulation (Lavine et al. 2005). Bien que ces peptides soient produits en grande quantité après une infection bactérienne ou par un champignon unicellulaire, et que les voies menant à leur synthèse aient été rapidement découvertes (passées en revue dans Hoffmann 2003), leur rôle exact et leur mode d'action restent méconnus.

Outre les effecteurs immunitaires largement étudiés, on retrouve au sein du système immunitaire des insectes d'autres effecteurs, tels que les lysozymes. Ces derniers sont des organites cellulaires qui ont une action antibactérienne, essentiellement dirigée contre les bactéries Gram-positives, et facilitent la phagocytose (Hultmark 1996). En effet, selon les lysozymes considérés, ceux-ci peuvent grâce à l'action de nombreuses enzymes, assurer la digestion intra ou extracellulaire. Enfin, on retrouve également au sein du système immunitaire des insectes, des composés cytotoxiques issus de la réponse inflammatoire, comme l'oxyde nitrique ou des formes actives d'oxygène, qui participent également à l'élimination des organismes pathogènes (passé en revue dans Rivero 2006).

Mémoire immunitaire et priming chez les invertébrés

La défense contre les organismes pathogènes se déroule normalement en deux étapes : la reconnaissance du « non-soi » et donc d'un potentiel danger pour l'hôte, suivie de l'activation des mécanismes visant à éliminer cet élément indésirable (Beutler 2004). Une fois activés, ces effecteurs immunitaires restent actifs un certain temps. Durant cette période, les parasites attaquant l'hôte ont une probabilité moins importante de l'infecter. Ce type de réponse persistante au cours du temps, qui peut apporter un bénéfice en termes de survie, comme il l'a été montré par Boman et al. (1972) chez *Drosophila melanogaster*, est à différencier du phénomène de mémoire immunitaire. En effet, celle-ci se définit par le stockage ou l'utilisation d'informations collectées suite à une première rencontre avec un parasite (au sens large du terme), lors d'une seconde rencontre avec ce dernier (Murphy 2016 ; Franck 2002). L'étude de Boman montre que des drosophiles challengées avec la bactérie *Enterococcus cloacae,* inactivée à la chaleur, survivent mieux à un second challenge ayant lieu 4 jours plus tard avec cette même bactérie, en comparaison à des individus qui n'avaient pas reçu le premier challenge. Ce bénéfice en termes de survie s'explique essentiellement par la persistance d'une activité antibactérienne maintenue 4 jours après le premier challenge. Ce maintien d'une importante activité immunitaire après une infection a été retrouvé chez d'autres modèles biologiques. C'est le cas du ténébrion meunier *Tenebrio molitor,* où l'activité antimicrobienne des individus est maintenue à un haut niveau au moins 7 jours après une stimulation avec du LPS (Moret & Siva-Jothy 2003).

Les réponses immunitaires induites au sein de ces études étant dues à la persistance d'une réaction après un premier contact avec un parasite, on ne peut parler de mémoire immunitaire. Néanmoins, même si le système immunitaire des invertébrés a longtemps été considéré comme uniquement de type inné, notamment du fait de l'absence des anticorps et des lymphocytes B et T, de nombreuses études ont récemment démontré l'existence de phénomènes proches de la mémoire immunitaire des vertébrés, au sein de onze clades différents d'invertébrés (passés en revue dans Pham & Schneider 2008 et Milutinovic & Kurtz 2016). Si les mécanismes de ce phénomène, appelé « priming immunitaire », restent méconnus, le résultat sur l'activation des effecteurs immunitaires et la survie des individus reste comparable à ceux observés chez les vertébrés. Le priming immunitaire peut d'ailleurs se montrer hautement spécifique, comme il l'a été décrit chez le bourdon, *Bombus terrestris* (Sadd & Schmid-Hempel 2006). La spécificité correspond à la capacité du priming immunitaire à discriminer différents agents pathogènes, entre la première infection et les suivantes. Chez *B. terrestris*, l'effet protecteur d'une bactérie à Gram négatif, *Pseudomonas fluorescens*, et de deux bactéries à Gram positif phylogénétiquement proches, *Paenibacillus alvei* et *Paenibacillus larvae*, a été testé à 8 et 22 jours post-priming. Ces bactéries ont été choisies car les doses létales et sub-létales correspondantes sont connues chez

le bourdon. Les primings avec *P. fluorescens* et *P. larvae* protègent tout deux les bourdons contre une dose létale de *P. fluorescens* délivrée 8 jours plus tard, faisant état d'une protection généraliste. Néanmoins, de la spécificité est observée au pas de temps de 22 jours. En effet, après ce délai de 22 jours entre le priming et l'infection, seul un priming avec *P. fluorescens* protège les bourdons d'une dose létale de cette même bactérie. Ce résultat est retrouvé avec les autres combinaisons homologues de priming et d'infection, réalisées avec *P. alvei* et *P. larvae.* La réponse immunitaire innée chez *B. terrestris* est donc non spécifique lors des phases de réponse les plus précoces, avant de devenir spécifique plus tardivement. L'augmentation de survie observée dans cette étude est corrélée avec le nombre d'individus qui ont réussi à éliminer les bactéries de leur hémolymphe. Il faut également noter que le bénéfice en termes de survie constaté lors d'une infection 8 jours après le priming, est associé à une activité antibactérienne persistante. Néanmoins, ces peptides antibactériens ne sont pas retrouvés 22 jours après le priming. La protection spécifique accordée par le priming immunitaire 22 jours post-priming n'est donc pas due à la persistance des peptides antimicrobiens. Il parait plus probable que les hémocytes soient spécialement activés lors de la seconde réponse immunitaire, ou qu'une activation différentielle du processus de mélanisation intervienne. D'ailleurs, chez *D. melanogaster*, il a été montré récemment que les hémocytes sont les acteurs majoritaires de la réponse spécifique associée au priming immunitaire (Pham et al. 2007). En effet, si aucune différence de production de peptides antimicrobiens et de mélanisation n'a été détectée entre les mouches naïves et celles qui ont été immunisées, le bénéfice de survie observée suite à un priming dans le cas d'une infection à *Streptococcus pneumoniae*, est bien due à l'action des hémocytes. Cela a été mis en évidence lors d'une expérience visant à inhiber l'action de la phagocytose en injectant des billes de polystyrène aux drosophiles. Les hémocytes ayant phagocyté les billes, ne peuvent plus participer à l'élimination de bactéries injectées ultérieurement à l'injection des billes. Les drosophiles primées et injectées avec les billes de polystyrènes n'expriment alors plus de bénéfice en termes de survie face à une infection par *S. pneumoniae*, soulignant l'importance des hémocytes dans la spécificité de la réponse immunitaire (Pham et al. 2007).

Si le priming permet de développer une réponse immunitaire plus efficace contre un parasite rencontré dans le passé, les coûts associés à cette réponse n'ont jamais été étudiés. Pourtant, développer une réponse immunitaire innée peut réduire la longévité (Moret & Schmid-Hempel 2000 ; Pursall & Rolff 2011) et la reproduction (Moret & Schmid-Hempel 2004,). De plus, Råberg et al. (2001) ont proposé que le développement d'une réponse immunitaire adaptative chez la souris soit énergétiquement avantageux par rapport à une réponse immunitaire innée. Pour cela, les auteurs ont comparé le taux métabolique basal de souris génétiquement modifiées pour une déficience en lymphocytes, capables uniquement de développer une réponse immunitaire innée, avec celui de souris normales capables de développer une réponse immunitaire innée et adaptative. Les auteurs ont alors montré que les souris déficientes en lymphocytes présentaient un

taux métabolique basal plus élevé que les souris normales. Il semble donc que, chez les vertébrés, l'évolution de la réponse immunitaire adaptative ait été aussi favorisée par l'économie d'énergie qu'elle confère par rapport à la seule réponse immunitaire innée (Råberg et al. 2001). Dans le cadre du priming immunitaire des invertébrés, on ne sait pas si la réponse secondaire (développée lors d'un second contact avec un pathogène) est elle aussi moins coûteuse que la réponse primaire (développée lors d'un premier contact avec un agent pathogène). Si tel est le cas, un coût plus faible associé à la réponse secondaire pourrait aussi avoir favorisé l'évolution d'un phénomène de mémoire immunitaire chez les invertébrés.

Transfert trans-générationnel d'immunité chez les invertébrés

En plus de pouvoir exprimer une mémoire immunitaire individuelle à travers le phénomène de priming, cet effet peut aussi perdurer d'une génération à l'autre. En effet, des parents peuvent transférer leur expérience immunologique à leur descendance, dans le but de les protéger d'infections récurrentes contre des bactéries ou des parasites présents dans leur environnement (Huang & Son 1999 ; 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 ; Boots & Roberts 2012 ; Trauer & Hilker 2013 ; Yue et al. 2013 ; Hernández López et al. 2014 ; Norouzitallab et al. 2015). Depuis sa première mise en évidence chez le crustacé *Penaeus monodon*, où des mères exposées à des β-glucans sont capables de protéger leur descendance contre le virus associé au syndrome des taches blanches (Huang & Son 1999), ce phénomène appelé « transfert trans-générationnel d'immunité » (TTGI) a été révélé au sein de 9 groupes phylogénétiques différents, que sont les Coléoptères, les Crustacés, les Diptères, les Hémiptères, les Hyménoptères, les Lépidoptères, les Mollusques, les Nématodes, ainsi que les Orthoptères.

De manière générale chez les invertébrés, les femelles ont tendance à plus investir dans l'approvisionnement de leur descendance que les mâles (Mousseau & Fox 1998). Partant de ce postulat, on pourrait s'attendre à uniquement observer du TTGI en provenance des mères au bénéfice de leur progéniture. Néanmoins, lorsque le TTGI n'est pas directement associé à des soins parentaux, comme c'est le cas avec les insectes qui abandonnent généralement leurs œufs, les deux sexes bénéficient de la protection attribuée à leur descendance. Ainsi, si dans la plupart des études le TTGI a été mis en évidence chez les femelles transmettant une meilleure immunocompétence à leur descendance, des effets paternels ont clairement été identifiés chez certaines espèces. C'est notamment le cas chez les coléoptères *Tribolium castaneum* (Roth et al. 2010 ; Eggert et al. 2014) et *T. molitor* (Zanchi et al. 2011). Cet effet paternel peut également être suspecté chez *Manduca sexta* (Trauer & Hilker 2013; Trauer-Kizilelma & Hilker 2015a; Trauer-Kizilelma & Hilker 2015b), *Crassostrea gigas* (Green et al. 2016) et *Teleogryllus oceanicus* (McNamara et al. 2014), car les deux parents ont été stimulés immunologiquement lorsque du TTGI a été observé chez leur descendance. Par contre, aucune étude

n'a pour l'instant démontré de relation additive ou en interaction, en ce qui concerne la protection transmise par les deux parents, même si l'on peut suspecter une meilleure protection globale contre les parasites, en comparaison à un TTGI provenant d'un seul parent.

Comme dans le cas du priming immunitaire, un niveau étonnant de spécificité a pu être mis en évidence chez certaines espèces dans l'expression du TTGI. C'est le cas par exemple chez *T. castaneum* (Roth et al. 2010), *Anoplophora glabripennis* (Fisher & Hajek 2015) ou encore chez *Artemia* (Norouzitallab et al. 2016). Mais, en plus de ne pas connaître les effecteurs immunitaires à l'origine de cette spécificité, cette dernière n'a été retrouvée que dans des conditions particulières qui traduisent certainement de l'écologie de ces espèces. Par exemple, chez *T. castaneum,* de la spécificité a été retrouvée chez la descendance de mères immunisées contre différentes souches de la bactérie Gram-positive, *Bacillus thuringiensis*, alors qu'aucune spécificité n'a été mise en évidence contre la bactérie à Gram-négatif, *Escherichia coli* (Roth et al. 2010). Chez *Anaplophora glabripennis,* de la spécificité a été trouvée pour le TTGI contre les champignons mais pas contre les bactéries (Fisher & Hajek 2015). Ces deux études suggèrent que pour mettre en évidence une réponse immunitaire spécifique chez les invertébrés, voire simplement mettre en évidence du TTGI, il semble nécessaire de considérer les parasites les plus à même d'infecter l'espèce hôte étudiée dans son environnement naturel.

Si les mécanismes responsables du TTGI restent méconnus, les effecteurs immunitaires qui paraissent être majoritairement impliqués dans ce phénomène, semblent être les mêmes que ceux à l'origine du priming immunitaire. Les effets sur ces effecteurs que sont les peptides antimicrobiens, les hémocytes ou encore la PO et la PPO, se retrouvent à tous les stades de vie de la descendance, que ce soit au niveau de l'œuf (Dubuffet et al. 2015; Moreau et al. 2012; Sadd & Schmid-Hempel 2007; Zanchi et al. 2012), pendant le développement larvaire (Freitak et al. 2009; Moret 2006; Rahman et al. 2004; Rosengaus et al. 2017; Tidbury et al. 2011) ou encore au stade adulte (Castro-Vargas et al. 2017; Roth et al. 2010; Sadd & Schmid-Hempel 2009; Zanchi et al. 2011). Par exemple, chez *M. sexta*, suite à l'injection des deux parents avec des peptidoglycanes, une plus forte concentration en PO et en peptides antimicrobiens est retrouvée dans les œufs produits (Trauer-Kizilelma & Hilker 2015a). Mais les effets du TTGI sur les effecteurs immunitaires ne résultent pas exclusivement de leur surexpression. En effet, chez l'abeille *Apis mellifera*, les larves descendantes de reines injectées avec la bactérie inactivée à la chaleur, *Paenibacillus larvae*, présentent des taux de différenciation des pro-hémocytes en hémocytes, plus important (Hernández López et al. 2014).

Contrairement au priming immunitaire, les coûts associés au TTGI ont été considérablement étudiés (Sadd & Schmid-Hempel 2009a ; Roth et al. 2010 ; Zanchi et al. 2011 ; McKean & Lazzaro 2011 ; Zanchi et al. 2012 ; Moreau et al. 2012 ; Trauer et al. 2013 ; Tate et al. 2015). En effet, comme les parents synthétisent et transmettent les effecteurs et/ou les éliciteurs de l'immunité de leur descendance, il est attendu qu'en plus du

prix de l'activation de leurs propres systèmes immunitaires, cette transmission soit aussi coûteuse pour eux. De la même manière qu'il existe des compromis associés à certains investissements maternels qui affectent la qualité de la descendance, tels que l'approvisionnement des œufs (Messina & Fox 2001; Roff 1992; Stearns 1992), le TTGI peut répondre à un trade-off entre l'immunité des parents, et celle transférée à leur descendance. Ce type de coût a notamment été trouvé chez le ténébrion meunier, *Tenebrio molitor*. Chez cette espèce, lorsque des femelles de petite taille sont stimulées avec du LPS, elles investissent moins dans la protection des œufs qu'elles produisent, au profit de leur propre immunité (Moreau et al. 2012). Mais les coûts associés au TTGI ne s'expriment pas seulement à travers un compromis opposant parents et descendants. En effet, des trade-off d'allocation des ressources entre deux paramètres immunitaires peuvent être retrouvés au sein même de la descendance de parents challengés. Chez *T. castaneum*, la descendance adulte issue de mâles stimulés avec *E. coli* voit son activité antimicrobienne diminuer, en comparaison avec celle provenant de pères contrôles. Néanmoins, ces individus bénéficient d'une augmentation de leur PO (Roth et al. 2010). Enfin, des coûts peuvent être observés au sein d'une descendance de parents immunologiquement stimulés, à travers des compromis entre l'immunité des individus et d'autres traits d'histoire de vie. Toujours chez *T. castaneum*, un challenge bactérien de la mère augmente le temps de développement larvaire de la descendance, alors que le TTGI est associé à un bénéfice en termes de survie pour la descendance adulte en cas de réinfection (Roth et al. 2010). Cela suggère qu'un bénéfice obtenu à travers le TTGI à un moment de la vie de la descendance, sera payé à un autre moment de la vie de cette même descendance.

Priming immunitaire et transfert trans-générationnel d'immunité chez *Tenebrio molitor*

Au cours de ma thèse, j'ai utilisé comme modèle biologique le ténébrion meunier chez qui le priming immunitaire et le TTGI ont été mis en évidence. En effet, il a été montré chez cette espèce de coléoptère que les larves ayant reçu une injection de LPS expriment un bénéfice en termes de survie par rapport aux individus témoins, lors d'une infection avec le champignon entomopathogène, *Metharizium anisopliae*, survenant 4 ou 7 jours plus tard. Cette protection immunitaire non spécifique semble être due à la persistance des peptides antimicrobiens produits suite à l'injection de LPS (Moret & Siva-Jothy 2003).

Concernant le TTGI chez cette espèce, il a été montré que celui-ci pouvait à la fois être d'origine maternelle ou paternelle, mais qu'en fonction du sexe stimulé, son expression chez la descendance varie. Ainsi, lorsqu'une femelle de ténébrion est injectée avec du LPS, celle-ci va produire une descendance adulte ayant une concentration hémocytaire plus importante que celle de femelles témoins. Par contre, les adultes provenant d'un mâle stimulé avec du LPS voient leur concentration en PO et en PPO augmenter, mais seulement après avoir été eux-mêmes stimulés immunologiquement avec ce même LPS (Zanchi et al. 2011).

Le TTGI d'origine maternelle a été plus étudié chez *T. molitor* que celui d'origine paternelle et montre que des femelles injectées de LPS produisent des œufs (Moreau et al. 2012 ; Zanchi et al. 2012) et des larves (Moret 2006) ayant une plus forte concentration en peptides antimicrobiens que ceux provenant de mères témoins. De plus, la protection transmise à la descendance ne semble pas concerner toutes les progénitures. En effet, suite à une injection de LPS à des femelles, il a été montré que la séquence de ponte allant de 2 à 8 jours post-injection est majoritairement protégée (65% des œufs exprimant de l'activité antibactérienne), alors qu'avant ou après cette période, peu d'œufs bénéficient du TTGI (de 0 à 30 % d'œufs exprimant de l'activité antibactérienne en fonction des séquences de pontes étudiées ; Zanchi et al. 2012). Au cours de cette période où le TTGI semble s'exprimer le plus fortement, on peut voir à travers le ratio entre le nombre d'œufs produits et le nombre d'œufs protégés (exprimant une activité antimicrobienne), que les stratégies d'investissement à la protection immunitaire de la descendance sont très variables en fonction des femelles (Zanchi et al. 2012). En effet, certaines femelles produisent très peu d'œufs par rapport à la moyenne et n'investissent pas dans leur protection. D'autres pondent un nombre d'œufs proche de la moyenne et tendent à en protéger une grande majorité. Enfin, un dernier type de femelle pond beaucoup plus d'œufs que la moyenne mais n'en protège que très peu (Zanchi et al. 2012). Cependant, il n'a pas encore été démontré que ces variations reflètent des différences génétiques dans la capacité des femelles à protéger leurs œufs suite à une infection maternelle. Dans tous les cas, que la stimulation immunitaire soit d'origine paternelle ou maternelle, aucun type de spécificité au sens propre n'a été décelé dans l'étude du TTGI chez *T. molitor*. Néanmoins, l'évolution de ce phénomène chez cette espèce aurait pu être contrainte par les bactéries à Gram-positif, plus à même de persister dans l'environnement que les bactéries à Gram-négatif. En effet, Dubuffet et al. (2015) ont démontré que quelle que soit la stimulation immunologique réalisée chez des femelles (bactérie à Gram-positif ou négatif et champignon), l'activité antibactérienne retrouvée dans les œufs est uniquement efficace contre les bactéries à Gram-positif. Ce phénomène semble être le résultat de l'action d'un peptide de la famille des défensines, la ténécine 1, qui a un spectre d'action exclusivement dirigé vers ces bactéries à Gram-positif. De plus, il a été montré au cours de cette étude que les champignons sont un faible inducteur de TTGI chez *T. molitor*, alors que les niveaux d'activité antimicrobienne retrouvés dans les œufs sont similaires lorsque le challenge maternel a été fait avec une bactérie à Gram-positif ou négatif (Dubuffet et al. 2015).

Enfin, comme attendu et malgré les avantages qu'il confère, le TTGI est coûteux pour les parents et leur descendance chez le ténébrion meunier. En effet, il a été montré que dans le cas d'un trade-off pour l'allocation aux ressources, que les parents peuvent ne pas investir dans leur descendance après un challenge immunitaire afin de probablement maximiser leur propre survie et de se reproduire plus tard lorsque l'environnement sera plus favorable. Cela se traduit pour les femelles de petite taille par un compromis entre leur activité antibactérienne et celle de leurs œufs, en faveur de leur propre immunité (Moreau et al. 2012). De plus, comme

chez l'espèce phylogénétiquement proche *T. castaneum*, un challenge maternel augmente le temps de développement larvaire des descendants (Roth et al. 2010 ; Zanchi et al. 2011). Cela est particulièrement coûteux pour la descendance. Dans un premier temps, cela augmente le délai pour atteindre l'âge de la reproduction et augmente également la compétition pour l'accès aux ressources dans le cas d'une importante densité larvaire (Koella & Boëte 2002). De plus, cela accroît la probabilité de mortalité (Bell 1980), surtout chez *T. molitor* où le cannibalisme envers les juvéniles est très important (Ichikawa & Kurauchi 2009).

Objectifs de ma thèse

Le but de cette thèse est de définir quelles sont les conditions écologiques qui ont favorisé l'évolution du priming immunitaire et du TTGI chez *T. molitor*, tout en examinant les bases génétiques du TTGI. L'étude des coûts et des bénéfices liés à ces phénomènes nous renseigne sur les pressions de sélection ayant conduit à son évolution chez les insectes.

Dans le premier chapitre, nous avons exploré les bénéfices en termes de survie liés au priming immunitaire et au TTGI. Nous avons réalisé des suivis de mortalité suite à des protocoles de priming et d'infections croisés. Le but était de tester l'hypothèse que chez les invertébrés, le priming immunitaire a très certainement évolué à la suite d'infections répétitives par des agents pathogènes de l'environnement local. Un tel scénario aurait pu favoriser l'évolution d'une certaine forme de spécificité aux microbes exposant l'hôte à la plus grande probabilité de ré-infection. Plus particulièrement, la réponse immunitaire ayant lieu à la suite d'un priming avec ces microbes devrait être plus efficace et moins coûteuse qu'avec d'autres agents pathogènes. Nous avons également recherché dans le cadre de cette expérimentation, quels effecteurs immunitaires étaient impliqués dans l'éventuel bénéfice en termes de survie accordé par le priming immunitaire ou le TTGI.

Dans le deuxième chapitre, nous avons cherché à mettre en évidence un bénéfice au TTGI d'origine maternelle sur les œufs et les jeunes larves juste après l'éclosion, en cas d'infection bactérienne. Pour ce faire nous avons exposé des femelles à deux bactéries différentes. Nous avons ensuite maintenu les œufs et les larves qui en étaient issues soit dans des conditions stériles, soit en présence de la bactérie ayant stimulé la mère. Nous avons suivi l'éclosion des œufs et la survie des larves placés dans ces conditions. Etant donné que l'origine de la protection, et notamment de l'activité antibactérienne, transmise à la descendance dans le cadre du TTGI n'a jamais été recherchée, nous avons également exposé des femelles aux deux mêmes bactéries que précédemment, afin de savoir si cette protection résulte d'un transfert passif de peptides aux œufs, ou si ces derniers sont capables de les produire eux-mêmes. En étudiant la dynamique de l'activité antibactérienne au sein des œufs tout au long de leur période d'incubation, nous pouvons obtenir des éléments de réponse quant à l'origine de la production de ces peptides antibactériens. De plus, ces deux mécanismes qui ne sont pas

obligatoirement exclusifs, sont censés avoir des impacts différents en termes de valeur sélective, et notamment de coût, pour la mère et sa descendance. Si le transfert est dû à un transfert passif de la mère à la descendance, nous pouvons nous attendre à ce que les coûts soient essentiellement supportés par celle-ci. Au contraire, si les œufs produisent eux-mêmes les peptides antimicrobiens, les coûts du TTGI devraient se retrouver essentiellement chez la descendance bénéficiant de cette protection.

Dans le troisième chapitre, nous avons cherché à savoir si le TTGI est déterminé génétiquement. En utilisant des lignées consanguines de *T. molitor*, nous avons quantifié la variation génétique trouvée chez les femelles quant à la protection de leurs œufs et de la descendance adulte dans le cadre du TTGI. Nous avons également mesuré d'autres traits de valeur sélective que ce soit pour les femelles (tels que leur masse, l'effort reproducteur, leur niveau de défense immunitaire et leur longévité) ou leur descendance (taux d'éclosion, survie larvaire, masse nymphale, masse à l'âge adulte et niveau de défense immunitaire). Si l'investissement au TTGI est déterminé génétiquement, ce trait devrait être héritable. Nous avons également estimé des corrélations phénotypiques entre l'investissement des femelles au TTGI et d'autres traits de fitness, que ce soit chez les femelles ou leur descendance. Le but étant de savoir si ces traits peuvent évoluer indépendamment ou s'ils sont génétiquement liés les uns aux autres, ce qui contraindrait leur évolution. De plus, cette approche permet de tester si la protection des œufs et de la descendance à des stades de développement plus tardifs sont génétiquement corrélées, ou si ces traits peuvent évoluer indépendamment.

Dans le quatrième chapitre, nous avons passé en revue l'ensemble des études concernant le TTGI chez les invertébrés. Le but étant de confronter l'ensemble des données actuellement existantes afin de mettre en exergue les principales caractéristiques et les mécanismes identifiés, en fonction de l'écologie et de l'évolution du phénomène. Pour ce faire nous avons décrit tous les articles ayant exploré le TTGI à l'heure actuelle, tout en proposant une analyse critique des procédures expérimentales utilisées. Nous avons fini ce travail de revue en proposant les scénarios mécanistiques hypothétiques à travers lesquels le TTGI pourrait se réaliser, ainsi qu'en proposant des pistes et des conseils pour les prochains travaux de recherche sur le sujet.

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 priming immunitaire et du TTGI, ainsi que sur les mécanismes mis en œuvre dans sa réalisation chez *T. molitor*.

Chapitre 1

Chapitre 1 : Recherche de spécificité dans le priming intra-individuel et trans-générationnel chez *T. molitor* : bactéries ayant contraint l'évolution du phénomène.

Dans ce premier chapitre, nous avons voulu tester l'hypothèse que chez les invertébrés, le priming immunitaire a très certainement évolué à la suite d'infections répétitives par des agents pathogènes de l'environnement local. Un tel scénario aurait pu favoriser l'évolution d'une certaine forme de spécificité aux microbes exposant l'hôte à la plus grande probabilité de ré-infection. Plus particulièrement, la réponse immunitaire ayant lieu à la suite d'un priming avec ces microbes, devraient être plus efficace et moins coûteuse qu'avec d'autres agents pathogènes.

De plus, il a récemment été démontré chez le cloporte *Porcellio scaber* (Roth & Kurtz 2009) et la drosophile *Drosophila melanogaster* (Pham et al 2007) que l'activité phagocytaire semble être responsable d'une réponse spécifique chez les invertébrés. Nous nous sommes donc intéressés à l'implication des cellules phagocytaires dans le priming chez *T. molitor*.

Afin de tester notre hypothèse, nous avons examiné la spécificité du priming immunitaire au sein d'une même génération et entre générations, chez le ténébrion meunier. Cela a été fait en comparant la survie d'individus infectés avec des bactéries en fonction de leur propre expérience immunologique, ou de celle de leur mère, avec ces mêmes bactéries.

Ici, les insectes primés avec des bactéries Gram-positives sont fortement protégés contre les infections des bactéries Gram-positives et Gram-négatives, ayant lieu 20 jours après le priming. Cela est essentiellement dû à l'induction d'une réponse antibactérienne persistante, qui n'existe pas lorsque les individus sont primés avec une bactérie Gram-négative. La survie des individus n'étant pas affectée lorsque l'activité des phagocytes est inhibée, cela minimise leur importance dans le priming.

Dans le cas du transfert trans-générationnel d'immunité, les descendances de mères primées avec des bactéries Gram-positives et Gram-négatives, montrent le même niveau d'amélioration de leur immunité, quel que soit la bactérie utilisée pour l'infection. Ce bénéfice exprimé en termes de survie à l'infection semble essentiellement dû à une augmentation de l'activité de la phénoloxydase lorsque les mères ont été stimulées avec une bactérie Gram-positive, alors que dans le cas d'une stimulation par une bactérie à Gram-négatif, c'est les capacités de tolérance qui semblent être augmentées chez la descendance. Cette protection maternelle est coûteuse en ce qui concerne le développement larvaire de la descendance, mais ce coût est plus faible pour les descendants de mères primées avec des bactéries Gram-positives.

En conclusion, même si *T. molitor* peut développer un certain niveau de protection suite à un priming avec des bactéries Gram-négatives, le priming immunitaire induit par des bactéries Gram-positives est plus efficace et moins coûteux. Les bactéries Gram-positives ont donc dû jouer un rôle majeur dans l'évolution du phénomène de priming immunitaire chez cet insecte.

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

Immune priming specificity within and across generations reveals the range of pathogens affecting evolution of immunity in an insect

Authors: J. Dhinaut, M. Chogne, Y. Moret

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Abstract

1. Many organisms can improve their immune response as a function of their immunological experience or that of their parents. This phenomenon, called immune priming, has likely evolved from repetitive challenges by the same pathogens during the host lifetime or across generation.

2. All pathogens may not expose host to the same probability of re-infection and immune priming is expected to evolve from pathogens exposing the host to the greatest probability of re-infection. Under this hypothesis, the priming response to these pathogens should be specifically more efficient and less costly than to others.

3. We examined the specificity of immune priming within and across generations in the mealworm beetle, *Tenebrio molitor*, by comparing survival of individuals to infection with bacteria according to their own immunological experience or that of their mother with these bacteria.

4. We found that insects primed with Gram-positive bacteria became highly protected against both Gram-positive and Gram-negative bacterial infections, mainly due to an induced persistent antibacterial response, which did not exist in insects primed with Gram-negative bacteria. Insects primed with Gram-positive bacteria also exhibited enhanced concentration of hemocytes, but their implication in acquired resistance was not conclusive because of the persistent antibacterial activity in the hemolymph. Offspring maternally primed with Gram-positive and Gram-negative bacteria exhibited similarly improved immunity, whatever the bacteria used for the infection. Such maternal protection was costly in the larval development of offspring, but this cost was lower for offspring maternally primed with Gram-positive bacteria.

5. While *T. molitor* can develop some levels of primed response to Gram-negative bacteria, the priming response to Gram-positive bacteria was more efficient and less costly. We concluded that Gram-positive bacterial pathogens were of paramount importance in the evolution of immune priming in this insect species.

I. Introduction

Immune responses are usually divided into adaptive and innate. The adaptive immune response is unique to vertebrates because it involves B and T lymphocytes, which can develop antigen-specific memory. Upon a secondary challenge with a previously encountered pathogen, the clonal expansion of memory T and/or B cells leads to a faster and stronger immune response (Pancer & Cooper 2006). In contrast, the innate immune response is thought to be non-specific, lacking immunological memory. Since invertebrates have no functional equivalent of T and B cells, their immune system relies solely on innate immune mechanisms (Theopold et al. 1996). The immune system of invertebrates recognizes a range of conserved non-self molecules associated with groups of pathogens, such as lipopolysaccharides (LPS), peptidoglycans, ß-1,3 glucans and other sugar moieties, through germ-line encoded immune receptors, which then activate various immune effector systems, including phagocytosis in some hemocytes (Kaaya 1993), the prophenoloxidase cascade (Sugumaran et al. 2000) and the synthesis of antimicrobial peptides (Fehlbaum et al. 1994). Because immunity in invertebrates relies on innate mechanisms, their immune responses were assumed to be essentially identical to repeated challenges with the same stimulus. However, there is now cumulative evidence demonstrating that the innate immune system of invertebrates is able to produce immune responses involving a memory-like phenomenon (see Pham & Schneider 2008; Milutinović & Kurtz 2016 for reviews) known as immune priming (Little & Kraaijeveld 2004). A spectacular characteristic of immune priming is that it also occurs across generations, a phenomenon called trans-generational immune priming (TGIP) (Little et al. 2003; Sadd et al. 2005; Moret 2006; Sadd & Schmid-Hempel 2007; Freitak et al. 2009; Roth et al. 2010; Tidbury, Pedersen, & Boots 2011; Boots & Roberts 2012; Trauer & Hilker 2013; Yue et al. 2013; Hernández López et al. 2014; Norouzitallab et al. 2015). Whether both phenomena involve the same mechanisms is not yet known. However, from an ecological and evolutionary perspective, these plastic adjustments of immune investment in response to individual immunological experience are likely to have evolved from multiple encounters with the same parasites within the host lifetime and across generations. Such a scenario could have allowed a certain level of immune priming specificity to evolve, either within or across generations.

Specificity refers to the degree to which immune priming discriminates pathogens at different levels of relatedness between the primary infection and the subsequent ones, either within or across generations. For both within and trans-generational immune priming, previous studies have shown that a wide degree of specificity occurs, from cross-reactive (non-specific) (Huang & Song 1999; Moret & Siva-Jothy 2003) to highly specific (Little et al. 2003; Roth et al. 2010; Sadd & Schmid-Hempel 2006; Pham et al. 2007; Roth et al. 2009). For instance, using natural bacterial pathogens of bees, Sadd and Schmid-Hempel (2006) found that primed workers of the bumblebee, *Bombus terrestris*, were non-specifically protected against subsequent bacterial infections that occurred up to 8 days post-priming, whereas immune protection became specific to

the bacteria used to prime the insects when the subsequent infections occurred 22 days post-priming. The early nonspecific phase of the primed response was associated with the presence of antimicrobial activity in the hemolymph, likely active toward a large range of pathogens, within the first 8 days post-priming, but which did not persist for 22 days. It seems therefore that other immune effectors than antimicrobial peptides might support the later specific phase of the primed response. Other studies suggest that hemocytes play a major role in specific immune priming (Pham et al. 2007; Roth & Kurtz 2009; Rodrigues et al. 2010). Similarly, trans-generationally primed individuals can exhibit cross-reactive elevation of their immunocompetence (Huang & Song 1999) when a maternal immune challenge with LPS from *Escherichia coli* in the shrimp, *Peneaus monodon*, protects the offspring against the virus of the white spot syndrome. Conversely, TGIP was found to be pathogen-specific in the red flour beetle, *Tribolium castaneum* (Roth et al. 2010), or the crustacean, *Daphnia magna* (Little et al. 2003), although the immune effectors involved were not identified.

The level of specificity associated with immune priming is likely dependent on the immune mechanisms involved. Since hemocytes were shown to be attractive candidate effectors for a specific primed response, a receptor possessing the degree of diversity accounting for the observed specificity must be involved to mediate faster recognition by phagocytes (Pham & Schneider 2008). Molecules such as Dscam (for Down syndrome cell adhesion molecule) and FREPS (for Fibrinogen-Related Proteins) were proposed as good candidates to generate receptor diversity in the invertebrate immune response (Watson et al. 2005; Dong, Taylor & Dimopoulos 2006; Hanington & Zhang 2011; Huang et al. 2015). However, the level of diversity possibly proposed by these molecules is still relatively limited, compared to that of the vertebrate acquired immune response, enabling recognition of an almost infinite diversity of antigens. Therefore, considering the limited ability of the invertebrate immune system to produce highly diversified pathogen receptors, and as pathogen recognition mostly relies on germ-line encoded genes, specificity or even the induction of immune priming should be restricted to a limited range of pathogens. Accordingly, out of five microbial pathogens, only *Streptococcus pneumonia* and *Beauveria bassiana* were able to elicit a protective effect in *D. melanogaster*, but this protection was highly specific (Pham et al. 2007). Similarly, in the beetle *T. castaneum*, specificity of immune priming could differentiate different strains of the bacterium *Bacillus thuringiensis*, but no such a result could be found for defense against *Escherichia coli* (Roth et al. 2009). As expected from the cost of immunity (Moret & Schmid-Hempel 2000), immune priming was found to bear fitness costs too (Sadd & Schmid-Hempel 2009; Zanchi et al. 2011; Moreau et al. 2012; Zanchi et al. 2012). It is therefore hypothesized that immune priming and the ability to develop a specific primed response should evolve from the most threatening pathogens (Dubuffet et al. 2015).

Here, we tested this hypothesis by investigating a specific primed response within and across generation in the mealworm beetle, *Tenebrio molitor*. In this insect, immune priming has previously been reported both within and across generations (Moret & Siva-Jothy 2003; Moret 2006; Zanchi et al. 2011; Moreau et al. 2012; Zanchi et al. 2012; Krams et al. 2013; Dubuffet et al. 2015). After a primary immune challenge with bacterial LPS, the immune response remains activated long enough to protect the insect against a subsequent infection with the entomopathogenic fungus, *Metharizium ansiopliae* (Moret & Siva-Jothy 2003). Such cross-reactive immune protection presumably results from increased levels of antimicrobial activity in the hemolymph of primed insects, which can last for 14 days after the immune challenge (Haine et al. 2008a, b). In addition, mothers similarly immune-challenged produce eggs containing enhanced antimicrobial activity (Moreau et al. 2012; Zanchi et al. 2012), the developing larvae exhibit increased levels of antimicrobial activity in the hemolymph (Moret 2006), and the young adults have higher concentrations of circulating hemocytes (Zanchi et al. 2011). The survival benefit and specificity associated with these changes in immune defenses among primed insects have not been tested so far. Intriguingly, whereas the maternal immune challenge with fungi did not increase antimicrobial activity in the eggs, whatever the bacterial type (Gram-positive or Gram-negative) used to prime mothers, the resulting antibacterial activity found in the eggs was only active against Gram-positive bacteria (Dubuffet et al. 2015). One explanation might be that Gram-negative bacteria might be preserved from the host immune response because this range of bacteria might play an important symbiotic role in the mealworm beetle. However, this is unlikely since the microbiota composition in *T. molitor* comprises similar number of taxa from Gram-positive and Gram-negative bacteria, with Gram-positive bacteria being the most abundant (Jung et al. 2014; Wang & Zhang 2015; Stoops et al. 2016). Alternatively, these results suggest that Gram-positive pathogens may have been an important selective force for the evolution of immune priming in *T. molitor*. Indeed, Gram-positive bacteria include important entomopathogenic pathogens such as *Bacillus thuringiensis*, which are also the most able to persist in the external environment of *T. molitor* by forming endospores (Jurat-Fuentes & Jackson 2012). The persistence of these bacterial pathogens exposes the insect to repeated infections within an individual lifetime and across generations.

In this study, we tested whether Gram-positive bacteria were a major selective force in the evolution of immune priming in *T. molitor*, at both the individual level and across generations. To this end, we first tested for specific immune priming of resistance to different bacteria in survival experiments. The bacteria used were Gram-positive and Gram-negative, comprising both an entomopathogenic and a non-entomopathogenic bacterium for each Gram type. Beetles were primed with inactivated bacteria and then they, or their offspring at the adult stage, were infected with live bacteria in a reciprocal design. We investigated the principal immune effectors involved in immune priming within and across generations. We especially focused on the role of

phagocytes, because they may play an important role in invertebrate immune memory (Pham et al. 2007; Roth & Kurtz 2009; Rodrigues et al. 2010). Under the hypothesis that Gram-positive bacteria are the most threatening bacterial pathogens of *T. molitor*, we expected to observe primed specific responses toward these bacteria, but not toward Gram-negative bacteria.

II. Material and Methods

II.1. Insect cultures

Virgin adult beetles of controlled age (10 ± 2 days post-emergence) were obtained from pupae taken at random from an outbred stock culture maintained in standard laboratory conditions at the University of Burgundy, Dijon, France. All the experimental insects were reared and maintained in an insectary at 24 ± 2°C, 70% RH in permanent darkness, and supplied *ad libitum* with bran flour and water, supplemented by apple. All the insects were weighed to the nearest 1 mg with an OHAUS balance (discovery series, DU114C) before use in the experiments. We conducted two separate sets of three successive experiments to study immune priming within and across generations. Each set of experiments aimed to test for specificity of resistance to different bacteria and examined the immune effectors involved in each type of immune priming.

II.2.Studying individual immune priming

A first experiment consisted of testing for specificity of resistance to four different bacteria in a survival experiment (Fig. S1a). We used the Gram-positive bacteria *Staphylococcus aureus* and *Bacillus thuringiensis*, and the Gram-negative bacteria *Escherichia coli* and *Serratia entomophila*. One bacterium within each Gram-type was entomopathogenic, known to infect coleopteran insects (*B. thuringiensis* and *S. entomophila*), while the other was not entomopathogenic (*S. aureus* and *E. coli*). There were five priming treatment groups, for a total of 965 adult beetles (512 females and 453 males). Each priming treatment group consisted of insects injected with a 5-µL suspension of one of the four inactivated bacteria in phosphate buffer saline (PBS 10 mM, pH 7.4) after being chilled on ice for 10 min, except for the fifth group, where the insects were treated in the same way but without bacteria, as a procedural control for the effect of the injection. Twenty days later, each priming treatment group was divided into four equal groups of beetles, for infection with a fine sterilized needle dipped into a pellet of live bacteria, either *S. aureus*, *B. thuringiensis*, *E. coli*, or *S. entomophila*. The 20-day delay between priming and infection was chosen to ensure that any long-lasting antibacterial immune response induced by the injection of bacteria had ceased (Haine et al. 2008), because any persistence or cross-reactivity of antibacterial peptides could prevent the detection of a pathogen-specific priming response (Sadd & Schmid-Hempel 2006). Survival to infection was recorded once a day for 14 days.

A second experiment tested the effect of priming with either a Gram-positive bacterium (*B. thuringiensis*) or a Gram-negative bacterium (*S. entomophila*) on various immune effectors, before and after a secondary immune challenge, to reveal their potential implication in the immune "memory" process (Fig. S1b). For this purpose, 50 adult beetles (25 males and 25 females) were primed by injection of inactivated *B. thuringiensis*,

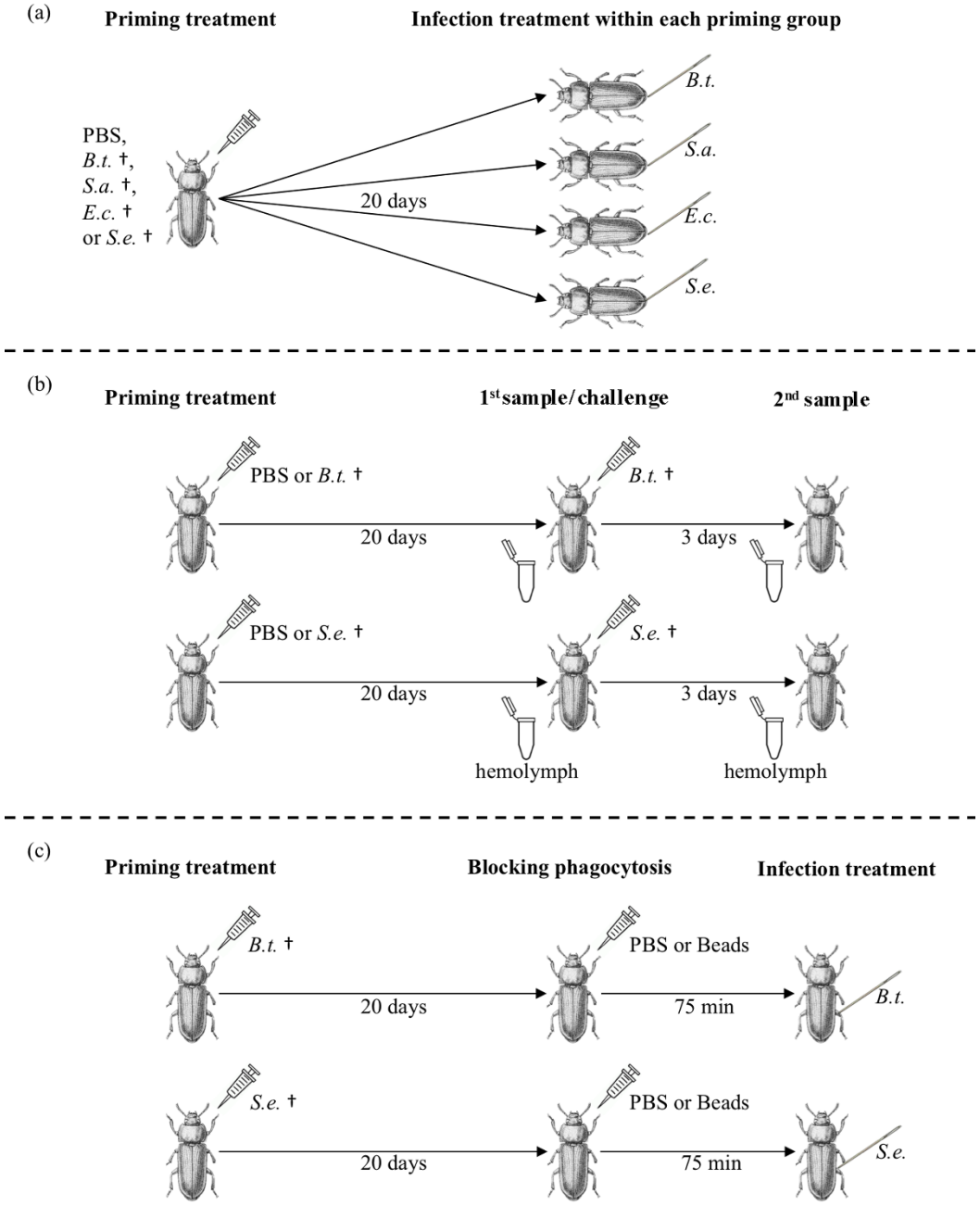

Fig. S1. Experimental design of the three experiments studying individual immune priming: (a) tested for specificity of the primed response on survival to bacterial infection, (b) investigated the immune effectors potentially involved in the primed response, and (c) tested the role of phagocytosis in the primed response. *Bacillus thuringiensis* (*B.t.*), *Staphylococcus aureus* (*S.a.*), *Escherichia coli* (*E.c.*) and *Serratia entomophila* (*S.e.*). † indicates that bacteria were inactivated. Infection treatment was followed by a survival survey of 14 days (see material and methods for details).

another 50 were primed with inactivated *S. entomophila*, and 100 beetles were sham-injected, as described above. Twenty days later, a 5µL-sample of hemolymph was collected from each beetle, to quantify the three main immune effectors: hemocyte concentration estimated by microscopy (Zanchi et al. 2011); antibacterial activity in the hemolymph tested using a standard zone of inhibition assay (Moret 2006) against the bacterium, *Arthrobacter globiformis* (which offers the best sensitivity to any antimicrobial activity developed in the mealworm beetle; Dubuffet et al. 2015); the activity of naturally activated phenoloxidase (PO) enzymes only (hereafter PO activity), and the activity of proenzymes (proPO) in addition to that of PO enzymes (hereafter total-PO activity) both measured by spectrophotometry, to test for the maintenance and use of the prophenoloxidase system (Zanchi et al. 2011). Immediately after collection of the first sample of hemolymph, the beetles were subjected to a secondary immune challenge, with the same inactivated bacterium as that used for priming. Half of the control beetles were challenged with inactivated *B. thuringiensis* and the other half with inactivated *S. entomophila*. A second 5µL-sample of hemolymph was collected from each beetle, three days after this challenge, corresponding to the peak of the secondary immune response (Haine et al. 2008) for hemocyte concentration, antibacterial activity, and phenoloxidase activity.

In a third experiment, we examined whether phagocytes were the main effectors of the individual primed response against a Gram-positive or a Gram-negative bacterium (Fig. S1c), with an adaptation of the method used by Pham *et al.* (2007). This assay consists of testing the contribution of phagocytes to bacterial killing by inhibiting phagocytosis in primed insects, through the injection, *in vivo*, of micro-beads, shortly before infecting them with bacteria. In principle, phagocytes having engulfed micro-beads will not participate in the phagocytosis of the bacteria subsequently spreading into the hemocoel. Hence, if the primed response is dependent on phagocytes, then the priming effect should vanish when primed insects are injected with micro-beads before being infected. Eighty adult beetles (40 males and 40 females) were primed by injection of inactivated *B. thuringiensis*, while another 80 beetles were primed with inactivated *S. entomophila*. Twenty days later, the beetles were infected with the same live bacterium as that used for priming. However, 75 min before infection, we blocked phagocytosis in half of the insects in each priming group by injection of carboxylate-modified YG fluorescent 2-µm-diameter polystyrene beads (Fluoresbrite® Polysciences, Inc, Warrington PA, USA), suspended in PBS (10^8.µL^{-1}). The other half of each group was treated in the same way, but without beads, as a procedural control for the effect of the injection. Survival was followed for 14 days.

II.3. Studying trans-generational immune priming

Studying TGIP used the same experimental approach as above to study individual immune priming. Specificity of resistance was tested against the same bacterial species as used above in a survival experiment (Fig. S2a). Fifty adult females were weighed and divided into five priming treatment groups, corresponding to immunization by injection with inactivated *S. aureus*, *B. thuringiensis*, *E. coli*, or *S. entomophila*, in addition to the control group, in which females were injected with sterile PBS only. Immediately after the priming injection, each female was paired with an immunologically naïve virgin male, and allowed to produced eggs in a plastic box (L x 1 x H, 17 x 11 x 9.5 cm) for 8 days, supplied with bran flour, water and small slices of apple. Only the eggs produced between day 2 and day 8 after maternal priming were kept, because more eggs will be protected during this period of time (Zanchi et al. 2012). Nine weeks after egg laying, 20 larvae per couple were randomly taken and individually isolated in grid boxes (boxes with 10 compartments; each compartment: L x 1 x H, 4.8 x 3.2 x 2.2 cm). For each individual, larval development time (duration in days from hatching to adult stage) and adult weight were recorded. Larval development time was measured because it has been shown to be affected by maternal priming (Zanchi et al. 2011). At 10 days old, adult offspring of each female were sexed and randomly infected with one of the 4 bacterial species, as described above. Survival to infection was recorded once a day for 14 days.

The effect of maternal priming with a Gram-positive or Gram-negative bacterium was tested on the same immune effectors as described above in the offspring at the adult stage before and after an immune challenge similar to the maternal challenge (Fig. S2b). For this purpose, 5 females were primed with inactivated *B. thuringiensis*, 5 females were primed with inactivated *S. entomophila*, and 10 females were injected with PBS only. Immediately after the priming treatment, females were paired with an immunologically naïve virgin male to produce offspring as described above. Ten larvae per couple were randomly taken and maintained individually in grid boxes. Offspring were sexed at adulthood (10 days old) and we sampled a 5µL-sample of hemolymph to test for hemocyte concentration, antibacterial activity and phenoloxidase activity. Immediately after this first sample of hemolymph, the beetles were immune-challenged with the same inactivated bacterium as that used for maternal priming. The offspring of five of the control mothers were challenged with inactivated *B. thuringiensis*, while the offspring of the other five control mothers were challenged with inactivated *S. entomophila*. The offspring beetles were tested again while developing an immune response, 3 days later, for hemocyte concentration, antibacterial activity, and phenoloxidase activity.

To test the contribution of phagocytes to TGIP, 8 females were primed with inactivated *B. thuringiensis*, while 8 females were primed with inactivated *S. entomophila* (Fig. S2c). Females were allowed to produce offspring as described above, and 10 larvae per mother were randomly taken and maintained individually in grid boxes. Adult offspring from each mother were sexed at 10 days old, and divided into two groups. One half (5 per

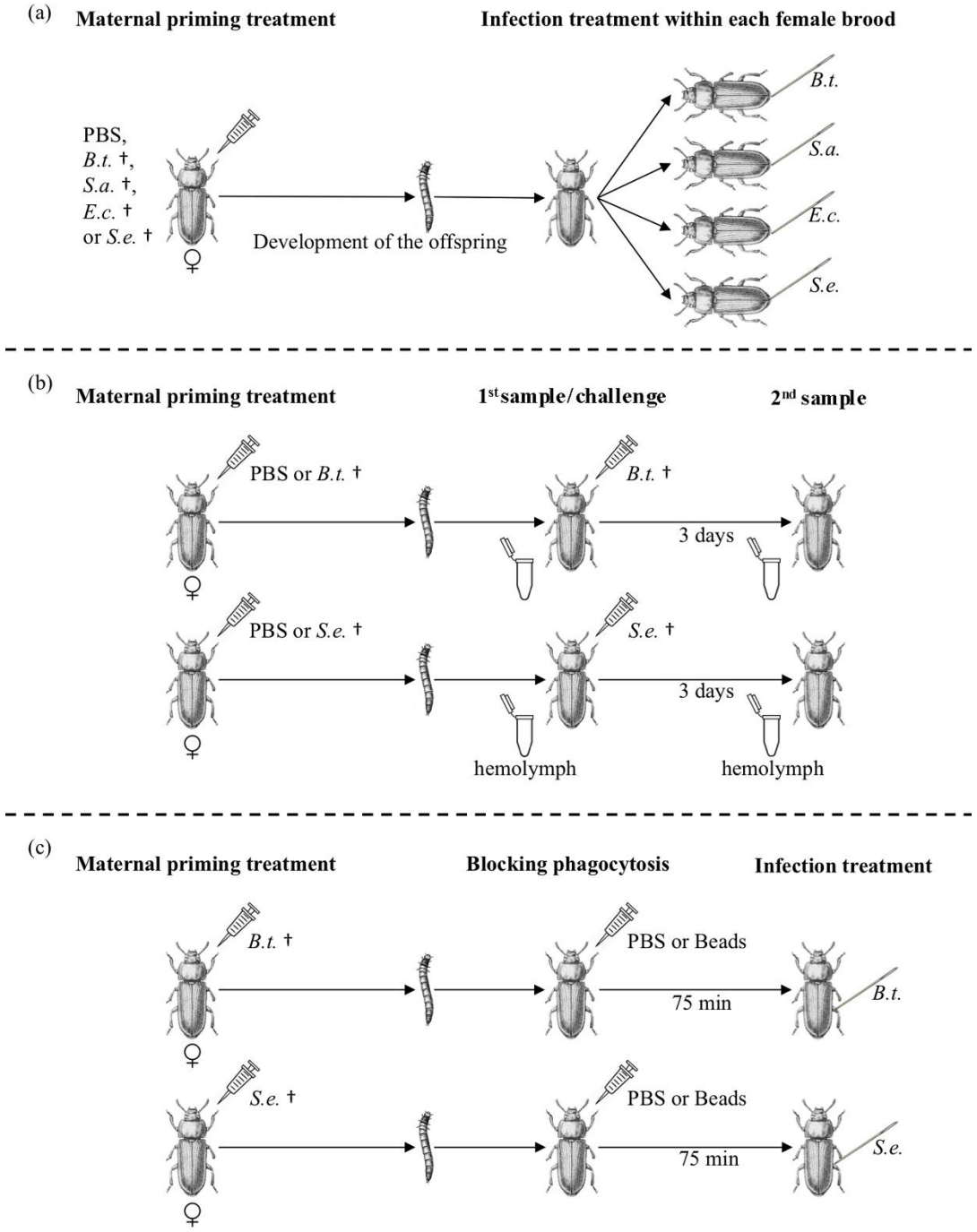

Fig. S2. Experimental design of the three experiments studying trans-generational immune priming: (a) tested for specificity of the primed response on survival of the offspring to bacterial infection, (b) investigated the immune effectors potentially involved in the primed response in the offspring, and (c) tested the role of phagocytosis in the primed response in the offspring. *Bacillus thuringiensis* (*B.t.*), *Staphylococcus aureus* (*S.a.*), *Escherichia coli* (*E.c.*) and *Serratia entomophila* (*S.e.*). † indicates that bacteria were inactivated. Infection treatment was followed by a survival survey of 14 days (see material and methods for details).

mother, i.e. 40 individuals) received a 5µL injection of micro-beads (10^8 beads per µL of PBS) to block phagocytosis, whereas the other half received a 5µL injection of PBS only. After 75 minutes, the offspring beetles were infected with the same live bacterium as that used for maternal priming and survival was followed for 14 days.

II.4. Bacterial cultures for priming and infections

All the bacteria used for experimental priming or infections were obtained from the Pasteur Institute: *S. aureus* (CIP52.149), *B. thuringiensis* (CIP53.1), *E. coli* (CIP103470) and *S. entomophila* (CIP102919). To perform priming treatments, bacteria were grown overnight at 28°C in liquid Broth medium (10 g bacto-tryptone, 5 g yeast extract, 10 g NaCl in 1000 mL of distilled water, pH 7). Bacteria were then inactivated in 0.5% formaldehyde prepared in PBS for 30 minutes, rinsed three times in PBS, and their concentration adjusted to 10^8 bacteria per mL using a Neubauer improved cell counting chamber. The success of the inactivation was tested by plating a sample of the bacterial solution on sterile Broth medium with 1% of bacterial agar and incubated at 28°C for 24 hours. Aliquots were kept at -20°C until use. Beetle priming was performed by injection through the pleural membrane between the second and third abdominal tergites using sterile glass capillaries that had been pulled out to a fine point with an electrode puller (Narashige PC-10). For infection treatments, overnight bacterial cultures (20 mL) were centrifuged at 4000 rpm at 4°C for 30 min. The supernatant was discarded and the bacteria pellet was used for infection. Beetles were infected by dipping a sterilized 0.03 mm diameter needle (Fine Science Tools® n° 26000-25) into the bacteria pellet and pricking the animal through the pleural membrane between the second and third abdominal tergites.

II.5. Hemolyph collection and immune parameters

Individual beetles were chilled on ice until 5 µL of hemolymph was collected from a wound in the beetle's neck and flushed into a microcentrifuge tube containing 25 µL of PBS. A 10-µL subsample was immediately used for the measurement of hemocyte concentration, using a Neubauer improved hemocytometer under a phase-contrast microscope (magnification x 400). Another 5-µL subsample was kept in an N-phenylthiourea-coated microcentrifuge tube (Sigma-Aldrich, St Louis, MO, USA, P7629) and stored at -80°C until later examination for antibacterial activity. The remaining hemolymph solution was diluted with 15 µL of PBS and immediately stored at -80°C for later measurement of phenoloxidase activity.

Antimicrobial activity in the hemolymph was measured using a standard zone of inhibition assay (Moret 2006). Samples were thawed on ice, and 2 µL of the sample solution was used to measure antimicrobial

activity on zone of inhibition plates seeded with *Arthrobacter globiformis* from the Pasteur Institute (CIP105365). An overnight culture of the bacterium was added to Broth medium containing 1% agar to achieve a final concentration of 10^5 cells per mL. Six millilitres of this seeded medium was then poured into a Petri dish and allowed to solidify. Sample wells were made using a Pasteur pipette fitted with a ball pump. Two microlitres of sample solution was added to each well, and a positive control (Tetracycline: Sigma-Aldrich, St Louis, MO, USA, T3383; 2.5 $mg.mL^{-1}$ in absolute ethanol) was included on each plate (Zanchi et al. 2011). Plates were then incubated overnight at 28 °C. The diameter of inhibition zones was then measured for each sample. As there was slight variation among plates for inhibition zones resulting from tetracycline addition, the diameter of the zone of inhibition of each sample was adjusted to take into account such a between plate variation. For this, the Petri dish in which the inhibition zone resulting from the tetracycline positive control was the largest was used as reference. Then, for each sample, inhibition zone was multiplied by the ratio between the inhibition zone due to tetracycline in the reference Petri dish and that due to tetracycline in the Petri dish in which the sample was measured.

For each individual hemolymph sample, both the activity of naturally activated phenoloxidase (PO) enzymes only (PO activity), and the activity of the proenzymes (proPO) in addition to that of the PO (total-PO activity), were measured using a spectrophotometer (Zanchi et al. 2011). The PO activity was quantified without further activation, while the total-PO activity required the activation of the proPO into PO with chymotrypsin. For this purpose, frozen hemolymph samples were thawed on ice and centrifuged (3500 g, 5 min, 4°C). Five microlitres of supernatant was added to a microplate well containing 20 µL of PBS, and either 140 µL of distilled water to measure PO activity only, or 140 µL of chymotrypsin solution (Sigma-Aldrich, St Louis, MO, USA, C-7762, 0,07 $mg.mL^{-1}$ of distilled water) to measure total-PO activity. Then 20 µL of L-Dopa solution (Sigma-Aldrich, St Louis, MO, USA, D-9628, 4 mg mL^{-1} of distilled water) was added to each well. The reaction was allowed to proceed at 30°C in a microplate reader (Versamax; Molecular Devices, Sunnyval, CA, USA) for 40 min. Readings were taken every 15 s at 490 nm and analyzed using the software SOFT-Max Pro 4.0 (Molecular Devices, Sunnyval, CA, USA). Enzyme activity was measured as the slope (V_{max} value: change in absorbance unit per min) of the reaction curve during the linear phase of the reaction and reported to the activity of 1 µL of pure hemolymph.

II.6. Injection with beads

To block phagocytosis, the method used by Pham *et al.* (2007) in *Drosophila melanogaster* was adapted as follows. Carboxylate fluorescent 0.2-µm-diameter polystyrene beads (Polysciences, http://www.polysciences.com) were washed three times in PBS and resuspended in the original volume to a

concentration (10^8.µL^{-1}), determined by injecting beetles with a 5-µL suspension of FITC-conjugated beads, and collecting hemolymph extracts at different time points post-injection, by washing the abdomen with 0.5 mL of ice-cold PBS into a 1.5 mL centrifuge tube (Barnes & Siva-Jothy 2000). Preliminary work using this adapted method showed that within 75 min after injection of 5 µL of bead solution at 10^8.µL^{-1}, more than 95 % of the hemocytes of immunologically naïve insects had internalized at least one bead. The proportion of phagocytosing hemocytes was estimated from a 5 µL subsample of hemolymph solution diluted with 5 µL of a 0.5% Trypan blue solution (suspended in PBS) using epifluorescence microscopy (magnification x 400).

II.7. Statistics

Cox regressions were used to analyze insect survival when testing immune priming specificity and the implication of phagocytes in immune memory. Depending on the experiment, survival was analyzed as a function of sex, body mass, immune priming, infection, and bead injection. Larval development time of offspring insect as a function of maternal priming was also analyzed using a Cox regression. The best statistical model was searched using a backward stepwise procedure from full models testing the main effect and two-way interactions between all the categorical explanatory factors (e.g., sex, immune priming, infection, and bead injection), but the effect of continuous covariables, such as insect body mass, was tested as a main effect only. Body mass of the offspring at the adult stage was analyzed using a univariate analysis of variance (ANOVA) with maternal priming and sex of the offspring as fixed factors.

Individual variation in hemocyte concentration, PO activity, total-PO activity and antimicrobial activity, before and after a secondary immune challenge (here with inactivated bacteria), was analyzed as a function of sex, immune priming (either individual or maternal immune priming, depending on the experiment), and challenge, as fixed factors, and insect body mass, as covariate. Data on hemocyte concentration of were natural log-transformed, whereas data on PO activity and total-PO activity were square-root-transformed to satisfy the conditions of parametric tests. All statistical analyses used IBM® SPSS® Statistics 19 for Macintosh.

III. Results

III.1. Individual priming and survival to infection

Survival to bacterial infection depended on both immune priming and infection treatment (Table 1). However, there was no interaction between these treatments, suggesting that insects were not specifically protected against the pathogen used for priming. All primed insects exhibited a survival benefit when inoculated with live bacteria compared to control insects (with the exception of insects primed with *S. entomophila*, for which survival was only marginally improved, Table 1). However, the survival benefit was greater for insects primed with Gram-positive bacteria than for insects primed with Gram-negative bacteria (Table 1, Fig. 1). The entomopathogenic bacteria (*B. thuringiensis* and *S. entomophila*) were more deadly than the non-entomopathogenic bacteria (*S. aureus* and *E. coli*), which did not differ in pathogenicity (Table 1). *Serratia entomophila* was more pathogenic than *B. thuringiensis* (Table 1). Bacterial pathogenicity was sex-dependent, mainly because infection by *B. thuringiensis* killed females more than males (Table 1). Finally, survival to bacterial infection was dependent on body mass (Table 1), with the heaviest insects surviving best.

III.2. Individual priming and immune parameters

Results for beetles challenged with *B. thuringiensis* showed that insects primed with *B. thuringiensis* had a higher concentration of hemocytes and greater antibacterial activity than control insects, whereas PO and total-PO activity levels were not affected by priming (Table 2a). Males had higher levels of both PO activity and total-PO activity than females, but this sex difference in PO activity was dependent on priming (Table 2a). Overall, PO activity was enhanced in primed males, but lowered in primed females (Fig. S3). The immune challenge with *B. thuringiensis* increased hemocyte concentration and antibacterial activity (Table 2a, Figs 2a and 2c). Increased antibacterial activity in response to the immune challenge only concerned control insects (Table 2), since primed insects already had strong levels of antibacterial activity circulating in their hemolymph (Fig. 2a).

By contrast, priming with *S. entomophila* had no lasting effect on immune defenses (Table 2b). The immune challenge with *S. entomophila* was the only source of variation among the immune parameters (Table 2b). Hemocyte concentration (Fig. 2d), total-PO activity, and antibacterial activity (Fig. 2b) were increased by the immune challenge, but PO activity was unaffected. Males tended to have a lower concentration of hemocytes (Fig. S4) and less antibacterial activity (Fig. S5) than females before the immune challenge, but reached similar levels of both immune parameters after the immune challenge (Table 2b).

Table 1. Survival of adult *T. molitor* to different bacteria, depending on individual priming treatment, sex and body mass. *S. aureus* (Sa), *B. thuringiensis* (Bt), *E. coli* (Ec) or *S. entomophila* (Se). The "simple" contrast was used for priming (survival of control insects was used as baseline), and sex (survival of control females was used as baseline), whereas the "deviation" contrast was used for infection (the average of all levels was used as baseline). The best model was searched using backward stepwise method utilizing likelihood ratio significance tests for evaluation of each effect. Procedure is available in COXREG procedure of SPSS statistical package. Model fitting was initiating with a model that included all main effect and two ways interactions, with the exception of body mass. The interaction term between priming and infection did not fit in the best model, suggesting that such an interaction does not explain survival (see variables not in the best model). Values where $p \leq 0.05$ are given in bold.

Variable in the best model	***Coeff. B***	***Standard error***	***Wald***	***df***	***P***	***Exp(B)***
Sex (males vs females)	-0.22	0.10	4.41	1	**0.036**	0.80
Priming			65.28	4	< **0.001**	
Priming (Sa vs PBS)	-1.07	0.15	48.00	1	< **0.001**	0.34
Priming (Bt vs PBS)	-0.83	0.14	33.59	1	< **0.001**	0.43
Priming (Ec vs PBS)	-0.47	0.13	12.22	1	< **0.001**	0.62
Priming (Se vs PBS)	-0.25	0.13	3.56	1	0.059	0.78
Priming (Bt vs Sa)	0.24	0.16	2.15	1	0.143	1.27
Priming (Ec vs Sa)	0.60	0.16	14.76	1	< **0.001**	1.82
Priming (Se vs Sa)	0.82	0.15	28.79	1	< **0.001**	2.27
Priming (Ec vs Bt)	0.36	0.15	6.18	1	**0.013**	1.43
Priming (Se vs Bt)	0.58	0.14	16.82	1	< **0.001**	1.78
Priming (Se vs Ec)	0.22	0.13	2.76	1	0.097	1.25
Infection			281.31	3	< **0.001**	
Infection (Sa)	-0.85	0.10	73.43	1	< **0.001**	0.43
Infection (Bt)	0.28	0.08	12.84	1	< **0.001**	1.33
Infection (Ec)	-0.60	0.10	38.91	1	< **0.001**	0.55
Infection (Se)	1.17	0.07	254.63	1	< **0.001**	3.22
Body mass	-0.02	0.00	83.08	1	< **0.001**	0.98
Priming*sex			8.15	4	0.086	
Infection*sex			9.92	3	**0.019**	
Sa*sex	0.25	0.20	1.55	1	0.212	1.28
Bt*sex	-0.45	0.16	7.96	1	**0.005**	0.64
Ec*sex	-0.01	0.19	0.001	1	0.975	0.99
Se*sex	0.21	0.14	2.15	1	0.143	1.23

Variables not in the best model	***Score***	***df***	***P***
Priming*Infection	17.86	12	0.120

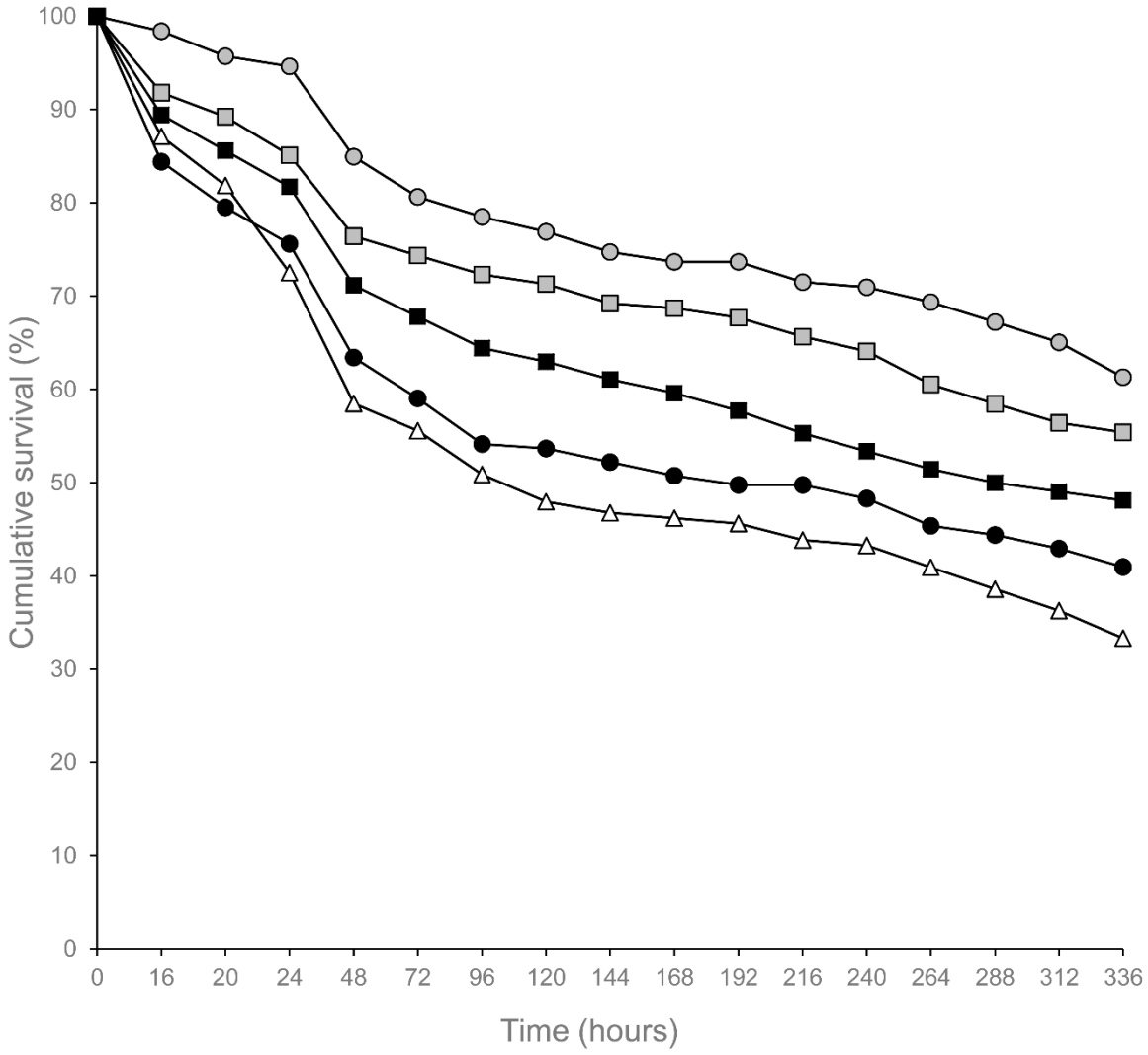

Figure 1. Survival of mealworm beetles, *T. molitor*, according to individual priming treatment whatever the bacterial infection treatment used. Each survival curve corresponds to one priming treatment that includes all the bacterial infection treatments together. Gray circles: primed with *S. aureus*. Gray squares: primed with *B. thuringiensis*. Black squares: primed with *E. coli*. Black circles: primed with *S. entomophila*. White triangles: control insects.

Table 2. Changes in immune parameters of *T. molitor* tested by repeated measures according to individual immune priming and immune challenge, with (a) *B. thuringiensis* or (B) *S. entomophila*. Changes in the hemolymph were tested, for hemocyte concentration (Hemocyte), PO enzyme activity (PO), proenzymes in addition to PO activity (Total-PO), and antibacterial peptide activity (Antibacterial), according to immunological experience (Priming: with either (A) *Bacillus thuringiensis*, or (b) *Serratia entomophila*), or Sex, or Priming and Sex. Individual changes (Within subjects) were tested before and 3 days after an immune challenge (Ch). N. R. refers to effects not retained by the stepwise procedure. Values where $p \leq 0.05$ are given in bold.

	(a) *Bacillus thuringiensis*				(b) *Serratia entomophila*			
Source	Hemocyte	PO	Total-PO	Antibacterial	Hemocyte	PO	Total-PO	Antibacterial
	Between subjects							
Priming	$F_{1, 60} = 21.68$	$F_{1, 91} = 0.48$	$F_{1, 71} = 0.03$	$F_{1, 71} = 302.30$	$F_{1, 70} = 1.67$	$F_{1, 91} = 1.05$	$F_{1, 84} = 1.77$	$F_{1, 82} = 0.16$
	$\mathbf{P < 0.001}$	$P = 0.490$	$P = 0.860$	$\mathbf{P < 0.001}$	$P = 0.200$	$P = 0.308$	$P = 0.187$	$P = 0.687$
Sex	$F_{1, 60} = 0.48$	$F_{1, 91} = 5.55$	$F_{1, 71} = 4.26$	$F_{1, 71} = 0.51$	$F_{1, 70} = 1.78$	$F_{1, 91} = 0.48$	$F_{1, 84} = 0.07$	$F_{1, 82} = 0.003$
	$P = 0.491$	$\mathbf{P = 0.021}$	$\mathbf{P = 0.043}$	$P = 0.477$	$P = 0.187$	$P = 0.488$	$P = 0.788$	$P = 0.955$
Priming*Sex	N. R.	$F_{1, 91} = 7.29$	$F_{1, 71} = 3.63$	N. R	N. R.	N. R.	N. R.	N. R.
		$\mathbf{P = 0.008}$	$P = 0.061$					
	Within subjects							
Ch	$F_{1, 60} = 39.49$	$F_{1, 91} = 1.05$	$F_{1, 71} = 0.23$	$F_{1, 71} = 402.91$	$F_{1, 70} = 37.12$	$F_{1, 91} = 0.65$	$F_{1, 84} = 11.71$	$F_{1, 82} = 1669.39$
	$\mathbf{p < 0.001}$	$p < 0.309$	$p = 0.629$	$\mathbf{p < 0.001}$	$\mathbf{p < 0.001}$	$p = 0.422$	$\mathbf{p = 0.001}$	$\mathbf{p < 0.001}$
Ch*Priming	$F_{1, 60} = 1.85$	$F_{1, 91} = 2.05$	$F_{1, 71} = 1.46$	$F_{1, 71} = 345.03$	$F_{1, 70} = 2.65$	$F_{1, 91} = 0.20$	$F_{1, 84} = 0.31$	$F_{1, 82} = 3.12$
	$p = 0.178$	$p = 0.156$	$p = 0.231$	$\mathbf{p < 0.001}$	$p = 0.108$	$p = 0.653$	$p = 0.579$	$p = 0.081$
Ch*Sex	$F_{1, 60} = 0.80$	$F_{1, 91} = 0.15$	$F_{1, 71} = 2.96$	$F_{1, 71} = 3.60$	$F_{1, 70} = 5.07$	$F_{1, 91} = 0.10$	$F_{1 ,84} = 0.51$	$F_{2, 82} = 6.20$
	$p = 0.376$	$p = 0.694$	$p = 0.090$	$p = 0.062$	$\mathbf{p = 0.028}$	$p = 0.750$	$p = 0.475$	$\mathbf{p = 0.015}$
Ch*Priming*Sex	N. R.	$F_{1, 91} = 3.33$	$F_{1, 71} = 0.01$	N. R	N. R.	N. R.	N. R.	N. R.
		$p = 0.071$	$p = 0.917$					

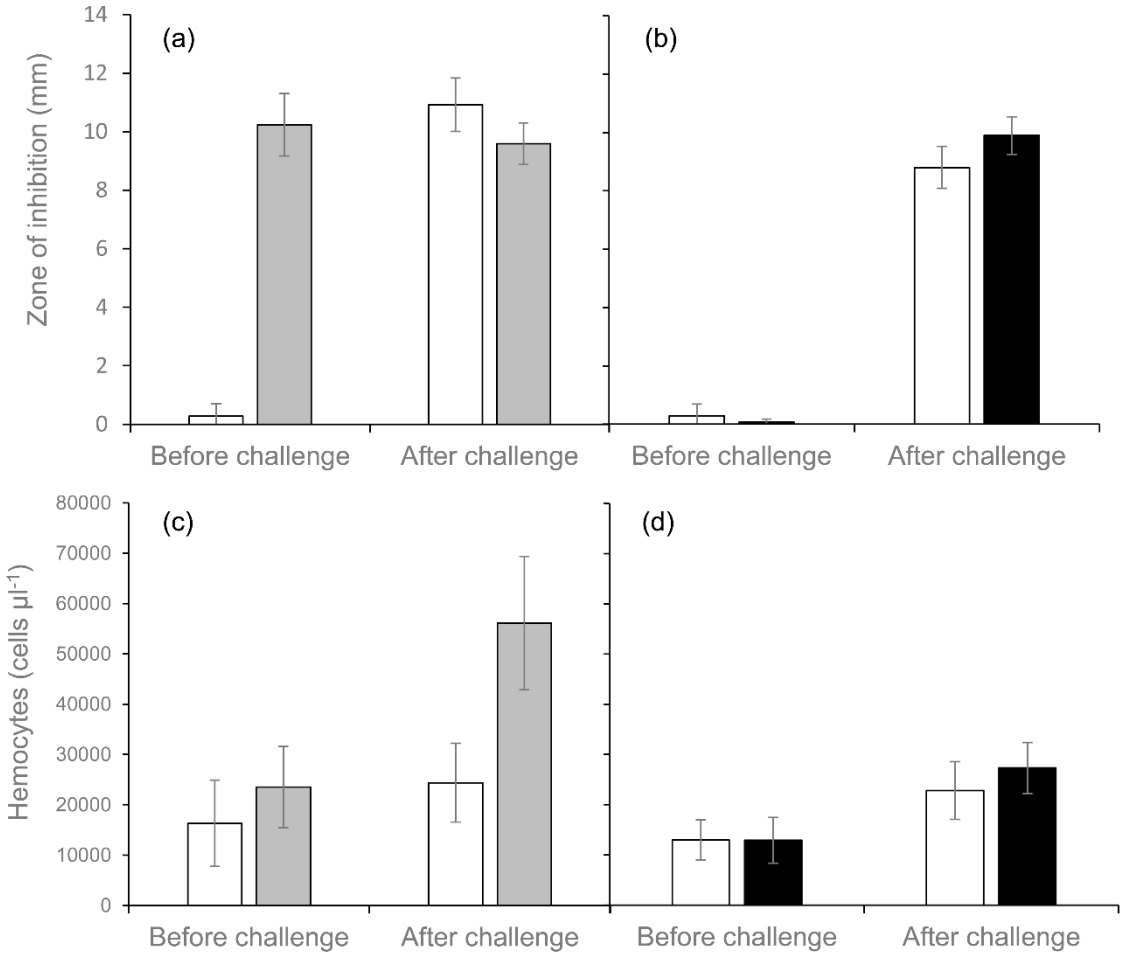

Figure 2. Changes in immune parameters of mealworm beetle, *T. molitor*, depending on individual priming treatment. (a-b) Antibacterial activity expressed as the zone of inhibition diameter (mean ± IC 95%) in the hemolymph of control insects (PBS; white bars) and (a) *B. thuringiensis* primed insects (gray bars), before and after an immune challenge with inactivated *B. thuringiensis*, or (b) *S. entomophila* primed insects (black bars), before and after an immune challenge with inactivated *S. entomophila*. (c-d) Hemocyte concentration (mean ± IC 95%), in the hemolymph of control insects (PBS; white bars) and (c) *B. thuringiensis* primed insects (gray bars), before and after an immune challenge with inactivated *B. thuringiensis* or (d) *S. entomophila* primed insects (black bars), before and after an immune challenge with inactivated *S. entomophila*.

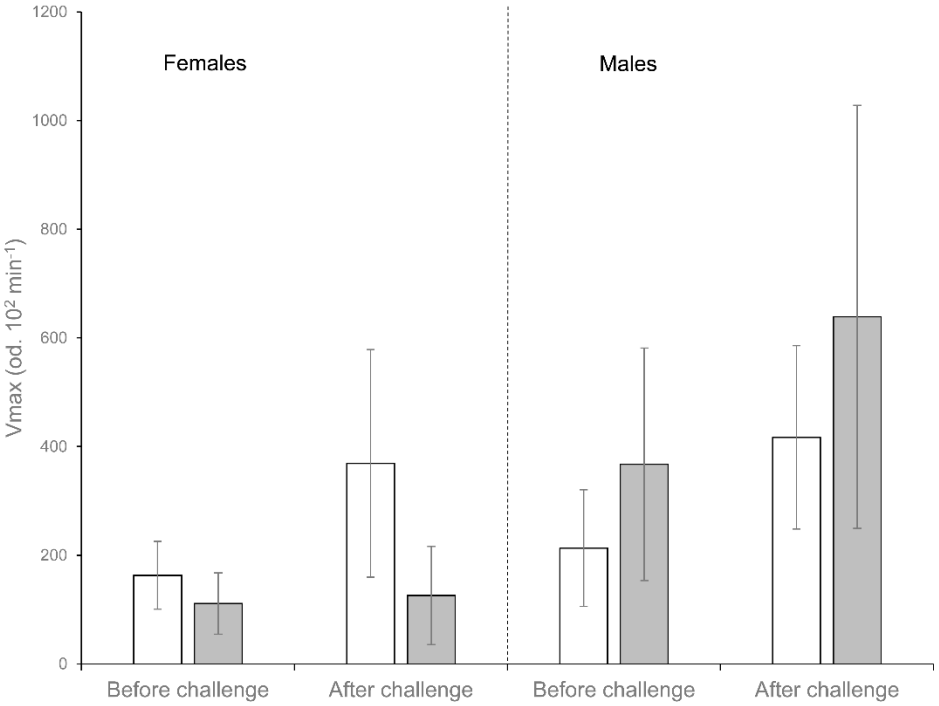

Fig. S3. Changes of phenoloxidase activity in mealworm beetle *T. molitor*, depending on sex and individual priming treatment.

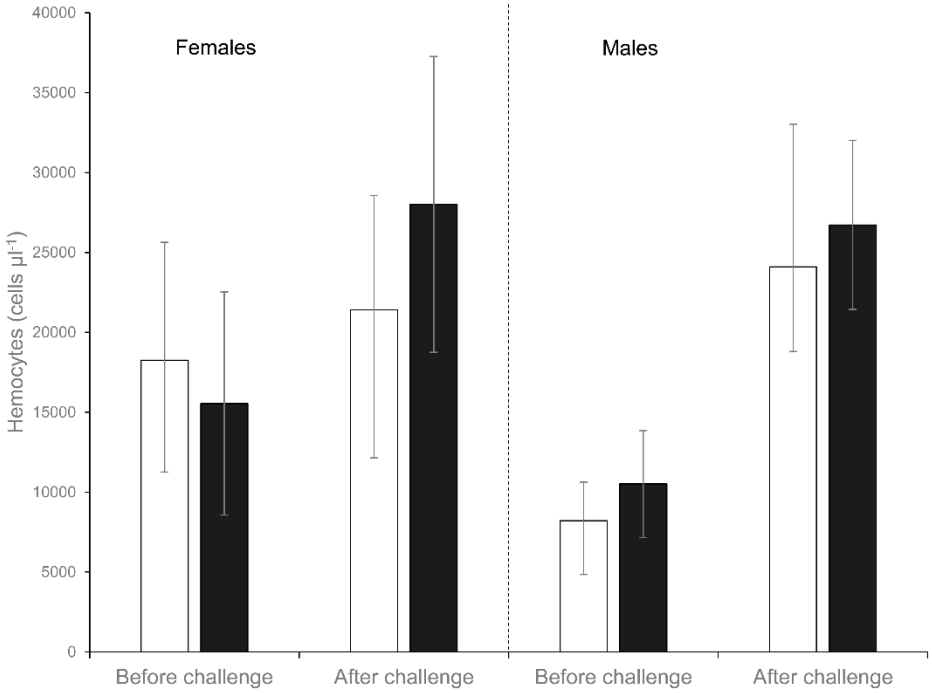

Fig. S4. Changes of hemocyte concentration in mealworm beetle *T. molitor*, depending on sex and individual priming treatment.

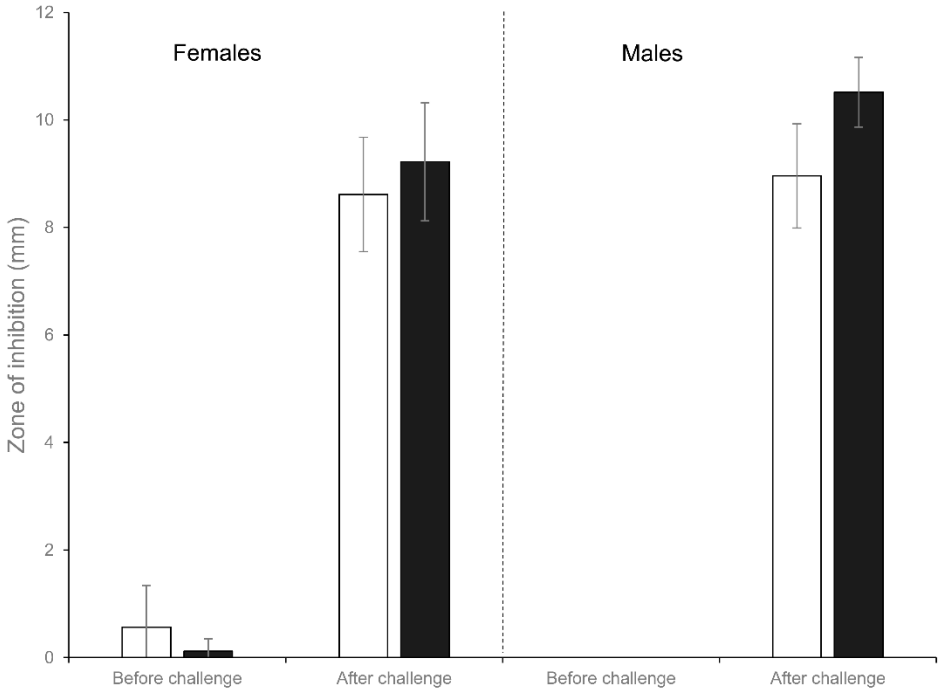

Fig. S5. Changes of antibacterial activity in the mealworm beetle *T. molitor*, depending on sex and individual priming treatment.

III.3. Individual immune priming and phagocytosis

Inhibiting phagocytosis did not remove the survival benefit of priming with *B. thuringiensis* (Table 3, Fig. 3a), suggesting that phagocytes were not the main effectors involved in this priming effect. However, the persistence of antibacterial activity found in the previous experiment in insects primed with *B. thuringiensis* could mask phagocyte involvement. Inhibiting phagocytosis in insects primed with *S. entomophila* decreased their survival to the infection (Table 3, Fig. 3b). This suggests that phagocytes could play an important role in clearing bacteria. However, as found earlier, the survival benefit of priming with *S. entomophila* remained extremely low compared to priming with *B. thuringiensis* (Fig. 3). Survival to either *B. thuringiensis* or *S. entomophila* infections was sex-dependent (Table 3). While males survived better than females to *B. thuringiensis*, they survived less than females to *S. entomophila*.

III.4. Maternal priming and offspring survival to infection

Survival of the offspring to the bacterial infection depended on both maternal immune priming and infection treatment, but not on their interaction (Table 4), suggesting that insects were not specifically protected against the pathogen used for maternal priming. All maternally primed insects, with the exception of those maternally primed with *E. coli*, exhibited a similar survival benefit when inoculated with live bacteria, compared to the offspring of control mothers (Table 4, Fig. 4). Infections with entomopathogenic bacteria (*B. thuringiensis* and *S. entomophila*) were more deadly than those with non-entomopathogenic bacteria (*S. aureus* and *E. coli*, Table 4). *Bacillus thuringiensis* was more pathogenic than *S. entomophila*, and *E. coli* was more pathogenic than *S. aureus* (Table 4). The pathogenicity of Gram-positive bacteria was sex-dependent. Males died less than females when infected with *B. thuringiensis*, whereas they died sooner than females when infected with *S. aureus* (Table 4).

As previously found (Zanchi et al. 2011), maternal immune priming was associated with a significant prolongation of larval development time in offspring, compared to controls. However, such a change in larval development time in maternally primed offspring was dependent on the bacteria used for the maternal priming (Table 5).

Table 3. Survival of primed adult *T. molitor* to bacterial infection, with phagocytosis blocked by bead injection (Beads), according to sex (Sex), and body mass (Mass). Insects were primed with either *B. thuringiensis* (*B.t.*) or *S. entomophila* (*S.e.*). The "simple" contrast was used for Beads (survival of sham-injected insects was used as baseline), and Sex (survival of females was used as baseline). The best model was searched using backward stepwise method utilizing likelihood ratio significance tests for evaluation of each effect. Procedure is available in COXREG procedure of SPSS statistical package. Model fitting was initiating with a model that included all main effect and two ways interactions, with the exception of body mass. Values where $p \leq 0.05$ are given in bold.

Priming with B.t.

Variables in the best model	***Coeff. B***	***Standard error***	***Wald***	***df***	***P***	***Exp(B)***
Sex	-2.24	0.75	8.93	1	**0.003**	0.11

Variables not in the best model	***Score***	***df***	***P***
Mass	0.38	1	0.538
Beads	0.84	1	0.361
Beads*Sex	3.64	1	0.056

Priming with S.e.

Variables in the best model	***Coeff. B***	***Standard error***	***Wald***	***df***	***P***	***Exp(B)***
Beads	0.48	0.25	3.83	1	**0.050**	1.62
Sex	0.95	0.27	12.90	1	< **0.001**	2.59

Variables not in the best model	***Score***	***df***	***P***
Mass	0.34	1	0.561
Beads*Sex	1.10	1	0.294

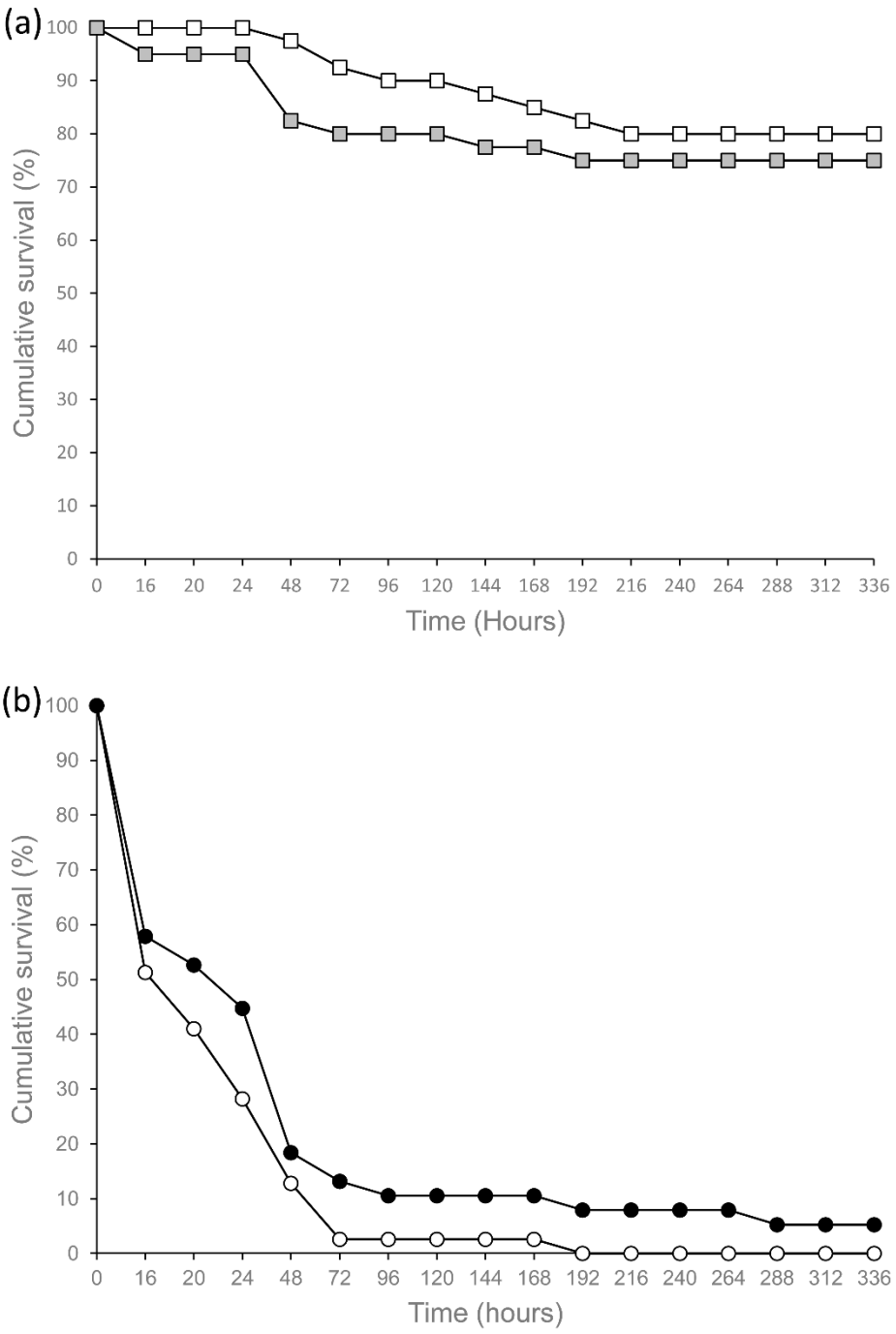

Figure 3. Survival of mealworm beetle, *T. molitor*, to bacterial infection, depending on individual priming treatment. (a) Priming and infection with *B. thuringiensis*. White squares: phagocytosis inhibited with bead injection. Gray squares: control insects injected with PBS. (b) Priming and infection with *S. entomophila*. White circles: phagocytosis inhibited with bead injection. Black circles: control insects injected with PBS.

Table 4. Survival of adult *T. molitor* to different bacteria, depending on maternal priming treatment, sex, and body mass. *S. aureus* (Sa), *B. thuringiensis* (Bt), *E. coli* (Ec) or *S. entomophila* (Se). The "simple" contrast was used for priming (survival of the offspring of control mothers was used as baseline), and sex (survival of control females was used as baseline), whereas the "deviation" contrast was used for infection (the average of all levels was used as baseline). Values where $p \leq 0.05$ are given in bold.

Variables in the best model	***Coeff. B***	***Standard error***	***Wald***	***df***	***P***	***Exp(B)***
Maternal priming			12.52	4	**0.014**	
Sa vs PBS	-0.36	0.14	6.83	1	**0.009**	0.70
Bt vs PBS	-0.39	0.14	7.58	1	**0.006**	0.68
Ec vs PBS	-0.07	0.14	0.27	1	0.606	0.93
Se vs PBS	-0.30	0.14	4.39	1	**0.036**	0.74
Bt vs Sa	-0.03	0.14	0.03	1	0.857	0.98
Ec vs Sa	0.29	0.14	4.24	1	**0.040**	1.33
Se vs Sa	0.06	0.14	0.17	1	0.678	1.06
Ec vs Bt	0.31	0.14	4.87	1	**0.027**	1.37
Se vs Bt	0.09	0.15	0.34	1	0.561	1.09
Se vs Ec	-0.23	0.15	2.48	1	0.116	0.79
Infection			326.63	3	< **0.001**	
Infection (Sa)	-2.89	0.25	138.93	1	< **0.001**	0.06
Infection (Bt)	1.65	0.11	241.71	1	< **0.001**	5.22
Infection (Ec)	-0.14	0.11	1.48	1	0.224	0.87
Infection (Se)	1.38	0.11	169.40	1	< **0.001**	3.97
Infection*Sex			22.25	3	< **0.001**	
Sa*Sex	0.76	0.26	8.48	1	**0.004**	2.14
Bt*Sex	-0.65	0.15	19.88	1	< **0.001**	0.52
Ec*Sex	-0.23	0.19	1.47	1	0.225	0.81
Se*Sex	0.12	0.14	0.68	1	0.411	1.12

Variables not in the best model	***Score***	***df***	***P***
Body mass	0.04	1	0.851
Sex	2.08	1	0.149
Maternal priming*Infection	8.24	12	0.766
Maternal priming*Sex	6.76	4	0.149

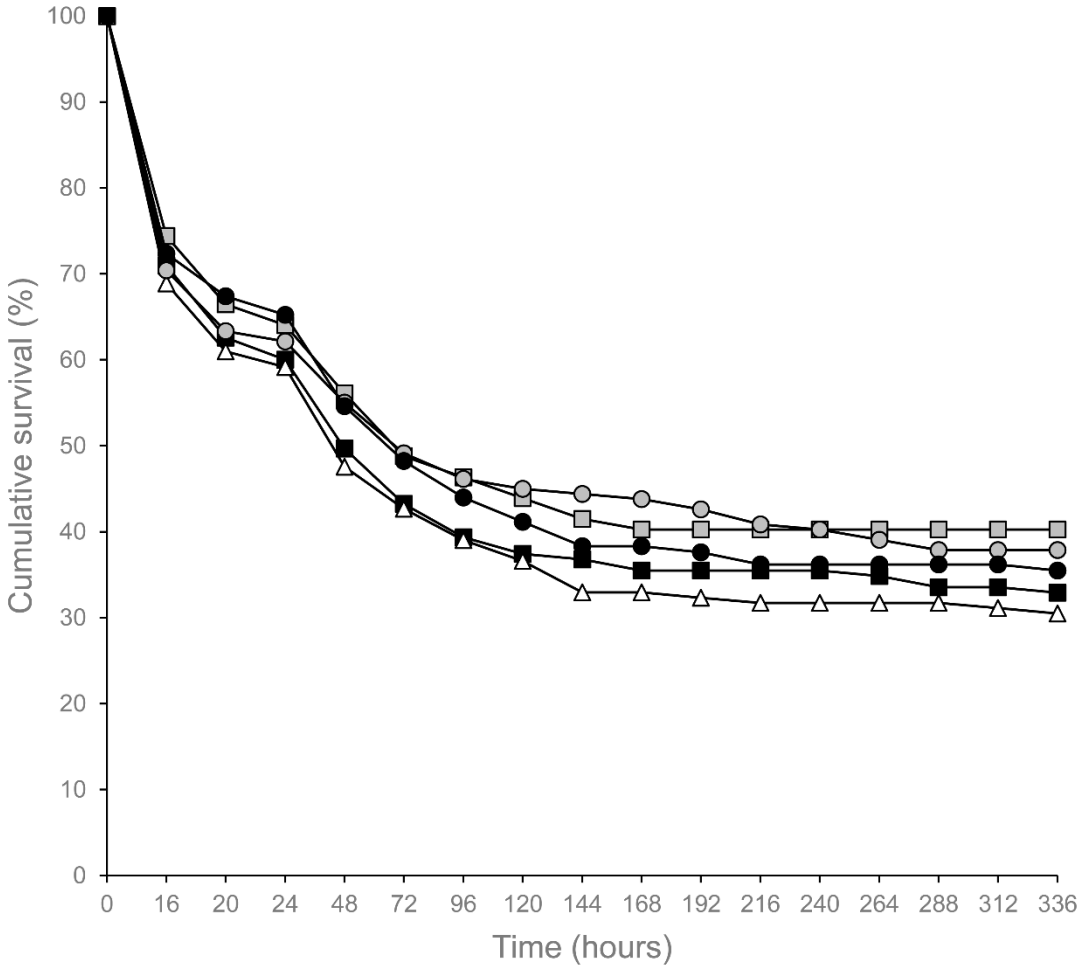

Figure 4. Survival of mealworm beetles, *T. molitor*, according to maternal priming treatment whatever the bacterial infection treatment used. Each survival curve corresponds to one maternal priming treatment that includes all the bacterial infection treatments together. Gray circles: mothers primed with *S. aureus*. Gray squares: mothers primed with *B. thuringiensis*. Black squares: mothers primed with *E. coli*. Black circles: mothers primed with *S. entomophila*. White triangles: insects from control mothers injected with PBS.

If offspring maternally primed with Gram-positive bacteria had a longer larval development time than controls (with the exception of the offspring of mothers immunized with *S. aureus*, with larval development time similar to controls), offspring maternally primed with Gram-negative bacteria exhibited the most prolonged larval development time (Table 5, Fig. 5). While no significant difference exists between *B. thuringiensis* and *E. coli* maternal priming treatments on larval development time in offspring, maternal treatment with *S. entomophila* induced the most important cost on offspring larval development time. Despite changes in larval developmental time, body mass of the resulting was not significantly affected by the maternal immune treatment (ANOVA, Global model $F_{9,765} = 2.14$, $P = 0.025$; Maternal Priming $F_{4,765} = 1.38$, $P = 0.240$). Only sex explained variation in mass of nymphs and adults, with males smaller than females ($F_{1,765} = 11.98$, $P = 0.001$).

III.5. Maternal priming and offspring immune parameters

The offspring of mothers primed with *B. thuringiensis* had higher PO activity and total-PO activity (although marginally significant for the latter) than control insects (Fig. 6a,c), whereas hemocyte concentration and antibacterial activity were not affected by maternal priming (Table 6a). Females had a higher concentration of hemocytes than males (Table 6a; females, mean ± IC 95%: 11050.50±3236.28 hemocytes.μL^{-1}, males: 10531.25±3235.12 hemocytes.μL^{-1}). Unsurprisingly, mother identity significantly explained variation in hemocyte concentration, PO activity, and total-PO activity (only marginally significant for total-PO). As expected, the immune challenge with *B. thuringiensis* increased antibacterial activity in the hemolymph (Table 6a). By contrast, maternal priming with *S. entomophila* had no effect on immune defenses of the offspring (Table 6b, Figs 6b and 6d). The immune challenge with *S. entomophila* was the only source of variation among the immune parameters, increasing hemocyte concentration and antibacterial activity (Table 6b). Overall, antibacterial activity was sex-dependent (Table 6b), with higher levels of antibacterial activity in males than in females (males, mean ± IC 95 %: 0.62±0.15 mm; females: 0.39±0.14 mm). Furthermore, males exhibited higher antibacterial activity than females after the immune challenge (mean ± IC 95 % in males before and after challenge: 0.02±0.04 and 1.35±0.19 mm; in females before and after challenge: 0.017±0.03 and 0.78±0.23 mm).

Table 5. Larval developmental time of *T. molitor*, depending on maternal immune priming. Mothers were primed either with *S. aureus* (Sa), *B. thuringiensis* (Bt), *E. coli* (Ec) or *S. entomophila* (Se); controls were injected with PBS. The "simple" contrast was used for maternal priming (survival of the offspring of control mothers was used as baseline). Values where $p \leq 0.05$ are given in bold.

Sources	***Coeff. B***	***Standard error***	***Wald***	***df***	***P***	***Exp(B)***
Maternal priming			31.89	4.00	< **0.001**	
Sa vs PBS	-0.11	0.11	1.01	1	0.315	0.90
Bt vs PBS	-0.32	0.11	9.13	1	**0.003**	0.73
Ec vs PBS	-0.39	0.11	13.35	1	< **0.001**	0.68
Se vs PBS	-0.54	0.11	24.10	1	< **0.001**	0.58
Bt vs Sa	-0.21	0.10	4.13	1	**0.042**	0.81
Ec vs Sa	-0.29	0.11	7.29	1	**0.007**	0.76
Se vs Sa	-0.43	0.11	15.99	1	< **0.001**	0.65
Ec vs Bt	-0.08	0.10	0.55	1	0.458	0.92
Se vs Bt	-0.22	0.11	4.40	1	**0.036**	0.80
Se vs Ec	-0.14	0.11	1.75	1	0.188	0.87

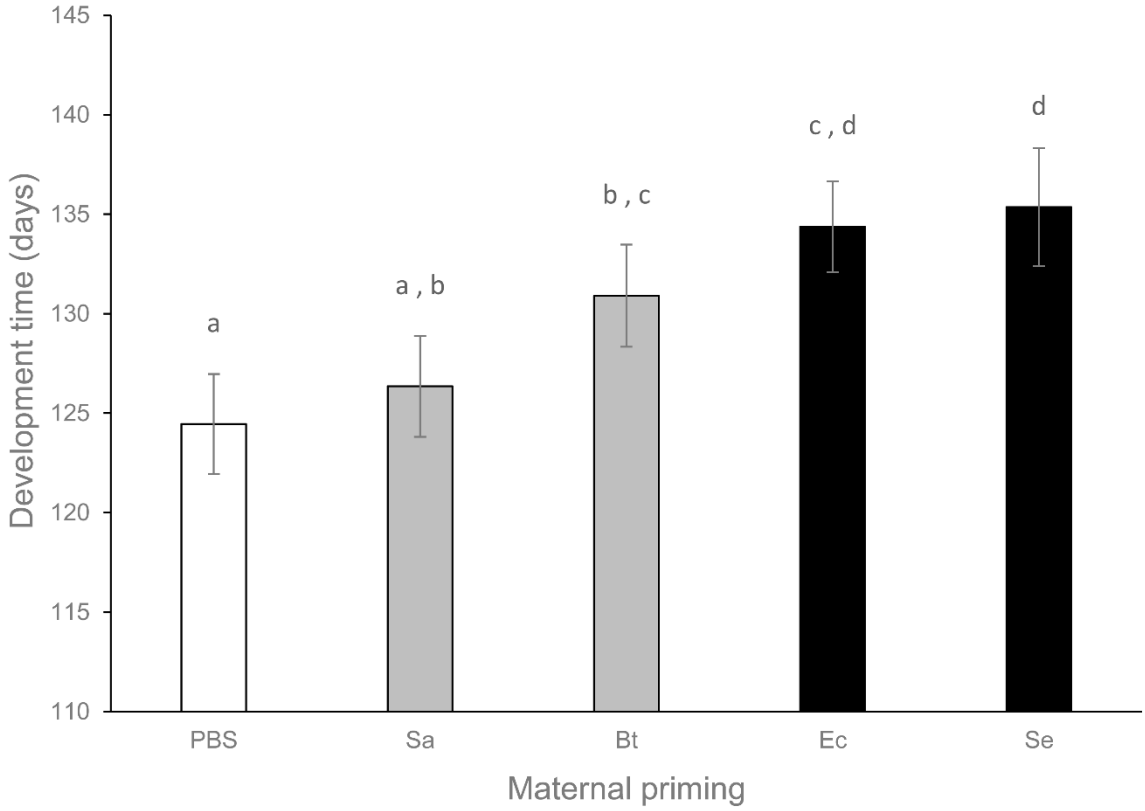

Figure 5. Larval developmental time from hatching to adult stage. Duration in days (mean ± IC 95%). White bars refer to control parental immune treatment (PBS), whereas gray bars refer to Gram-positive immune challenge of parents, and black bars to Gram-negative immune challenge of parents. *S. aureus* (Sa), *B. thuringiensis* (Bt), *E. coli* (Ec) or *S. entomophila* (Se). Different letters indicate significant differences between maternal treatments, based on Cox regression ($p \leq 0.05$).

Table 6. Changes in immune parameters of *T. molitor* tested by repeated measures according to maternal immune priming and immune challenge, with (a) *B. thuringiensis* or (B) *S. entomophila*. Changes in the hemolymph were tested, for hemocyte concentration (Hemocyte), PO enzyme activity (PO), proenzymes in addition to PO activity (Total-PO), and antibacterial peptide activity (Antibacterial), according to maternal immunological experience (Maternal priming: with either (A) *Bacillus thuringiensis*, or (b) *Serratia entomophila*), or Sex, or Maternal priming and Sex. Individual changes (Within subjects) were tested before and 3 days after an immune challenge (Ch). N. R. refers to effects not retained by the stepwise procedure. Values where $p \leq 0.05$ are given in bold.

	(a) *Bacillus thuringiensis*				(b) *Serratia entomophila*			
Source	Hemocyte	PO	Total-PO	Antibacterial	Hemocyte	PO	Total-PO	Antibacterial
	Between subjects							
Maternal priming	$F_{1, 66} = 2.44$	$F_{1, 83} = 8.80$	$F_{1, 86} = 3.44$	$F_{1, 89} = 0.54$	$F_{1, 67} = 0.21$	$F_{1, 87} = 0.01$	$F_{1, 95}= 0.01$	$F_{1, 95} = 0.07$
	$P = 0.123$	$\mathbf{P = 0.004}$	$P = 0.067$	$P = 0.463$	$P = 0.65$	$P = 0.914$	$P = 0.934$	$P = 0.793$
Sex	$F_{1, 66} = 4.30$	$F_{1, 83} = 1.39$	$F_{1, 86} = 0.48$	$F_{1, 89} = 0.02$	$F_{1, 67} = 5.84$	$F_{1, 87} = 0.02$	$F_{1, 95} = 0.01$	$F_{1, 95} = 13.19$
	$\mathbf{P = 0.042}$	$P = 0.242$	$P = 0.49$	$P = 0.900$	$P = 0.18$	$P = 0.899$	$P = 0.922$	$\mathbf{P < 0.001}$
Mother	$F_{1, 66} = 14.51$	$F_{1, 83} = 5.33$	$F_{1, 86} = 3.75$	$F_{1, 89} = 1.97$	$F_{1, 67} = 0.04$	$F_{1, 87} = 1.19$	$F_{1, 95} = 1.82$	$F_{1, 95} = 0.63$
	$\mathbf{P < 0.001}$	$\mathbf{P = 0.023}$	$P = 0.056$	$P = 0.164$	$P = 0.833$	$P = 0.278$	$P = 0.180$	$P = 0.431$
Maternal priming*Sex	N. R.	N. R.	N. R.	N. R	N. R.	N. R.	N. R.	N. R.
	Within subjects							
Ch	$F_{1, 66} = 2.69$	$F_{1, 83} = 0.07$	$F_{1, 86} = 1.70$	$F_{1, 89} = 21.52$	$F_{1, 67} = 13.82$	$F_{1, 87} < 0.001$	$F_{1, 95} = 0.68$	$F_{1, 95} = 58.47$
	$P = 0.106$	$P < 0.786$	$P = 0.196$	$\mathbf{P < 0.001}$	$\mathbf{P < 0.001}$	$P = 0.983$	$P = 0.411$	$\mathbf{P < 0.001}$
Ch*Maternal priming	$F_{1, 66} = 0.11$	$F_{1, 83} = 0.11$	$F_{1, 86} = 0.66$	$F_{1, 89} = 0.54$	$F_{1, 67} = 0.16$	$F_{1, 87} = 0.14$	$F_{1, 95} = 0.05$	$F_{1, 95} = 0.24$
	$P = 0.742$	$P = 0.739$	$P = 0.418$	$P = 0.463$	$P = 0.694$	$P = 0.71$	$P = 0.821$	$P = 0.627$
Ch*Sex	$F_{1, 66} = 2.81$	$F_{1, 83} = 0.70$	$F_{1, 86} = 0.004$	$F_{1, 89} = 0.02$	$F_{1, 67} = 1.39$	$F_{1, 87} = 0.08$	$F_{1 ,95} = 0.34$	$F_{1, 95} = 14.43$
	$P = 0.099$	$P = 0.406$	$P = 0.949$	$P = 0.900$	$P = 0.243$	$P = 0.78$	$P = 0.561$	$\mathbf{P < 0.001}$
Ch*Mother	$F_{1, 66} = 1.04$	$F_{1, 83} = 0.14$	$F_{1, 86} = 0.21$	$F_{1, 89} = 1.97$	$F_{1, 67} <0,001$	$F_{1,87} = 2.17$	$F_{1 ,95} = 1.41$	$F_{1, 95} = 0.53$
	$P = 0.311$	$P = 0.712$	$P = 0.648$	$P = 0.164$	$P = 0.994$	$P = 0.145$	$P = 0.237$	$P = 0.470$
Ch*Maternal priming*Sex	N. R.	N. R.	N. R.	N. R	N. R.	N. R.	N. R.	N. R.

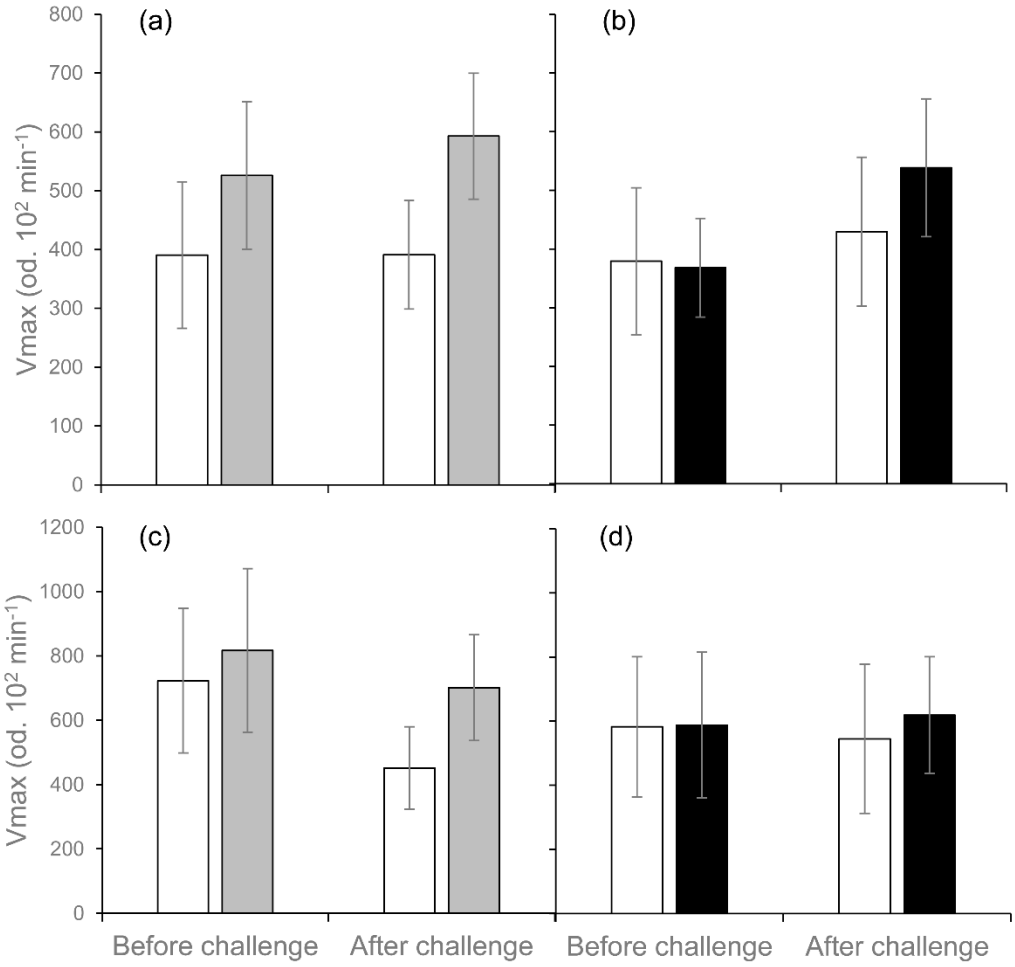

Figure 6. Changes in immune parameters of mealworm beetle *T. molitor*, depending on maternal priming treatment. (a-b) PO activity expressed as the zone of inhibition diameter (mean ± IC 95%) in the hemolymph of offspring from control mothers (PBS; white bars) and (a) *B. thuringiensis* primed mothers (gray bars), before and after an immune challenge with inactivated *B. thuringiensis*, or (b) *S. entomophila* primed mothers (black bars), before and after an immune challenge with inactivated *S. entomophila*. (c-d) Total-PO activity (mean ± IC 95%), in the hemolymph of offspring from control mothers (PBS; white bars) and (c) *B. thuringiensis* primed mothers (gray bars), before and after an immune challenge with inactivated *B. thuringiensis*, or (d) *S. entomophila* primed mothers (black bars), before and after an immune challenge with inactivated *S. entomophila*.

III.6. Maternal priming and offspring phagocytosis

The inhibition of phagocytosis did not remove the survival benefit in offspring maternally primed with *B. thuringiensis* or *S. entomophila* (Table 7, Fig. 7), suggesting that phagocytes are not the main immune effectors involved in this trans-generational acquired resistance.

Table 7. Survival of maternally primed adult *T. molitor* to bacterial infection, with phagocytosis blocked by bead injection (Beads), according to sex (Sex), and body mass (Mass). Insects were the offspring of mothers primed with either *B. thuringiensis* (*B.t.*) or *S. entomophila* (*S.e.*). The "simple" contrast was used for Beads (survival of sham-injected insects was used as baseline), and Sex (survival of females was used as baseline). The best model was searched using backward stepwise method utilizing likelihood ratio significance tests for evaluation of each effect. Procedure is available in COXREG procedure of SPSS statistical package. Model fitting was initiating with a model that included all main effect and two ways interactions, with the exception of body mass.

Maternal priming with B.t.

Variables in the best model	***Coeff. B***	***Standard error***	***Wald***	***df***	***P***	***Exp(B)***
Beads*Sex	0.88	0.56	2.51	1	0.113	2.42

Variables not in the best model	***Score***	***df***	***P***
Mass	0.33	1	0.568
Beads	1.94	1	0.163
Sex	0.30	1	0.586

Maternal priming with S.e.

Variables in the best model	***Coeff. B***	***Standard error***	***Wald***	***df***	***P***	***Exp(B)***
Beads*Sex	0.80	0.51	2.40	1	0.121	2.22

Variables not in the best model	***Score***	***df***	***P***
Mass	0.11	1	0.742
Beads	2.32	1	0.127
Sex	0.06	1	0.808

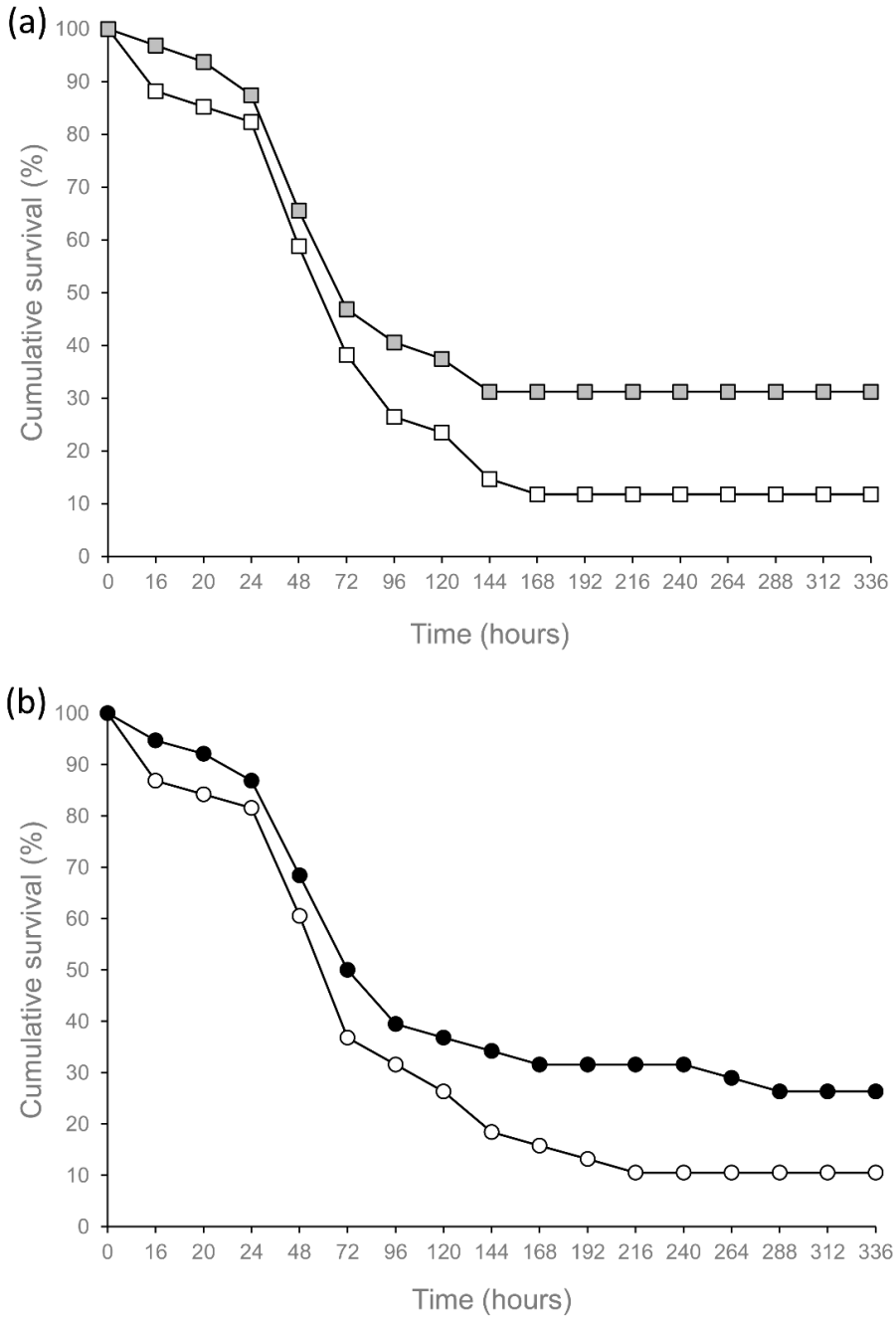

Figure 7. Survival of mealworm beetle *T. molitor* to bacterial infection, depending on maternal priming treatment. (a) Maternal priming and infection with *B. thuringiensis*. White squares: phagocytosis inhibited with bead injection. Gray squares: control insects injected with PBS. (b) Maternal priming and infection with *S. entomophila*. White circles: phagocytosis inhibited with bead injection. Black circles: control insects injected with PBS.

IV. Discussion

This study tested for specific immune priming of resistance to various Gram-negative and Gram-positive bacteria at both individual and trans-generational levels. Under the hypothesis that Gram-positive bacteria might have been a relatively important selective force for the evolution of immune priming in *T. molitor*, we expected to observe primed specific responses toward these bacteria, but not toward Gram-negative bacteria. Overall, our results provide reasonable evidence that our hypothesis is true.

At the individual level, beetles primed with inactivated bacteria were shown to survive subsequent infection with live bacteria better than control beetles, whatever the bacteria used for priming, confirming previous observation in this system (Moret & Siva-Jothy 2003). However, survival was not necessarily better when the beetles were infected with the same bacterium as that used for priming. We therefore failed to gain evidence that immune priming in *T. molitor* could exhibit pathogen-specific protection similar to that produced by the acquired immune response of vertebrates. Nevertheless, we found that priming with Gram-positive bacteria, whether entomopathogenic or not, provided much more effective protection against bacterial reinfection than priming with Gram-negative bacteria. Measurements of immune parameters revealed that insects primed with the Gram-positive bacterium *B. thuringiensis* exhibited higher hemocyte concentration and greater antibacterial activity before and after immune stimulation. No such result was found in insects primed with the Gram-negative bacterium *S. entomophila*, although some protection was found. Such a protection may either result from the action of unmeasured immune effectors or increased tolerance to the bacterial infection, as defined by Schneider and Ayres (2008). It seems therefore that the immune protection induced by priming with Gram-positive bacteria results from both the long-lasting antibacterial immune response and the enhanced concentration of circulating hemocytes. Such a long-lasting antibacterial immune response induced by Gram-positive bacteria, over 14 days post immunization, was unsuspected from previous results (Haine et al. 2008), but is supported by the recent study of Makarova et al. (2016), showing that a large number of antimicrobial peptides remained abundant 21 days after an immune challenge of *T. molitor* beetles with heat-killed *S. aureus*. It has also been shown that phagocytes play a key role in providing a pathogen-specific priming response in the fruit fly, *D. melanogaster* (Pham et al. 2007), the woodlice, *Porcellio scaber* (Roth & Kurtz 2009) or the mosquito, *Anopheles gambiae* (Rodrigues et al. 2010). The enhanced hemocyte concentration observed in *T. molitor* could perhaps be similarly involved in the priming response induced by Gram-positive bacteria, but further investigation

is indicated, because of the unexpectedly long-lasting antibacterial immune response identified in this study and by Makarova et al. (2016). Our results clearly show that individual priming responses induced by Gram-positive bacteria are stronger and more protective than those induced by Gram-negative bacteria, suggesting that Gram-positive bacteria may have played an important evolutionary role in shaping the immune system of *T. molitor*.

At the trans-generational level, the adult offspring of bacterially immunized mothers survived bacterial infection better than the adult offspring of control mothers, whatever the bacteria used to prime the mothers, except for offspring from mothers primed with the non-entomopathogenic bacteria, *E. coli*, confirming previous results with this insect (Zanchi et al. 2011). Unexpectedly, the immune protection of the offspring was not dependent on the bacterium used to prime the mother, showing that trans-generational immune protection was not pathogen specific. In addition, maternal priming with Gram-positive bacteria did not better protect the offspring against bacterial infection compared to maternal priming with Gram-negative bacteria. These results contrast with those obtained from the phylogenetically and ecologically related beetle, *Tribolium castaneum*, for which TGIP exhibited a certain level of pathogen specificity (Roth et al. 2010). This suggests that the degree of pathogen specificity of TGIP can differ even between closely related species.

The TGIP, whether with *B. thuringiensis* or *S. entomophila*, was associated with almost no change in base levels of immune defenses in offspring, apart from increased PO activity in the offspring of mothers primed with *B. thuringiensis*. From the work of Hernández López et al. (2014), showing changes in the hemocyte population in the offspring workers of immunized honeybee queens, and that of Zanchi et al. (2011), showing increased hemocyte concentration in the offspring of *T. molitor* mothers immunized with bacterial cell wall lipopolysaccharides, strong involvement of these immune cells was expected in the enhanced immunity of maternally primed offspring. However, we were not able to identify any contribution of hemocytes to TGIP induced by *B. thuringiensis* or *S. entomophila*, since we found no change in base concentration of these immune cells, yet blocking phagocytosis did not remove the survival benefit of primed offspring upon infection. Increased PO activity is only involved in the immune protection of the offspring of mothers primed with *B. thuringiensis*. As for individual immune priming with *S. entomophila*, the offspring of mothers primed with *S. entomophila* may in fact be enhancing unmeasured immune effectors or showing increased tolerance to the bacterial infection.

In line with previous results (Zanchi et al. 2011), maternally primed offspring had a prolonged larval development time. Longer developmental time is likely costly in insects because it might lower competitive ability for food under higher larval densities (Koella & Boëte 2002) and delay access to reproduction. Moreover, it could increase the probability of mortality (Bell 1980), especially in insects like *T. molitor*, which exhibits cannibalism on juveniles (Ichikawa & Kurauchi 2009). In this case, prolonged development time is likely a real fitness cost as long as it is not associated to increased body size or mass, known to improve fitness by, for instance, increasing fecundity (Thornhill & Alcock 1983). Here we found that prolonged offspring development time was dependent on the bacteria used for maternal priming, while body mass of the offspring was not affected. Offspring maternally primed with Gram-positive bacteria exhibited the shortest developmental time, whereas offspring maternally primed with Gram-negative bacteria exhibited the longest time. Therefore, for a similar survival benefit upon bacterial infection, the cost of maternal priming was lower for offspring maternally primed with Gram-positive bacteria than for offspring maternally primed with Gram-negative bacteria. Despite its cost, immune defense is an important trait in hosts and selection should act to minimize costs under high infection pressure (Moret 2003). Here, we propose that Gram-positive bacterial pathogens might have been highly prevalent in the evolutionary history of *T. molitor*, constraining the development of maternal priming responses at relatively lower cost. Therefore, as with individual immune priming, Gram-positive bacteria may have played a key role in the evolution of maternal priming in *T. molitor*.

Overall, males survived bacterial infections better than females. In addition, and consistently with previous results in this system (Zanchi et al. 2011), males often exhibited stronger levels of immune defenses than females. These results appear in contradiction with the theory proposed by Rolff (2002), which predicted that females should invest more in immunity than males, because males are expected to gain fitness by increasing their mating success, while females are expected to increase fitness through longevity because their reproductive effort is much higher. These predictions are coherent with numerous observations in many vertebrate (Zuk 1990; Folstad & Karter 1992; Poulin 1996; Zuk & McKean 1996; Schalk & Forbes 1997; Moore & Wilson 2002) and invertebrate models (Gray 1998; Wedekind & Jakobsen 1998; Adamo, Jensen & Younger 2001; Schwarzenbach et al. 2005; Hangartner et al. 2013). However, our experimental setup did not allow reproduction to occur in our focal insects, which might have created particular conditions with respect to Rolff's predictions, and which are therefore beyond the scope of this study.

To summarize, we found that priming in the mealworm beetle, *T. molitor*, resulted in enhanced immune protection of the insects both within and across generations. Insects primed with Gram-positive bacteria exhibited the best survival benefit upon re-infection, whatever the bacteria used for re-infection. The immune protection of insects primed with Gram-positive bacteria mainly resulted from a long-lasting antibacterial immune response, which did not exist in insects primed with Gram-negative bacteria. While insects primed with Gram-positive bacteria also exhibited enhanced hemocyte concentration, its involvement in acquired resistance could not be demonstrated because of unexpectedly persistent antibacterial activity in the hemolymph. Offspring survival of mothers primed whether with Gram-positive or Gram-negative bacteria was not pathogen specific. While improved resistance to infection in the offspring of mothers primed with Gram-positive bacteria could be attributed to enhanced PO activity, enhanced protection in the offspring of mothers primed with Gram-negative bacteria could not be attributed to any of the immune parameters measured in our study. As previously found (Zanchi et al. 2011), TGIP was costly in terms of larval development time of the offspring. However, such a cost was lower for the offspring of mothers primed with Gram-positive bacteria than for those from mothers primed with Gram-negative bacteria. These results, taken with those of Dubuffet et al. (2015), showing that immune-challenged *T. molitor* mothers, whether with Gram-positive or Gram-negative bacteria, produced eggs exhibiting enhanced antibacterial activity against Gram-positive bacteria, clearly suggest that Gram-positive pathogens may have been relatively important selective force for the evolution of immune priming in *T. molitor*. Gram-positive bacteria include major entomopathogenic pathogens, which are also the best able to persist in the external environment of *T. molitor* (Jurat-Fuentes & Jackson 2012; Du & Laing 2011). Their persistence, exposing the insect to repeated infections both within an individual lifetime and across generations, might have played a relatively important role in the evolution of immune priming in *T. molitor*.

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Chapitre 2

Chapitre 2 : Recherche de l'origine de l'activité antibactérienne des œufs dans le cadre du transfert trans-générationnel d'immunité et caractérisation des bénéfices et des coûts associés pour la descendance.

Chez *T.molitor*, un des moyens d'expression du TTGI consiste en un transfert d'activité antimicrobienne aux œufs (Moreau et al. 2012 ; Zanchi et al. 2012). Celui-ci pourrait servir à protéger la descendance dans le cas de pathogènes qui ne serait pas transmis verticalement via la mère, mais qui seraient capables de persister dans le milieu naturel du ténébrion meunier. D'ailleurs, il a été récemment démontré que chez *T.molitor*, la protection transmise aux œufs est uniquement efficace contre les bactéries Gram-positives (Dubuffet et al. 2015), capables pour la plupart de persister dans l'environnement sous forme d'endospore. Cette protection résulte essentiellement de l'action d'une protéine particulière : la Ténécine 1 (Dubuffet et al. 2015).

Néanmoins, ces études récentes ne permettent pas de savoir si la protection transmise aux œufs est efficace pour lutter contre un pathogène persistant, permettant soit un meilleur taux d'éclosion des œufs protégés par rapport aux non protégés, soit une meilleure survie des jeunes larves à l'éclosion. De même, ces études ne donnent pas d'éléments de réponse quant à l'origine de la protection transmise. Cette dernière pourrait être le résultat d'une transmission passive de peptides antimicrobien de la mère à l'œuf. Elle pourrait aussi être due à l'expression de gènes codant pour la synthèse de peptides antimicrobiens dans les œufs, qui fluctuerait en fonction de l'existence d'un challenge maternel. Ces deux mécanismes qui ne sont pas obligatoirement exclusifs, sont censés avoir des impacts différents en termes de valeur sélective, et notamment de coût, pour la mère et sa descendance. Si le transfert est dû à un transfert passif de la mère à la descendance, nous pouvons nous attendre à ce que les coûts soient essentiellement supportés par celle-ci. Au contraire, si les œufs produisent eux-mêmes les peptides antimicrobiens, les coûts du TTGI devraient se retrouver essentiellement chez la descendance bénéficiant de cette protection.

En étudiant la dynamique de l'activité antibactérienne au sein des œufs tout au long de leur période d'incubation, nous avons démontré que les deux mécanismes existent, mais sont

pathogène-dépendant. En effet à peine pondus, les œufs venant de femelles stimulées avec *A. globiformis* présentent une forte activité antibactérienne, qui diminue ensuite jusqu'au moment de l'éclosion. Cela suggère que dans ce cas, le TTGI résulte d'un transfert passif de la mère à sa descendance. A contrario, les œufs issus de femelles challengées par *B. thuringiensis* sont très faiblement protégés au moment de la ponte, mais l'activité antimicrobienne augmente chez ces derniers et se maintient à un niveau important, jusqu'à leur éclosion. Cela révèle la capacité des œufs à produire eux-mêmes leurs peptides antimicrobiens dans le cadre du TTGI.

Si des coûts en termes de valeur sélective étaient attendus pour les descendants de mères challengées, que ce soit au niveau du temps d'incubation, de la taille des œufs, du succès à l'éclosion ou de la survie des jeunes larves issues de ces œufs, notre étude n'en a pas relevé. Au contraire, les œufs issus de femelles challengées, quel que soit la bactérie utilisée pour la stimulation, avaient un meilleur succès à l'éclosion que les œufs issus de femelles témoins, tandis que les larves provenant de femelles primées avec *B. thuringiensis* exprimaient une meilleure survie à la fois dans des conditions stériles et lorsqu'elles étaient exposées à la bactérie. Ces résultats contrastes avec ceux d'études précédentes révélant différents coûts associés au TTGI, notamment chez le ténébrion meunier à des stades de vie plus avancés. En effet, comme nous l'avons vu dans le chapitre précédent, le TTGI tend à allonger le temps de développement de la descendance issue de femelles primées (Zanchi et al. 2011 ; Dhinaut et al. 2017).

Nos résultats démontrent ici que les premiers stades de vie de la descendance d'une mère immunologiquement stimulée, sont critiques en termes d'optimisation de différents traits d'histoire de vie pour les ténébrions exposés à des pathogènes capables de persister dans l'environnement.

Trans-generational immune priming in the mealworm beetle protects eggs through pathogen-dependent mechanisms imposing no immediate fitness cost for the offspring

Authors: J. Dhinaut, M. Chogne, Y. Moret

Submitted

Abstract

Immune-challenged mothers can improve their offspring immunity through trans-generational immune priming (TGIP). In insects, TGIP endows the offspring with lifetime immunity, including the eggs, which are likely exposed soon after maternal infection. Egg protection may rely on the transfer of maternal immune effectors to the egg or/and the induction of egg immune genes. These respective mechanisms are assumed to have early-life fitness costs of different magnitude for the offspring. We provide evidence in the mealworm beetle *Tenebrio molitor* that enhanced egg immunity following a maternal immune challenge is achieved by both of these mechanisms but in a pathogen-dependent manner. While previously found having late-life fitness costs for the offspring, TGIP here improved egg-hatching success and early larval survival, in addition of improving offspring immunity. These results suggest that early-life of primed offspring is critical in the optimization of life history trajectory of this insect under trans-generational pathogenic threats.

I. Introduction

In many animals, the immunological experience of mothers can be transferred to otherwise naïve offspring and can protect them against infection. This plastic modulation of offspring immunity is likely adaptive by preparing them for the prevailing parasite environment. Maternal transfer of immunity has been well studied in vertebrates, in which antibodies produced by infected females can be transferred to the foetus through the placenta and in milk during lactation in mammals, or via the egg yolk in birds, reptiles and fish (Hasselquist & Nilsson 2009). Since newborn vertebrates have limited abilities to produce their own antibodies, this maternal help protects them pending the development of their own immune defence.

Invertebrates lack the antibodies that vertebrate mothers pass to their offspring. However, such a trans-generational effect, also called trans-generational immunity (TGIP), has been observed in many invertebrates too (Boots & Roberts 2012; Freitak et al. 2009; Hernández López et al. 2014; Huang & Song 1999; Little et al. 2003; Moret 2006; Norouzitallab et al. 2015; Roth et al. 2010; Sadd et al. 2005; Sadd & Schmid-Hempel 2007; Tidbury et al. 2011; Trauer & Hilker 2013; Yue et al. 2013). TGIP effects were revealed through enhanced immune activity and/or increased survival to infection in primed eggs (Dubuffet et al. 2015; Moreau et al. 2012; Sadd & Schmid-Hempel 2007; Zanchi et al. 2012), juveniles (Freitak et al. 2009; Moret 2006; Rahman et al. 2004; Rosengaus et al. 2017; Tidbury et al. 2011) and adult offspring (Castro-Vargas et al. 2017; Roth et al. 2010; Sadd & Schmid-Hempel 2009; Zanchi et al. 2011).

While beneficial against repeated parasite attacks across generations, TGIP is also associated to fitness costs. Indeed, investment in immunity is costly on multiple levels (Schmid-Hempel 2003), and its enhanced activity, due to TGIP, was found negatively impacting primed offspring growth (Lorenz & Koella 2011; Roth et al. 2010) and reproduction (Trauer & Hilker 2013). However, the magnitude of the cost paid by the offspring to TGIP could be dependent on the bacterial pathogens that immune challenged the mothers (Dhinaut et al. 2017). For instance, the offspring of the mealworm beetle, *Tenebrio molitor*, paid a lower cost to TGIP in terms of growth when their mothers were immune challenged by Gram-positive bacteria, which are believed to have being an important part of the selection behind the evolution of TGIP in this insect species (Dubuffet et al. 2015; Dhinaut et al. 2017). Furthermore, in addition of paying the usual immune activation costs, immune-challenged mothers were also found to pay a cost to TGIP when producing and transferring immune products to the offspring (Moreau et al. 2012). However, the division of TGIP costs between mothers and their offspring might depend

on the mechanisms through which the immune protection of the offspring is achieved, which is currently unknown.

In insects, bacterially immune-challenged females produced eggs containing enhanced level of antibacterial activity (Dubuffet et al. 2015; Moreau et al. 2012; Sadd & Schmid-Hempel 2007; Trauer-Kizilelma & Hilker 2015; Zanchi et al. 2012). This TGIP effect on eggs may result from two non-exclusive mechanisms, which should have different fitness outcomes for mothers and their progeny. On the one hand, primed eggs might be passively supplied with antibacterial substances from the mother (Berry 1982). Therefore, eggs should exhibit strong levels of antibacterial activity soon after being laid. This antibacterial activity may then persist or decline during incubation, as the antibacterial substances are metabolized. Since the mother mostly ensures the egg immune protection, they may pay a significant cost to TGIP, which may take the form of a reduced fecundity (Moreau et al. 2012). However, as eggs do not need to spend resources for their defense, they are not expected to pay a cost to TGIP. On the other hand, immune-challenged mothers may elicit the activation of immune genes in the developing embryo as insect eggs were shown to activate their own immune response (Freitak et al. 2014; Jacobs et al. 2014; Jacobs et al. 2017; Jacobs & van der Zee 2013). The egg immune response could either be induced by the maternal transfer of bacteria or bacterial fragments into the egg (Freitak et al. 2014; Knorr et al. 2015; Salmela et al. 2015) or by epigenetic mechanisms such as DNA methylation or histone modification (Castro-Vargas et al. 2017; Sysoev et al. 2016). In this case, eggs should exhibit temporal increase in level of antibacterial activity with time after oviposition as the immune response of the embryo is activated. Because immune responses are costly (Moret & Schmid-Hempel 2000), eggs raising their own immune activity are expected to pay a cost to TGIP too. Indeed, eggs have limited resources and nutrients do develop into larvae and those consumed by the immune response could be then missing for the developing embryo. As resource shortage in insect eggs affects the development of the embryo and the survival of the resulting larvae (Geister et al. 2008), resources used by the egg immune response may reduce hatching success rate, extend incubation time or decrease survival of young larvae.

Here, we used the mealworm beetle, *Tenebrio molitor*, to first decipher the mechanisms through which immune-challenged mothers protect their eggs. We then tested the fitness consequences that these mechanisms may have on egg success when exposed or not to the same bacterial pathogen used to immune challenge the mother. To this purpose, we characterized the temporal dynamics of antibacterial activity in eggs laid by immune-challenged mothers with

different Gram-positive bacterial pathogens. Changes in antibacterial activity levels with time after oviposition should reveal the main process involved for egg protection. In particular, increased levels of antibacterial activity with time post-oviposition should indicate that eggs are producing themselves their antibacterial substances. By contrast, maintained or decreased levels of antibacterial activity with time post-oviposition would indicate that the mother is mostly providing antibacterial substances to the eggs, although both mechanisms are likely not exclusive. In addition, to estimate fitness costs associated to egg immune protection, we compared the success of eggs of control and immune-challenged mothers with different Gram-positive bacteria in terms of egg size, hatching rate and incubation time when exposed or not to the same bacterial pathogen that immune challenged the mother. When eggs successfully hatched, we further compared the survival of the resulting larvae under food privation, while prolonging the infectious treatment to which they were exposed at the egg stage. We expected that primed eggs and young larvae should respectively show improved hatching success rate and survival when exposed to the bacterial infection. Furthermore, in pathogen free condition, we did not expect to see any cost of TGIP resulting from a passive transfer of antibacterial substances from the mother. Conversely, eggs protected through the activation of their own immune response were expected to be smaller, to exhibit lower hatching success rate and/or to show prolonged incubation time. In addition, larvae that successfully hatched were expected to suffer from a lower survival to starvation.

II. Material and Methods

II.1. Insect cultures

Virgin adult beetles of controlled age (10 ± 2 days post-emergence) were obtained from pupae taken at random from an outbred stock culture maintained in standard laboratory conditions at the University of Burgundy, Dijon, France. All the experimental insects were reared and maintained in an insectary at 24 ± 2°C, 70% RH in permanent darkness, and supplied *ad libitum* with bran flour and water, supplemented by apple, unless specified. All the insects were weighed to the nearest 1 mg with an OHAUS balance (discovery series, DU114C) before use in the experiments. We conducted two separate experiments to study the impact of TGIP on produced eggs. The first experiment aimed to characterize the temporal dynamics of antibacterial activity in eggs after being laid by immune-challenged females with two Gram-positive bacteria: *Arthrobacter globiformis* and *Bacillus thuringiensis*. *B. thuringiensis* is a known entomopathogenic bacterium of beetles, whereas *A. globifomis* is opportunistic. The second experiment tested the effect of maternal priming with either *A. globifomis* or *B. thuringiensis* on egg size, egg hatching success rate, egg incubation time and survival to starvation of the resulting larvae, while exposed or not to the same bacteria that was used for the maternal challenge. These experiments used these two Gram-positive bacteria because antibacterial activity found in *T. molitor* eggs was shown to be active against Gram-positive bacteria only (Dubuffet et al. 2015).

II.2. Temporal dynamics of antibacterial activity in eggs

Groups of 40 virgin females were anaesthetized for 10 min on ice and injected with a 5-µL suspension of either inactivated *A. globiformis* or inactivated *B. thuringiensis* at a concentration of 10^8 cells per mL of phosphate buffer saline solution (PBS 10 mM, pH 7.4). An additional group of 40 females was treated in the same way but without bacteria, as a procedural control for the effect of the injection. Immediately after their immune treatment, the females were paired with a virgin and immunologically naïve male of the same age and allowed to produce eggs in a Petri dish supplied with wheat flour, apple and water in standard laboratory conditions (24 ± 2°C, 70% RH; permanent darkness). Males were removed from the Petri dishes two days after being paired with the females. Only the eggs produced at the third day after maternal priming were collected to test their antibacterial activity, because females protect most of their eggs from day 2 to day 8 after immunization (Zanchi et al. 2012). Three eggs per female were

collected and randomly allocated to be tested for their antibacterial activity at either day 1 (between 1 and 24 hours after being laid), day 3 (between 48 and 72 hours after being laid), day 5 (between 96 and 120 hours after being laid) or day 7 (between 144 and 168 hours after being laid) after being laid. Hence, within each immune treatment group, 3 eggs of 10 females independently contributed to each age point of the eggs. When reaching their allocated age (1, 3, 5 or 7 days after being laid), eggs were snap frozen into liquid nitrogen and stored at – 80°C, awaiting measurement of their antibacterial activity using a standard zone of inhibition assay (Zanchi et al. 2012) against *A. globiformis*. Previous work showed that egg antibacterial activity of immune-challenged mothers was only active against Gram-positive bacteria (Dubuffet et al. 2015). In addition, since antibacterial activity of a same egg extract tested on *A. globiformis* and *B. thuringiensis* were highly positively correlated, zone inhibition tests, in this study, were only done on *A. globiformis*, which also offers the best sensitivity to any antimicrobial activity developed in the mealworm beetle (Dubuffet et al. 2015).

II.3. Egg size, egg hatching rate, incubation time and larval survival

Assessment of primed egg success in terms of egg size, egg hatching success rate, egg incubation time and larval survival while exposed or not to the same bacterial pathogen that immune challenged mothers used 40 immune-challenged females with *A. globiformis*, 40 immune-challenged females with *B. thuringiensis* and 60 control females, all treated and allowed to produce eggs as described above. Three eggs per female were stored at -80°C for later measurement of their size. Egg size was estimated by measuring egg area under microscope (magnification x20) SMZ1500 connected to a camera and a computer running NIS-Elements-AR. Egg size is expected to reveal difference in egg quality, with larger eggs believed to be of better quality (Capinera et al. 1977). Three additional eggs per female were isolated into 12-wells micro-titer plates. Within each maternal immune treatment group, eggs were randomly assigned for being exposed to the living bacterium used to challenge their mother or to pathogen free condition. Eggs of control mothers were randomly divided into three equal groups allocated to exposure to living *A. globiformis*, living *B. thuringiensis* and free pathogen condition. Hatching success and incubation time (time required from the moment the egg has been laid to the moment it hatched) were recorded. Four days after hatching, young larvae were transferred into a new well to measure their survival while starved. Larvae resulting from eggs exposed to bacterial pathogen were further exposed to the same bacterium as the one used at the egg stage. Survival to starvation is expected to reveal difference in body condition post

hatching. Larvae with the best body condition at hatching are expected to show the longest survival to starvation. Survival was recorded for 31 days post-hatching.

II.4. Bacterial cultures for maternal treatment and offspring exposure

All the bacteria used in this study were obtained from the Pasteur Institute: *A. globiformis* (CIP105365) and *B. thuringiensis* (CIP53.1) and cultured as described in Dubuffet et al. (2015) and Dhinaut et al. (2017). Briefly, bacteria were grown overnight at 28°C in liquid Broth medium (10 g bacto-tryptone, 5 g yeast extract, 10 g NaCl in 1000 mL of distilled water, pH 7.5). To perform the maternal immune treatment, the bacteria were then inactivated in 0.5% formaldehyde prepared in PBS for 30 minutes, rinsed three times in PBS, and their concentration adjusted to 10^8 bacteria per mL using a Neubauer improved cell counting chamber. The success of the inactivation was tested by plating a sample of the bacterial solution on sterile Broth medium with 1% of bacterial agar and incubated at 28°C for 24 hours (Dhinaut et al., 2017). Aliquots were kept at -20°C until use. Maternal treatment was done by injection of 5 µL of the bacterial suspension through the pleural membrane between the second and third abdominal tergites using sterile glass capillaries that had been pulled out to a fine point with an electrode puller (Narashige PC-10). For egg and larvae bacterial treatments, overnight cultures (20 mL) were centrifuged at 3500 g at 4°C for 30 min. The supernatant was discarded and the bacteria pellet was used to spread them, using a small paintbrush, on the bottom of wells hosting eggs or larvae.

II.5. Egg antibacterial activity

Antibacterial activity of the eggs was measured using a standard zone of inhibition assay (Dubuffet et al. 2015; Zanchi et al. 2012). Samples were thawed on ice, and egg extracts were prepared by smashing eggs into an acetic acid solution (0.05%, 5 µl per egg). After centrifugation (3500 g, 2 min, 4°C) 2 µL of the supernatant was used to measure antibacterial activity on zone of inhibition plates seeded with *A. globiformis*. An overnight culture of the bacterium was added to Broth medium containing 1% agar to achieve a final concentration of 10^5 cells per mL. Six millilitres of this seeded medium was then poured into a Petri dish and allowed to solidify. Sample wells were made using a Pasteur pipette fitted with a ball pump. Two microlitres of sample solution was added to each well, and a positive control (Tetracycline:

Sigma-Aldrich, St Louis, MO, USA, T3383; 2.5 $mg.mL^{-1}$ in absolute ethanol) was included on each plate (Zanchi et al. 2011). Plates were then incubated overnight at 28 °C. The diameter of inhibition zones was then measured for each sample. As there was slight variation among plates for inhibition zones resulting from tetracycline addition, the diameter of the zone of inhibition of each sample was adjusted to take into account such a between plate variation. For this, the Petri dish in which the inhibition zone resulting from the tetracycline positive control was the largest was used as reference. Then, for each sample, inhibition zone was multiplied by the ratio between the inhibition zone due to tetracycline in the reference Petri dish and that due to tetracycline in the Petri dish in which the sample was measured (Dhinaut et al. 2017).

II.6. Statistics

Variation in egg antibacterial according to maternal immune treatment and time after oviposition was analyzed using a Generalized Linear Model (GLM) fitted with a gamma distribution and a Log link function. Differences between each female treatment at each time point after oviposition (or egg age) were highlighted by estimating the degree of overlap of the 95% confidence intervals (CI), following recommendations by Cumming and Finch (2005). The difference was considered significant when the 95% CI did not overlap on more than half of their length ($P < 0.05$, Fig. 1).

Data on egg number and egg size were analyzed using univariate analyses of variance (ANOVAs), according to maternal immune treatment, mother body mass and female identity (when required) as main effects.

Data on egg hatching success and egg incubation time were respectively analyzed using a binary logistic regression analysis and an ANOVA, according to maternal immune treatment, egg bacterial exposure, mother body mass and female identity. For these analyses, the best statistical models were searched using a backward stepwise procedure from a full model that included all the main effects and the interaction term between maternal treatment and egg bacterial exposure.

Larval survival was analysed by using a Cox regression analysis, according to maternal immune treatment, larvae bacterial exposure, mother body mass and female identity. The best statistical model was searched using a backward stepwise procedure from a full model including all the

main effect and the interaction between maternal immune treatment and larvae bacterial exposure.

All statistical analyses used IBM® SPSS® Statistics 19 for Macintosh.

III. Results

III.1. Temporal dynamics of antibacterial activity in eggs

Overall, eggs of immune-challenged females with either *A. globiformis* or *B. thuringiensis* exhibited higher levels of antibacterial activity than those from control females (GLM χ^2 = 154.84, df = 2, N = 432, P < 0.001, Fig. 8). Egg antibacterial activity in primed eggs was also dependent on egg age ($\chi^2_{3, 420}$ =21.74, P = <0,001, Fig. 8). However, pattern of co-variation between egg antibacterial activity and egg age highly differed according to the bacteria used to immune challenge mothers (Maternal treatment*Egg age $\chi^2_{6, 420}$ = 55.52, P < 0.001, Fig. 8). Indeed, egg antibacterial activity of *B. thuringiensis*-immunized females increased with egg age. Antibacterial activity of 1-day old eggs is significantly lower than that of 3, 5 or 7-days old eggs (Fig. 8). By contrast, antibacterial activity of 1, 3 and 5-days old eggs from immune-challenged females with *A. globiformis* was significantly higher than that of 7-days old eggs (Fig. 8). Egg antibacterial activity from immune-challenged mothers either by *B. thuringiensis* or *A. globiformis* was significantly dependent on mother body mass ($\chi^2_{1, 420}$ = 16.09, P = 0.001), but not on mother identity ($\chi^2_{1, 420}$ = 0.25, P = 0.61).

III.2. Egg size, hatching rate, incubation time and larval survival

Number of eggs produced by females was independent of the maternal immune treatment and female body mass (Table 8). Conversely egg size was dependent on the maternal immune treatment (Table 8). Immune-challenged mothers with *A. globiformis* produced the largest eggs, whereas immune-challenged mothers with *B. thuringiensis* produced the smallest (Fig. 9).

Hatching success was about 8 to 12 times (see odd ratios in Table 9) higher for eggs of immune-challenged mothers with respectively either *A. globiformis* and *B. thuringiensis* than for eggs of control mothers (Table 9, Fig. 10). Egg bacterial exposure with either *A. globiformis* or *B. thuringiensis* did not explain any variation in hatching rate either as main effect or in interaction with the maternal immune treatment (Table 9). Hatching success of eggs produced by large females tended to decrease than that of eggs produced by small females in the experiment that used *A. globiformis* (Table 9A). Female identity explained part of the variation in egg hatching success too (Table 9).

Variation in egg incubation time could not be significantly explained by the statistical models comprising maternal immune treatment, egg bacterial exposure, mother body mass and mother

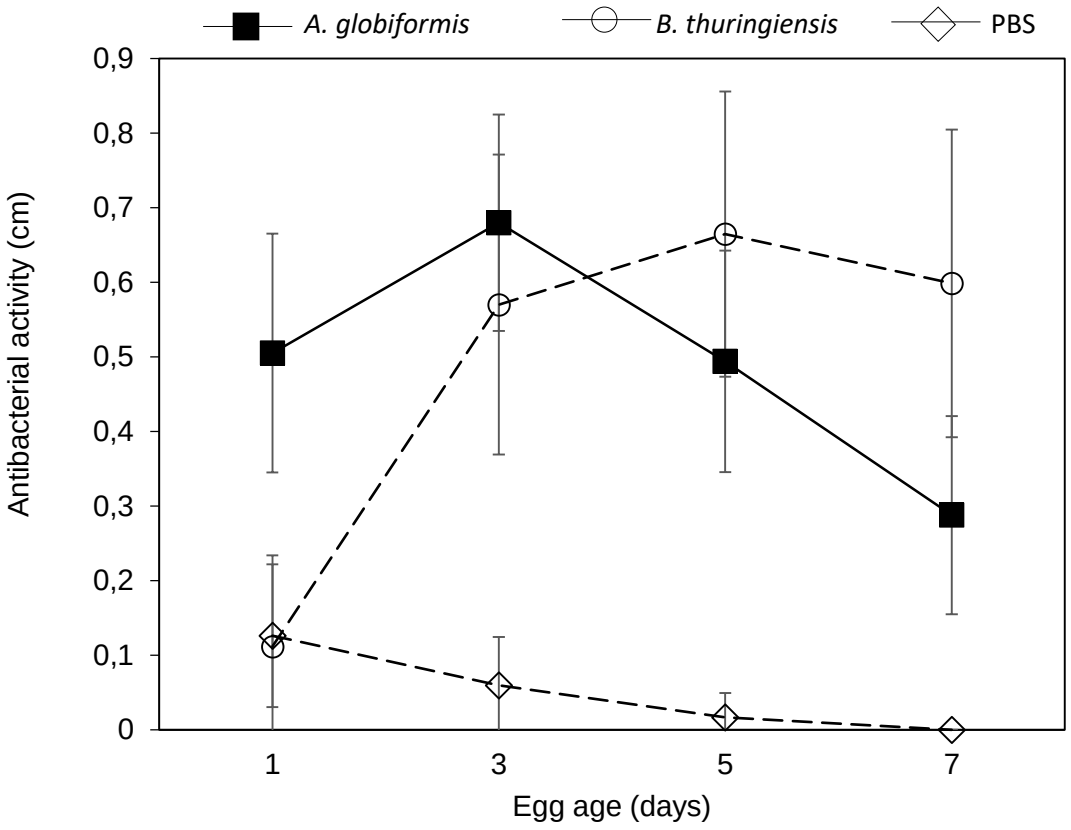

Figure 8. Antibacterial activity in eggs of *T. molitor* expressed as the zone of inhibition diameter (mean in cm ± IC 95%) along time (in days) post oviposition, according to maternal immune treatment (PBS: open diamond; *B. thuringiensis*: open circle; *A. globiformis*: filled square) and age of eggs.

Table 8. Univariate analyses of egg number and egg size produced by *T. molitor* females immune challenged with either *A. globiformis* or *Bacillus thuringiensis* (Maternal treatment), controlling for mother body mass and mother identity (when required). Values where P ≤ 0.05 are given in bold.

Dependent variables	Source of variation	F	d.f.	P
Egg number	Model	0.98	3, 167	0.405
	Maternal treatment	0.76	2, 167	0.467
	Mother body mass	1.64	1, 167	0.203
Egg Size	Model	9.60	4, 481	< **0.001**
	Maternal treatment	11.68	2, 481	< **0.001**
	Mother body mass	12.91	1, 481	< **0.001**
	Mother identity	6.37	1, 481	**0.012**

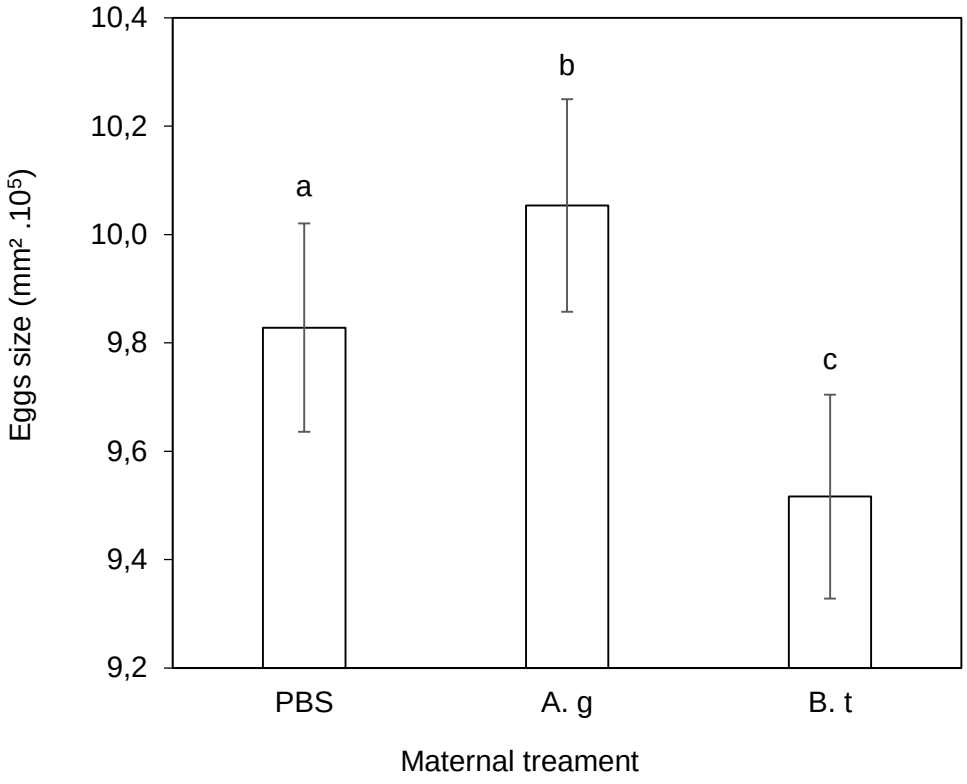

Figure 9. Egg size of *T. molitor* (mean in mm^2 ± IC 95%) according to maternal immune treatment (PBS; *B. thuringiensis*: B.t; *A. globiformis*: A.g). Different letters indicate significant differences between maternal treatments, based on ANOVA ($P \leq 0.05$).

Table 9. Binary logistic regression analysis of hatching rate of eggs produced by control and immune-challenged *T. molitor* females either with (A) *A. globiformis* ('Maternal treatment': control females *n* = 40; challenged females *n* = 40) or (B) *Bacillus thuringiensis* (Maternal treatment': control females *n* = 40; challenged females *n* = 40) when exposed or not to the same bacteria used for the maternal treatment ('Egg exposure'), controlling for mother body mass and mother identity. The "simple" contrast was used for maternal immune treatment (hatching rate of eggs of control mothers was used as baseline) and egg bacterial exposure (hatching rate of eggs unexposed to bacteria was used as baseline). Values where $P \leq 0.05$ are given in bold.

A *Maternal immune challenge and egg exposure with A. globiformis*

Variables in the best model	*B*	S.E.	*Wald*	d.f.	*P*	*Odd ratio*
Maternal treatment	2.04	0.53	14.66	1	< **0.001**	7.71
Mother identity	0.01	0.004	6.18	1	**0.013**	1.01
Mother body mass	-0.02	0.009	4.98	1	**0.026**	0.98

Variables not in the best model	Score	d.f.	*P*
Egg exposure	0.58	1	0.446
Maternal treatment* Egg exposure	0.05	1	0.823

B *Maternal immune challenge and egg exposure with B. thuringiensis*

Variables in the best model	*B*	S.E.	*Wald*	d.f.	*P*	*Odd ratio*
Maternal treatment	2.46	0.90	7.55	1	**0.006**	11.77
Mother identity	0.01	0.005	4.48	1	**0.034**	1.01

Variables not in the best model	Score	d.f.	*P*
Egg exposure	0.03	1	0.862
Mother body mass	0.07	1	0.786
Maternal treatment* Egg exposure	0.92	1	0.338

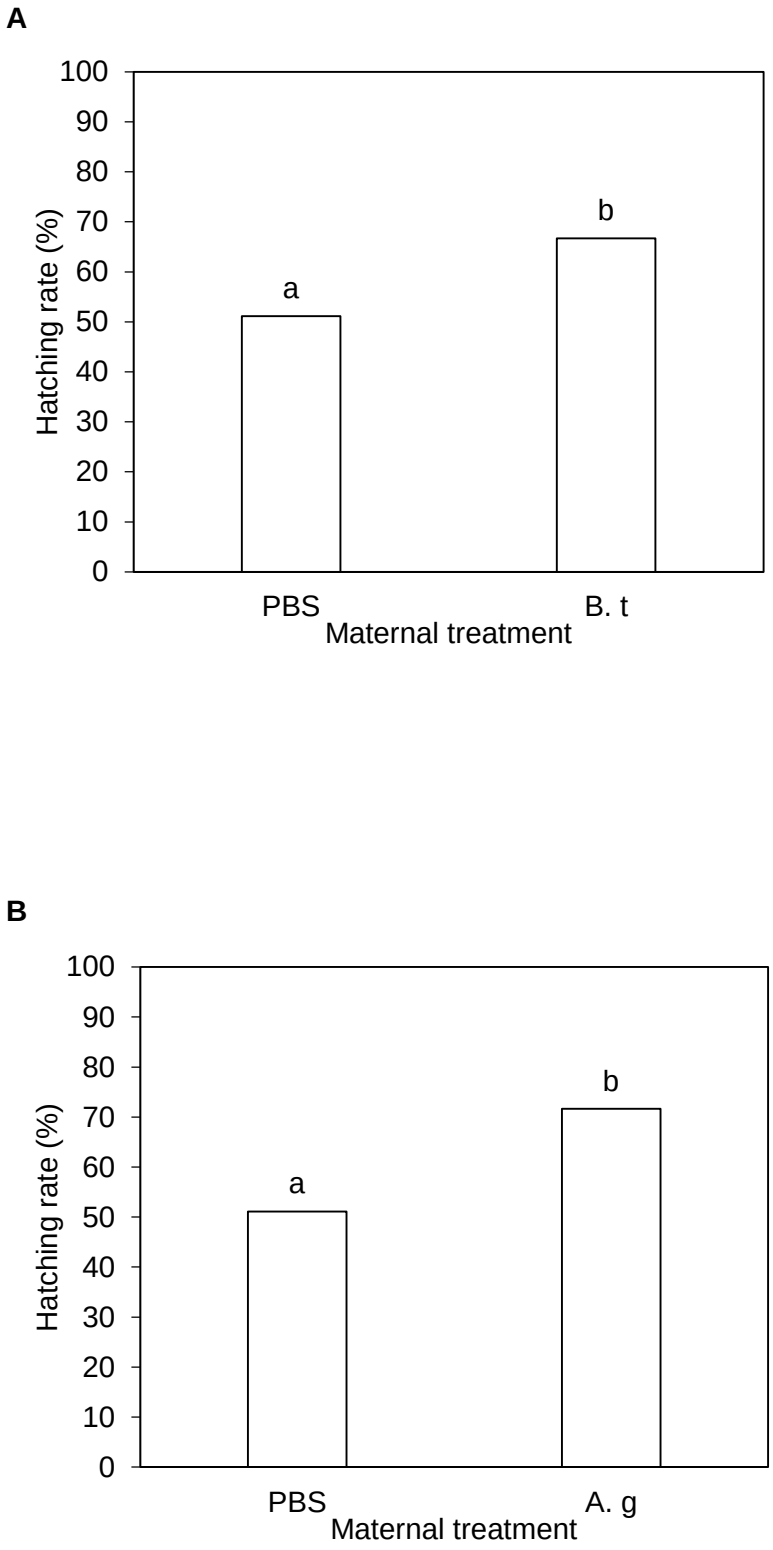

Figure 10. Proportion of eggs of *T. molitor* that successfully hatched according to maternal immune treatment with (A) *A. globiformis* and (B) *B. thuringiensis* independently of the bacterial exposure treatment of the eggs, which had no statistically effect (PBS; *B. thuringiensis*: B.t; *A. globiformis*: A.g). Different letters indicate significant differences between maternal treatments, based on binary logistic regression analysis ($P \leq 0.05$).

identity as main effects either when comparing eggs of control mothers with those of challenged mothers with *A. globiformis* (Global model: $F_{4, 141} = 1.43$ $P = 0.226$) or those of challenged mothers with *B. thuringiensis* (Global model: $F_{4, 136} = 2.03$ $P = 0.094$). However, for the data from the experiment that used *B. thurigiensis* as bacterial pathogen for priming and egg bacterial exposure, removing maternal immune treatment from the latter statistical model (this factor was the one that explained the less variation in incubation time: $F_{1, 136} = 0$), succeeded in explaining variation in egg incubation time (Global model: $F_{3, 137} = 2.73$ $P = 0.047$), essentially because of the egg bacterial exposure treatment ($F_{41\ 137} = 7.19$ $P = 0.008$). Eggs exposed to the bacterium *B. thuringiensis* exhibited a slight prolonged incubation time (mean in days ± 95% IC: 9.10 ± 0.12) than non-exposed ones (mean in days ± 95% IC: 8.9 ± 0.08). A similar procedure for the analysis of the data from the experiment that used *A. globiformis* failed to improve variance explanation (Global model: $F_{3, 132} = 1.25$ $P = 0.295$).

In the experiment using *A. globiformis* for the maternal treatment and offspring bacterial exposure, survival of larvae was only explained by the bacterial exposure treatment, which imposed a 1.6-folds survival reduction of the larvae (Table 10A, Fig. 11A). In the experiment that used *B. thuringiensis*, larval exposure to the bacterium imposed a 2.7-folds survival reduction (Table 10B, Fig. 11B). However, larvae hatched from eggs of immune-challenged mothers with *B. thuringiensis* exhibited improved survival, irrespectively of the bacterial exposure treatment (Table 10B). Mother identity significantly explained survival of newborn larvae, but not mother body mass (Table 10B).

Table 10. Cox regression survival analyses for the young larvae hatched from eggs produced by control and immune-challenged *T. molitor* females either with (A) *A. globiformis* or (B) *B. thuringiensis* ('Maternal treatment') when exposed or not to the same bacteria used for the maternal treatment ('Larvae exposure'), controlling for mother body mass and mother identity. The "simple" contrast was used for maternal immune treatment (hatching rate of eggs of control mothers was used as baseline) and egg bacterial exposure (hatching rate of eggs unexposed to bacteria was used as baseline). Values where $P \leq 0.05$ are given in bold.

A *Maternal immune challenge and larvae exposure with A. globiformis*

Variables in the best model	*B*	S.E.	*Wald*	d.f.	*P*	*Odd ratio*
Larvae exposure	0.48	0.18	7.20	1	**0.007**	1.62

Variables not in the best model	Score	d.f.	*P*
Maternal treatment	0.32	1	0.570
Mother identity	0.20	1	0.652
Mother body mass	0.87	1	0.350
Maternal treatment* Larvae exposure	0.45	1	0.504

B *Maternal immune challenge and larvae exposure with B. thuringiensis*

Variables in the best model	*B*	S.E.	*Wald*	d.f.	*P*	*Odd ratio*
Maternal treatment	-3.07	0.74	17.42	1	< **0.001**	0.05
Larvae exposure	1.00	0.20	24.70	1	< **0.001**	2.72
Mother identity	0.01	0.004	16.09	1	< **0.001**	0.985
Maternal priming* Larvae exposure	0.63	0.38	2.77	1	0.096	1.88

Variables not in the best model	Score	d.f.	*P*
Mother body mass	0.001	1	0.976

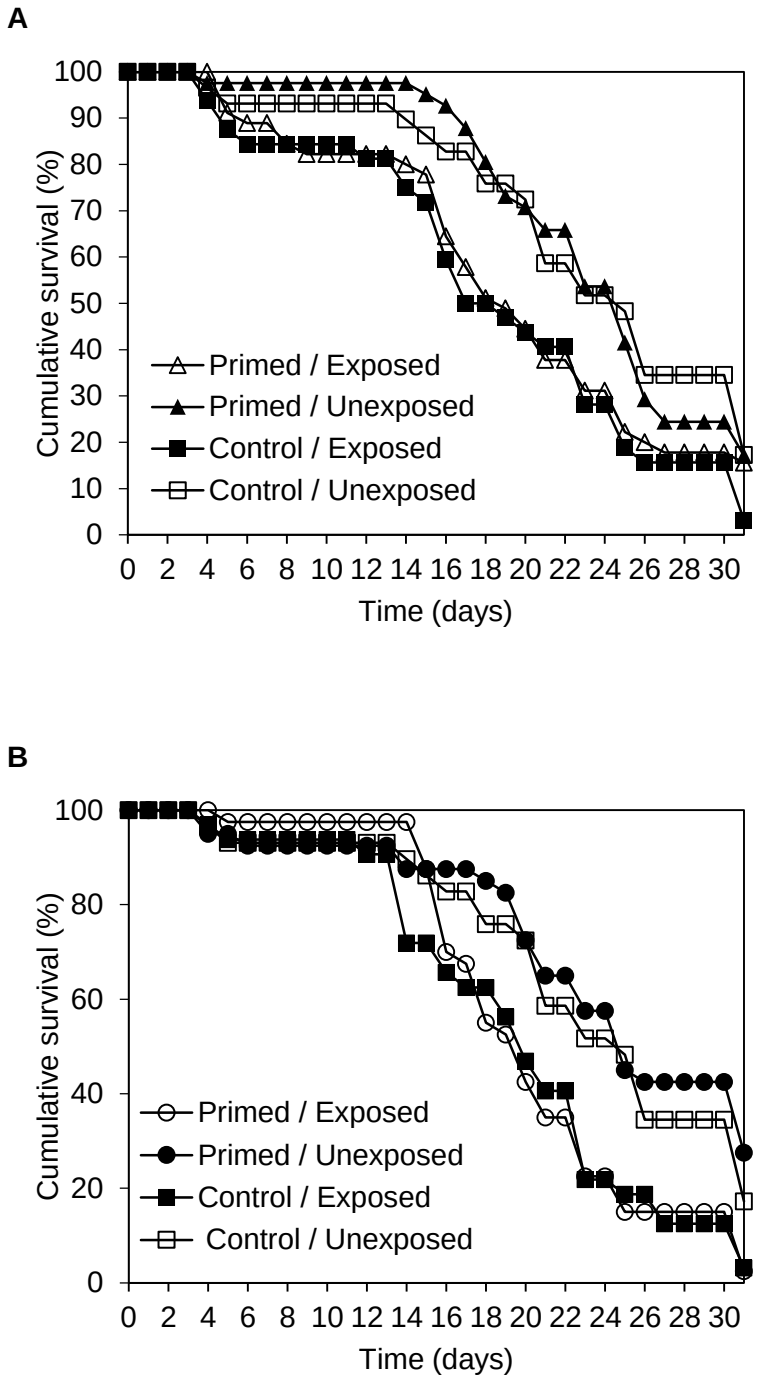

Figure 11. Early survival of *T. molitor* larvae according to the maternal immune treatment of mothers: (A) control or Primed with *A. globiformis* and (B) Control or Primed with *B. thuringiensis*, when the larvae were either exposed or unexposed to the living bacteria used to prime their mothers.

IV. Discussion

Our results show that eggs produced by immune-challenged females of the mealworm beetle, *T. molitor*, with different bacterial pathogens exhibited contrasted patterns of variation in antibacterial activity with time post-oviposition. In particular, primed eggs of immune-challenged mothers with an opportunistic bacterial pathogen, such as *A. globiformis*, exhibited strong levels of antibacterial activity soon after being laid, which then progressively decreased until hatching (Fig. 8). Such a pattern of variation in antibacterial activity with egg age is consistent with the hypothesis that mothers passively supply their eggs with antibacterial substances, which are then metabolized as eggs develop. By contrast, primed eggs of immune-challenged mothers with a known entomopathogenic bacteria of *T. molitor*, such as *B. thuringiensis* (Du Rand & Laing 2011) exhibited very low levels of antibacterial activity soon after being laid. Antibacterial activity then increased to reach a plateau from the third day post-oviposition to hatching (Fig. 8). Such a pattern of variation clearly suggests that antimicrobial substances are synthesized in the eggs and are not directly provided by the mother. The production of immune effectors by the egg themselves is probably not surprising as eggs in some insect species can activate their own immune response (Freitak et al. 2014; Gorman et al. 2004; Jacobs & van der Zee 2013; Tingvall et al. 2001). This is also true in *T. molitor* (Jacobs et al. 2017). However, while our results clearly suggest that antibacterial substances, which are proteins (Dubuffet et al. 2015), are produced by the eggs, we are unable to state whether the transcripts at the origin of these antibacterial proteins are synthesized in the eggs too or produced and transferred by mothers to the eggs. Further work is needed to decipher the complete mechanisms of egg immune protection. Nevertheless, we found evidence that the mechanisms through which eggs become immune protected after a maternal immune challenge are pathogen-dependent. The existence of these different mechanisms suggests that at least one of them may have specifically evolved from a tight coevolutionary history between *T. molitor* immune system and one of the pathogen used in our experiment. Compared to *A. globiformis*, *B. thuringiensis* is a known bacterial entomopathogen of *T. molitor* (Du Rand & Laing 2011), characterised by a strong ability to persist in the environment between insect generations, by forming endospores (Jurat-Fuentes & Jackson 2012). It is therefore tempting to speculate that the mechanism of egg protection after a maternal infection with *B. thuringiensis* could be specific to that range of bacterial pathogens, whereas the one involved in the response to a maternal infection with *A. globiformis* could be more generic. However, further studies are required to confirm or refute such a hypothesis.

These mechanisms were assumed to have different implications about fitness costs associated to egg immune protection resulting from the maternal immune challenge. Indeed, mothers were expected to mostly support the cost of the egg immune protection when producing and transferring immune effectors to the eggs. Conversely, eggs were expected to mostly support the cost of their immune protection when induced to produce themselves their immune effectors. However, our results did not reveal any evidence of cost associated to the egg immune protection for mothers, whatever the mechanisms involved. Indeed, control and immune-challenged females, with either *A. globiformis* or *B. thuringiensis*, produced a similar number of eggs. This suggests that immune-challenged females did not pay an immediate cost on fecundity to the immune challenge it-self and for supplying immune effectors to the eggs. The latter is particularly true for immune-challenged females with *A. globiformis*, which, despite directly supplying antibacterial substances to their eggs, and producing larger eggs, did not show any reduced fecundity. It is nevertheless possible that while fecundity was not affected at this particular egg-laying sequence of the female beetles, it could be reduced during subsequent egg-laying sequences as previously found (Moreau et al. 2012). Furthermore, provisioning eggs with antibacterial substances could be done at the expense of the own immune response of the mother (Moreau et al. 2012), which was not estimated in this study.

We found that the maternal immune challenge had no negative effect on egg success either. On the contrary, eggs of immune-challenged mothers by either *A. globiformis* or *B. thuringiensis* even exhibited higher hatching success rates than those of control mothers, and this, whether eggs were exposed or not to the maternal bacterial pathogen. This result confirms a recent observation in *Manduca sexta*, where the hatching success of eggs of immune-challenged mothers was improved (Rosengaus et al. 2017). In addition, primed eggs did not exhibit longer incubation time, which could have been expected when eggs are using limited resources to produce their own immune effectors. Here, eggs of immune-challenged mothers by *B. thuringiensis*, which we found producing themselves their own antibacterial substances, did not traded-off their development against their immunity. This suggests that primed eggs might be sufficiently provisioned with the necessary metabolites, such as carbohydrates, proteins and lipids (Kinsella 1966; Pant et al. 1979), to develop normally and even exhibit higher hatching success. While incubation time of eggs exposed to *A. globiformis* was not affected, it was slightly increased in eggs exposed to *B. thuringiensis*. This result shows that eggs could somehow specifically sense the presence of pathogens, which presumably affects their

development. However, the mechanisms through which the eggs respond to the presence of certain bacterial pathogens remain unknown.

While exposure to living bacteria did not affect egg success, it strongly reduced the early survival performance of the larvae. However, survival of larvae of immune-challenged mothers with *B. thuringiensis* was higher than survival of those of control mothers whether they were exposed or not to the living bacterium. Such a survival improvement was not found among larvae of immune-challenged mothers with *A. globiformis*. This confirms that the priming response leading to the immune protection of the eggs and then of the larvae is dependent on the bacterial pathogens that previously immune challenged the mothers. Again, in pathogen free conditions, *B. thuringiensis*-primed larvae performed better than control larvae, suggesting that larvae pay no cost to TGIP during their early life. In this study, larvae were not fed and therefore could not reach the adult stage. Previous studies have evidenced that the offspring of immune-challenged mothers with various bacterial pathogens, including *B. thuringiensis*, are paying a cost to TGIP by exhibiting a prolonged larval developmental time (Zanchi et al. 2011; Dhinaut et al. 2017). Longer developmental time is costly because it reduced competitive ability for food under higher densities (Koella & Boëte 2002) and should delay access to reproduction. Moreover, it could increase the probability of mortality (Bell 1980), especially in insects like *T. molitor*, which exhibits cannibalism on juveniles (Ichikawa & Kurauchi 2009). Costs of TGIP on larval development of the primed offspring were evidenced in other insect models, such as flour beetles (Roth et al. 2010) or mosquitoes (Lorenz & Koella 2011). In *M. sexta*, adult offspring derived from immune-challenged parents have a lower fecundity than those derived from control parents (Trauer & Hilker 2013). This suggests that, while TGIP may show no cost early in life of the offspring and may even provide benefits beyond enhanced immunity to the current pathogenic threat, it is nevertheless costly and its costs appear later in life of the offspring. Such long-term trade-offs are classical in the study of maternal effects (Clark & Galef 1995; Lindström 1999; Qvarnström & Price 2001), to which TGIP belongs. Hence TGIP may have strong consequences on individual life-history trajectories, which evolutionary and ecological impacts on population dynamics and life history evolution are still underexplored.

To summarize, this study reports three novel findings about TGIP in the eggs of the mealworm beetle *T. molitor* following a maternal bacterial immune challenge. First, we found that enhanced antibacterial activity in eggs of immune-challenged mothers relies on different mechanisms that are dependent on the bacterial pathogen challenging the mother. On the one

hand, females produce and transfer antibacterial substances to the eggs and, on the other hand, eggs are allowed to produce them-selves their antibacterial substances. Whether these various mechanisms, affecting the temporal dynamics of egg immunity have evolved from relatively specific coevolutionary history between the insect immune system and pathogens remain unknown. Second, TGIP provided fitness benefits to the offspring beyond enhanced of immunity as it increased egg hatching success and early larval survival while exposed or not to the infection by the bacteria that previously immune challenged the mother. This suggests that TGIP does not only enhance immunity of the offspring but also implies the provision of extra metabolites or nutrients to the offspring, increasing their individual quality. Third, we failed to evidence any immediate fitness cost associated to TGIP for the eggs and early larval stages, whereas numerous previous studies have evidence fitness costs for the offspring later in life (Lorenz & Koella, 2011; Roth et al. 2010; Trauer & Hilker 2013; Zanchi et al. 2011; Dhinaut et al. 2017). This suggests that early life of primed offspring appear critical in the optimization of life history trajectory of the beetles exposed to pathogens exhibiting high probability of persistence between insect generations.

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Chapitre 3

Chapitre 3: Recherche de bases génétiques pour le transfert trans-générationel d'immunité

Il est maintenant prouvé que la réponse immunitaire chez les insectes est génétiquement variable (Rolff et al. 2005 ; Hammerschmidt et al. 2012). Récemment, il a été mis en évidence une grande variation interindividuelle parmi les femelles de *T. molitor*, préalablement immunisées, pour leur investissement à la protection immunitaire de leurs œufs (Zanchi et al. 2012). Pour l'heure, il est difficile de statuer sur les composantes génétique et environnementale de cette variation. Pour comprendre l'évolution du TTGI et de son potentiel de réponse à la sélection, il convient d'étudier la composante génétique de sa variabilité. Cependant, comme pour tous les effets maternels, la sélection naturelle agit à la fois sur le phénotype de la mère et le phénotype de la descendance (Kirkpatrick & Lande 1989). Par conséquent, il convient d'examiner aussi les covariations génétiques potentielles entre le niveau d'investissement maternel et le phénotype de la descendance (Grindstaff et al. 2003). La réponse à la sélection dépendra du signe de ces covariations. Par exemple, une covariance génétique négative indiquera que les gènes qui augmentent la valeur du TTGI sont aussi contre balancés par des gènes qui diminuent l'aptitude de la descendance (Grindstaff et al. 2003). Dans ce cas, l'évolution du TTGI ne serait pas aussi rapide que prévu.

Ce chapitre a pour but de déterminer comment l'évolution du TTGI aux œufs affecte l'évolution des caractères de la valeur sélective de la mère et de sa descendance chez *T. molitor*. En utilisant des lignées consanguines (Archer et al. 2012), nous avons quantifié la variation génétique de l'investissement maternel à la protection des œufs et mesuré d'autres traits associés à la valeur sélective des mères (comme la condition corporelle, l'effort de reproduction, l'activité immunitaire et la longévité) et de la descendance (comme la survie larvaire, la croissance larvaire, la masse nymphale, la condition corporelle des adultes et leur activité immunitaire). Si l'investissement dans le TTGI est déterminé génétiquement, alors ce trait devrait être héritable. Ensuite, l'existence de corrélations génétiques entre l'investissement au TTGI et les autres traits de fitness de la mère et de la descendance a été recherchée afin de savoir si ces traits évoluent de façon indépendante ou s'ils sont génétiquement liés les uns aux autres.

Malheureusement, il nous a été impossible de conclure quant à l'existence de bases génétiques associées au TTGI. Si nos premiers résultats tendent à réfuter leur existence, une trop grande variance est associée à l'héritabilité calculée pour le TTGI. Cette importante variance semble être dû à un nombre trop faible de lignées utilisées au sein de notre expérimentation (9 au lieu

des 12 initialement prévues) ainsi qu'à un nombre trop faible de femelles représentant certaines des lignées (parfois 8 ou 10 au lieu des 20 femelles envisagées). Ainsi, même si l'étude des corrélations semble montrer des relations essentiellement positives entre les traits de fitness de la mère et de sa descendance dans le cadre du TTGI, suggérant que celui-ci pourrait être sélectionné positivement, notre priorité sera de renforcer nos effectifs afin d'affiner notre calcul d'héritabilité. Cela nous permettra de ne pas rejeter de manière hâtive l'existence de bases génétiques associées au TTGI.

I. Introduction

The phenotype of an individual has long been viewed as the result of the interaction between its genome and the environment it has experienced. However, individual phenotype expression was often, and sometimes dramatically, influenced by the environmental experience of other individuals in the population (Mousseau & Fox 1998). In particular, the maternal experience of the environment plays an important role in the phenotype of the offspring through maternal effects (Bernado 1996). Maternal effects affect many traits of the offspring phenotype with, sometimes, important impacts on offspring fitness. Although not always beneficial to the offspring (Wiklund & Sundelin 2001; Moya-Laraño 2002; Sram et al. 2005; Gibbs et al. 2010), maternal effects can constitute a mechanism for adaptive trans-generational phenotypic plasticity in response to environmental heterogeneity when mothers adjust the phenotype of their offspring to match the environment that offspring are likely to experience (Mousseau & Fox 1998; Fox et al. 1997; Agrawal et al. 1999; Benton et al. 2005; Marshall & Uller 2007; Plaistow et al. 2007; Allen et al. 2008). The extent to which maternal environment influences the offspring phenotype and fitness should determine the likelihood that such maternal effects themselves are shaped by natural selection (Mousseau & Fox 1998). A particular case of maternal effects corresponds to trans-generational effects on offspring immunity as a result of the maternal immunological experience. When pathogens and parasites become prevalent in the environment and offspring are likely to experience the same conditions, the offspring will benefit from mother providing them with enhanced immunity through a phenomenon called "trans-generational immune priming" (TGIP). This maternal effect should increase the mother's reproductive success by increasing her offspring survival to the pathogenic threat. TGIP has been particularly well studied in vertebrates, in which infected females can transfer specific antibodies to the offspring, providing them with a temporal protection awaiting for their own immune system to mature (Grindstaff et al. 2003; Boulinier & Staszewski 2008; Hasselquist & Nilsson 2009). Invertebrates lack the antibodies that vertebrate females transfer to their offspring. However, TGIP occurs in invertebrates too (Huang & Song 1999; Moret & Schmid-Hempel 2001; Little et al. 2003; Sadd et al. 2005; Moret 2006; Sadd & Schmid-Hempel 2007; Freitak et al. 2009; Sadd & Schmid-Hempel 2009; Roth et al. 2010; Tidbury et al. 2010; Zanchi et al. 2011; Moreau et al. 2012; Zanchi et al. 2012; Dhinaut et al. 2017), suggesting that it has to be achieved by other, yet unknown, mechanisms. Evidences of TGIP in invertebrates are largely phenomenological and await the elucidation of their evolutionary and ecological implications. Furthermore, maternal effect on immunity is believed to have important

ecological and evolutionary implications on the dynamics of host-parasite interactions and thus on the epidemiology of wildlife diseases (Grindstaff et al. 2003; Boulinier & Staszewski 2008; Sadd & Schmid-Hempel 2009). However, much remains to be learned about individual differences within species to clearly infer about the ecological and evolutionary implications of this phenomenon. For instance whether TGIP in invertebrates is genetically variable is not known. Such information is yet crucial to know whether maternal transfer of immunity is subject to selection driven either by pathogens or by genetic pleiotropic effects acting on other important life history traits. Hence estimating the heritability of TGIP as well as examining its genetic correlation with other important fitness traits appears to be crucial to understand the evolution of such a maternal effect.

There is now extensive evidence about genetic variation in the immune response of insects (Rolff et al. 2005; Hammerschmidt et al. 2012). Moreover, it was found that immune-challenged females of *Tenebrio molitor* exhibit variation in their investment into the immune protection of their eggs in relation to their fecundity (Zanchi et al. 2012). Indeed, females of *T. molitor* mainly protect eggs produced between day 2 and day 8 following a bacterially based benign immune challenge. Within this period, great variation in the number of eggs protected was found between immune challenged females (Zanchi et al. 2012). Whether such a variation reflects genetic differences in the ability to protect the eggs upon maternal infection is not known. Investigating whether there is genetically based variation among females in immunity transmission also requires the investigation of potential genetic covariations of this maternal investment with fitness related traits of the resulting offspring. Indeed response to selection will depend of the sign of these covariations (Kirkpatrick & Lande 1989; Wolf et al. 1998; Grindstaff 2010). For instance, a negative genetic covariance will indicate that genes that increase the value of maternal transfer of immunity will be counteracted by genes that decrease the value of traits associated with fitness in the offspring (Grindstaff 2010).

Here we have investigated how the evolution of TGIP affects the evolution of fitness related traits of females and that of their progeny in the mealworm beetle, *T. molitor*. Using inbred lines, we have quantified genetic variation in female investment into the protection of the eggs and adult offspring after a maternal challenge and measures other fitness related traits of females (e.g. body size, reproductive effort, immune defences and longevity) as well as measures of fitness related traits of the offspring (e.g. larval survival, larval growth, nymph mass, adult body size and immune defences). If investment into maternal transfer of immunity

is genetically based, we predict that this trait will be heritable. We have then estimated the phenotypic correlations between investment into TGIP and other female and offspring fitness related traits to examine whether these traits could evolve independently or whether they are genetically linked to each other, constraining their relative evolution. In addition, this approach also tested whether the protection of the eggs and that of the offspring at later developmental stages are phenotypically correlated or can evolve independently.

II. Material and Methods

II.1. Insect cultures

Tenebrio molitor used in this study come from an outbred stock culture maintained in standard laboratory conditions at the University of Burgundy, Dijon, France, allowed to breed panmictically. Twelve inbred lines were created by subjecting a random subset of beetles from this population to 5 generations of full-sib mating followed by free mating in panmixis within each line. In addition, we also maintained a culture of mealworm beetles originating from Netherland, which was raised isolated from the laboratory stock cultures used to generate our inbred lines. All the experimental insects were reared and maintained in an insectary at 24 ± 2°C, 70% RH in permanent darkness, and supplied *ad libitum* with bran flour and water, supplemented by apple. Beetles used in this study were virgin adult beetles of controlled age (10 ± 2 days post-emergence) obtained from pupae taken at random from the inbred lines, the outbred stock culture or the Dutch population. All the insects were weighed to the nearest 1 mg with an OHAUS balance (discovery series, DU114C) before being used for the study.

II.2. Experimental design

For practical reasons and when it was possible, the study used three groups of 20 females per inbred lines, the stock culture and the Dutch population to respectively characterize maternal investment into TGIP after a standard maternal immune challenge, immune defenses and fitness related traits of mothers, and immune defenses and fitness related traits of the offspring of each inbred line, the stock culture and the Dutch population. This allowed the quantification of genetic variation among insect lines for investment into TGIP, immune defenses and life history traits of mothers and the offspring through the calculation of their respective heritability and test for their correlation. We must specify that outbred and Dutch beetles are not included in any of our statistical analyses but are provided in our figures only for visual comparison.

Maternal investment into offspring immune protection among insect lines following a standard benign bacterial immune challenge was estimated on the number of eggs produced exhibiting antibacterial activity (Zanchi et al. 2012). The Gram-positive bacteria *Bacillus thuringiensis*, known to be a common bacterial pathogen of coleopteran insects was used to immune challenge insect mothers. For each inbred lines a maximum of 20 virgin adult females were immune challenged by injection of a 5 µL suspension of inactivated *B. thuringiensis* in phosphate-

buffered saline (PBS 10 mM, pH 7.4) after being chilled on ice for 10 min. Immediately after the immune challenge, females were paired with a virgin and immunologically naïve male taken from the outbred stock culture, and allowed to produced eggs for 8 days after the immune challenge in Petri dishes supplied with wheat flour, apple and water in standard laboratory conditions (24 ± 2°C, 70% RH; permanent darkness). Similarly, 20 virgin adult females from the outbred stock culture and the Dutch population were used as control groups. The total number of eggs produced by each female during the 8 days following their immune challenge was recorded, but only the eggs produced between day 2 and day 8 after the maternal immune challenge were used to test their antibacterial activity because immune challenge females of *T. molitor* were shown to mainly protect their eggs within this period of time (Zanchi et al., 2012). Furthermore, eggs of immune-challenged females with *B. thuringiensis* require 3 days of development after being laid to exhibit antibacterial activity (Dhinaut et al. *in prep*; cf. chapter 2). They were therefore kept in standard laboratory conditions for 3 days post oviposition before to be frozen in liquid nitrogen and stored at – 80°C, awaiting for measurement of their antibacterial activity. On the 8th day after their immune challenge, females were isolated in grid boxes (boxes with 10 compartments; each compartment: L x l x H, 4.8 x 3.2 x 2.2 cm) supplied with bran flour and apple, and checked once a week for their survival to estimate longevity. Hence this approach allowed the estimation of female fecundity within 8 days post challenge, investment into egg protection and female longevity.

Concomitantly, other females from the inbred lines, the stock culture and the Dutch population were used to characterize their investment into immune defenses and other fitness associated traits. As described above, a maximum of 20 virgin adult females per inbred line, the stock culture and the Dutch population, were similarly immune challenged with inactivated *B. thuringiensis* and then paired with a virgin and immunologically naïve male from the outbred stock in a Petri dish supplied with wheat flour, apple and water in standard laboratory conditions (24 ± 2°C, 70% RH; permanent darkness). Four days after immunization, a sample of haemolymph was collected from each female to quantify the circulating concentration of haemocytes, level of antibacterial activity in the haemolymph and the activity of the phenoloxidase enzyme. As above, 8 days after their immune challenge, the females were isolated in grid boxes, supplied with bran flour and apple and checked once a week for their survival to estimate longevity. Hence this group of females allowed measuring female immunity after their immune challenge and their longevity.

At the same time, a last group of females were used to characterize immune defenses and other fitness traits of the offspring of immune-challenged mothers from each line, the stock culture and the Dutch population to study phenotypic covariation between immunity and fitness traits of the offspring and that of mothers among insect lines. As previously described, a maximum of 20 virgin adult females of our 12 inbred lines, outbred stock and the Dutch population were immune challenged with inactivated *B. thuringiensis*. Females were then paired with a virgin and immunologically naïve male from the outbred stock in a Petri dish supplied with wheat flour, apple and water in standard laboratory conditions. All the eggs produced between day 2 and day 8 after the maternal challenge were counted and transferred into a larger plastic box (L x l x H, 17 x 11 x 9.5 cm) supplied with bran flour, water and apple, allowing the eggs to hatch and the larvae to grow. As above the females were isolated on the 8^{th} days post challenge in grid boxes, supplied with bran flour and apple and checked once a week for their survival to estimate longevity. Nine weeks after oviposition, larvae were counted and 6 larvae per couple were randomly taken and individually isolated in 6 wells-microplate, supplied with water, bran flour and apple to measure their larval development time (duration in days from hatching to adult stage), their weight at the nymph and the adult stages. At 10 days old, adult offspring of each female were sexed and a first 5-µL sample of haemolymph was collected to quantify the circulating concentration of haemocytes, level of antibacterial activity in the haemolymph and that of the phenoloxidase enzyme, while non-immune challenged. Immediately after collection of the first sample of haemolymph, the beetles were subjected to an immune challenge with *B. thuringiensis*. Three days later, a second 5-µL sample of haemolymph was collected from each beetle to measure again the circulating concentration of haemocytes, level of antibacterial activity in the haemolymph, and that of the phenoloxidase enzyme, but while the beetles were developing an immune response. Three days post-challenge usually corresponds to time at which the immune response of *T. molitor* reachs its peak (Haine et al. 2008a et b). After this second sample of haemolymph longevity of these insects was checked once a week until death. In the mean time, offspring insects that were not isolated were checked once a week to know the number of them that reach the adult stage. Hence, this last group of insects allowed estimate the reproductive success of immune challenged females, survival, mass, immunity and longevity of the offspring among insect lines.

II.3. Bacterial cultures for immune challenges

The bacterium *B. thuringiensis* (CIP53.1), used for the immune challenges was obtained from the Pasteur Institute. Bacteria were grown overnight at 28°C in liquid Broth medium (10 g

bacto-tryptone, 5 g yeast extract, 10 g NaCl in 1000 mL of distilled water, pH 7). Bacteria were then inactivated for 30 minutes in 0.5% formaldehyde prepared in PBS, rinsed three times in PBS, and its concentration adjusted to 10^8 bacteria per mL using a Neubauer improved cell counting chamber under a phase-contrast microscope (magnification x 400).. The success of the inactivation was checked by plating a sample of the bacterial solution on sterile Broth medium with 1% of bacterial agar and incubated at 28°C for 24 hours. Aliquots of the bacterial suspension were kept at -20°C until use. Immune challenges of the beetles were performed by injection through the pleural membrane between the second and third abdominal tergites using sterile glass capillaries that had been pulled out to a fine point with an electrode puller (Narashige PC-10).

II.4. Hemolymph collection and immune parameters

Individual beetles were chilled on ice for ten min after which a 5-µL sample of haemolymph was collected from a wound in the beetle's neck with a sterile glass capillary (Hirschmann®Laborgeräte, Ringcaps®, Eberstadt, Germany) and flushed into a microcentrifuge tube containing 25 µL of ice cold PBS. A 10-µL subsample was immediately used for the measurement of haemocyte concentration, using a Neubauer improved hemocytometer under a phase-contrast microscope (magnification x 400). Another 5-µL subsample was kept in an N-phenylthiourea-coated microcentrifuge tube (Sigma-Aldrich, St Louis, MO, USA, P7629) and stored at -80°C until later examination for antibacterial activity. The remaining haemolymph solution was diluted with 15 µL of PBS and immediately stored at -80°C for later measurement of PO and total-PO activities.

Antimicrobial activity of the eggs and the haemolymph was measured using a standard zone of inhibition assay (Moret 2006). Samples were thawed on ice, and egg extracts were prepared by smashing eggs into an acetic acid solution (0.05%, 5 µl per egg). After centrifugation (3500 g, 2 min, 4°C), 2 µL of the supernatant was used to measure antibacterial activity on zone of inhibition plates seeded with *Arthrobacter globiformis* from the Pasteur Institute (CIP105365). For antibacterial activity of haemolymph samples, 2 µL of the solution was directly used on zone of inhibition plates. Zone inhibition plates were prepared from overnight culture of the bacterium which was added to Broth medium containing 1% agar to achieve a final concentration of 10^5 cells per mL. Six millilitres of this seeded medium was then poured into Petri dishes and allowed to solidify. Sample wells were made using a Pasteur pipette fitted with

a ball pump. Two microlitres of sample solution was added to each well, and a positive control (Tetracycline: Sigma-Aldrich, St Louis, MO, USA, T3383; 2.5 $mg.mL^{-1}$ in absolute ethanol) was included on each plate as positive control (Zanchi et al. 2011). Plates were then incubated overnight at 28 °C. The diameter of inhibition zones was then measured for each sample. As there was slight variation among plates for inhibition zones resulting from tetracycline addition, the diameter of the zone of inhibition of each sample was adjusted to take into account such a between plate variation. For this, the Petri dish in which the inhibition zone resulting from the tetracycline positive control was the largest was used as reference. Then, for each sample, inhibition zone was multiplied by the ratio between the inhibition zone due to tetracycline in the reference Petri dish and that due to tetracycline in the Petri dish in which the sample was measured (Zanchi et al. 2011; Dhinaut et al. 2017).

For each individual haemolymph sample, both the activity of naturally activated phenoloxidase (PO) enzymes only (PO activity), and the activity of the proenzymes (proPO) in addition to that of the PO (total-PO activity), were measured using a spectrophotometer (Zanchi et al. 2011). PO activity was quantified without further activation, while total-PO activity required the activation of the proPO into PO with chymotrypsin. To this purpose, frozen hemolymph samples were thawed on ice and then centrifuged (3500 g, 5 min, 4°C). Five microlitres of supernatant was added to a microplate well containing 20 µL of PBS, and either 140 µL of distilled water to measure PO activity only, or 140 µL of chymotrypsin solution (Sigma-Aldrich, St Louis, MO, USA, C-7762, 0,07 $mg.mL^{-1}$ of distilled water) to measure total-PO activity. Then 20 µL of L-Dopa solution (Sigma-Aldrich, St Louis, MO, USA, D-9628, 4 mg mL^{-1} of distilled water) was added to each well. The reaction was allowed to proceed at 30°C in a microplate reader (Versamax; Molecular Devices, Sunnyval, CA, USA) for 40 min. Readings were taken every 15 s at 490 nm and analyzed using the software SOFT-Max Pro 4.0 (Molecular Devices, Sunnyval, CA, USA). Enzyme activity was measured as the slope (V_{max} value: change in absorbance unit per min) of the reaction curve during the linear phase of the reaction and reported to the activity of 1 µL of pure hemolymph.

II.5. Statistics

We estimated heritability of maternal investment into offspring immune protection (corresponding to the number of eggs produced exhibiting antibacterial activity, divided by the

number off eggs produced from each females of our inbred lines) as described by Archer et al. (2012) by calculating the coefficient of intraclass correlation (t) as:

$$t = \frac{nVb - Vw}{nVb + (n-1)Vw}$$

where n is the number of lines and V_b and V_w are the between-line and within-line variance components, respectively estimated directly from an analysis of variance (ANOVA) including line as the main effect. The standard error of the intraclass correlation (SE(t)) was calculated according to Becker (1984) as:

$$SE(t) = \sqrt{\frac{2(1-t)^2[1+(k-1)t]^2}{k(k-1)(n-1)}}$$

where k is the average number of individuals sampled within each line. The heritability (h^2) of maternal investment into offspring immune protection in TGIP was then estimated according to Hoffmann and Parsons (1988) as:

$$h^2 = \frac{2}{(\frac{1}{t} - 0.5)}$$

The standard error of this estimate, (SE(h^2)), was calculated according to Hoffmann and Parsons (1988) as:

$$SE(h^2) = \frac{2}{\left(1 - \frac{t}{2}\right)^2} SE(t)$$

We estimated phenotypic correlations (and their standard errors) between traits of mothers and their offspring, by using Pearson correlations with a bootstrap re-sampling of 1000 replicates. Heritability estimates and genetic correlations were considered statistically significant if the estimates divided by their standard errors were greater than 1.96, rejecting the null hypothesis of no correlation based on a two-tailed t-distribution with infinite degrees of freedom.

III. Results

Only 9 inbred lines out of 12 could be used to estimate maternal investment into egg protection through TGIP. Indeed, lines exhibited different developmental times and it took longer for some of the lines to obtain 5 generations of full-sib mating followed by free mating in panmixis. Furthermore, not all the 9 inbred lines could provide 20 females to estimate maternal investment into egg protection through TGIP as five of them could use the data of 8, 10, 12, 14 and 18 females per insect line. Hence heritability of investment into egg protection through TGIP was calculated using an average number of individuals of k =16 per insect line. Despite observing a large variation of investment into egg protection by females among inbred lines (Fig. 12; ANOVA: $F_{8,\ error} = 5.5$ $P < 0{,}001$), the heritability estimate found that trait was not found significant ($h^2 \pm SE = 0.38 \pm 0.24$ $h^2/se < 1.96$).

We found no consistent phenotypic correlations between maternal investment into egg protection through TGIP and other fitness traits of the mothers, with the exception of a negative correlation with mother body mass (Table 11). In the context of our study where mothers were immune challenged, there were consistent phenotypic positive correlations between immune defence of the offspring and that of their mothers, especially for PO and PPO (Table 11). This positive correlation exists also between antimicrobial activity of offspring and their immune-challenged mothers. By contrast, hemocyte concentration and the antibacterial immune response of the offspring were negatively associated to hemocyte concentration of mothers (Table 11). Survival and developmental time of the offspring was negatively correlated with the fecundity of their mothers (Table 11). Furthermore, larval developmental time of the offspring was also negatively correlated to their mother body mass (Table 11).

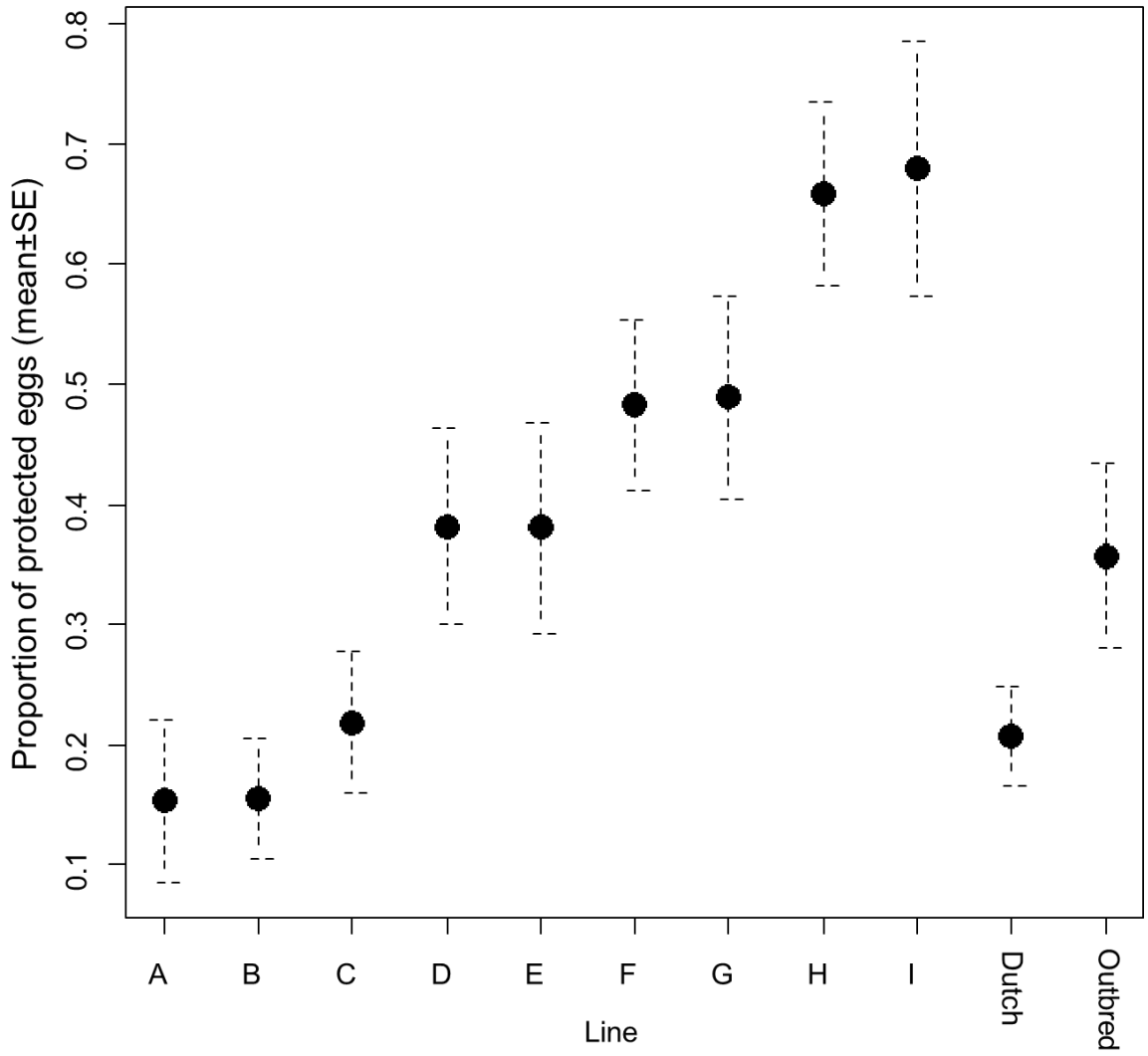

Figure 12: Proportion of protected eggs (mean±SE) depending of 9 inbred lines, Dutch our outbred control group.

Table 11: Phenotypic correlations (and SEs) between maternal and offspring fitness traits. Values below phenotypic correlation correspond to ratio between correlation and SE. Significant value (> 1.96) are in bold. Effect that have no significant correlation are not shown in the table. Proportion of protected eggs can be consider as part of immunity of offspring or as maternal investment. This trait is present in column and in line to estimate correlation with maternal and offspring fitness traits.

		Offspring											
		Proportion of protected eggs	Propoportion of surviving larvae	Larval Developmental time	Adult body mass	Longevity	Hemocytes post-challenge	PO pre-challenge	PO post-challenge	Total-PO pre-challenge	Total-PO post-challenge	Level of antibacterial activity pre-challenge	Proportion which exprimed antibacterial activity post-challenge
Mother	Proportion of protected eggs		-0.11±0.46 0.23	0.08±0.46 0.18	-0.34±0.40 0.84	0.41±0.47 0.87	-0.16±0.48 0.33	0.32±0.50 0.66	0.50±0.43 1.16	0.06±0.50 0.11	0.44±0.43 1.02	0.25±0.48 0.53	0.32±0.35 0.93
	Body mass	**-0.60±0.28** **2.15**	-0.49±0.45 1.08	**-0.75±0.28** **2.71**	0.07±0.42 0.16	**-0.86±0.36** **2.37**	0.45±0.40 1.12	**0.66±0.16** **4.15**	0.54±0.31 1.76	**0.72±0.22** **3.24**	**0.56±0.27** **2.10**	0.03±0.49 0.06	-0.33±0.39 0.85
	Fecundity	-0.24±0.42 0.57	**-0.82±0.17** **4.95**	-0.57±0.28 2.04	**0.81±0.35** **2.31**	-0.13±0.31 0.42	0.01±0.44 0.32	-0.12±0.36 0.32	0.32±0.41 0.78	-0.19±0.35 0.53	0.26±0.43 0.62	-0.25±0.39 0.64	-0.19±0.41 0.47
	Hemocytes	0.04±0.42 0.10	0.01±0.49 0.02	-0.56±0.44 1.27	0.36±0.37 0.96	0.31±0.37 0.83	**-0.74±0.16** **4.79**	-0.15±0.55 0.26	0.01±0.60 0.01	-0.10±0.47 0.21	-0.07±0.60 0.11	**-0.65±0.19** **3.42**	**-0.73±0.24** **3.00**
	PO	0.37±0.38 0.96	-0.57±0.33 1.74	-0.16±0.45 0.35	-0.10±0.45 0.22	-0.24±0.42 0.56	-0.27±0.45 1.08	**0.80±0.20** **4.06**	**0.78±0.30** **2.59**	**0.80±0.20** **4.06**	**0.78±0.30** **2.59**	-0.26±0.43 0.60	-0.46±0.39 1.17
	Total-PO	0.59±0.31 1.95	-0.47±0.38 1.23	0.17±0.44 0.38	-0.14±0.51 0.27	0.22±0.42 0.51	-0.55±0.41 1.36	0.55±0.30 1.84	0.60±0.31 1.91	0.55±0.30 1.84	0.60±0.31 1.91	-0.32±0.40 0.81	-0.38±0.38 0.99
	Antibacterial activity	0.37±0.41 0.90	-0.09±0.50 0.17	0.05±0.41 0.12	**-0.64±0.32** **2.03**	-0.23±0.49 0.48	**0.73±0.23** **3.17**	0.41±0.49 0.85	0.21±0.60 0.36	0.29±0.48 0.61	0.25±0.62 0.41	**0.90±0.18** **4.98**	**0.79±0.24** **3.26**

Discussion

TGIP is an increasingly studied phenomenon by testing for its existence in increasing number of invertebrate species and for its potential physiological and molecular mechanisms (Tetreau et al. in preparation; Chapter 4). However, the processes by which TGIP evolved and is maintained are still poorly studied. Particularly, much remains to be learned about individual differences within species to clearly infer about the ecological and evolutionary implications of this phenomenon. For instance, while the insect immune response is not known to be genetically variable (Rolff et al. 2005; Hammerschmidt et al. 2012), whether natural variation of TGIP in invertebrates has a genetic origin has never been tested to far. Here, using inbred lines, we studied the variation in egg immune protection by immune-challenged females of *T. molitor* previously found by Zanchi and colleagues (2012). If variation in investment into maternal transfer of immunity is genetically based, we predicted that this trait should be heritable. Then we tested potential phenotypic correlations between investment into TGIP and other female and offspring fitness related traits to examine whether these traits could evolve independently or whether they are genetically linked to each other, either facilitating or constraining their relative evolution. To our knowledge, this is the first study that quantified phenotypic covariance between maternal immune transfer of immunity, fitness of mothers and fitness of the offspring in an animal system.

The heritability value calculated from our data for maternal investment into egg protection was relatively high (h^2= 0.38), although not statistically significant as this value is associated to a large variance component (SE = 0.24). This may suggest that variation in TGIP in our study population cannot be not explained by genetics. However, such a conclusion might be hasty for two main reasons. First, our number of 9 inbred lines available (instead of 12) to calculate the heritability value is probably too low and may not cover the whole expected variation from the stock culture from which these lines originate. The addition of the 3 missing lines, which seem, at least, to have contrasted generation time compare to the 9 we used may stretch among inbred lines variance. Furthermore, based on the equation through the intraclass correlation coefficient is calculated, increasing the number of lines should automatically increase heritability value (Hoffmann & Parsons 1988; Archer et al. 2012). Second, only a relatively low number of females could be used in 3 of the available inbred lines (8, 10 and 12 females instead of 20, for the lines I, H and E, respectively), to estimate maternal investment into eggs protection. Large sample size within lines is also expected to decrease the standard error of the intraclass

correlation coefficient (SE(t)), which therefore should reduce the standard error of the heritability value ((SE(h^2)) (Becker 1984; Hoffmann & Parsons 1988). In addition, lines H and I are the lines that exhibited the most intense investment into egg protection through TGIP (Fig. 12) and are also those from which egg protection measurements were estimated on a relatively low number of females per lines (10 and 8 females, respectively). Adding data from additional females in these lines (to complete them to 20 females) may give them equivalent weight to compare to the other inbred lines and should therefore decrease the variance component of the heritability value. In other words, only a sufficient number of lines and females per lines will allow us to conclude on the heritability of egg protection through TGIP in our *T. molitor* population.

The correlation between line means is assumed to be an estimate of genetic correlation indicating whether quantitative traits have the same or different genetic bases (David et al., 2005). Since maintaining and using enhanced levels of immune defenses are costly (Moret & Schmid-Hempel 2000), enhanced levels of immune defense in primed offspring are expected to show trade-offs with others fitness related traits (Schmid-Hempel 2005), and can impact response to selection for TGIP, if this trait is genetically based (Grindstaff 2010). This might be particularly expected because TGIP was previously evidenced to have fitness costs (Sadd & Schmid- Hempel 2009; Roth et al. 2010; Zanchi et al. 2011; Moreau et al. 2012; Dhinaut et al. 2017). Here, we found no consistent phenotypic correlations between maternal investment into egg protection through TGIP and other fitness traits of mothers, with the exception of a negative correlation with mother body mass. Therefore, lines of light females are protecting a higher proportion of their eggs than lines of heavy females. In general, larger or heavier insect females are also those exhibiting higher fecundity (Thornhill and Alcock 1983). Here, lighter immune-challenged females appear to maximize the immune protection of their relatively low number of offspring compare to larger females. This results also suggest that investment into egg protection was not necessary costly for females. Indeed, it would be expected that smaller or lighter females would invest less into the immune protection of the eggs than larger females; the latter are expected to have more resources than the former. However, it is possible that the cost of maternal protection of eggs after an immune challenge with *B. thuringiensis* could be limited for mothers because antibacterial activity in the eggs does not result from a passive transfer of antibacterial substances from the mother to the eggs. Instead, eggs of *B. thuringiensis* immune-challenged females are producing themselves their own antibacterial activity (Dhinaut et al. submitted; Chapter 2). In this case, the cost of TGIP might be therefore mostly supported

by the offspring. Consistent with this hypothesis, we did not find any correlation between antibacterial activity of immune-challenged mothers and that of their eggs, either in terms of proportion of protected eggs or levels of antibacterial activity per egg.

Immunity of the offspring at the adult stage was associated to numerous traits of mothers. In particular we found contrasted correlations between immune defenses of the mothers and those of the offspring. While cellular immune defenses (hemocyte concentration) of mothers and the offspring after being immune challenged were negatively correlated, humoral defenses (phenoloxidase and antibacterial activity) of mothers and the offspring after being immune challenged were positively correlated. This suggests that while the expression of cellular defense in the offspring is traded off against that of their mothers, the use of humoral defenses in the context of TGIP could be rapidly selected. However, this might not be true for the expression of antibacterial activity in the offspring as it is also negatively associated to the cellular immune response of mothers. Only the offspring phenoloxidase activity appears to be not constrained by any of the immune defenses of the mothers. This suggests that PO activity could play an important role in the immune protection of primed offspring after a maternal immune challenge by *B. thuringiensis*. This is consistent with previous results showing that primed offspring of *T. molitor* mothers immune challenged by *B. thuringiensis* exhibit enhanced levels of PO activity (Dhinaut et al. 2017; Chapter 1).

Other traits of the offspring than immune defenses were found associated to mother fitness traits. Lines in which females are producing strong antibacterial immune response are also those producing lighter offspring, suggesting a cost of the maternal antibacterial immune response on offspring size. However, lines in which females were the most fecund were also those producing the heaviest offspring at the adult stage that developed into adult the most rapidly but at the expense of lower larval survival. Lines with heavy females also produced offspring that developed rapidly into adult, but the latter exhibited shorter longevity.

To summarize, we failed to conclude on the existence of genetic basis of TGIP because of large variance component associated to the calculated heritability value, possibly because of a too low number inbred lines available and with an insufficient sample size in some of the inbred lines used, to properly estimate heritability. Moreover, we did not find any correlation between egg protection through TGIP with any fitness traits of mothers and the offspring that would suggest that egg protection may not evolve at costs. However, while the expression of immune defenses in the offspring at the adult stage seems constrained by maternal immunity or the

expression of other mother fitness traits, the use of the phenoloxidase system (which does not appear to be costly on other fitness traits in our study) may have the potential to evolve rapidly in the context of TGIP against repeated infection by *B. thuringiensis*. This is consistent with previous finding showing that phenoloxidase plays an important role in the immune protection of the offspring of *T. molitor* mothers previously immune challenged with *B. thuringiensis* (Dhinaut et al. 2017; Chapter 1).

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Chapitre 4

Chapitre 4: Revue des études sur le transfert trans-générationel d'immunité

L'existence du TTGI a été testée chez de nombreuses espèces d'invertébrés. Cela a été fait en utilisant des approches et des méthodes d'études variées et différentes, que ce soit d'un point de vue fonctionnel ou évolutif.

Dans ce quatrième chapitre, nous avons passé en revue l'ensemble des études concernant le TTGI chez les invertébrés. Le but étant de confronté l'ensemble des données actuellement existantes afin de mettre en exergue les principales caractéristiques et les mécanismes identifiés, en fonction de l'écologie et de l'évolution du phénomène. Pour ce faire nous avons décrit tous les articles ayant explorés le TTGI à l'heure actuelle, tout en proposant une analyse critique des procédures expérimentales utilisées. Nous avons notamment identifié trois paramètres majeurs qui varient entre les études, alors que ceux sont des facteurs clés pouvant permettre de comparer les résultats obtenus par différents groupes de chercheurs, ou ceux résultant de l'étude d'une espèce hôte face à différents agents pathogènes. Ces paramètres d'études sont le procédé d'infection, le genre des individus étudiés et leur stade de développement.

Nous avons ensuite détaillé les quatre scénarios mécanistiques hypothétiques à travers lesquels le TTGI pourrait se réaliser. Cela pourrait se faire par un transfert de « signal » de la femelle à sa descendance, la provision d'ARNm dans les œufs codant pour des effecteurs immunitaires, le transfert d'effecteurs immunitaires directement à l'œuf, ou alors par la transmission d'une modification épigénétique suite à une infection maternelle.

Nous avons conclu ce travail en suggérant les méthodes expérimentales à appliquer afin de pouvoir déterminer quel scénario mécanistique est juste pour chaque espèce hôte et en proposant des conseils pour les prochains travaux de recherche sur le sujet.

Trans-generational immune priming in invertebrates: where are we now and where must we go?

Authors: G. Tetreau[$], J. Dhinaut[$], B. Gourbal, Y. Moret

[$]Authors contributed equally to this work

Submitted

Abstract

Trans-generational immune priming (TGIP) refers to the transfer of an individual immunological experience to its progeny, protecting it from repeated encounters with pathogens that may persist across generations. Although extensively studied in vertebrates for over a century, this phenomenon has only been identified seventeen years ago in invertebrates. Since then, invertebrate TGIP has been the focus of an exponentially increasing interest, with half of studies published during the last three years. TGIP has now been tested in numerous invertebrate systems using contrasting experimental approaches and various different measures to study it at both the functional and the evolutionary levels. However, drawing an overall picture of TGIP from available studies still appears a difficult task. Here, we provide the first extensive review of TGIP in invertebrates with the objective of confronting all the data generated to date to highlight the main features and mechanisms identified in the context of its ecology and evolution. To this purpose, we describe all the articles reporting experimental investigation of TGIP in invertebrates and propose a critical analysis of the experimental procedures performed to study this phenomenon. We then investigate the outcome of TGIP in the offspring and its ecological and evolutionary relevance before reviewing the potential molecular mechanisms identified to date. In the light of this review, we build hypothetical scenarios of the mechanisms through which TGIP might be achieved, and propose guidelines for future investigations.

I. Introduction

All living organisms developed defense strategies to protect themselves against potential pathogens, from the antiviral CRISPR-Cas system of prokaryotes to the complex immune system of vertebrates (Akira et al. 2006; Barrangou & Marraffini, 2014). The immune system of each species is the result of a long term evolution in response to its environment and to co-evolving pathogens (Sheldon & Verhulst 1996). This innate immunity generally allows organisms to be rapidly protected against a wide range of pathogens by recognizing a restricted set of molecules conserved through evolution and shared by several infectious agents, such as peptidoglycans (PGN) and lipopolysaccharides (LPS) from bacterial cell walls (Janeway & Medzhitov 2002). Historically, this generalist innate immunity based on non-clonal cells was opposed to adaptive immunity, which exhibits a high pathogen specificity through the production of antibodies by clonal cells in vertebrates (Janeway et al. 2002). However, this dichotomic view of immunity as either innate or adaptive has been challenged and the line between them became more and more blurry as the number of empirical evidence for an innate immune memory increased over the last decade (Kvell et al. 2007; Netea et al. 2016; Vivier & Malissen 2005). In vertebrates, innate immune cells, such as macrophages and natural killer (NK) cells, can be "trained" to trigger an improved immune response after a second encounter with the same and/or with other pathogens than the one used for priming (Saeed et al. 2014; Sun et al. 2014). Immune memory in vertebrates is therefore far more complex than just restricted to antibody production and can notably involve epigenetic modifications, such as histone acetylation and microRNA that target molecular actors of the innate immune system (Netea et al. 2011).

Such trained innate immunity also exists in invertebrates (Kurtz 2005). Indeed, sublethal doses of *Streptococcus pneumoniae* induced a protection of *Drosophila melanogaster* to a second infection with otherwise lethal doses of the same pathogen by a mechanism dependent on phagocytes (Pham et al. 2007). This phenomenon can also be nonspecific as antimicrobial peptides production triggered upon exposure to bacterial LPS provided an increased survival to fungal infection in *Tenebrio molitor* (Moret & Siva-Jothy 2003). Nevertheless, invertebrate immunity does not solely rely on the innate immune system as previously thought, considering that it also possesses immune receptors with the potential for somatic diversification similar to antibodies in vertebrates (Du Pasquier 2006). These include notably the fibrinogen-related proteins (FREPs) in mollusks (Adema 2015) and the Down Syndrome Cell Adhesion Molecule (Dscam) in arthropods (Brites & Du Pasquier 2015). Generally, this heightened response upon

a second infection relies on either the sustained or the recalled response involving the same immune actors as for the primary response (Coustau et al. 2016). Nevertheless, recent work on the snail *Biomphalaria glabrata* revealed a shift from a primary cellular immune response, involving encapsulation of *Schistosoma mansoni* by haemocytes, to a secondary humoral response, consisting in *S. mansoni* elimination mediated by FREPs and cytotoxic/cytolytic molecules from plasma (Pinaud et al. 2016). This highlights how the invertebrate immune memory can be mediated by simple mechanisms as well as by complex processes with a high variability across different invertebrate taxa, probably due to the large variety of contrasting organisms that constitute the group of invertebrates (Milutinovic & Kurtz 2016).

Beyond the establishment of an immune memory in individuals (within-generation), parents can also transfer their immunological experience to their offspring with the aim of protecting them against recurrent pathogens present in their environment. This trans-generational parental effect has been first identified and extensively studied in vertebrates in which offspring enhanced immunity can be mediated by maternal antibodies, hormones and nutrients provided via placenta and milk in mammals and via the egg yolk in birds and reptiles (Hasselquist & Nilsson 2009). This parental effect can also be induced by an epigenetic reprogramming of the fetus that can last for several generations (Ho & Burggren 2010). In the light of these different processes and considering the parallels highlighted previously between the trained immunity of vertebrates and the immune memory of invertebrates, it is not surprising that this phenomenon of trans-generational immune priming (TGIP) has also been discovered in invertebrates (Huang & Son 1999; Moret 2006). In invertebrates, TGIP specifically refers to the vertical transmission of the immunological experience from the parent(s) to the offspring and is therefore a specific case of social immunization, which is widespread in many social insects and also comprises horizontal transfers between adults and between adults and other parents' offspring (Masri & Cremer 2014). Many articles have been published on TGIP in invertebrates on a wide range of biological models using a large variety of approaches. Therefore, there is a need to confront all the data generated to date and to highlight the main features and mechanisms identified. The present work aims at being the first extensive review of TGIP in invertebrates. To do so, after a description of all the articles reporting experimental investigation of TGIP in invertebrates (section II), we propose a critical analysis of the experimental procedures performed to study TGIP (section III). We also investigate the outcome of TGIP in the offspring and its ecological and evolutionary relevance (section IV) followed by the different potential molecular mechanisms identified to date (section V). Altogether, this allows us to build hypothetical

scenarios of the mechanisms leading to TGIP based on empirical data (section VI) and to propose guidelines for future investigations (section VII).

II. TGIP, an increasingly studied phenomenon

Since its first discovery in 1999 in the crustacean *Penaeus monodon* from mothers exposed to β-glucans with offspring protected against the white spot syndrome associated virus (WSSV) (Huang et al. 1999), a total of 44 articles investigating TGIP in invertebrates has been published (Figure 13). The number of articles published on this topic remained low for more than a decade and then exhibited an exponential increase, with half of the articles published during the last three years, reflecting the recent craze for this new field of research in invertebrate immunity (Figure 13). So far, TGIP has been investigated on 24 different invertebrate species belonging to 10 different phylogenetic groups, including 2 articles published on Bivalvia, 4 on Brachiopoda, 11 articles on Coleoptera, 5 on Diptera, 1 on Hemiptera, 6 on Hymenoptera, 10 on Lepidoptera, 1 on Malacostraca, 3 on Nematoda and 1 on Orthoptera (Figure 14). Although some mollusks and nematode have been studied, TGIP studies are strongly biased toward arthropods, representing 89% of all TGIP studies, and many invertebrate groups have not been investigated yet (Figure 14).

While TGIP has been clearly evidenced in all coleopteran, crusteacean, hymenopteran, orthopteran and mollusk species investigated to date, some other phylogenetic groups exhibit more contrasted patterns (Figure 14). Indeed, only one out of the five articles on Diptera provided evidence for TGIP. TGIP was found in *Anopheles gambiae* larvae to the microsporidia *Vavraia culicis* (Lorenz & Koella 2011), while exposure of *D. melanogaster* mothers to bacteria and of three mosquito species to *Plasmodium sp.* and to negatively-charged beads did not trigger any increased immune protection of the offspring (Linder & Promislow 2009; Pigeault et al. 2015; Vantaux et al. 2014; Voordouw et al. 2008). Similarly, for Lepidoptera, two articles focusing on *Plodia interpunctella* exposed to the bacteria *B. thuringiensis* and on *Trichoplusia ni* challenged with *Autographa californica multiple nucleopolyhedrovirus* did not find evidence of TGIP (Littlefair et al. 2016; Shikano et al. 2016). Interestingly, parental challenge of the same species to different pathogens, *P. interpunctella* to the *granulosis virus (PiGV)* and *T. ni* to the bacteria *Escherichia coli* and *Micrococcus luteus*, did provide immune protection of the offspring (Boots & Roberts 2012; Freitak et al. 2009; Tidbury et al. 2011). This suggests that TGIP might depend on the pathogen used for priming and/or on the procedure used for infection.

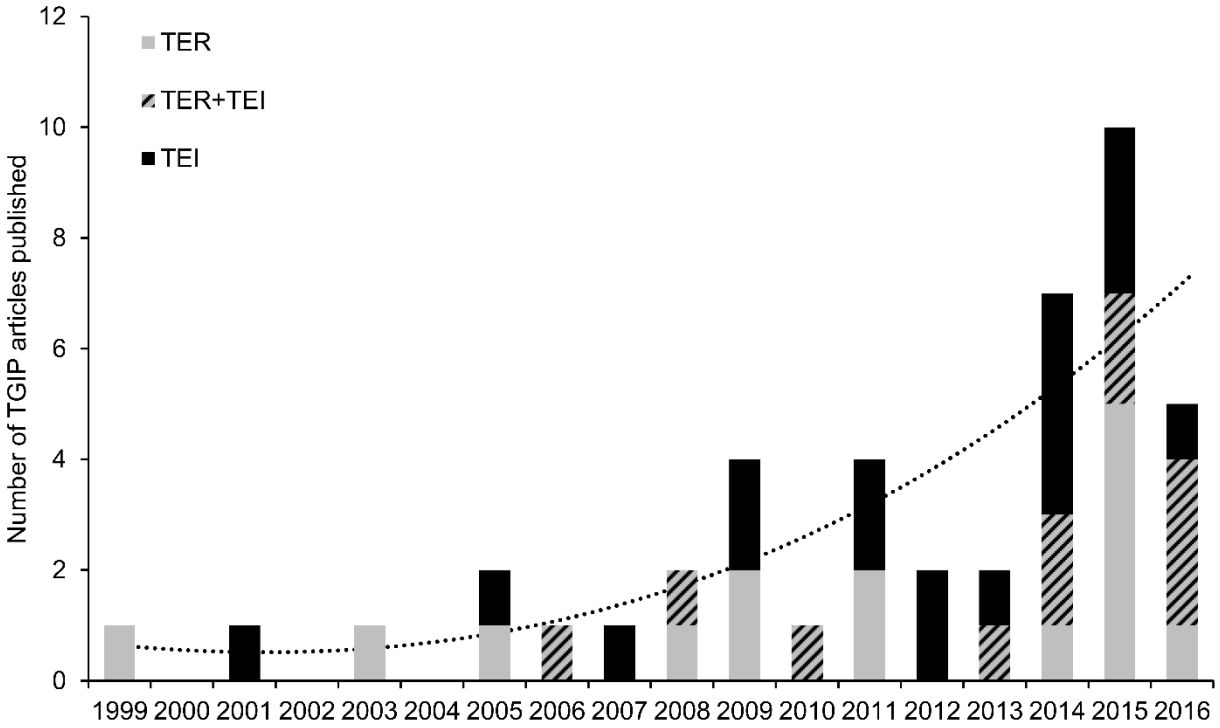

Figure 13. Total number of articles published in peer-reviewed journals that investigated TGIP in invertebrates. Articles evaluating the physiological consequence on offspring of parental pathogen exposure (*e.g.,* parasite prevalence & intensity, host fitness & survival) are indicated as TER (transgenerational effect on resistance; grey color) while those focusing on the impact on offspring immunity (*e.g.*, number of haemocytes, modified expression or activity of AMPs, PPO or immune pathways) are indicated as TEI (transgenerational effect on immunity; black color), following the updated nomenclature proposed by Pigeault *et al.* (2016). Articles that evaluated both parameters are hatched in black and grey and indicated as TER+TEI.

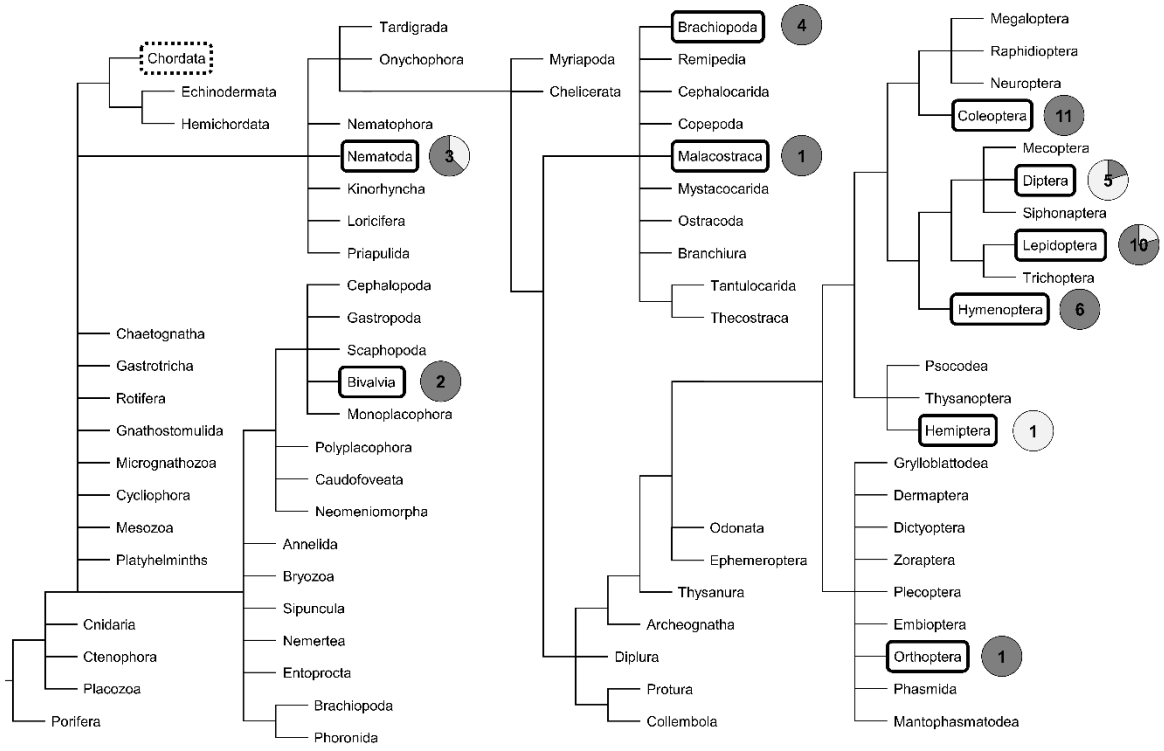

Figure 14. Phylogeny of invertebrates, adapted from Tree of Life Web Project (Maddison & Schulz, 2007). Taxa in which transgenerational immune priming has been investigated are boxed. The circle charts indicate the proportion of TGIP studies that reported the presence (dark grey) and the absence (light grey) of TGIP for each phylogenetic group boxed. The number indicated inside the circle chart is the number of TGIP studies reported to date for each phylogenetic group. The group of Chordata, which includes the vertebrates, is highlighted by a dotted box.

It is noteworthy that the reports of TGIP are essentially biased toward model species and positive results, although 8 out of the 44 articles published to date reported an absence of offspring immune protection upon parental priming. Such effort of publishing negative results is essential and provides key information in recent fields like TGIP as it allows deciphering between real absence of TGIP and lack of experiments performed on some taxa (Torgerson 2010). Considering that TGIP is protecting offspring from pathogens experienced by their parents, it is expected to be a selected process in species with long life-span (over 60 days) and limited dispersion, increasing the chances for the offspring to encounter a pathogen diversity similar to their parents' one (Pigeault et al. 2016). This should be taken into account while investigating TGIP in new species and discussed accordingly.

By building a network of all the authors that published empirical reports of TGIP, two main categories of studies arise based on the research group that published them (Figure 15). It reveals that few research teams extensively work on immune priming, and by extension on TGIP. They produced several articles on their model with the objective of testing different hypothesis and deciphering details of the TGIP process. However, the network is still sparse and not highly connected and it reflects the relative novelty of this field of research. This is probably going to evolve with the development of this research theme and considering that few different teams already published on the same species without collaborating yet. On the other side, many articles have been published by teams whose primary objective was to determine if TGIP is present in their model species rather than to identify the underlying mechanisms at play. This has consequences on the way the experiments were designed and therefore, it can complicate the comparisons between different studies from different research groups.

Another clustering can be made based on the approach used to identify TGIP, with articles evaluating the physiological consequence of pathogen infection (*e.g.*, parasite prevalence & intensity, host fitness & survival) while others focused on the impact on host immunity (*e.g.*, number of haemocytes, modified expression or activity of antimicrobial peptides (AMPs), prophenoloxidase (PPO) or immune pathways) (Figure 13).

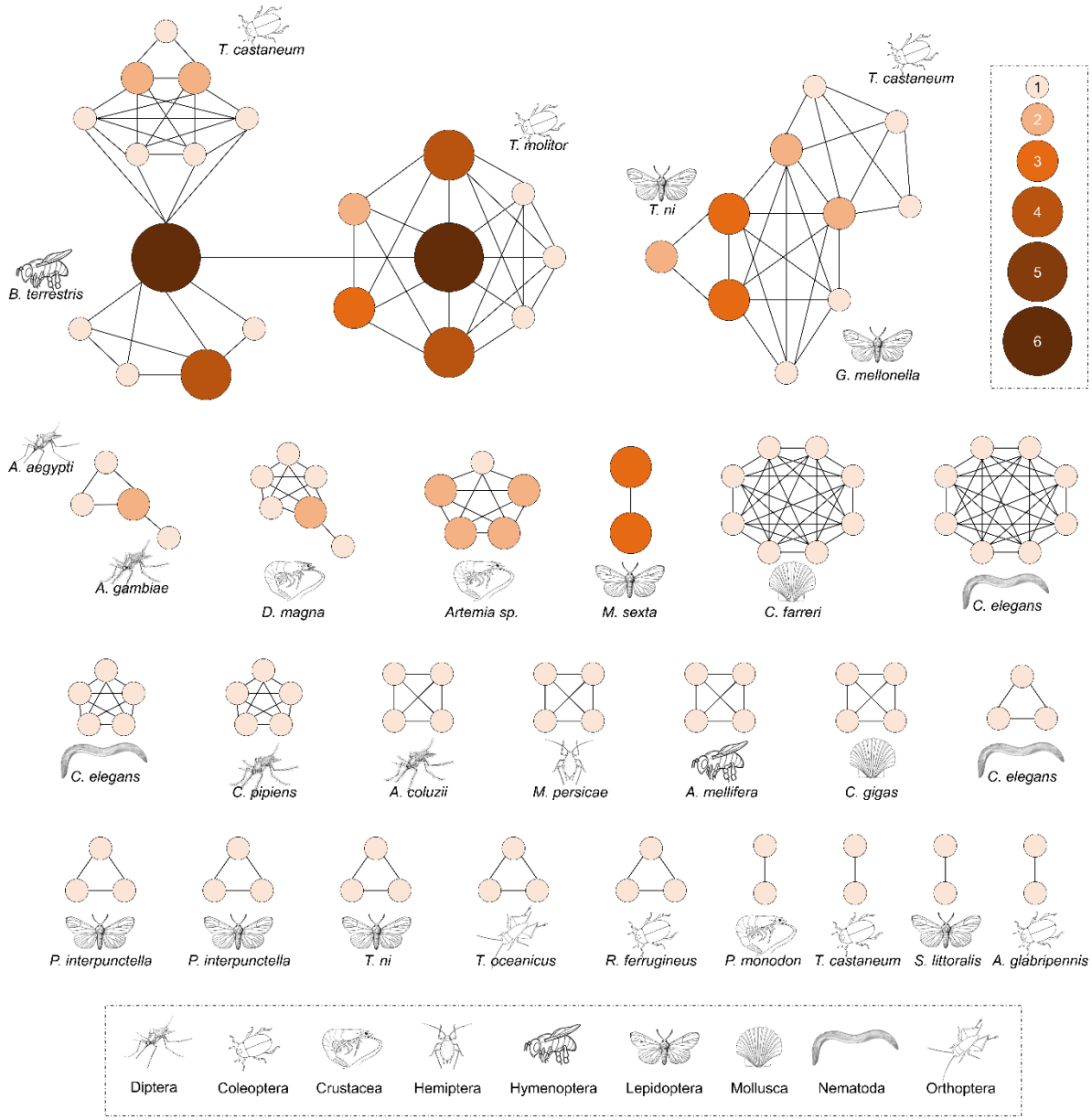

Figure 15. Network of all authors that published articles reporting experimental investigation of TGIP in invertebrates. Each author is represented by a sphere which size and color indicate the number of TGIP articles he published, from 1 (light brown, small) to 6 (dark brown, big). When two authors co-published at least one article on TGIP, their spheres are linked by a dark line. Therefore, two clusters of spheres with no line connecting them indicate that these two groups of scientists didn't publish any article together. The species name on which a group of authors published is indicated, together with a pictogram for the phylogenetic group of the species.

TGIP is a generic term that has been historically given based on phenotypic observations to describe the process of immune transfer, irrespective of the molecular mechanism involved (Moret 2006). Due to the identification of some mechanisms and to the multiplication of contrasting experiments performed, it might need to be redefined and clarified as part of a global brainstorming about the nomenclature used in invertebrate immunity. A first attempt was recently made by Pigeault et al. (2016) who named these two categories transgenerational effect on resistance (TER) and transgenerational effect on immunity (TEI), respectively, both referring to an overall transgenerational protection (OTP). The large diversity of approaches used can therefore render the comparison of the results obtained complex, even for the same species, and the relevance of the choice of key parameters needs to be evaluated.

III. Critical analysis of approaches used to study TGIP

The different TGIP studies exhibited a high variation in the procedure that they used to investigate TGIP (Table 12). Most notably, we identified three major parameters that showed a high variability between studies and that we believe are key to be able to compare the results obtained from different groups of scientists, between different pathogens from the same study and to properly discuss the relevance of the results obtained: the infection procedure, the gender of the insect host and the developmental stage studied. The influence of some of these parameters have already been discussed in the context of immune priming and within-generation immune memory in invertebrates (Milutinovic et al. 2016) but we are adopting another angle of view as we propose to add another layer, which is the temporal and cross-generational dimension.

III.1. The infection procedure

The method used to infest the host with the pathogen is chronologically the first step of any experiment but the choice of the procedure of infection and of the pathogen studied also orientate and determine the extent to which the analysis and conclusions can be drawn from it. Two interacting notions will be discussed here: the injection *versus* ingestion of the pathogen by the host and the use of inactivated *versus* living pathogens for the exposure of the parents and/or offspring.

Table 12. Summary of the main features of TGIP identified in different phylogenetic groups and species.

Group studied	Species studied	Parental priming	Priming way	TER benefit	TEI benefit	Costs
Coleoptera	*Anoplophora glabripennis*	Bacteria† and fungi°†	Injected	Survival (adults)	Not tested	Not tested
	Rhynchophorus ferrugineus	Bacteria°	Injected	Not tested	Enhanced PO and antibacterial activity (larvae)	Not tested
	Tenebrio molitor	Bacteria†, fungi† or LPS	Injected	Not tested	Enhanced antibacterial activity (larvae). Enhanced antimicrobial activity (eggs). Enhanced haemocyte concentration or PO activity (adults).	Trade-off between maternal immune response and egg protection (antibacterial activity). Longer offspring development time.
	Tribolium castaneum	Bacteria°† and/or parasite°	Injected, ingested or parasitized	Survival (adults)	Modified gene expression (eggs). Enhanced expression of PGRP receptors and enhanced PO activity (adults)	Lower antibacterial activity in adults. Longer develomental time. Lower offspring fecundity
Crustacea	*Artemia sp.*	Bacteria°	Ingested	Survival (larvae)	Enhanced gene expression (larvae)	Not tested
	Daphnia magna	Bacteria°	Ingested	Lower susceptibilty (larvae)	Not tested	Not tested
	Penaeus monodon	b-1,3-1,6-glucan	Injected and ingested	Survival (larvae)	Not tested	Not tested
Diptera	*Anopheles gambiae*	Microsporidia°	Ingested	Lower susceptibilty (adults)	Not tested	Longer offspring developmental time
Hemiptera	*Myzus persicae*	Parasitoid°	Parasitized	Lower susceptibilty (nymphs)	Not tested	Not tested

Footnotes: ° = alive pathogen; † = inactivated pathogen

Group studied	Species studied	Parental priming	Priming way	TER benefit	TEI benefit	Costs
Hymenoptera	*Apis mellifera*	Bacteria†	Injected	Survival (larvae)	Enhanced prohaemocytes-to-haemocytes differenciation (larvae)	Not tested
	Bombus terrestris	Bacteria† or LPS	Injected	Not tested	Enhanced antibacterial activity (worker adults, eggs). Enhanced PO activity (male adults). Enhanced gene expression (worker adults)	Parents produced less offspring. Decreased PO in offspring adults workers. Increased susceptibility in adults to a parasite unrelated to the maternal challenge
Lepidoptera	*Galleria mellonella*	Bacteria°	Ingested	Not tested	Modified gene expression (eggs)	Not tested
	Manduca sexta	Peptidoglycan	Injected	Reduce parasitoid development and its emergence (eggs)	Enhanced PO and antibacterial activity (eggs, larvae). Enhanced gene expression (eggs)	Faster reduction of antibacterial activity in adult offspring. Reduced offspring fecundity
	Plodia interpunctella	Virus° (not efficient with bacteria° and fungi°)	Ingested	Lower susceptibilty (adults)	Not tested	Not tested
	Trichoplusia ni	Bacteria° (not efficient with virus°)	Ingested	No (but just tested with one virus)	Enhanced PO activity (larvae). Modified gene expression (eggs, larvae)	Not tested
Mollusca	*Chlamys farreri*	Bacteria†	Injected	Survival (all stages)	Increased number of proteins and mRNA (eggs, larvae and adults)	Not tested
	Crassostrea gigas	Poly(I:C)	Injected	Survival (larvae)	Modified gene expression (larvae)	Not tested
Nematoda	*Caenorhabditis elegans*	Virus°	Ingested	Not tested	Parental RNAi transfer (larvae)	Not tested
Orthoptera	*Teleogryllus oceanicus*	Bacteria°	Injected	Not tested	Enhanced antibacterial activity (male adults)	Reduced son's sperm viability and daughter's ovary mass

Footnotes: ° = alive pathogen; † = inactivated pathogen

III.1.a. Injection *versus* ingestion

Most TGIP articles reported parental priming by injection (60%) while animals were fed with the pathogen in 40% of cases. Far from being trifling, the immune response of the host can greatly vary depending on the infection route of the pathogen (Behrens et al., 2014). Moreover, the choice of the infection procedure must take into account the pathogen biology and ecology, and must be driven by the co-evolutionary interactions between the host and the pathogen (Martins et al. 2013; Tate 2016). If we take the example of the bacterium *Bacillus thuringiensis*, which is a widely used pathogen for immune priming of invertebrates; *B. thuringiensis* is able to produce pore-forming toxins as a crystal during its sporulation, which are toxic for insects after ingestion of both crystals and spores (Schnepf et al. 1998). After solubilization of the crystal, toxins are released, bind to specific receptors at the surface of epithelial cells, which leads to the perforation of the gut to allow the spores to reach the hemolymph to germinate and the bacteria to proliferate (Vachon et al. 2012). Insect hosts and *B. thuringiensis* subspecies coevolved, one to survive to the toxins produced and to the bacterial infection and the other by adjusting the cocktail of toxins and its infectivity (Masri et al. 2015). Therefore, a realistic approach would be to use both spores and toxins of *B. thuringiensis* to allow the entire process to occur during the priming. But in this case, there could be confounding effects, as exposure to toxins alone can trigger a modified immune response that can bias the measurement of the host response toward the bacteria (Ericsson et al. 2009; Rahman et al. 2004). At the opposite, directly injecting *B. thuringiensis* bacteria into the hemolymph mimics the proliferation step of the bacteria but it bypasses the gut disruption step and cancel any potential influence that these damages could have on the immune response capacity of the host (Behrens et al. 2014; Matzinger 1994; Moreno-Garcia et al. 2014). Simplifying the exposure is of course mandatory to disentangle potential interacting factors, but the results must be critically discussed in regard with the infection procedure and its relevance to the ecology of the pathogen used.

Furthermore, many TGIP studies compared different pathogens while using the same infection procedure and usually drive general conclusions on different pathways in response to the different pathogens, without taking into account the adequacy between the infection procedure and the pathogens studied. This particular point requires a specific attention to ensure that no overstated conclusions are driven that could be misleading and lead to an erroneous view of the universality of TGIP process and mechanisms.

When studying TGIP, which is an evolutionary selected process, the choice of the infection procedure is even more important than for within-generation priming and it could drastically change the outcome of the priming and bias the resulting conclusions. One good example comes from *Caenorhabditis elegans* exposed to *Orsay virus*, which is a virus that specifically infects *C. elegans* nematodes by oral route (Felix et al. 2011). Two articles have been published to study TGIP upon exposure of *C. elegans* to *Orsay virus*: one reported the presence of TGIP after parental larval ingestion of the virus (Sterken et al. 2014) while the other did not present any evidence for improved offspring immunity when *Orsay virus* was injected to adult parents (Ashe et al. 2015).

III.1.b. Inactivated *versus* living pathogen

Half of TGIP studies used living pathogens for priming the parents while one quarter used specific immunogens, such as PGN and LPS, and the other quarter inactivated pathogens, mostly by heating treatment. Living pathogens are generally used at a sublethal dose to avoid confounding TGIP with the effect of selection, which happened in one TGIP study on *D. melanogaster* (Linder et al. 2009). At the opposite, high concentration of inactivated pathogen is generally injected to the host, which is supposed to mimic an infection in the hemolymph with a massive load of antigens but is lacking all the response of the host to its pathogenicity. The inactivation procedure itself, notably by heat treatment, can also affect the immunogenicity of the pathogen and the corresponding response of the host either by increasing the release of antigens or by altering their three-dimensional structure (Milutinovic et al. 2016). Although the presence of antigens from uncommon pathogen might trigger some immune response (Pradeu et al. 2013), this response might not be complete and might lack all damage-associated immune mechanisms of response of the host (Matzinger 1994; Moreno-Garcia et al. 2014; Pradeu & Cooper 2012). Moreover, if TGIP is triggered by an antigen dose-dependent mechanism, sublethal doses of living pathogen might not be sufficient to induce a full within-generation and trans-generational immune priming (Milutinovic et al. 2016; Wu et al. 2016).

III.2. The gender of parents & offspring

III.2.a. The gender of parents

Although mothers and fathers have been shown to both participate to offspring's immunity, this protection can be qualitatively and quantitatively different between the two sexes. This has been evidenced in studies investigating the effect of both mother and father, exposed separately to

bacteria or LPS, on the offspring immune status of the lepidopteran *T. ni* (Freitak et al. 2009), the orthopteran *Teleogryllus oceanicus* (McNamara et al. 2014) and the coleopteran *Rhynchophorus ferrugineus* (Shi et al. 2014), *T. molitor* (Zanchi et al. 2011) and *Tribolium castaneum* (Roth et al. 2010).

Nevertheless, in 29% of TGIP studies, parents were not separated according to gender, essentially because they were exposed at the larval stage at which sex identification can be tricky in invertebrates. The main problem is that maternal and paternal effects might be confounded. If only one of the two parents is providing most, if not all, trans-generational immune protection, this effect might be diluted and potentially not detected or underestimated. An additional factor could bias the experiments performed on unseparated sex. In *Drosophila*, males are known to enhance female immunity after mating, notably by activating Imd and Toll pathways and by promoting antimicrobial peptide (AMP) gene expression in females due to the transfer of male seminal fluid proteins (SFPs) (Domanitskaya et al. 2007; Peng et al. 2005). These SFPs can also affect *Drosophila* female's behavior by decreasing their receptivity to further mating and by increasing egg laying (Chen et al. 1988; Liu & Kubli 2003), which could also affect the extent of egg immune protection. A role of SFPs on females' immunity and physiology has also been evidenced in several other invertebrate species such as *Aedes aegypti*, *An. gambiae* and *Apis mellifera* (Avila et al. 2011). To date, the consequence of paternal priming on offspring through mating-associated increased maternal immunity has only been indirectly investigated once. It revealed that lytic activity in unchallenged females was similar if they were mating with a challenged male or an unchallenged one, and that no modified lytic activity of offspring was observed after challenge of any of the two parents (McNamara et al. 2014). This parameter might not be the good proxy for characterizing such effect of the father on mother's immunity and their offspring's one. The same procedure followed by McNamara et al. (2014), *i.e.* mating unchallenged females with either challenged or unchallenged males and measuring immune parameters in both the mother and the offspring, must be applied to other species. Other parameters should be monitored, such as immune gene expression, prophenoloxidase and antimicrobial activities that are generally more responsive to TGIP.

Furthermore, data on TGIP are essentially biased toward the mother effect, as 56% of total TGIP articles focused on mother-transferred immunity only. Even if female TGIP is consistent with parental investment theory because mothers invest more into rearing their offspring than fathers, immune priming is not directly linked to parental care, as it is often the case in insects that abandon their eggs (Jokela 2010). After oviposition, both sexes might benefit from

protecting their offspring. This protection that offspring receives from mothers and fathers may be more than additive and could result in a general better protection against pathogens. There is a need to increase the number of studies including paternal effect to re-equilibrate the balance and provide a more comprehensive view of the sex-dependent TGIP process (Eggert et al. 2014).

III.2.b. The gender of offspring

In the oceanic field cricket, *T. oceanicus*, the antibacterial immune response of male offspring was mediated by a complex interaction between maternal and paternal immune status (McNamara et al. 2014). Moreover, a sexually dimorphic TGIP was found, as female offspring did not exhibit immune protection when male offspring did (McNamara et al. 2014). These observations suggest that both parental and offspring genders can induce contrasting TGIP phenotypes and that they should be taken into account when studying TGIP.

III.3. The developmental stage

The life cycle of invertebrates is constituted of a sequence of several developmental stages that strongly differ in term of metabolism, physiology and immunity. Therefore, the choice of the developmental stage of the parents for priming and of the offspring for measuring the outcome of TGIP is far from being trivial. The choice of a specific developmental stage for the priming of the parents has often been driven by the adequacy to the pathogens used and by the easiness of their manipulation. For the choice of the offspring developmental stage, most articles focused on a unique specific stage, which can have consequences on the phenotype observed and on the conclusion of the study. Generally, offspring were studied at the same stage at which parents were exposed, which is the most ecologically relevant, or in the egg to study the effect of TGIP at the very first steps of offspring development.

In the mollusk *Chlamys farreri*, the immunity of the offspring from mothers exposed to the bacterial pathogen *Vibrio anguillarum* was studied at different ontogenic stages (4-cell, blastula, gastrula, trochophore), from egg to larva (Yue et al. 2013). It showed that, antibacterial activities, the expression of some immune genes, and that of an enzyme of the antioxidant system, the superoxide dismutase (SOD), differed depending on the stage at which they were measured (Yue et al. 2013). In the moth, *Manduca sexta*, the monitoring of the melanisation index, lysozyme and antimicrobial activities in offspring from PGN-primed parents revealed that there was a high fluctuation (from 2 to 100 fold) of these parameters between different larval instars, the pupal and the adult stages (Trauer & Hilker 2013). As a consequence, focusing

only on a limited number of offspring developmental stages increases the risk of missing the main TGIP effect. A good example comes from the Gastropoda *B. glabrata* in which no TGIP has been found after parental exposure to the metazoan parasite *S. mansoni* in 10-day and 60-day old offspring (unpublished data). However, TGIP should be a selected mechanism in this species as it exhibits a low dispersion and can live up to several months (Pigeault et al. 2016) and that evidence for maternal protection of the eggs by transfer of immune proteins have already been reported (Baron et al. 2013; Wang et al. 2015). One could then argue that studies reporting absence of TGIP might just have missed the developmental stage at which it is expressed. This highlights the importance of following offspring's immunity at different life stages, from egg to adult.

Another important factor to take into account is the time elapsed since the parental priming was performed, as the effect of priming in the mother might not be stable over time and so might impact the transfer of this immune experience to the offspring. Such phenomenon has been characterized in *T. molitor* in which the antibacterial activity in mother's hemolymph decreased every day since the priming occurred until the tenth day (Zanchi et al. 2012). They also observed that the transfer of this antibacterial activity to the eggs was one-day delayed and that eggs older than nine days exhibited a significant decreased antibacterial activity until none was detected at eleven days (Zanchi et al. 2012). Therefore, TGIP must be considered as a dynamic process with a temporal dimension that experiments focusing on a unique life stage might miss.

IV. An ecological and evolutionary perspective of TGIP

IV.1. Is TGIP a specific or generalist mechanism?

Previous studies on TGIP have revealed that a wide range of specificity occurs, from cross-reactive (non-specific) to highly specific. Specificity refers to the degree to which immune priming discriminates pathogens at different levels of relatedness between the primary infection and the subsequent ones, either within or across generations. Trans-generationally primed individuals can exhibit cross-reactive elevation of their immunocompetence, as it was observed when a maternal immune challenge of the shrimp *P. monodon* with glucans from yeast protected the offspring against the white spot syndrome virus (Huang et al. 1999). Conversely, TGIP was found to be pathogen-specific in the red flour beetle, *T. castaneum* (Roth et al. 2010), and in the crustacean *Daphnia magna* (Little et al. 2003) and *Artemia sp.* (Norouzitallab et al. 2016), although the immune effectors involved were not identified. In *T. castaneum*, TGIP was dependent on the combination of bacteria (*B. thurinigiensis* or *E. coli*) used for the parental

priming and the offspring challenge. The offspring exposed to the same bacteria as their parents exhibited lower mortality than the offspring exposed to a different bacteria as their parents, indicating that TGIP was specific to the bacterial species (Roth et al. 2010). In the Asian longhorned beetle, *Anoplophora glabripennis*, maternal exposure to the bacterium *Serratia marcescen*s provided non-specific protection to offspring against a fungal pathogen. However, TGIP in response to the fungus *Metarhizium* only occurred when offspring were exposed to the same fungal species that was used to prime mothers, indicating a degree of specificity at the species level within the same genus of pathogen (Fisher & Hajek 2015). Such a level of specific TGIP revealed by these two above studies has been shown in offspring at the adult stage only and its occurrence at earlier ontogenetic stages is currently unknown. A study using the mealworm beetle, *T. molitor*, tested the existence of such a level of specificity of TGIP in eggs (Dubuffet et al. 2015). Mothers were exposed to a large range of microbial pathogens and resulting antimicrobial activity of their eggs was tested against several microorganisms, including those used for mothers' exposure, using inhibition zone assays. The antimicrobial activity found in the eggs was only active against Gram-positive bacteria, even when mothers were exposed to Gram-negative bacteria or to fungi (Dubuffet et al. 2015). This seems to be due to an antibacterial peptide from the defensin family (tenecin 1), which spectrum of activity is exclusively directed toward Gram-positive bacteria. Contrary to the phylogenetically close species *T. castaneum* (Roth et al. 2010), the induction of immune response was only directed against one type of pathogens, irrespective of the maternal immune challenge. Moreover, in this study, fungi were weak inducers of TGIP, whereas similar levels of anti-Gram-positive activity were found in eggs of mothers exposed to Gram-positive or Gram-negative bacteria (Dubuffet et al. 2015). This is really surprising considering that in another Coleoptera species, *A. glabripennis*, specific TGIP was found only against fungi but not against bacteria (Fisher et al. 2015). This highlights the diversity of TGIP, with host-specific and pathogen-specific characteristics, and it suggests that the mechanisms at play are likely multiple and complex.

IV.2. Is TGIP always costly for the fitness of parents & offspring?

Although TGIP is beneficial by conferring the offspring enhanced protection to pathogens that persist in the environment over the parental generation, it may also come at costs. Testing fitness costs of TGIP is important because it informs us about the adaptive nature of the phenomenon and tells us about the conditions of its evolution. Indeed, under the assumption that organisms are selected to optimally allocate limited resources among fitness-associated traits (Roff 1992; Stearns 1992), the presence of trade-offs involving TGIP might be an

important limit to adaptation. Several lines of evidence suggest that TGIP is costly. First, TGIP requires the immune challenge of the parents to be induced. Such an inducible aspect of defense in the offspring is assumed to evolve when it is costly to use, preventing negative impacts on fitness in the absence of threat (Agrawal et al. 1999). In the absence of costs, primed levels of immunity would be expected across all the offspring, independently of the parental experience. Another potential indirect evidence of the cost of TGIP may come from studies that failed to demonstrate its occurrence in some invertebrate models (*e.g.*, (Linder et al. 2009; Vorburger et al. 2008)). Under particular ecological conditions, the fitness cost of TGIP might be too high for being selected in these species. Costs associated with TGIP may take the form of trade-offs between the protection of the offspring and other important fitness traits of both the parents and the offspring, which will presumably have a selective disadvantage if infection risks do not persist over the parental generation.

In addition of paying the usual immune activation costs (Moret & Schmid-Hempel 2000), immune-challenged parents also expected to pay a cost to TGIP when synthesizing and transmitting effectors and/or elicitors of immunity to their offspring. Such a cost is relatively difficult to demonstrate as it might be confounded with that of the immune response produced by immune-challenged parents. A prerequisite for attributing cost to TGIP is that it should be associated with enhanced immunity and/or resistance in the offspring, as it would otherwise only reflect the cost of the parental immune challenge. After a bacterial immune challenge, females of *T. molitor* exhibited a transient transfer of different levels of antibacterial activity to the eggs and a relatively large number of eggs were not protected, suggesting that maternal transfer of immunity is costly for females (Zanchi et al. 2012). Furthermore, the level of antibacterial activity found in the eggs was negatively correlated to the maternal immune response, suggesting that mothers are traded-off their own immunity against that of their eggs, especially among small females that are inherently of lower quality (Moreau et al. 2012).

Since immunity imposes fitness costs (Schmid-Hempel 2003; Siva-Jothy et al. 2005), improved immunity in the offspring through TGIP is expected to bear costs too. These costs could be revealed by trade-offs between investment into immunity and other important fitness traits in the offspring. In line with this, related fitness consequences of TGIP have been found in primed offspring. In the beetles *T. castaneum* and *T. molitor,* and in the mosquito *An. gambiae*, maternally-primed offspring exhibited enhanced immunity but a prolonged larval development time (Roth et al. 2010; Zanchi et al. 2011). In *M. sexta*, larvae derived from immune-challenged parents grew faster and gained more weight than those derived from control parents, in addition

to exhibiting stronger immune reactivity upon challenge (Trauer et al. 2013). However, at the adult stage, the offspring derived from immune-challenged parents were also those with the lowest fecundity (Trauer et al. 2013). TGIP may also impose trade-offs between the efficacy of offspring defense against diverse pathogens (McKean & Lazzaro 2011). For instance, while workers of the bumblebee, *B. terrestris*, developed higher levels of antibacterial protection when their mother had been immune-challenged by bacteria, they also became more susceptible to infection by their trypanosome parasite, *Crithidia bombi* (Sadd & Schmid-Hempel 2009a). Similarly, flour beetles were shown to exhibit TGIP against the bacterial pathogen *B. thuringiensis* but this TGIP effect was found inhibited by maternal co-infection with the prevalent gut protozoan parasite, *Gregaria minuta* (Tate & Graham 2015). Despite the negative correlation observed between immunity and other important fitness traits within the offspring, one cannot entirely exclude that such a negative correlation is actually causal. Indeed, reduced fitness in the offspring can still reflect the parental cost of the immune challenge on offspring quality. For instance, paternally-primed offspring in *T. molitor* were all found lighter than control offspring at the pupal stage. However, among these primed offspring, only those born from the first laid eggs had enhanced phenoloxidase activity and they were not further lighter than those born from laid eggs later on, suggesting here that the cost of the paternal challenge was predominant compared to that of TGIP (Zanchi et al. 2011).

The majority of the above studies that have characterized TGIP and revealed associated costs have been carried out in carefully controlled laboratory conditions. They may therefore provide little insight as to whether protective effects always occur in more natural conditions. Diet abundance and quality, infection with multiple pathogens and population density are known to affect individual immune response (Freitak et al. 2009; Mitchell & Read 2005; Singer et al. 2014; Sternberg et al. 2015; Triggs et al. 2012; Tseng 2006), Since the up-regulation of immunity and, as seen above, the induction of TGIP are costly traits, the manifestation of TGIP is expected to be altered by such environmental factors. For instance, under poor food conditions, animals will have less resource available to concomitantly sustain investment in TGIP and other important life history traits. Therefore, poor food source is likely to be associated with reduced investment in TGIP. In lines with this, poor parental food conditions in the moth *Plodia interpunctella* was found to induce a strong reduction of immune reactivity in the offspring (Triggs et al. 2012). By contrast, in the same insect model, poor maternal resource conditions resulted in offspring exhibiting stronger immune reactivity and being more resistant to the *granulosis virus* (*PiGV*) (Boots et al. 2012). These results emphasize that the

outcomes of maternal effects in response to parental food restriction are not trivial and may complicate expected predictions on TGIP occurrence in such a condition (Littlefair et al. 2016).

Such a complexity is also true when considering other environmental factors. In *T. castaneum*, a cold shock of the parental generation enhanced offspring immunity and resistance to infection by *B. thuringiensis*, while a heat shock decreased offspring resistance to the bacterium and immunity (Eggert et al. 2015). Population density can also affect TGIP, following the gregarious or solitary development of *S. littoralis* parents and/or offspring (Wilson & Graham 2015). These results connecting a stressful environment with parents and offspring immunity highlights the importance of considering the parameters that can affect TGIP for understanding to which extent TGIP can be modulated by the environmental context.

Finally, multiple infections of the parents can induce additional costs for the offspring. Indeed, as mentioned earlier in *T. castaneum*, maternal co-infection with the gut parasite, *G. minuta*, inhibited TGIP against bacteria (Tate et al. 2015). This cost highlights the complex interactions between host and pathogens and it raises the question of the extrapolation from laboratory-discovered TGIP to natural environment.

IV.3. Consequence of TGIP for the evolution of host-pathogens interaction.

While immune priming, either within or across generations, have implications for individual hosts, it is also expected to have implications on host-pathogen interactions and population dynamics. Mathematical models available found that immune priming is predicted to reduce disease prevalence but destabilize population dynamics (Tidbury et al. 2012). The relative importance of TGIP on diseases prevalence depends on stage-specific host-pathogen interaction (Tate & Rudolf 2012). Indeed, when larvae drive infection, but both larvae and adults can contract the parasites, TGIP is predicted to have the greatest positive impact on infection prevalence and population size. Conversely, when adults cannot contract the pathogen that kills their larvae, both within-generation immune priming and TGIP are needed to reduce infection prevalence and increase host population size. Hence, immune priming and TGIP could have substantial consequences for population-level phenomena that could be misinterpreted if immune priming is not considered (Tate et al. 2012). However, reliable empirical data on disease transmission, waning of immunity and recovery rates are still crucially missing to improve estimates from models.

Beside, affecting population dynamics, the inducible nature of TGIP, and in a broader extent immune priming, may decrease the rate of evolution of constitutive levels of defense against

pathogens (Sadd & Schmid-Hempel 2009b). Immune priming represents an acquired element of host resistance that is only induced upon the experience of a primary pathogen infection, which may contrast to genetically-based level of constitutive resistance existing in naïve individuals. Assuming a population of hosts varying in its range of basal level of constitutive resistance, from highly resistant to susceptible, and that an initial infection do not clear all susceptible genotypes from that population, then immune priming is expected to maintain these susceptible genotypes (Sadd et al. 2009b). Furthermore, since constitutive defenses are expected to be more costly than those that are inducible (Agrawal et al. 1999; Harvell 1990), advantageous acquired resistance may even select against genotypes exhibiting the highest levels of constitutive resistance. This hypothesis is in line with modelling results based on vertebrate systems showing that beneficial acquired resistance could decrease the rate of evolution of innate resistance traits (Harding et al. 2005).

If immune priming has evolved from selective pressure imposed by parasites, it should then, in return, impact the evolution of pathogens too. Immune responses are acting as predator for pathogens, and enhanced immune capabilities resulting from immune priming is expected to favor the evolution counter-measures, such as immune evasion and faster replication rates among pathogens, as found in vertebrates (André & Gandon 2006; Fenton et al. 2006; Mackinnon & Read 2004). These traits are usually believed to increase pathogen virulence (Sadd et al. 2009b), although it is probably not always the rule (Schmid-Hempel 2009).

V. Identifying the molecular mechanisms of TGIP: are we there yet?

Many molecular actors of the innate and acquired immunity of invertebrates have been identified. As a consequence, many TGIP studies monitored several of these known mechanisms by measuring their activity, such as lysozyme, antimicrobial or phenoloxidase (PO) activities, or their gene expression by RT-qPCR (reverse transcription quantitative PCR) (Table 1). Few studies used global approaches to unravel the role of potential other genes and proteins, by using next-generation sequencing (NGS) transcriptomic approach by RNA-seq (Barribeau et al. 2016), or by proteomic profiling using 1-dimension (Dubuffet et al. 2015; Freitak et al. 2009) or 2D polyacrylamide gel electrophoresis (SDS-PAGE) coupled with mass spectrometry (MS) analysis (Freitak et al. 2014). In future studies, such global approaches should be considered as they might help identifying additional and potentially new candidates that could be specific of TGIP and not identified in within-generation immune priming yet. It would also be helpful to identify potential metabolic reorganization following an increased

immunity, notably due to energy reallocation processes. In this section, we will describe some of these major immune actors and detail the supporting studies.

V.1. Prophenoloxidase & encapsulation

In arthropods, melanisation is a major innate immune response to a pathogen. The conversion of prophenoloxidase (PPO) into PO eventually leads to the production of melanin that will encapsulate and partially or completely inactivate the pathogen. Considering the large over-representation of the groups of insects among the invertebrate species studied for TGIP (Figure 14), it is not surprising that PPO/PO activity and gene expression have been largely monitored. An increased PPO/PO activity has been recorded in the progeny from parents exposed to *E. coli* and *M. luteus* in the lepidopteran *T. ni* and *G. mellonella* (Freitak et al. 2009; Freitak et al. 2014) and after parental exposure to *B. thuringiensis* (Eggert et al. 2014; Roth et al. 2010) and *E. coli* in the coleopteran *T. castaneum* and *R. ferrugineus* (Eggert et al. 2014; Shi et al. 2014). In *T. castaneum*, this increased PPO/PO activity was not associated with an increase of PO gene expression (Eggert et al. 2014). Exposure of workers from the parental generation to LPS also triggered an increased PPO/PO activity in offspring males of *B. terrestris* (Moret & Schmid-Hempel 2001). Exposure of *M. sexta* parents to PGN induced an increased PPO/PO activity only in the hemolymph of offspring unexposed to PGN (Trauer et al. 2013) and in eggs parasitized by *Trichogramma evanescens* (Trauer-Kizilelma & Hilker 2015a). This was consistent with an increased PO gene expression in parasitized eggs from challenged parents as compared to naïve parents (Trauer-Kizilelma et al. 2015a). In contrary, stimulation of mother's melanisation by negatively-charged beads did not lead to an increased melanisation in offspring of *Ae. aegypti* (Voordouw et al. 2008). *In T. molitor*, the procedure of injection itself (injection of saline buffer PBS) induced a similar effect as LPS injection, which is an increased PPO/PO activity as compared to the uninjected control in *T. molitor* (Moret 2006). In *Artemia sp.*, the modification of PO gene expression was stochastic across generations, with a significant increase at F1, then decrease at F2 and no difference at F3 between offspring from challenged parents and those from unchallenged ones (Norouzitallab et al. 2016). Considering that PO is a major actor of the generic innate immune response and that its activity seems to be induced in several cases, it might play a role in TGIP but the specificity of the conditions upon which it is stimulated together with its potential instability across generation suggests that additional mechanisms might be at play.

V.2. Epigenetic modifications

The involvement of epigenetic reprogramming in animal response to pathogens has been increasingly reported, and it is a central process in innate immune memory (Netea et al. 2016; Vilcinskas 2016). In invertebrates, modifications of gene methylation and histone acetylation are major epigenetic factors that could boost or impair immune response toward bacteria, viruses or fungi (Galbraith et al. 2015; Mukherjee et al. 2012). Surprisingly, only two studies investigated the role of epigenetics in TGIP, one focusing on histone acetylation in the response of the crustacean *Artemia sp.* to *Vibrio campbelli* (Norouzitallab et al. 2016) and the other on gene methylation after bacterial exposure of *T. castaneum* (Knorr et al. 2015). While TGIP was identified in both cases, no link between epigenetic modifications and TGIP was found. The involvement of microRNA has not been investigated in TGIP yet, despite its known role in invertebrate immunity and host-pathogens interaction (Asgari 2013; Harris et al. 2013). Considering the prominent role of epigenetics in many transgenerational adaptation process in animals and its implication in the modulation of several immune response pathways, its involvement in TGIP must be more deeply and widely investigated (Vilcinskas 2016; Youngson & Whitelaw 2008). It could notably be a good candidate to explain at least a part of the paternal effect and of the sustenance of TGIP effect over multiple successive generations (Norouzitallab et al. 2015).

V.3. Antimicrobial peptides (AMPs)

Invertebrates can produce a wide range of AMPs that act against a large number of pathogens and the majority of AMPs have been found in more than two invertebrate orders (Vizioli & Salzet 2002; Yi et al. 2014). In arthropods, AMPs are mostly synthesized by the haemocytes (heterometabolous insects) or by the fat body (holometabolous insects) in response to infection and secreted into the hemolymph while they are essentially produced by the albumen gland in mollusks (Bulet & Stöcklin 2005; Takamatsu et al. 1995). Several studies reported the transfer and storage of AMPs from mothers into the eggs (Bouts et al. 2007; Esteves et al. 2009; Marchini et al. 1997). Mother-derived AMPs have notably been shown to drive the colonization of the embryo by symbiotic bacteria (Fraune et al. 2010). The involvement of AMPs in TGIP has only been investigated in Coleoptera, Lepidoptera and Hymenoptera after parental exposure to LPS, PGN, bacteria or fungi.

The role of AMPs was essentially investigated by RT-qPCR experiments that measured the level of expression of a selected set of genes encoding AMPs. This revealed that increased AMP

gene expression is not triggered by all pathogen challenges and that the set of AMPs differentially regulated differs from one pathogen to another in offspring from challenged mother as compared to offspring from unchallenged mother (Eggert et al. 2014; Freitak et al. 2009; Freitak et al. 2014; Green et al. 2016; Knorr et al. 2015; Trauer-Kizilelma & Hilker, 2015b). Interestingly, one specific AMP, the gloverin, was found over-expressed in *M. sexta* eggs from mothers primed with PGN (Trauer-Kizilelma et al. 2015b), in *Galleria mellonella* eggs from mothers exposed to *S. entomophila* (Freitak et al. 2014) and in *T. ni* larval offspring from mothers fed with a mixture of *E. coli* and *M. luteus* (Freitak et al. 2009). Gloverin is a lepidopteran-specific AMP that has been implicated in antibacterial and antifungal response in several lepidopteran species (Yi et al. 2014) but its exact function in the context of TGIP would require further experiments.

AMP gene expression data analyzed by RT-qPCR are restricted to a very limited set of candidate genes. In a recent study, gene expression in adult bumblebee workers from challenged queens as compared to unchallenged ones was analyzed by next-generation RNA sequencing (RNA-seq), which allows to access to the entire transcriptome of the species (Barribeau et al. 2016). The authors showed that several AMPs were found over-expressed in offspring from queens injected with the bacteria *Arthrobacter globiformis*, such as hymenoptaecin, defensin, abaecin, apidaecin and battenin (Barribeau et al. 2016). This gives a more extensive view of AMP regulation and expression in TGIP and provides a list of candidate genes to be further validated.

To be noted, the induction of AMP production is triggered by pathogen recognition through the Imd and Toll pathways (Lazzaro 2008; Yokoi et al. 2012). No modification in gene expression related to these pathways was observed in offspring from exposed parents as compared to offspring from naïve ones, neither by RT-qPCR (Eggert et al. 2014), nor by RNA-seq (Barribeau et al. 2016). This could indicate a transient activation of these pathways that is not detected by these techniques and/or that the activation of these pathways is not induced upon TGIP in these specific host-pathogen couples and with the experimental procedures used (Barribeau et al. 2016; Eggert et al. 2014).

In *T. molitor*, an antimicrobial peptide, a tenecin, was identified after acid extraction of all small peptides from eggs analyzed on an AU-PAGE gel (acid urea polyacrylamide gel electrophoresis) (Dubuffet et al. 2015). Tenecin was systematically found in egg extracts from mothers injected with different bacteria (*A. globiformis*, *B. thuringiensis*, *E. coli* and *S. entomophila*) but was absent in eggs from unchallenged mother, which makes it a good

candidate for the general antibacterial response in *T. molitor* eggs, without excluding the implication of other AMPs undetected by these approaches and yet to be discovered (Dubuffet et al. 2015).

V.4. Vitellogenin, a multi-tool protein responsible for an "actively passive" TGIP?

Vitellogenin is a precursor of vitellin, which is the major egg storage protein in invertebrates. It is produced by the fat body and then secreted into the hemolymph to be stored in oocytes via receptor-mediated endocytosis (Raikhel & Dhadialla 1992). It is a highly evolutionary conserved protein whose main role is to provide the embryo with sufficient energetic resources for its proper development within the egg (Tufail & Takeda 2008). Nevertheless, vitellogenin can play many additional roles, notably in the defense of invertebrates against stress and infections. In response to an oxidative stress, honeybees (*A. mellifera*) are synthesizing a high quantity of vitellogenin that is able to recognize damaged cells and to bind to living cells to protect them from reactive oxygen species (Havukainen et al. 2013; Seehuus et al. 2006). Vitellogenin has also been implicated in the modulation of the immune response of invertebrates, notably indirectly due to shared gene expression regulation regions with AMPs, such as defensins (Fischer et al. 2012; Raikhel et al. 2002). Vitellogenin can also directly act as a multivalent pattern recognition receptor (PRR) with an opsonic and antibacterial activity (Li et al. 2008; Singh et al. 2013).

A recent study reported an additional somewhat intriguing property of vitellogenin, which is the mediation of the translocation of bacterial proteins from the gut of *A. mellifera* females to the eggs (Salmela et al. 2015). Vitellogenin would recognize bacteria by specifically binding to pathogen-associated molecular patterns (PAMPs), such as PGN and LPS, to trigger the transfer of cell-wall fragments of bacteria into the eggs (Salmela et al. 2015). Interestingly, such bacterial protein translocation from the midgut to the eggs has already been characterized and visualized by fluorescence microscopy in *G. mellonella* and *T. castaneum* mothers exposed to bacteria and it was associated with an increased expression of immune genes in the eggs (Freitak et al. 2014; Knorr et al. 2015). Although this bacterial transfer was not associated with vitellogenin at this time, the route followed by the bacterial proteins had been identified (*i.e.*, crossing the midgut epithelium then being entrapped into nodules in the hemocoel followed by an accumulation in the ovaries ended by a deposition in the eggs) and could match with the tropism and mechanism of translocation of vitellogenin into the eggs (Freitak et al. 2014; Raikhel et al. 1992). Such mechanism would expose the developing embryo within the egg to

antigens from pathogens that his mother encountered during her life. It would be an easy way to induce an immune priming in the offspring in order to boost its innate immunity and increase its capacity to respond to the pathogen community that it might be exposed to when it will hatch. It is however yet to be understood if this mechanism is strictly passive, *i.e.* costless in term of energy allocation for the mother, or if the mother can actively stimulate vitellogenin production and/or activity in response to pathogen exposure, and if it can be facilitated by a potential paternal effect. Moreover, similar experiments should be performed with other pathogens than bacteria such as viruses, fungi, microsporidia and protozoan parasites, when relevant, to know if translocation of pathogen proteins by vitellogenin is a generalist or a bacteria-specific TGIP mechanism.

By its ability to translocate bacterial proteins to the eggs and its involvement in immunity and stress response, vitellogenin could play both direct and indirect major roles in the protection of embryos in eggs from challenged mothers. It is therefore a strong candidate for TGIP that will require further investigation, notably to detail its gene expression regulation in parents and to decipher its exact function in the offspring.

VI. The many roads to TGIP: hypothetical scenarios based on empirical data

In the light of the different potential mechanisms identified and discussed in section V (summarized and illustrated in Figure 16), we propose four different hypothetical scenarios to explain how TGIP can happen and how these mechanisms can be characterized. Although there might be as many mechanisms as there are invertebrate species and pathogens, the objective of such scenarios is to highlight common features and to provide a baseline to facilitate further brainstorming about TGIP mechanisms and processes.

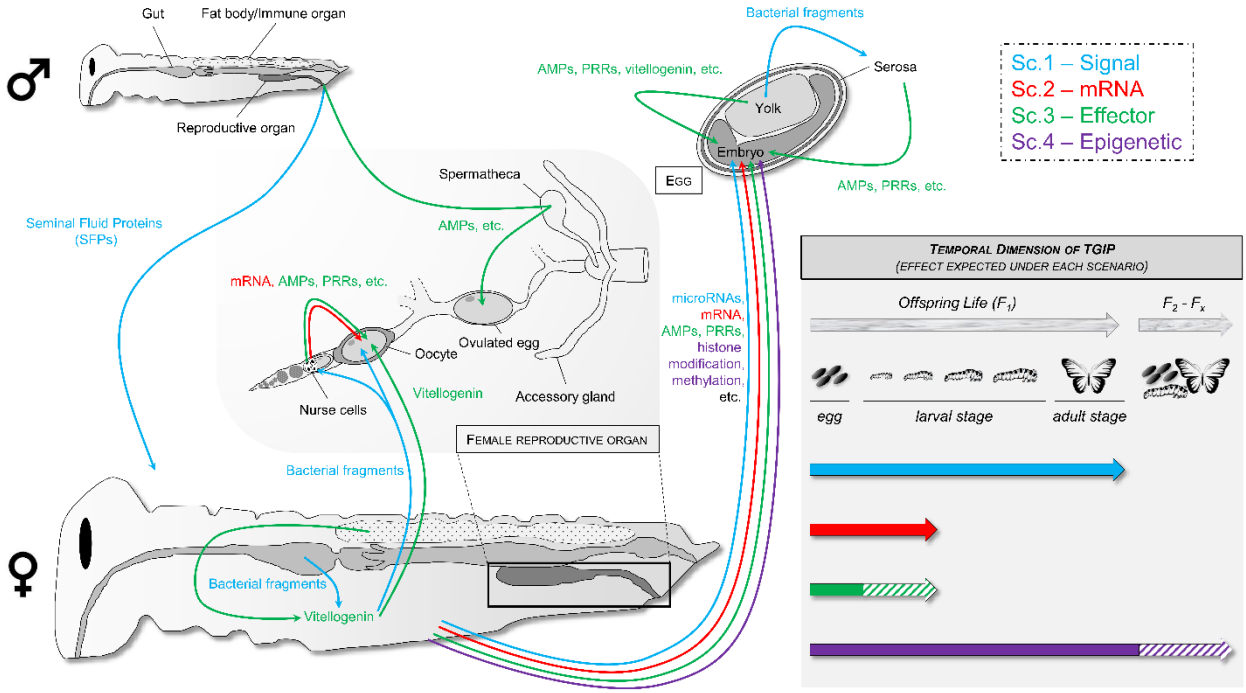

Figure 16. Hypothetical molecular mechanisms responsible for TGIP in invertebrates following the four described scenarios highlighted in blue, red, green and purple for scenarios 1, 2, 3 and 4, respectively.

VI.1. Description of the scenarios

In the first scenario, females may transmit a “signal” to their progeny, which could be an eliciting substance transferred in the developing eggs. Such signal can notably be bacterial peptides transferred by vitellogenin from mother’s gut to the egg (Salmela et al. 2015) or maternal microRNAs that directly act on offspring gene expression (Vilcinskas 2016). They could induce the activation of immune-related genes in the developing embryo inside the egg and/or by the extraembryonic serosa, which is a frontier epithelium able to express many immune genes and provide the insect egg with a full-range innate immune response (Jacobs & van der Zee 2013; Jacobs et al. 2014).

In the second scenario, females may provide their eggs with mRNAs coding for the antimicrobial immune effectors, which are then produced by the developing embryo. Transfer of maternal mRNAs in developing eggs during oogenesis has been characterized in many species, including insects (Berry 1982). In insects with polytrophic meroistic (*e.g.*, Hymenoptera, Lepidoptera, and Diptera) and telotrophic ovaries (*e.g.*, Hemiptera and Coleoptera), these maternal mRNAs are synthetized by nurse cells and provided to oocytes via the trophic cord (Biczkowski & Dittman 1995; Capco & Jeffery 1979; Johnstone & Lasko 2001). Although these mRNAs are mostly known to be involved in the control of development (Becalska & Gavis 2009), they may also serve for early immune protection of embryos. In the fish *Cyprinus carpio* L. for example, maternal mRNAs encoding immune-related genes have been identified in unfertilized eggs (Huttenhuis et al. 2006).

In the third scenario, females could transfer immune effector proteins to their eggs, either passively through the diffusion or sequestration of proteins present in the mother’s hemolymph into the egg (scenario 3a), or actively via the provision of eggs by specialized cells, such as nurse cells that are known to produce proteins that are transferred to oocytes (Berry 1982) (scenario 3b). Transfer of immune effectors is well known in vertebrates where antibodies are transmitted through the yolk in birds, fishes and reptiles, or through the placenta or milk in mammals (Hasselquist et al. 2009). Although antibodies do not exist in invertebrates, other immune effectors can be transferred to the offspring such as AMPs, lectins or LBP/BPI (LPS-binding proteins/bactericidal permeability-increasing proteins) (Baron et al. 2013; Wang et al. 2015). In hydras for example, AMPs that originate from the mothers control the proliferation of bacterial symbionts during the first stages of embryogenesis (Fraune et al. 2010).

In the fourth and last scenario, females exposed to a pathogen would experience an epigenetic reprogramming (*e.g.*, by acetylation/deacetylation of histone and/or by methylation/demethylation of immune genes) and would transfer this reshaped epigenetic state to their offspring. This could lead to a modified expression of immune genes at different developmental stages and be stable over few successive generations (Vilcinskas 2016).

These scenarios are by essence not mutually exclusive and could act simultaneously at the same developmental stage of the offspring and/or act sequentially at different moment of offspring life (Figure 16).

VI.2. How to experimentally disentangle the different scenarios?

Under these four different scenarios, transcripts coding for the immune effector(s) found in the eggs and the localization of the effectors themselves are expected to localize in distinct parts of the mother's and egg's tissues (Table 2).

In the case of a maternal transfer of immune effectors (scenario 3), transcripts of the effector(s) should be absent in the eggs laid by immune-challenged females, while under the first two other scenarios, transcripts should be detected in the eggs (Table 13). However, in the case of a maternal transfer of mRNAs, transcripts should be absent from the oocyte nucleus (*i.e.* the site of transcription in oocytes), but should be detectable in maternal tissues, such as the nurse cells and trophic cords, and/or in more systemic mother's organs.

Besides, absence of transcripts in females would favor the hypothesis of a transfer of a maternal signal (scenario 1), while their presence may not help to distinguish between the scenarios 2 and 3 (Table 13). The precise localization of these transcripts may however be informative. Many antimicrobial effectors are known in insects to be expressed in the fat body and in haemocytes following an immune challenge (Hoffman 2003; Tsakas & Marmaras 2010), but some can be expressed in other tissues. For example, the AMP drosocin is expressed in the calyx and oviducts of mated *D. melanogaster* females that have started to lay eggs (Charlet et al. 1996) and in the medfly, *Ceratitis capitata*, ceratotoxin A and B are expressed constitutively within the female's accessory glands (Marchini et al. 1995). In case of a transfer of maternal mRNAs (scenario 2) or active transfer of effectors (scenario 3b), high levels of transcripts are expected to be observed in the ovaries, especially in the nurse cells, known to provide both maternal mRNAs and maternal proteins to developing oocytes, and in the trophic cords, which connect the nurse cells to the oocytes.

Table 13. Expected presence of transcripts and proteins in immune-challenged females and their eggs according to the four different scenarios.

	Transfer of a signal (scenario 1)	Transfer of mRNA (scenario 2)	Transfer of effectors (scenario 3)		Epigenetic shaping (scenario 4)
			a. Passive diffusion	b. Active transfer	
Gene expression in mothers	Not necessarily	Yes, in ovaries	Yes, in fat body and/or haemocytes	Yes, in ovaries	Not necessarily
Presence of the protein in mothers	Not necessarily	Not necessarily	Yes, in the hemolymph	Yes, in ovaries	Not necessarily
Transcripts in embryo	Yes, in nuclei of embryo cells and/or in serosa	Yes (maternal origin) but not necessarily in nuclei of embryo cells	No	No	Not necessarily
Presence of the protein in eggs	Yes	Yes	Yes	Yes	Not necessarily

Also, the presence of large amounts of effector proteins in female tissues would rather favor the third scenario while their absence would clearly favor the first two others (Table 13). Under the third scenario, the presence of these molecules in the mother's hemolymph would favor the hypothesis of a passive transfer of proteins (scenario 3a) while a concentration within ovarian tissues would rather indicate an active transfer (scenario 3b).

The outcome of the fourth scenario, involving epigenetic reshaping, is difficult to predict in term of transcript and protein presence in mother and offspring as it would largely depend on the genes that are affected (Table 13). It would require a specific investigation through dedicated approaches (*e.g.*, chromatine immunoprecipitation sequencing (ChIP-seq) and bisulfite sequencing (BS-seq) for studying DNA-chromatine interaction and methylation, respectively). Nevertheless, if the immune protection is maintained across several successive generations, this would strongly indicate that an epigenetic factor is involved (Figure 16). However, it would not completely exclude the other scenarios, as the increased immune status of the offspring due to an increased amount of proteins could be transferrable to the next generation(s), which would support the involvement of the transfer of effectors too (scenario 3).

VII. Conclusions

(1) Trans-generational immune priming corresponds to the plastic adjustment of offspring immunity as a result of parental immune experience. It represents a recent field of research (17 years old) and it has been increasingly studied for the last years. Based on authors' network, one can argue that further studies will investigate into more details TGIP underlying mechanisms through an increase in the number of collaborations between different teams of research, which will result in a more interactive network. In addition, new research teams will continue to publish unique TGIP articles on new invertebrate species, providing key information on the occurrence of TGIP in the tree of life.

(2) Considering that TGIP is an evolutionary conserved phenomenon that can be costly for fitness of both parents and offspring, it is expected to occur principally against the most threatening pathogens from their environment. Therefore, characterizing the ecology of the host before studying TGIP is an important prerequisite to select the most appropriate pathogen(s) for studying TGIP and to avoid missing the phenotype due to an inadequate host-pathogen combination.

(3) The infection procedure (ingestion/injection & inactivated/living pathogen) and the dose applied must be chosen based on their adequacy to the biology and ecology of both the host and the pathogen studied. When comparing two pathogens, these pathogens must share some common features in term of infection route and pathogenicity to be comparable through the same infection procedure.

(4) When possible, immune parameters and associated fitness costs should be measured separately in females and males in both parents and offspring to disentangle sex-biased TGIP. Ideally, the paternal influence should be investigated more in-depth, notably its impact on mother's immunity and its consequence on offspring protection.

(5) Immune status and fitness costs of offspring from challenged parents should be investigated at different developmental stages to account for potential stage-specificity of TGIP and avoid missing its expression. Moreover, different mechanisms might be at play at the different developmental stages and investigating a too limited number of stages could bias the analysis of TGIP process. The age of the mother (and potentially father) should also be monitored considering that older females might not invest as much in offspring protection as younger ones.

(6) Several successive generations should be monitored to see if TGIP is a sustained process or if it is restricted to the first generation, which could help deciphering the underlying mechanisms.

(7) Last but not least, all articles investigating TGIP mechanisms by the mean of molecular approaches such as transcriptomic, proteomic or enzymatic activities (TEI), should also systematically monitor the enhanced offspring resistance (TER), notably by measuring the offspring survival to the studied pathogen(s) and parasite load. This is mandatory to be able to properly compare different studies and to decipher all the complexity of trans-generational immune priming because, as Tom J. Little and collaborators wrote in 2005, "*without analogous experiments, mechanism-driven work may not demonstrate the full richness of invertebrate immunity*" (Little et al. 2005).

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Discussion

Discussion

Le but de cette thèse était de définir quelles sont les conditions écologiques qui ont favorisé l'évolution du priming immunitaire et du TTGI chez *T. molitor*, tout en caractérisant les mécanismes associés et en examinant les bases génétiques du TTGI. Nous avons mis en évidence que les insectes primés avec des bactéries Gram-positives sont fortement protégés contre les infections ultérieures, ayant lieu 20 jours après le priming. Cela est essentiellement dû à l'induction d'une réponse antibactérienne (impliquant la production de peptides antibactériens) persistante, qui n'existe pas lorsque les individus sont primés avec une bactérie Gram-négative. Dans le cas du TTGI, la descendance de mères primées avec des bactéries Gram-positives et Gram-négatives, montre le même niveau d'amélioration de leur immunité, quelle que soit la bactérie utilisée pour l'infection. Ce bénéfice exprimé en termes de survie à l'infection semble essentiellement dû à une augmentation de l'activité de la phénoloxydase lorsque les mères ont été stimulées avec une bactérie Gram-positive, alors que dans le cas d'une stimulation par une bactérie à Gram-négatif, ce sont les capacités de tolérance qui semblent être augmentées chez la descendance. Cette protection maternelle est coûteuse en ce qui concerne le développement larvaire de la descendance, mais ce coût est plus faible pour les descendants de mères primées avec des bactéries Gram-positives. Nous avons vu également que la dynamique temporelle de l'activité antibactérienne au sein des œufs issus de femelles stimulées par une bactérie à Gram-positif dépendait de l'espèce bactérienne utilisée pour la stimulation immunitaire maternelle. Les œufs issus de femelles challengées, quelle que soit la bactérie utilisée pour la stimulation, avaient un meilleur succès à l'éclosion que les œufs issus de femelles témoins. Les larves provenant de femelles primées avec *B. thuringiensis* exprimaient quant à elles une meilleure survie à la fois dans des conditions stériles et lorsqu'elles étaient exposées à la bactérie. Par contre, aucun coût en termes de valeur sélective n'a été mis en évidence pour les descendants de mères challengées. Enfin, nous n'avons pas pu établir si des bases génétiques déterminent l'expression du TGIP, du fait d'un nombre de lignées trop bas et d'un effectif parfois trop faible pour certaines des lignées utilisées, provoquant une variance trop importante dans notre calcul d'héritabilité.

Dans un premier temps, je discuterai la nature du priming et du TTGI sur la protection de la descendance, et des mécanismes potentiellement impliqués lors de leur expression. Dans un second temps, je discuterai de l'existence d'une forme de spécificité dans la réponse immunitaire de *T. molitor*, qui nous renseigne sur les pressions de sélection ayant mené à l'évolution du priming immunitaire individuel et transgénérationel chez cet insecte.

Priming immunitaire et TTGI : expression et bénéfices

Dans les chapitres 1 et 2, nous avons montré différents bénéfices associés au priming individuel (aussi dit ontogénique) et au TTGI. Dans le chapitre 1, nous avons vu qu'en fonction de la stimulation immunitaire, le priming individuel et le TTGI pouvaient induire une augmentation de la charge hémocytaire, accompagnée d'une importante concentration en peptide antibactérien, ou induire une augmentation de la concentration en phénoloxydase, ou encore n'être accompagnée d'aucun effet visible sur les effecteurs immunitaires que nous avons étudiés. Dans tous les cas, le challenge immunitaire était associé à un bénéfice en termes de survie lors d'une ré-infection, qu'elle concerne l'individu lui-même ou sa descendance. Dans le chapitre 2, nous avons vu qu'une stimulation maternelle par une bactérie à Gram-positif se traduisait par la présence de peptides antibactériens dans les œufs, même si leur origine semble différer en fonction de la bactérie utilisée pour le challenge maternel. Cette stimulation induit des bénéfices pour la descendance. Cela se traduit par une meilleure éclosion des œufs et une meilleure survie pour les jeunes larves issues de femelles stimulées par *B. thuringiensis.* Ces études mettent en évidence l'implication de mécanismes et d'effecteurs immunitaires différents, à trois stades de vie distincts des individus immuno-stimulés et de leur descendance.

Dans le cadre du priming immunitaire individuel, l'augmentation de la charge hémocytaire pouvait être attendue. Surtout depuis qu'il a été montré chez *D. melanogaster* que les hémocytes sont responsables chez cette espèce du bénéfice de survie observé lors d'une seconde infection par *Streptococcus pneumoniae* (Pham et al. 2017). Toutefois, au vu de ces résultats, leur rôle pouvait être supposé plus important dans le priming chez *T. molitor*. Nous avons montré dans le chapitre 1 que c'est essentiellement l'activité antibactérienne persistante après un premier challenge avec une bactérie Gram-positive qui explique le bénéfice en survie observé lors d'une seconde infection. De plus, les résultats obtenus chez les descendants de femelles stimulées avec une bactérie Gram-positive sont également inattendus car il avait été montré par Zanchi et al. (2011) que c'était la concentration hémocytaire qui augmentait chez les descendants de femelles stimulées et non la phénoloxydase. Néanmoins, ces résultats avaient été obtenus en stimulant les femelles avec du LPS, qui pourrait activer d'autres voies immunitaires que celles activées par les bactéries à Gram-positif que nous avons utilisé. Mais ce qui reste le plus énigmatique, c'est l'absence d'effet sur les effecteurs immunitaires que nous avons testés lors d'une stimulation avec une bactérie à Gram-négatif, que ce soit dans le cas du priming ou du TTGI. S'il est possible que d'autres effecteurs immunitaires puissent jouer un rôle dans les bénéfices en termes de survie observés, la piste d'une plus grande tolérance est à explorer. Le

phénomène de tolérance consiste à limiter les effets négatifs sur la santé qu'entraîne une maladie, tout en ne diminuant pas le nombre de pathogènes présents au sein de l'hôte (Raberg et al. 2009 ; Schneider & Ayres 2008). Cela peut se faire en agissant sur certains effecteurs immunitaires qui en induisant un mécanisme de résistance, peuvent causer des dégâts chez l'hôte lui-même et diminuer sa tolérance. La tolérance et la résistance sont donc liées et leurs effets opposés. Par exemple, les espèces réactives de l'oxygène qui sont produites durant la réponse immunitaire sont importantes pour lutter contre l'infection, mais leur activité peut aussi induire de graves pathologies allant parfois jusqu'à la mort de l'hôte, diminuant ainsi la tolérance. (Lambeth et al. 2007 ; Lambeth 2007). Ce phénomène a déjà été montré chez des invertébrés et notamment chez *D. melanogaster*, où onze génotypes différents ont été infectés avec *Pseudomonas aeruginosa* (Corby-Harris et al. 2007). Ces génotypes variaient significativement que ce soit en termes de charge bactérienne (une mesure de la résistance) ou de survie. Mais il n'y avait pas de corrélation entre ces deux paramètres, indiquant que les individus avec la charge bactérienne la plus faible n'étaient pas forcément ceux en meilleure santé. Ces résultats suggèrent donc que d'autres mécanismes que la résistance servent à lutter contre le stress de l'infection et que la tolérance est un déterminant crucial de la survie contre les agents pathogènes. Pour confirmer cette hypothèse chez *T. molitor* et déterminer si le priming individuel et le TTGI suite à une stimulation avec une bactérie Gram-négative s'effectuent essentiellement par tolérance, il serait judicieux de mesurer la charge bactérienne des individus dans les premières heures suivant une infection, avant d'explorer plus profondément les mécanismes de cette éventuelle tolérance. De plus, il faut savoir que la balance entre résistance et tolérance est complexe. En effet, des changements altérant la tolérance vis-à-vis d'un microbe peuvent avoir l'effet inverse concernant la tolérance face à un autre microbe, ou peuvent même affecter la résistance de manière négative ou positive (Ayres & Schneider 2008 ; Ayres & Schneider 2009). Il serait intéressant d'étudier si les mécanismes de résistance développés dans le cadre d'un priming individuel ou d'un TTGI suite à un challenge par une bactérie Gram-positive, sont corrélés à une plus grande tolérance dans le cas d'une stimulation avec une bactérie Gram-négative chez *T. molitor*.

Dans le chapitre 1, nous avons mis en évidence que le TTGI chez la descendance adulte issue de femelles stimulées par *B. thuringiensis* s'exprimait par une augmentation de l'activité de la phénoloxydase. Même si l'étude conduite dans le chapitre 3 ne nous a pour le moment pas permis de conclure quant à l'existence de bases génétiques associées au TTGI, l'étude des corrélations phénotypiques entre différents traits d'immunité de la mère et de sa descendance,

associée aux résultats du chapitre 1, souligne l'importance de l'action de la phénoloxydase au sein du TTGI. En effet, même si des corrélations positives et négatives existent entre activité antimicrobienne et hémocytes, à la fois de la mère et de la descendance, l'activité de la phénoloxydase n'est corrélée négativement à aucun autre paramètre immunitaire. Par contre une corrélation positive existe pour ce trait entre la mère et sa descendance. De plus, nous avons vu dans le chapitre 1 que lorsque l'on inhibe l'action des hémocytes par l'utilisation de billes de latex, la survie des individus issus de femelles primées n'est pas modifiée. La phénoloxydase semble donc être un acteur majeur du TTGI dans le cadre d'une stimulation avec une bactérie Gram-positive.

Concernant, le TTGI chez les œufs, nous avons vu dans le chapitre 2 qu'il peut résulter de deux mécanismes non exclusifs : un transfert passif de peptides antibactériens de la mère à la descendance, ou la transmission d'un signal permettant aux œufs de synthétiser leurs propres peptides antibactériens. Pour cette seconde possibilité, il existe plusieurs mécanismes, pas nécessairement exclusifs, qui peuvent être à l'origine de la production de peptides antimicrobiens par l'œuf. Premièrement, la translocation de bactéries ou de fragments bactériens de la mère à ses œufs, ce qui pourrait éliciter la réponse immunitaire embryonnaire de manière bactérie spécifique (Freitak et al. 2014 ; Knorr et al. 2015). Il serait intéressant de tester cette hypothèse chez *T. molitor*, par une expérimentation utilisant des *B. thuringiensis* marquées par fluorescence. Une seconde hypothèse pourrait consister en l'incorporation de nutriments préfabriqués par la mère, ou de composés liés à l'immunité, directement dans les œufs, tels que des enzymes, des ARNm, des protéines de liaison de l'ARN, ce qui pourrait, comme le transfert direct de peptides antimicrobien, protéger la descendance avant que sa propre machinerie transcriptionnelle soit active (Sysoev et al. 2016). Ce type de « cadeaux » maternels a d'ailleurs été référencé à la fois chez les vertébrés et les invertébrés (Broggi et al. 2016 ; Grindstaff et al. 2003; Hasselquist & Nilsson 2009 ; Rossiter 1991 ; Sadd & Schmid-Hempel 2007 ; Seppola et al. 2009 ; Trauer-Kizilelma & Hilker 2015a, 2015b). Un dernier moyen pour les mères de moduler l'expression des gènes de sa descendance consisterait en une méthylation de l'ADN et/ou à l'acétylation d'histones (Oldroyd et al. 2014).

Quel que soit le mécanisme à l'origine de la synthèse des peptides antimicrobiens par les œufs, ceux-ci peuvent être un élément d'explication de la meilleure survie des jeunes larves exposées à *B. thuringiensis*. Néanmoins, l'action de ces peptides n'explique pas l'amélioration globale de survie pour les jeunes larves venant de femelles stimulées par *B. thuringiensis* et placées dans des conditions stériles. L'activité antimicrobienne associée au TTGI n'explique pas non

plus l'augmentation du taux d'éclosion observée pour les œufs issus de mères immuno-stimulées. On peut ainsi se demander quelle est la contribution maternelle qui permet d'augmenter le taux d'éclosion des œufs et d'améliorer la survie globale de ses descendants ? Il est possible que des mères faisant face à un agent pathogène approvisionnent plus abondamment leurs descendants avec des métabolites importants, tels que des carbohydrates, des protéines ou des lipides (Kinsella 1966 ; Pant et al. 1979) qui amélioreraient le taux d'éclosion. Une hypothèse alternative pourrait consister en une modification d'autres gènes à effets maternels que ceux liés à l'immunité. 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 & Scüpbach 1989). Par ailleurs, le développement embryonnaire et l'immunité empruntent les mêmes récepteurs et voies de transduction du signal. Stein et al. (1998) ont par exemple montré que la protéine Dif (Dorsal-related immunity factor), impliquée dans la réponse immunitaire chez *Drosophila*, joue aussi un rôle dans le bon développement de l'axe dorso-ventral chez des embryons, en activant et réprimant les gènes responsables de la mise en place de cet axe dorso-ventral. Il est donc possible que chez *T. molitor*, l'augmentation constatée suite à une stimulation maternelle, du taux d'éclosion, ainsi que de l'immunocompétence de la descendance, résultent d'un mécanisme commun. On pourrait ainsi supposer qu'une même protéine ou un même gène, dont l'expression serait activée par un challenge maternel, soit responsable du développement embryonnaire chez *T. molitor*, mais aussi de la synthèse d'effecteurs immunitaires. En conséquence, les différents bénéfices observés dans le cadre du TTGI sur les stades de développement précoces du ténébrion meunier, pourraient résulter de la pléiotropie des gènes de l'immunité.

Priming immunitaire et TTGI : une immunité antibactérienne spécifique anti-Gram-positive mais une immunité limitée tout de même ?

Dans leur étude récente, Dubuffet et al. (2015) ont proposé que le TTGI, chez *T. molitor*, aurait principalement évolué en réponse aux pressions sélectives des bactéries Gram-positives, capables de persister dans l'environnement de l'insecte. Les résultats obtenus dans le chapitre 1 viennent conforter cette hypothèse en montrant que le priming immunitaire avec une bactérie Gram-positive induisait une meilleure protection dans le cas d'une infection secondaire, et que

cette première stimulation, dans le cadre du TTGI, se révélait moins coûteuse pour la descendance. L'hypothèse que l'immunité de *T. molitor* aurait évolué en réponse aux agents pathogènes capables de persister dans l'environnement est renforcée par les résultats du chapitre 2. En effet, si une stimulation maternelle par des bactéries à Gram-positif améliore le taux d'éclosion des œufs, quelle que soit la bactérie utilisée, le bénéfice en termes de survie pour les jeunes larves n'est observé que dans le cas où la stimulation a été faite avec *B. thuringiensis*, la bactérie capable de sporuler et donc de persister dans l'environnement. Tester la réponse induite par d'autres bactéries Gram-positives, capables ou non de sporuler, permettrait de confirmer davantage cette hypothèse. De manière générale, l'immunité des invertébrés a dû évoluer en réponse aux agents pathogènes les plus dangereux pour eux. C'est-à-dire ceux qui ont la plus grande probabilité de les infecter et d'avoir des effets délétères. Nos résultats sur les paramètres immunitaires de la descendance adulte issue de femelles primées avec *B. thuringiensis* viennent renforcer cette hypothèse. En effet, nous avons observé dans le chapitre 1 un investissement supérieur de cette descendance dans l'immunité constitutive (de par une importante activité de la phénoloxydase qui n'est pas modifiée par un challenge immunitaire) et non dans la part inductible de l'immunité. Or, l'investissement dans les défenses constitutives 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 agents 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 également 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, et plus globalement à l'immunité des invertébrés, est la persistance de l'infection au cours des générations, comme proposé par Sadd et Schmid-Hempel (2005). Cette hypothèse est cohérente avec les connaissances actuelles du TTGI, puisqu'elle n'a été retrouvée que chez des espèces à générations chevauchantes et essentiellement envers des agents bactériens ou viraux, c'est-à-dire ayant un taux de réplication rapide et étant susceptibles de persister dans l'environnement. L'existence du TTGI n'a, par exemple, pas été rapportée 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 (Sadd et al. 2005).

Le développement d'une immunité propre aux bactéries Gram-positives chez *T. molitor*, et de manière plus générale aux agents pathogènes persistant dans l'environnement, a dû être imposé

par les capacités limitées des invertébrés à produire des récepteurs diversifiés. En effet, une réponse spécifique aux bactéries Gram-positives ne correspond pas nécessairement à une immunité capable de spécificité au sens strict. Cela sous-entend plutôt que l'espèce hôte a co-évolué avec un agent infectieux particulier. Même si dans le chapitre 2 nous avons montré que le TTGI aux œufs pouvait s'exprimer par des réponses pathogènes spécifiques (différence d'expression au sein des dynamiques d'activité antibactérienne en fonction de l'âge de l'œuf, dépendant de la bactérie utilisée pour la stimulation maternelle), notre étude menée chez des individus adultes n'a pas révélé de spécificité, même au sein des bactéries Gram-positives. L'étude du priming immunitaire, toujours chez des individus adultes, n'a également pas révélé cette spécificité. L'augmentation du nombre d'hémocytes après un second challenge bactérien dans le cas d'un priming avec *B. thuringiensis*, pouvait laisser à penser que de la spécificité, basée sur l'action des hémocytes (Pham et al. 2007), existait chez *T. molitor*. Celle-ci aurait pu être masquée par la persistance des peptides antibactériens. Cette hypothèse pourrait être testée en inactivant l'action des peptides antibactériens, ou en attendant assez longtemps entre le priming et le challenge pour que les peptides antimicrobiens soient absents de l'hémolymphe des individus adultes. Cette seconde option a été choisie dans un projet de notre laboratoire en effectuant le priming à l'état larvaire (mais en conservant les infections à l'état adulte) et en utilisant comme bactéries trois bacilles : *B. thuringiensis, Bacillus cereus* et *Brevibacillus laterosporus*. Ces bactéries à Gram-positif ont été choisies car c'est à l'heure actuelle, les seules qui ont été décrites comme étant pathogènes de *T. molitor* (Du Rand & Laing 2011). Si un bénéfice en termes de survie associé au priming à l'état larvaire a été observé en cas de réinfection avec l'un des trois agents pathogènes, aucune forme de spécificité n'est associée à cette protection (données non publiées). Cela semble suggérer qu'aucune spécificité au sens strict du terme n'intervient chez les individus adultes de *T. molitor*.

Conclusions

Dans le chapitre 1, j'ai proposé que l'immunité des invertébrés ait pu évoluer en réponse aux agents pathogènes capables de persister dans l'environnement, et puisse répondre de manière spécifique à ces derniers. Malgré les résultats des chapitres 1 et 2, montrant que le système immunitaire de *T. molitor* est capable de discriminer différents agents pathogènes et d'adapter dans une certaine mesure sa réponse en fonction de celle-ci, la diversité des réponses développées semble limitée. En effet, le système immunitaire du ténébrion meunier semble avoir co-évolué avec les bactéries Gram-positives, et particulièrement les bacilles, capables de persister dans l'environnement en formant des endospores. On peut ainsi observer que les réponses contre ces agents pathogènes sont faiblement coûteuses et offrent les bénéfices les plus importants. Néanmoins, il serait intéressant de confirmer que ces bactéries sont bien la principale menace auquel est exposé le ténébrion meunier. Une possibilité serait d'identifier la communauté bactérienne à laquelle *T. molitor* est exposé dans son environnement naturel. Si en premier lieu il faut déterminer à quoi correspond « l'habitat naturel » de cette espèce, cela pourrait être réalisé par une approche en séquençage massif des microbes issus de son environnement. Ensuite, si le priming immunitaire et le TTGI ont évolué en réponse aux pressions imposées par les microbes, ces phénomènes doivent également impacter l'évolution des agents pathogènes eux-mêmes. Les réponses immunitaires intervenant comme des prédateurs pour les parasites, il est attendu que les défenses les plus importantes, résultant du priming et du TTGI, favorisent l'évolution de contre-mesures de la part des parasites. Cela pourrait se manifester par des mécanismes d'évasion immunitaire ou des taux de réplication plus importants (André & Gandon 2006 ; Fenton et al. 2006 ; Mackinnon & Read 2004). Sachant que ces traits sont habituellement associés à une augmentation de la virulence (Sadd & Schmid-Hempel 2009b), il sera intéressant de constater l'impact réel du priming et du TTGI sur l'évolution des agents pathogènes. Dans le cas de *T. molitor* il faudrait alors se concentrer essentiellement sur les bacilles. Enfin, si l'on pouvait penser que le priming immunitaire et le TTGI se basaient essentiellement sur l'action des hémocytes (Pham et al. 2007), à l'image des lymphocytes des vertébrés, il n'en est rien. Les deux phénomènes, que ce soit le priming immunitaire ou le TTGI, ne semblent pas associés à des effecteurs définis. Au contraire, l'évolution de ces phénomènes paraît plutôt associée à un ensemble de traits d'histoire de vie interdépendants, qui peuvent favoriser l'émergence et la persistance d'investissements parentaux et individuels coûteux.

Références

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Adamo, S. A., Jensen, M. & Younger, M. (2001) Changes in lifetime immunocompetence in male and female *Gryllus texensis* (formerly *G. integer*): trade-offs between immunity and reproduction. *Animal Behaviour*, 62, 417-425.

Adema, C. M. (2015) Fibrinogen-Related Proteins (FREPs) in Mollusks. *Results and Problems in Cell Differentiation*, 57**,** 111-129.

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.

Agrawal, A. A., Laforsch, C. & Tollrian, R. (1999) Transgenerational induction of defences in animals and plants. *Nature*, 401**,** 60-63.

Akira, S., Uematsu, S. & Takeuchi, O. (2006) Pathogen recognition and innate immunity. *Cell*, 124**,** 783-801.

Allander, K. & Schmid-Hempel, P. (2000) Immune defence reaction in bumble-bee workers after a previous challenge and parasitic coinfection. *Functional Ecology*, 14, 711–717.

Allen, R. M., Buckley, Y. M. & Marshall, D. J. (2007) Offspring size plasticity in response to intraspecific competition: an adaptive maternal effect across life-history stages. *The American Naturalist*, 171, 225-237.

André, J.-B., & Gandon, S. (2006) Vaccination, within-host dynamics, and virulence evolution. *Evolution*, 60, 13.

Archer, C. R., Zajitschek, F., Sakaluk, S. K., Royle, N. J. & Hunt, J. (2012) Sexual selection affects the evolution of lifespan and ageing in the decorated cricket *Gryllodes sigillatus*. *Evolution*, 66, 3088-3100.

Ayres, J. S. & Schneider, D. S. (2008) A signaling protease required for melanization in *Drosophila* affects resistance and tolerance of infections. *PLoS Biology*, 6, 2764-2773.

Ayres, J. S. & Schneider, D. S. (2009) The role of anorexia in resistance and tolerance to infections in *Drosophila*. *PLoS Biology*, 7, e1000150.

Asgari, S. (2013) MicroRNA functions in insects. *Insect Biochemistry and Molecular Biology*, 43, 388-397.

Ashe, A., Sarkies, P., Le Pen, J., Tanguy, M. & Miska, E. A. (2015) Antiviral RNA interference against Orsay virus is neither systemic nor transgenerational in *Caenorhabditis elegans*. *Journal of Virology,* 89, 12035-12046.

Avila, F. W., Sirot, L. K., LaFlamme, B. A., Rubinstein, C. D. & Wolfner, M. F. (2011) Insect seminal fluid proteins: identification and function. *Annual Review of Entomology*, 56, 21-40.

Bascuñán-García, A. P., Lara, C. & Córdoba-Aguilar, A. (2010) Immune investment impairs growth, female reproduction and survival in the house cricket, *Acheta domesticus*. *Journal of Insect Physiology*, 56, 204-211.

Baron, O. L., van West, P., Industri, B., Ponchet, M., Dubreuil, G., Gourbal, B., Reichhart, J. M. & Coustau, C. (2013) Parental transfer of the antimicrobial protein LBP/BPI protects *Biomphalaria glabrata* eggs against oomycete infections. *PLoS Pathogens*, 9, e1003792.

Barnes, A. I. & Siva-Jothy, M. T. (2000) Density–dependent prophylaxis in the mealworm beetle *Tenebrio molitor* L. (Coleoptera: Tenebrionidae): cuticular melanization is an indicator of investment in immunity. *Proceedings of the Royal Society of London Series B: Biological Sciences*, 267, 177-182.

Barrangou, R. & Marraffini, L. A. (2014) CRISPR-Cas systems: Prokaryotes upgrade to adaptive immunity. *Molecular Cell*, 54, 234-244.

Barribeau, S. M., Schmid-Hempel, P. & Sadd, B. M. (2016) Royal decree: Gene expression in trans-generationally immune primed bumblebee workers mimics a primary immune response. *PLoS One*, 11, e0159635.

Becalska, A. N. & Gavis, E. R. (2009) Lighting up mRNA localization in *Drosophila* oogenesis. *Development*, 136, 2493-2503.

Becker, W.A. (1984) Manual of Quantitative Genetics, 3rd ed. Washington State University, Washington.

Behrens, S., Peuss, R., Milutinovic, B., Eggert, H., Esser, D., Rosenstiel, P., Schulenburg, H., Bornberg-Bauer, E. & Kurtz, J. (2014) Infection routes matter in population-specific responses of the red flour beetle to the entomopathogen *Bacillus thuringiensis*. *BMC Genomics*, 15, 445.

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

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

Berry, S.J. (1982) Maternal direction of oogenesis and early embryogenesis in insects. *Annual Review of Entomology*, 27, 205-227.

Bernardo, J. (1996) Maternal effects in animal ecology. *American Zoologist*, 36, 83-105.

Beutler, B. (2004) Innate immunity: an overview. *Molecular immunology*, 40, 845-859.

Biczkowski, M. & Dittman, F. (1995) Translation of maternal mRNA during early oogenesis in a telotrophic-meroistic insect (*Dysdercus intermedius*). *Journal of Insect Physiology*, 41, 1133-1139.

Boman, H. G., Nilsson, I. & Rasmuson, B. (1972) Inducible antibacterial defence system in *Drosophila*. *Nature*, 237, 232-235.

Boots, M. & Roberts, K.E. (2012) Maternal effects in disease resistance: poor maternal environment increases offspring resistance to an insect virus. *Proceedings of the Royal Society B: Biological Sciences*, 279, 4009-4014.

Boulinier, T. & Staszewski, V. (2008) Maternal transfer of antibodies: raising immuno-ecology issues. *Trends in Ecology & Evolution*, 23, 282-288.

Bouts, D. M., Melo, A. C., Andrade, A. L., Silva-Neto, M. A., Paiva-Silva Gde, O., Sorgine, M. H., da Cunha Gomes, L. S., Coelho, H. S., Furtado, A. P., Aguiar, E. C., de Medeiros, L. N., Kurtenbach, E., Rozental, S., Cunha, E. S. N. L., de Souza, W. & Masuda, H. (2007) Biochemical properties of the major proteins from *Rhodnius prolixus* eggshell. *Insect Biochemistry and Molecular Biology*, 37, 1207-1221.

Brites, D. & Du Pasquier, L. (2015) Somatic and germline diversification of a putative immunoreceptor within one phylum: Dscam in Arthropods. *Results and Problems in Cell Differentiation*, 57, 131-158.

Broggi, J., Soriguer, R., & Figuerola, J. (2016) Transgenerational effects enhance specific immune response in a wild passerine. *PeerJ*, 4, e1766.

Bulet, P. & Stöcklin, R. (2005) Insect antimicrobial peptides: Structures, properties and gene regulation. *Protein and Peptide Letters*, 12, 3-11.

Capco, D. G. & Jeffery, W. R. (1979) Origin and spatial distribution of maternal messenger RNA during oogenesis of an insect, *Oncopeltus fasciatus*. *Journal of Cell Science*, 39, 63-76.

Capinera, J., Barbosa, P. & Hagedorn, H. (1977) Yolk and yolk depletion of Gypsy moth eggs: Implications for population quality 1, 2. *Annals of the Entomological Society of America*, 70, 40-42.

Castro-Vargas, C., Linares-López, C., López-Torres, A., Wrobel, K., Torres-Guzmán, J.C., Hernández, G.A.G., Wrobel, K., Lanz-Mendoza, H. & Contreras-Garduño, J. (2017) Methylation on RNA: A potential mechanism related to immune priming within but not across generations. *Frontiers in Microbiology*, 8, 473.

Cerenius, L. & Söderhäll, K. (2004) The prophenoloxidase-activating system in invertebrates. *Immunological Rev*iews, 198, 116–126.

Charlet, M., Lagueux, M., Reichhart, J. M., Hoffman, D., Braun, A. & Meister, M. (1996) Cloning of the gene encoding the antibacterial peptide drosocin involved in *Drosophila* immunity: expression studies during the immune response. *European Journal of Biochemistry*, 241, 699-706.

Chase, M., Raina, K., Bruno, J. & Sugumaran, M. (2000) Purification, characterization and molecular cloning of prophenoloxidase from *Sarcophaga bullata*. *Insect Biochemestry and Molecular Biology*, 30, 953–967.

Chen, P. S., Stumm-Zollinger, E., Aigaki, T., Balmer, J., Blenz, M. & Böhlen, P. (1988) A male accessory gland peptide that regulates reproductive behavior of female *D. melanogaster*. *Cell*, 54, 291-296.

Clark, M. M. & Galef, B. G. (1995) Prenatal influences on reproductive life history strategies. *Trends in Ecology & Evolution*, 10, 151-153.

Corby-Harris, V., Habel, K. E., Ali, F. G. & Promislow, D. E. (2007) Alternative measures of response to *Pseudomonas aeruginosa* infection in *Drosophila melanogaster*. *Journal of Evolutionary Biology*, 20, 526–533.

Coustau, C., Kurtz, J. & Moret, Y. (2016) A novel mechanism of immune memory unveiled at the invertebrate-parasite interface. *Trends in Parasitology*, 32, 353-355.

Cumming, G. & Finch, S. (2005) Inference by eye: confidence intervals and how to read pictures of data. *American Psychologist*, 60, 170.

David, J.R., Gibert, P., Legout, H., Pétavy, G., Capy, P. & Moreteau, B. (2005) Isofemale lines in *Drosophila*: an empirical approach to quantitative trait analysis in natural populations. *Heredity,* 94, 3–12.

Dettloff, M., Wittwer, D., Weise, C. & Wiesner, A. (2001) Lipophorin of lower density is formed during immune responses in the lepidopteran insect *Galleria mellonella*. *Cell Tissue Research,* 306, 449–458.

Dhinaut, J., Chogne, M. & Moret, Y. (2017) Immune priming specificity within and across generations reveals the range of pathogens affecting evolution of immunity in an insect. *Journal of Animal Ecology,* doi:10.1111/1365-2656.12661.

Dhinaut, J., Chogne, M. & Moret, Y. (2017) Immune priming specificity within and across generations reveals the range of pathogens affecting evolution of immunity in an insect. *Dryad Digital Repository,* doi:10.5061/dryad.t2850.

Dhinaut, J., Chogne, M. & Moret, Y. (submitted) Trans-generational immune priming in the mealworm beetle protects eggs through pathogen-dependent mechanisms imposing no immediate fitness cost for the offspring.

Domanitskaya, E. V., Liu, H., Chen, S. & Kubli, E. (2007) The hydroxyproline motif of male sex peptide elicits the innate immune response in *Drosophila* females. *The FEBS Journal*, 274, 5659-5668.

Dong, Y., Taylor, H. E. & Dimopoulos, G. (2006) AgDscam, a hypervariable immunoglobulin domain-containing receptor of the *Anopheles gambiae* innate immune system. *PLoS Biology*, 4, e229.

Du, R. N. & Laing, M. (2011) Determination of insecticidal toxicity of three species of entomopathogenic spore-forming bacterial isolates against *Tenebrio molitor L*. (Coleoptera: Tenebrionidae). *African Journal of Microbiology Research*, 5, 2222-2228.

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

Du Pasquier, L. (2006) Germline and somatic diversification of immune recognition elements in Metazoa. *Immunology Letters,* 104, 2-17.

Dubuffet, A., Zanchi, C., Boutet, G., Moreau, J., Teixeira, M. & Moret, Y. (2015) Trans-generational immune priming protects the eggs only against gram-positive bacteria in the mealworm beetle. *PLoS Pathogens,* 11, e1005178.

Eggert, H., Diddens-de Buhr, M. F. & Kurtz, J. (2015) A temperature shock can lead to trans-generational immune priming in the Red Flour Beetle, *Tribolium castaneum*. *Ecology and Evolution,* 5, 1318-1326.

Eggert, H., Kurtz, J. & Diddens-de Buhr, M. F. (2014) Different effects of paternal trans-generational immune priming on survival and immunity in step and genetic offspring. *Proceedings of the Royal Society of London Series B: Biological Sciences,* 281, 20142089.

Ericsson, J. D., Janmaat, A. F., Lowenberger, C. & Myers, J. H. (2009) Is decreased generalized immunity a cost of *Bt* resistance in cabbage loopers *Trichoplusia ni*? *Journal of Invertebrate Pathology,* 100, 61-67.

Esteves, E., Fogaca, A. C., Maldonado, R., Silva, F. D., Manso, P. P., Pelajo-Machado, M., Valle, D. & Daffre, S. (2009) Antimicrobial activity in the tick *Rhipicephalus* (Boophilus) microplus eggs: Cellular localization and temporal expression of microplusin during oogenesis and embryogenesis. *Developmental & Comparative Immunology,* 33, 913-919.

Fehlbaum, P., Bulet, P., Michaut, L., Lagueux, M., Broekaert, W.F., Hetru, C. & Hoffmann J.A. (1994) Insect immunity. Septic injury of Drosophila induces the synthesis of a potent antifungal peptide with sequence homology to plant antifungal peptides. *Journal of Biological Chemistry*, 269, 33159-33163.

Felix, M. A., Ashe, A., Piffaretti, J., Wu, G., Nuez, I., Belicard, T., Jiang, Y., Zhao, G., Franz, C. J., Goldstein, L. D., Sanroman, M., Miska, E. A. & Wang, D. (2011) Natural and experimental infection of *Caenorhabditis* nematodes by novel viruses related to nodaviruses. *PLoS Biology,* 9, e1000586.

Fenton, A., Lello, J. & Bonsall, M. B. (2006) Pathogen responses to host immunity: the impact of time delays and memory on the evolution of virulence. *Proceedings of the Royal Society B: Biological Sciences,* 273, 2083-2090.

Fischer, M., Regitz, C., Kahl, M., Werthebach, M., Boll, M. & Wenzel, U. (2012) Phytoestrogens genistein and daidzein affect immunity in the nematode *Caenorhabditis elegans* via alterations of vitellogenin expression. *Molecular Nutrition & Food Research,* 56, 957-965.

Fisher, J. J. & Hajek, A. E. (2015) Maternal exposure of a beetle to pathogens protects offspring against fungal disease. *PLoS One*, 10, e0125197.

Folstad, I. & Karter, A. J. (1992) Parasites, bright males, and the immunocompetence handicap. *The American Naturalist*, 139, 603-622.

Fox, C. W., Thakar, M. S. & Mousseau, T. A. (1997) Egg size plasticity in a seed beetle: an adaptive maternal effect. *The American Naturalist*, 149, 149-163.

Frank, S. A. (2002) *Immunology and evolution of infectious disease*. Princeton University Press.

Fraune, S., Augustin, R., Anton-Erxleben, F., Wittlieb, J., Gelhaus, C., Klimovich, V. B., Samoilovich, M. P. & Bosch, T. C. (2010) In an early branching metazoan, bacterial colonization of the embryo is controlled by maternal antimicrobial peptides. *Proceedings of the National Academy of Sciences of the United States of America*, 107, 18067-18072.

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

Freitak, D., Schmidtberg, H., Dickel, F., Lochnit, G., Vogel, H. & Vilcinskas, A. (2014) The maternal transfer of bacteria can mediate trans-generational immune priming in insects. *Virulence*, 5, 547-554.

Galbraith, D. A., Yang, X., Nino, E. L., Yi, S. & Grozinger, C. (2015) Parallel epigenomic and transcriptomic responses to viral infection in honey bees (*Apis mellifera*). *PLoS Pathogens*, 11, e1004713.

Geister, T. L., Lorenz, M. X., Hoffmann, K. H. & Fisher, K. (2008) Adult nutrition and butterfly fitness: effects of diet quality on reproductive output, egg composition, and egg hatching success. *Frontiers in Zoology*, 5, 10.

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.

Glupov, V. V., Khvoshevskaya, M. F., Lozinskaya, Y. L., Dubovski, I. M., Martemyanov, V. V. & Sokolova, J. Y. (2001) Application of the nitroblue tetrazolium-reduction method for studies on the production of reactive oxygen species in insect haemocytes. *Cytobios 106* (Suppl 2), 165–178.

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.

Gorman, M. J. & Paskewitz, S. M. (2001) Serine proteases as mediators of mosquito immunity. *Insect Biochemestry and Molecular Biology*, 31, 257–262.

Gray, D.A. (1998) Sex differences in susceptibility of house crickets, *Acheta domesticus*, to experimental infection with *Serratia liquefaciens*. *Journal of Invertebrate Pathology*, 71, 288-289.

Green, T. J., Helbig, K., Speck, P. & Raftos, D. A. (2016) Primed for success: Oyster parents treated with poly(I:C) produce offspring with enhanced protection against Ostreid herpesvirus type I infection. *Molecular Immunology*, 78, 113-120.

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 of London B: Biological Sciences*, 270, 2309-2319.

Grindstaff, J. L. (2010) Initial levels of maternally derived antibodies predict persistence time in offspring circulation. *Journal of Ornithology*, 151, 423-428.

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

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

Hammerschmidt, K., Deines, P., Wilson, A. J. & Rolff, J. (2012) Quantitative genetics of immunity and life history under different photoperiods. *Heredity*, 108, 569-576.

Hangartner, S., Sbilordo, S. H., Michalczyk, Ł., Gage, M. J. G. & Martin, O. Y. (2013) Are there genetic trade-offs between immune and reproductive investments in *Tribolium castaneum*? *Infection, Genetics and Evolution*, 19, 45-50.

Hanington, P. C. & Zhang, S. M. (2011) The primary role of fibrinogen-related proteins in invertebrates is defense, not coagulation. *Journal of Innate Immunity*, 3, 17-27.

Hao, Z. G., Kasumba, I. & Aksoy, S. (2003). Proventriculus (cardia) plays a crucial role in immunity in tsetse fly (Diptera: *Glossinidiae*). *Insect Biochemestry and Molecular Biology*, 33, 1155–1164.

Harding, K. C., Hansen, B. J. L. & Goodman, S. J. (2005) Acquired immunity and stochasticity inepidemic intervals impede the evolution of host disease resistance. *The American Naturalist*, 166, 722-730.

Harris, J. F., Micheva-Viteva, S., Li, N. & Hong-Geller, E. (2013) Small RNA-mediated regulation of host-pathogen interactions. *Virulence*, 4, 785-795.

Harvell, C. D. (1990) The ecology and evolution of inducible defenses. *Quaterly Review of Biology*, 65, 323-340.

Hasselquist, D. & Nilsson, J.-Å. (2009) Maternal transfer of antibodies in vertebrates: trans-generational effects on offspring immunity. *Philosophical Transactions of the Royal Society B-Biological Sciences*, 364, 51-60.

Havukainen, H., Munch, D., Baumann, A., Zhong, S., Halskau, O., Krogsgaard, M. & Amdam, G. V. (2013) Vitellogenin recognizes cell damage through membrane binding and shields living cells from reactive oxygen species. *The Journal of Biological Chemistry*, 288, 28369-28381.

Hernández López, J., Schuehly, W., Crailsheim, K. & Riessberger-Gallé, U. (2014) Trans-generational immune priming in honeybees. *Proceedings of the Royal Society B: Biological Sciences*, 281, 20140454.

Ho, D. H. & Burggren, W. W. (2010) Epigenetics and transgenerational transfer: A physiological perspective. *The Journal of Experimental Biology*, 213, 3-16.

Hoffmann, A. A. & Parsons, P. A. (1988) The analysis of quantitative variation in natural populations with isofemale strains. *Génétique, Sélection, Evolution*, 20, 87-98.

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

Huang, B., Zhang, L., Li, L., Tang, X. & Zhang, G. (2015) Highly diverse fibrinogen-related proteins in the Pacific oyster *Crassostrea gigas*. *Fish & Shellfish Immunology*, 43, 485-490.

Huang, C. C. & Song, Y. L. (1999) Maternal transmission of immunity to white spot syndrome associated virus (WSSV) in shrimp (*Penaeus monodon*). *Developmental & Comparative Immunology*, 23, 545-552.

Hultmark, D. (1995). Insect lysozymes. *Exs*, 75, 87-102.

Huttenhuis, H. B., Grou, C. P., Taverne-Thiele, A. J., Taverne, N. & Rombout, J. H. (2006) Carp (*Cyprinus carpio* L.) innate immune factors are present before hatching. *Fish & Shellfish Immunology*, 20, 586-596.

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

Jacobs, C., Wang, Y., Vogel, H., Vilcinskas, A., van der Zee, M. & Rozen, D. (2014) Egg survival is reduced by grave-soil microbes in the carrion beetle, *Nicrophorus vespilloides*. *BMC Evolutionary Biology*, 14, 208.

Jacobs, C. G. C., Gallagher, J. D., Evison, S. E. F., Heckel, D. G., Vilcinskas, A. & Vogel, H. (2017) Endogenous egg immune defenses in the yellow mealworm beetle (*Tenebrio molitor*). *Developmental & Comparative Immunology*, 70, 1-8.

Jacobs, C. G. C., Spaink, H. P. & van der Zee, M. (2014) The extraembryonic serosa is a frontier epithelium providing the insect egg with a full-range innate immune response. *Elife*, 3, e04111.

Jacobs, C.G.C. & van der Zee, M. (2013) Immune competence in insect eggs depends on the extraembryonic serosa. *Developmental & Comparative Immunology*, 41, 263-269.

Janeway, C. A., Jr. & Medzhitov, R. (2002) Innate immune recognition. *Annual Review of Immunology*, 20, 197-216.

Johnstone, O. & Lasko, P. (2001) Translational regulation and RNA localization in *Drosophila* oocytes and embryos. *Annual Review of Genetics*, 35, 365-406.

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

Jung, J., Heo, A., Park, Y. W., Kim, Y. J., Koh, H. & Park, W. (2014) Gut microbiota of *Tenebrio molitor* and their response ton environmental change. *Journal of Microbiology and Biotechnology*, 24, 888-897.

Jurat-Fuentes, J. L. & Jackson T. (2012) Bacterial entomopathogens. *Insect Pathology*, 2nd edn (eds H. Kaya & F. Vera), pp. 265-349. Elsevier, Academic Press, Cambridge, MA, USA.

Kaaya, G. P. (1993) Inducible humoral antibacterial immunity in insects. *Insect immunity*, (eds JPN. Pathak), pp. 69-89. Dordrecht Springer, Netherlands.

Kinsella, J. E. (1966) Metabolic patterns of the fatty acids of *Periplanteta americana* (L.) during its embryonic development. *Canadian Journal of Biochemistry*, 44, 247-258.

Kirkpatrick, M. & Lande, R. (1989) The evolution of maternal characters. *Evolution*, 43, 485-503.

Knorr, E., Schmidtberg, H., Arslan, D., Bingsohn, L. & Vilcinskas, A. (2015) Translocation of bacteria from the gut to the eggs triggers maternal transgenerational immune priming in *Tribolium castaneum*. *Biology Letters*, 11, 20150885.

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.

Krams, I., Daukste, J., Krama, T. & Rantala, M.J. (2013) Previous encapsulation response enhances within individual protection against fungal parasite in the mealworm beetle *Tenebrio molitor*. *Insect Science* 20, 771-777.

Kurtz, J. (2005) Specific memory within innate immune systems. *Trends in Immunology*, 26, 186-192.

Kvell, K., Cooper, E. L., Engelmann, P., Bovari, J. & Nemeth, P. (2007) Blurring borders: innate immunity with adaptive features. *Clinical & Developmental Immunology*, 2007, 83671.

Lackie, A. M., Takle, G. & Tetley, L. (1985) Haemocytic encapsulation in the locust *Schistocerca gregaria* (Orthoptera) and the cockroach *Periplaneta americana*. *Cell and Tissue Research*, 240, 343–351.

Lambeth, J. D. (2007) Nox enzymes, ROS and chronic disease: an example of antagonistic pleiotropy. *Free Radical Biology and Medicine*, 43, 332–347.

Lambeth, J. D., Kawahara, T. & Diebold, B. (2007) Regulation of Nox and Dox enzymatic activity and expression. *Free Radical Biology and Medicine*, 43, 319–331.

Lavine, M. D., Chen, G. & Strand, M. R. (2005) Immune challenge differentially affects transcript abundance of three antimicrobial peptides in hemocytes from the moth *Pseudoplusia includens*. *Insect Biochemistry and Molecular Biology*, 35, 1335-1346.

Lazzaro, B. P. (2008) Natural selection on the *Drosophila* antimicrobial immune system. *Current Opinion in Microbiology*, 11, 284-289.

Lemaître, B., Reichart, J. M. & Hoffmann, J. A. (1997) Drosophila host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. *Proceedings of the National Academy of Sciences of USA*, 94, 14614–14619.

Li, Z., Zhang, S. & Liu, Q. (2008) Vitellogenin functions as a multivalent pattern recognition receptor with an opsonic activity. *PLoS One*, 3, e1940.

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

Lindström, J. (1999) Early development and fitness in birds and mammals. *Trends in Ecology & Evolution*, 14, 343-348.

Little, T. J., Hultmark, D. & Read, A. F. (2005) Invertebrate immunity and the limits of mechanistic immunology. *Nature Immunology*, 6, 651-654.

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

Little, T. J., O'Connor, B., Colegrave, N., Watt, K. & Read, A.F. (2003) Maternal transfer of strain-specific immunity in an invertebrate. *Current Biology*, 13, 489-492.

Littlefair, J. E., Laughton, A. M. & Knell, R. J. (2016) Maternal pathogen exposure causes diet- and pathogen-specific transgenerational costs. *Oikos*, doi**:** 10.1111/oik.03430.

Liu, H. & Kubli, E. (2003) Sex-peptide is the molecular basis of the sperm effect in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America*, 100, 9929-9933.

Lorenz, L. M. & Koella, J. C. (2011) Maternal environment shapes the life history and susceptibility to malaria of *Anopheles gambiae* mosquitoes. *Malaria Journal*, 10, 382.

Luckhart, S., Vodovotz, Y., Cui, L. & Rosenberg, R. (1998) The mosquito *Anopheles stephensi* limits malaria parasite development with inducible synthesis of nitric oxide. *Proceedings of the National Academy of Sciences*, 95, 5700-5705.

Mackinnon, M. J. & Read, A. F. (2004) Immunity promotes virulence evolution in a malaria model. *PLoS Biology*, 2, E230.

Maddison, D. R. & Schulz, K. S. (2007) The Tree of Life Web Project. http://tolweb.org.

Makarova, O., Rodriguez-Rojas, A., Eravci, M., Weise, C., Dobson, A., Johnston, P. & Rolff, J. (2016) Antimicrobial defence and persistent infection in insects revisited. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 371, 20150296.

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. O., 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 horion of the medfly *Ceratitis capitata*. *Biochemical and Biophysical Research Communications*, 240, 657-663.

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

Martins, N. E., Faria, V. G., Teixeira, L., Magalhaes, S. & Sucena, E. (2013) Host adaptation is contingent upon the infection route taken by pathogens. *PLoS Pathogens*, 9, e1003601.

Masri, L., Branca, A., Sheppard, A. E., Papkou, A., Laehnemann, D., Guenther, P. S., Prahl, S., Saebelfeld, M., Hollensteiner, J., Liesegang, H., Brzuszkiewicz, E., Daniel, R., Michiels, N. K., Schulte, R. D., Kurtz, J., Rosenstiel, P., Telschow, A., Bornberg-Bauer, E. & Schulenburg, H. (2015) Host-pathogen coevolution: The selective qdvantage of *Bacillus thuringiensis* virulence and its cry toxin genes. *PLoS Biology*, 13, e1002169.

Masri, L. & Cremer, S. (2014) Individual and social immunisation in insects. *Trends in Immunology*, 35, 471-482.

Matzinger, P. (1994) Tolerance, danger and the extended family. *Annual Review of Immunology*, 12, 991-1045.

McKean, K. A. & Lazzaro, B. P. (2011) The costs of immunity and the evolution of immunological defense mechanisms. Oxford University Press Oxford.

McNamara, K. B., van Lieshout, E. & Simmons, L. W. (2014) The effect of maternal and paternal immune challenge on offspring immunity and reproduction in a cricket. *Journal of Evolutionary Biology*, 27, 1020-1028.

Milutinović, B. & Kurtz, J. (2016) Immune memory in invertebrates. *Seminars in Immunology*, 28, 328-342.

Mitchell, S. E. & Read, A. F. (2005) Poor maternal environment enhances offspring disease resistance in an invertebrate. *Proceedings of the Royal Society of London Series B: Biological Sciences*, 272, 2601-2607.

Moore, S. L. & Wilson, K. (2002) Parasites as a viability cost of sexual selection in natural populations of mammals. *Science*, 297, 2015-2018.

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.

Moreno-Garcia, M., Conde, R., Bello-Bedoy, R. & Lanz-Mendoza, H. (2014) The damage threshold hypothesis and the immune strategies of insects. *Infection, Genetics and Evolution,* 24, 25-33.

Moret, Y. (2003) Explaining variable costs of the immune response: selection for specific versus non-specific immunity and facultative life history change. *Oikos*, 102, 213-215.

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: Biological Sciences*, 273, 1399-1405.

Moret, Y. & Schmid-Hempel, P. (2000) Survival for immunity: The price of immune system activation 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 of London Series B: Biological Sciences*, 270, 2475-2480.

Mousseau, T. A. & Fox, C. W. (1998) Maternal effects as adaptations. Oxford University Press.

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

Mukherjee, K., Fischer, R. & Vilcinskas, A. (2012) Histone acetylation mediates epigenetic regulation of transcriptional reprogramming in insects during metamorphosis, wounding and infection. *Frontiers in Zoology*, 9, 25.

Murphy, K. & Weaver, C. (2016) *Janeway's Immunobiology*. Garland Science.

Nathan, C. F. & Hibbs, J. B. Jr. (1991) Role of nitric oxide synthesis in macrophage antimicrobiol activity. *Current Opinion in Immunology*, 3, 65–70.

Netea, M. G., Joosten, L. A., Latz, E., Mills, K. H., Natoli, G., Stunnenberg, H. G., O'Neill, L. A. & Xavier, R. J. (2016) Trained immunity: A program of innate immune memory in health and disease. *Science*, 352, aaf1098.

Netea, M. G., Quintin, J. & van der Meer, J. W. (2011) Trained immunity: a memory for innate host defense. *Cell Host & Microbe*, 9, 355-361.

Norouzitallab, P., Baruah, K., Biswas, P., Vanrompay, D. & Bossier, P. (2016) Probing the phenomenon of trained immunity in invertebrates during a transgenerational study, using brine shrimp *Artemia* as a model system. *Scientific Reports*, 6, 21166.

Norouzitallab, P., Biswas, P., Baruah, K. & Bossier, P. (2015) Multigenerational immune priming in an invertebrate parthenogenetic Artemia to a pathogenic *Vibrio campbellii*. *Fish & Shellfish Immunology*, 42, 426-429.

Oldroyd, B. P., Allsopp, M. H., Roth, K. M., Remnant, E. J., Drewell, R. A., & Beekman, M. (2014) A parent-of-origin effect on honeybee worker ovary size. *Proceedings of the Royal Society of London B: Biological Sciences*, 281, 20132388.

Pancer, Z. & Cooper, M. D. (2006) The evolution of adaptive immunity. *Annual Review of Immunology*, 24, 497-518.

Pant, R., Kumar, S. & Singh, S. D. (1979) Changes in carbohydrates and lipids during embryonic development of *Antheraea mylitta* (Lepidoptera). *Journal of Biosciences*, 1, 27-33.

Peng, J., Zipperlen, P. & Kubli, E. (2005) *Drosophila* sex-peptide stimulates female innate immune system after mating via the Toll and Imd pathways. *Current Biology*, 15, 1690-1694.

Pham, L. N., Dionne, M. S., Shirasu-Hiza, M. & Schneider, D. S. (2007) A specific primed immune response in *Drosophila* is dependent on phagocytes. *PLoS Pathogens*, 3, e26.

Pham, L. N. & Schneider, D. S. (2008) Evidence for specificity and memory in the insect innate immune response. *Insect Immunology*, (eds N.E. Beckage), pp. 97-127. Boston Academic Press, Boston.

Pigeault, R., Garnier, R., Rivero, A. & Gandon, S. (2016) Evolution of transgenerational immunity in invertebrates. *Proceedings of the Royal Society of London Series B: Biological Sciences*, 283, 20161136.

Pigeault, R., Vezilier, J., Nicot, A., Gandon, S. & Rivero, A. (2015) Transgenerational effect of infection in *Plasmodium*-infected mosquitoes. *Biology Letters*, 11, 20141025.

Pinaud, S., Portela, J., Duval, D., Nowacki, F. C., Olive, M. A., Allienne, J. F., Galinier, R., Dheilly, N. M., Kieffer-Jaquinod, S., Mitta, G., Theron, A. & Gourbal, B. (2016) A Shift from cellular to humoral responses contributes to innate immune memory in the vector snail *Biomphalaria glabrata*. *PLoS Pathogens*, 12, e1005361.

Plaistow, S. J., St. Clair, J. J., Grant, J. & Benton, T. G. (2007) How to put all your eggs in one basket: empirical patterns of offspring provisioning throughout a mother's lifetime. *The American Naturalist*, 170, 520-529.

Poulin, R. (1996) Sexual inequalities in helminth infections: a cost of being a male? *The American Naturalist*, 147, 287-295.

Pradeu, T. & Cooper, E. L. (2012) The danger theory: 20 years later. *Frontiers in Immunology*, 3, 287.

Pradeu, T., Jaeger, S. & Vivier, E. (2013) The speed of change: towards a discontinuity theory of immunity? *Nature Reviews Immunology*, 13, 764-769.

Pursall, E. R. & Rolff, J. (2011) Immune responses accelerate ageing: proof-of-principle in an insect model. *PLoS One*, 6, e19972.

Qvarnström, A. & Price, T. D., (2001) Maternal effects, paternal effects and sexual selection. *Trends in Ecology & Evolution*, 16, 95-100.

Råberg, L., Graham, A. L. & Read, A. F. (2009) Decomposing health: tolerance and resistance to parasites in animals. *Philosophical Transactions of the Royal Society B-Biological Sciences*, 364, 37-49.

Råberg, L., Vestberg, M., Hasselquist, D., Holmdahl, R., Svensson, E. & Nilsson, J.N. (2002) Basal metabolic rate and the evolution of the adaptive immune system. *Proceedings of the Royal Society B: Biological Sciences*, 269, 817-821.

Rahman, M. M., Roberts, H. L. S., Sarjan, M., Asgari, S. & Schmidt, O. (2004) 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.

Raikhel, A. S. & Dhadialla, T. S. (1992) Accumulation of yolk proteins in insect oocytes. *Annual Review of Entomology*, 37, 217-251.

Raikhel, A. S., Kokoza, V. A., Zhu, J. S., Martin, D., Wang, S. F., Li, C., Sun, G. Q., Ahmed, A., Dittmer, N. & Attardo, G. (2002) Molecular biology of mosquito vitellogenesis: from basic studies to genetic engineering of antipathogen immunity. *Insect Biochemistry and Molecular Biology*, 32, 1275-1286.

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

Robinson, J. M. & Badwey, J. A. (1994) Production of active oxygen species by phagocytic leukocytes. *Immunology*, 60, 159–178.

Rodrigues, J., Brayner, F.A., Alves, L.C., Dixit, R. & Barillas-Mury, C. (2010) Hemocyte differentiation mediates innate immune memory in *Anopheles gambiae* mosquitoes. *Science*, 329, 1353-1355.

Roff, D. A. (1992) *The evolution of life histories*. Chapman and Hall, New York, NY.

Rolff, J., Armitage, S. A. O. & Coltman, D. W. (2005) Genetic constraints and sexual dimorphism in immune defense. *Evolution*, 59, 1844-1850.

Rolff, J. (2002) Bateman's principle and immunity. *Proceedings of the Royal Society of London Series B: Biological Sciences*, 269, 867-872.

Rosengaus, R. B., Hays, N., Biro, C., Kemos, J., Zaman, M., Murray, J., Gezahegn, B. & Smith, W. (2017) Pathogen-induced maternal effects result in enhanced immune responsiveness across generations. *Ecology and Evolution*, 7, 2925-2935.

Rossiter, M. C. (1991) Maternal effects generate variation in life history: consequences of egg weight plasticity in the Gypsy Moth. *Functional Ecology*, 5, 386–393.

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.

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

Roth, O., Sadd, B. M., Schmid-Hempel, P. & Kurtz, J. (2009) Strain-specific priming of resistance in the red flour beetle, *Tribolium castaneum*. *Proceedings of the Royal Society B: Biological Sciences*, 276, 145-151.

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. (2006) Insect immunity shows specificity in protection upon secondary pathogen exposure. *Current Biology*, 16, 1206-1210.

Sadd, B. M. & Schmid-Hempel, P. (2007) Facultative but persistent trans-generational immunity via the mother's eggs in bumblebees. *Current Biology*, 17, R1046-R1047.

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

Sadd, B. M. & Schmid-Hempel, P. (2009) Ecological and evolutionary implications of specific immune responses. In *Insect Infection and Immunity: Evolution, Ecology, and Mechanisms* (ed. J. Rolff and S. Reynolds), pp. 272. Oxford University Press, Oxford, UK.

Saeed, S., Quintin, J., Kerstens, H. H. D., Rao, N. A., Aghajanirefah, A., Matarese, F., Cheng, S. C., Ratter, J., Berentsen, K., van der Ent, M. A., Sharifi, N., Janssen-Megens, E. M., Ter Huurne, M., Mandoli, A., van Schaik, T., Ng, A., Burden, F., Downes, K., Frontini, M., Kumar, V., Giamarellos-Bourboulis, E. J., Ouwehand, W. H., van der Meer, J. W. M., Joosten, L. A. B., Wijmenga, C., Martens, J. H. A., Xavier, R. J., Logie, C., Netea, M. G. & Stunnenberg, H. G. (2014) Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. *Science*, 345, 1251086.

Salmela, H., Amdam, G. V. & Freitak, D. (2015) Transfer of immunity from mother to offspring is mediated via egg-yolk protein vitellogenin. *PLoS Pathogens*, 11, e1005015.

Schalk, G. & Forbes, M. R. (1997) Male biases in parasitism of mammals: effects of study type, host age, and parasite taxon. *Oikos*, 78, 67-74.

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

Schmid-Hempel, P. (2005) Evolutionary ecology of insect immune defenses. *Annual Review of Entomology*, 50, 529-551.

Schmid-Hempel, P. (2009) Immune defence, parasite evasion strategies and their relevance for 'macroscopic phenomena' such as virulence. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 364, 85-98.

Schneider, D. S. & Ayres, J. S. (2008) Two ways to survive infection: what resistance and tolerance can teach us about treating infectious diseases. *Nature Reviews Immunology*, 8, 889-895.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D. R. & Dean, D. H. (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiology and Molecular Biology Reviews*, 62, 775-806.

Schwarzenbach, G. A., Hosken, D. J. & Ward, P. I. (2005) Sex and immunity in the yellow dung fly *Scathophaga stercoraria*. *Journal of Evolutionary Biology*, 18, 455-463.

Seehuus, S. C., Norberg, K., Gimsa, U., Krekling, T. & Amdam, G. V. (2006) Reproductive protein protects functionally sterile honey bee workers from oxidative stress. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 962-967.

Seppola, M., Johnsen, H., Mennen, S., Myrnes, B., & Tveiten, H. (2009) Maternal transfer and transcriptional onset of immune genes during ontogenesis in Atlantic cod. *Developmental Comparative Immunology*, 33, 205–211.

Sheldon, B. C. & Verhulst, S. (1996) Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. *Trends in Ecology & Evolution*, 11, 317-321.

Shi, Z. H., Lin, Y. T. & Hou, Y. M. (2014) Mother-derived trans-generational immune priming in the red palm weevil, *Rhynchophorus ferrugineus* Olivier (Coleoptera, Dryophthoridae). *Bulletin of Entomological Research*, 104, 742-750.

Shikano, I., Hua, K. N. & Cory, J. S. (2016) Baculovirus-challenge and poor nutrition inflict within-generation fitness costs without triggering transgenerational immune priming. *Journal of Invertebrate Pathology*, 136, 35-42.

Singer, M. S., Mason, P. A. & Smilanich, A. M. (2014) Ecological immunology mediated by diet in herbivorous insects. *Integrative and Comparative Biology*, 54, 913-921.

Singh, N. K., Pakkianathan, B. C., Kumar, M., Prasad, T., Kannan, M., Konig, S. & Krishnan, M. (2013) Vitellogenin from the silkworm, *Bombyx mori*: an effective anti-bacterial agent. *PLoS One*, 8, e73005.

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

Söderhäll, K. & Cerenius, L. (1998) Role of the prophenoloxidase-activating system in invertebrate immunity. *Current Opinion in Immunology*, 10, 23–28.

Šrám, R. J., Binková, B., Dejmek, J. & Bobak, M. (2005) Ambient air pollution and pregnancy outcomes: a review of the literature. *Environmental Health Perspectives*, 113, 375-382.

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

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.

Sterken, M. G., Snoek, L. B., Bosman, K. J., Daamen, J., Riksen, J. A., Bakker, J., Pijlman, G. P. & Kammenga, J. E. (2014) A heritable antiviral RNAi response limits Orsay virus infection in *Caenorhabditis elegans* N2. *PLoS One*, 9, e89760.

Sternberg, E. D., de Roode, J. C. & Hunter, M. D. (2015) Trans-generational parasite protection associated with paternal diet. *The Journal of Animal Ecology*, 84, 310-321.

Stoops, J., Crauwels, S., Waud, M., Claes, J., Lievens, B. & Van Campenhout, L. (2016) Microbial community assessment of mealworm larvae (*Tenebrio molitor*) and grasshoppers (*Locusta migratoria migratorioides*) sold for human consumption. *Food Microbiology*, 53, 122-127.

Sugumaran, M., Nellaiappan, K. & Valivittan, K. (2000) A new mechanism for the control of phenoloxidase activity: inhibition and complex formation with quinone isomerase. *Archives of Biochemistry and Biophysics*, 379, 252-260.

Sugumaran, M. (2002) Comparative biochemistry of eumelanogenesis and the protective roles of phenoloxidase and melanin in insects. *Pigment Cell Research*, 15, 2–9.

Sun, J. C., Ugolini, S. & Vivier, E. (2014) Immunological memory within the innate immune system. *The EMBO Journal*, 33, 1295-1303.

Sysoev, V. O., Fischer, B., Frese, C. K., Gupta, I., Krijgsveld, J., Hentze, M.W., Castello, A. & Ephrussi, A. (2016) Global changes of the RNA-bound proteome during the maternal-to-zygotic transition in *Drosophila*. *Nature Communication*s, 7, 12128.

Takamatsu, N., Shiba, T., Muramoto, K. & Kamiya, H. (1995) Molecular cloning of the defense factor in the albumen gland of the sea hare *Aplysia kurodai*. *FEBS Letters*, 377, 373-376.

Tate, A. T. (2016) The interaction of immune priming with different modes of disease transmission. *Frontiers in Microbiology*, 7, 1102.

Tate, A. T. & Graham, A. L. (2015) Trans-generational priming of resistance in wild flour beetles reflects the primed phenotypes of laboratory populations and is inhibited by co-infection with a common parasite. *Functional Ecology*, 29, 1059-1069.

Tate, A. T. & Rudolf, V. H. W. (2012) Impact of life stage specific immune priming on invertebrate disease dynamics. *Oikos*, 121, 1083-1092.

Tetreau, G., Dhinaut, J., Gourbal, B. & Moret, Y. (submitted) Trans-generational immune priming in invertebrates: where are we now and where must we go?

Theopold, U., Ekengren, S. & Hultmark, D. (1996) HLH106, a Drosophila transcription factor with similarity to the vertebrate sterol responsive element binding protein. *Proceedings of the National Academy of Sciences*, 93, 1195-1199.

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

Thornhill, R. & Alcock, J. (1983) The evolution of insect mating system. Harvard University Press, Cambridge, MA, USA.

Tidbury, H. J., Best, A. & Boots, M. (2012) The epidemiological consequences of immune priming. *Proceedings of the Royal Society of London B: Biological Sciences*, 279, 4505-4512.

Tidbury, H. J., Pedersen, A. B. & Boots, M. (2011) Within and transgenerational immune priming in an insect to a DNA virus. *Proceedings of the Royal Society B: Biological Sciences*, 278, 871-876.

Tingvall, T. Ö., Roos, E. & Engström, Y. (2001) The GATA factor Serpent is required for the onset of the humoral immune response in *Drosophila* embryos. *Proceedings of the National Academy of Sciences U.S.A*, 98, 3884-3888.

Torgerson, C. J. (2010) Publication Bias: The Achilles' heel of systematic reviews? *British Journal of Educational Studies*, 54, 89-102.

Trauer-Kizilelma, U. & Hilker, M. (2015) Impact of transgenerational immune priming on the defence of insect eggs against parasitism. *Developmental & Comparative Immunol*ogy, 51, 126-133.

Trauer-Kizilelma, U. & Hilker, M. (2015) Insect parents improve the anti-parasitic and anti-bacterial defence of their offspring by priming the expression of immune-relevant genes. *Insect Biochemistry and Molecular Biology,* 64, 91-99.

Trauer, U. & Hilker, M. (2013) Parental legacy in insects: variation of transgenerational immune priming during offspring development. *PLoS One*, 8, e63392.

Triggs, A. M., Knell, R. J. & Martin, L. (2012) Parental diet has strong transgenerational effects on offspring immunity. *Functional Ecology*, 26, 1409-1417.

Tsakas, S. & Marmaras, V. J. (2010) Insect immunity and its signalling: an overview. *Invertebrate Survival Journal*, 7, 228-238.

Tseng, M. (2006) Interactions between the parasite's previous and current environment mediate the outcome of parasite infection. *The American Naturalist,* 168, 565-571.

Tufail, M. & Takeda, M. (2008) Molecular characteristics of insect vitellogenins. *Journal of Insect Physiology*, 54, 1447-1458.

Vachon, V., Laprade, R. & Schwartz, J. L. (2012) Current models of the mode of action of *Bacillus thuringiensis* insecticidal crystal proteins: a critical review. *Journal of Invertebrate Pathology*, 111, 1-12.

Vantaux, A., Dabire, K. R., Cohuet, A. & Lefevre, T. (2014) A heavy legacy: offspring of malaria-infected mosquitoes show reduced disease resistance. *Malaria Journal*, 13, 442.

Vilcinskas, A. (2016) The role of epigenetics in host-parasite coevolution: lessons from the model host insects *Galleria mellonella* and *Tribolium castaneum*. *Zoology (Jena),* 119, 273-280.

Vivier, E. & Malissen, B. (2005) Innate and adaptive immunity: specificities and signaling hierarchies revisited. *Nature Immunology,* 6, 17-21.

Vizioli, J. & Salzet, M. (2002) Antimicrobial peptides from animals: focus on invertebrates. *Trends in Pharmacological Sciences,* 23, 494-496.

Voordouw, M. J., Lambrechts, L. & Koella, J. C. (2008) No maternal effects after stimulation of the melanization 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.

Wang, L., Yue, F., Song, X. & Song, L. (2015) Maternal immune transfer in mollusc. *Developmental & Comparative Immunology,* 48, 354-359.

Wang, Y. & Zhang, Y. (2015) Investigation of gut-associated bacteria in *Tenebrio molitor* (Coleoptera: Tenebrionidae) larvae using culture-dependent and DGGE methods. *Annals of the Entomological Society of America,* 108, 941-949.

Watson, F. L., Püttmann-Holgado, R., Thomas, F., Lamar, D. L., Hughes, M., Kondo, M., Rebel, V. I. & Schmucker, D. (2005) Extensive diversity of Ig-superfamily proteins in the immune system of insects. *Science,* 309, 1874-1878.

Wedekind, C. & Jakobsen, P. J. (1998) Male-biased susceptibility to helminth infection: an experimental test with a copepod. *Oikos,* 81, 458-462.

Weiske, J. & Wiesner, A. (1999) Stimulation of NO synthase activity in the immune-competent lepidopteran *Estigmene acraea* hemocyte line. *Nitric Oxide Biology and Chemestry,* 3, 123–131.

Whitten, M. M. A. & Ratcliffe, N. A. (1999) In vitro superoxide activity in the haemolymph of the West Indian leaf cockroach, *Blaberus discoidalis*. *Journal of Insect Physiology,* 45, 667–675.

Wiklund, A. K. E. & Sundelin, B. (2001) Impaired reproduction in the amphipods *Monoporeia affinis* and *Pontoporeia femorata* as a result of moderate hypoxia and increased temperature. *Marine Ecology Progress Series*, 222, 131-141.

Wilson, K. & Graham, R. I. (2015) Transgenerational effects modulate density-dependent prophylactic resistance to viral infection in a lepidopteran pest. *Biology Letters*, 11, 20150012.

Wolf, J. B., Brodie I, E. D., Cheverud, J. M., Moore, A. J. & Wade, M. J. (1998) Evolutionary consequences of indirect genetic effects. *Trends in Ecology & Evolution*, 13, 64-69.

Wu, G., Xu, L. & Yi, Y. (2016) *Galleria mellonella* larvae are capable of sensing the extent of priming agent and mounting proportionatal cellular and humoral immune responses. *Immunology Letters*, 174, 45-52.

Yue, F., Zhou, Z., Wang, L., Ma, Z., Wang, J., Wang, M., Zhang, H. & Song, L. (2013) Maternal transfer of immunity in scallop *Chlamys farreri* and its trans-generational immune protection to offspring against bacterial challenge. *Developmental & Comparative Immunology*, 41, 569-577.

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.

Zanchi, C., Troussard, J. P., Moreau, J. & Moret, Y. (2012) Relationship between maternal transfer of immunity and mother fecundity in an insect. *Proceedings of the Royal Society B: Biological Sciences*, 279, 3223-3230.

Zuk, M. (1990) Reproductive strategies and disease susceptibility: an evolutionary viewpoint. *Parasitology Today*, 6, 231-233.

Zuk, M. & McKean, K. A. (1996) Sex differences in parasite infections: patterns and processes. *International Journal for Parasitology*, 26, 1009-1024.

Annexes

A dietary carotenoid reduces immunopathology and enhances longevity through an immune depressive effect in an insect model

Authors: J. Dhinaut, A. Balourdet, M. Teixeira, M. Chogne, Y. Moret

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Abstract

Immunopathology corresponds to self-damage of the inflammatory response, resulting from oxidizing molecules produced when the immune system is activated. Immunopathology often contributes to age-related diseases and is believed to accelerate ageing. Prevention of immunopathology relies on endogenous antioxidant enzymes and the consumption of dietary antioxidants, including carotenoids such as astaxanthin. Astaxanthin currently raises considerable interest as a powerful antioxidant and for its potential in alleviating age-related diseases. Current in vitro and short-term in vivo studies provide promising results about immune-stimulating and antioxidant properties of astaxanthin. However, to what extent dietary supplementation with astaxanthin can prevent long-term adverse effects of immunopathology on longevity is unknown so far. Here, using the mealworm beetle, Tenebrio molitor, as biological model we tested the effect of lifetime dietary supplementation with astaxanthin on longevity when exposed to early life inflammation. While supplementation with astaxanthin was found to lessen immunopathology cost on larval survival and insect longevity, it was also found to reduce immunity, growth rate and the survival of non immune-challenged larvae. This study therefore reveals that astaxanthin prevents immunopathology through an immune depressive effect and can have adverse consequences on growth.

Introduction

Immunopathology is a remarkably common cause of disease resulting from inflammatory responses of the innate immune system elicited by trauma or infection[1-3]. Inflammation is a phenomenon known from both vertebrates[4] and invertebrates[5-6], corresponding to a fast but non-specific response characterised by the delivery of fluids, cytotoxic chemicals and cells to damaged and infected tissues, in order to combat infectious agents and initiate tissue repair. Cytotoxic chemicals released at the focal site of injury or infection comprise highly reactive oxygen species (ROS) and nitrogen species (RNS) destructive to both pathogens and hosts, leading to immunopathology[7]. When damaged tissues are not fully repaired and that homeostasis is not restored, inflammation can further develop into a chronic condition, with inevitable long-term debilitating consequences, such as increased rates of morbidity and mortality at older age[8-10].

As defence mechanisms, organisms produce a number of endogenous antioxidants capable of scavenging these harmful free radicals and prevent an imbalance between pro- and anti-inflammatory status. However, under conditions of high oxidative stress, the ability of these antioxidants to eliminate free radicals are often exceeded and, therefore, dietary sources of antioxidants are required[11]. These mostly include vitamin E (tocopherol), vitamin C (ascorbate), polyphenolic antioxidants, and carotenoid pigments, which animals obtain from food[12-13]. Carotenoids were reported in many physiological functions, with beneficial effects on survival, growth and immunity[14-15]. They have the ability to scavenge free radicals produced by immune activity[16-17], and the potential to interact with endogenous antioxidant enzymes[18-20]. By contrast, carotenoids were also suggested to have detrimental effects[21]. Such negative effects were reported on skeletal muscles and reproduction of birds when provided at high doses, mainly under relatively non-stressful conditions, suggesting context-dependent effects of carotenoids[22-23]. Beneficial effects of carotenoids were often attributed to the conversion of these pigments such as β carotene into vitamin A. However, similar effects were found using nonprovitamin A carotenoids such as astaxanthin[11, 15, 24]. Astaxanthin is a xanthophyll carotenoid mainly produced by fungi and algae, acquired and stored in large amount by aquatic animals, in which the pigment enhances immune activity and limits short term immunopathology effects[20, 25-26]. Astaxanthin currently raises considerable interest as a powerful antioxidant and for its potential in alleviating age related diseases[11, 15, 24, 27]. Limiting immunopathology to prevent its negative long-term consequences is currently an important contemporary health issue. For instance, it has been proposed that reduced inflammatory exposure during childhood may have contributed to increased lifespan in human industrialized societies[8].

While numerous studies support that astaxanthin might be beneficial against immunopathology[11, 15, 24, 27], yet no study has actually assessed experimentally to what extent dietary supplementation with such a pigment prevents adverse effects of immunopathology on longevity. Insect models offer a great opportunity for such an experiment because they can be easily assessed in large numbers for their whole lifespan in highly controlled laboratory conditions for their diet and immune status. They were therefore proposed as useful model organism to screen for dietary effects on health with relevance for stress resistance and lifespan[28]. The immune system of insects is innate, comprising constitutive defences relying on hemocyte immune cells and several rapidly activated enzyme cascades such as the prophenoloxidase cascade that is at the core of the inflammatory response[5-6, 29-30]. Upon infection, hemocytes produce ROS and RNS, which while participating in parasite killing can damage a large range of molecules in cells, inducing apoptotic or necrotic cell death[31-33]. Phenoloxidase enzymes catalyse the formation of toxic quinone intermediates, which undergo further non-enzymatic reactions to form melanin that heals wounds, immobilises invading microbial pathogens through clotting, and encapsulates pathogens in melanised immune cells[29]. Melanin production is also accompanied by the production of ROS and RNS, helping to kill invading organisms[29, 31, 34-35]. However, such an immune response was also shown to cause damage to self-tissues and organs in the mealworm beetle, *Tenebrio molitor*[36]. The immunopathology resulting from such an immune response early in the life of the mealworm beetle was also found to reduce longevity[7, 37].

Using *T. molitor* as biological model, we tested whether lifetime food supplementation with astaxanthin helps insects to reduce immunopathology costs in the short and the long term on survival after being exposed to early life inflammation. Mealworm beetles are originally notorious scavengers and decomposers living in leaf-litter and under rocks. They mostly fed on fungi and yeasts growing on decaying vegetables and other organic matter[38] among which some are producing astaxanthin (e.g., *Phaffia sp.* or *Xanthophyllomices sp.*)[39]. Fresh water microalgae colonizing temporary puddles (e.g., *Haemoatococcus sp.*)[40] might also be a source of the pigment that the beetles may consume by drinking water or when grazing remains of the algae when the puddle has dried. Nevertheless, the frequency and amount of astaxanthin mealworm beetles may get from their food or drinking water are currently unknown. In this study, we first assessed the phenotypic impact of a controlled immune challenge performed at the larval stage by injection of an inactivated bacterium on larval survival, larval growth and adult longevity of supplemented and non-supplemented insects with astaxanthin. Assuming a strong antioxidant effect of astaxanthin, thus reducing the costs of immunopathology, we predicted that lifetime food supplementation should have positive effects on larval survival after the immune challenge, insect growth and adult longevity. By contrast, assuming context dependent effects of carotenoids, detrimental effects of the food supplementation with astaxanthin might also be

observed among non-immune-challenged insects. In addition, since carotenoids were often reported to have a broad immune stimulating effect, we further examined the influence of food supplementation with astaxanthin on important cellular and humoral immune effectors in larvae, after a controlled immune challenge. We also further tested whether food supplementation with astaxanthin improves the resistance of larvae to an infection with living bacterial pathogens in survival experiments. Assuming a general immune stimulating effect of astaxanthin, food supplementation with this pigment was expected to increase levels of immune defence and resistance to infection.

Results

Larval survival, growth and longevity after an immune challenge

To know whether astaxanthin has indeed the potential to prevent immunopathology costs and improve longevity, we first tested experimentally whether dietary supplementation with this pigment influences larval survival, larval development and insect longevity after being subjected to an immune challenge. To this purpose, 9 weeks old larvae that were supplemented or not with astaxanthin for 3 weeks, and for which the food treatment was carried on for the entire life of the insects, were either or not immune challenged with a suspension of inactivated *Bacillus thuringiensis*, mimicking a bacterial infection and stimulating the immune response.

Survival of the larvae was found dependent on the interaction between the dietary supplementation and the immune challenge (Table 1). While the food treatment had no main statistical effect on survival on its own, the immune challenge marginally reduced larval survival (Table 1). To explain such a statistical interaction between the dietary treatment and the immune challenge, we tested the effect of the immune challenge on survival for larvae supplemented and non-supplemented separately. Among non-supplemented larvae, the immune challenge was associated with a 4 folds survival reduction (W = 8.90, p = 0.003, Odd ratio = 4.40, n = 195, Figure 1a), whereas the immune challenge slightly improved the survival of supplemented larvae with astaxanthin (W = 4.83, p = 0.028, Odd ratio = 0.35, n = 195, Figure 1b).

Beyond the larval stage, insect longevity was significantly increased by the supplementation with astaxanthin, whereas it was unaffected by the immune challenge (Table 1, Figure 1B). The positive effect of the dietary pigment on insect longevity slightly declined with time (see Food * T-Cov in Table 1). Male insects were more long-lived than females, independently of the immune challenge or their diet (Table 1, Figure 1b).

Table 1. Results of time-dependent Cox regression analyses for larval survival and the whole longevity of *Tenebrio molitor* (n = 385) according to food supplementation with asatxanthin (Food) and the immune challenge (Challenge). A time-dependent covariate (T-Cov.) was specified and included in interaction with the explanatory variables to account for their time-dependent effect. The "simple" contrast was used for Food (survival of non-supplemented larvae was used as baseline), and challenge (survival of larvae injected with saline solution only was used as baseline). The best model was searched using backward stepwise method utilizing likelihood ratio significance tests for evaluation of each effect. Procedure is available in COXREG procedure of SPSS statistical package. Model fitting was initiating with a model that included all main effect and two ways interactions, with the exception of Box. Values $p \leq 0.05$ are given in bold.

Larval survival

Variables in the best model	B	s.e.	Wald	df	p	Odd ratio
Challenge	0.73	0.38	3.63	1	0.057	2.07
Food * Challenge	-2.58	0.69	14.15	1	< **0.001**	0.76
Challenge * T-Cov	-0.04	0.02	3.19	1	0.074	0.96

Variables not in the best model	Score	df	p
Food	0.94	1	0.760
Food * T-Cov	0.23	1	0.634
Challenge * Food * T-Cov	0.36	1	0.549

Whole insect survival

Variables in the best model	B	s.e.	Wald	df	p	Odd ratio
Food	-1.53	0.46	10.87	1	**0.001**	0.22
Sex	-0.23	0.12	4.88	1	**0.027**	0.772
Food * T-Cov	0.008	0.003	8.16	1	**0.004**	1.01

Variables not in the best model	Score	df	p
Challenge	1.15	1	0.284
Challenge * Food	0.15	1	0.696
Food * Sex	1.29	1	0.253
Challenge * T-Cov	0.66	1	0.416
Challenge * Sex	0.44	1	0.506
Sex * T-Cov	0.46	1	0.498
Challenge * Food * T-Cov	0.001	1	0.997
Food * Sex * T-Cov	1.53	1	0.216
Challenge * Sex * T-Cov	0.32	1	0.568

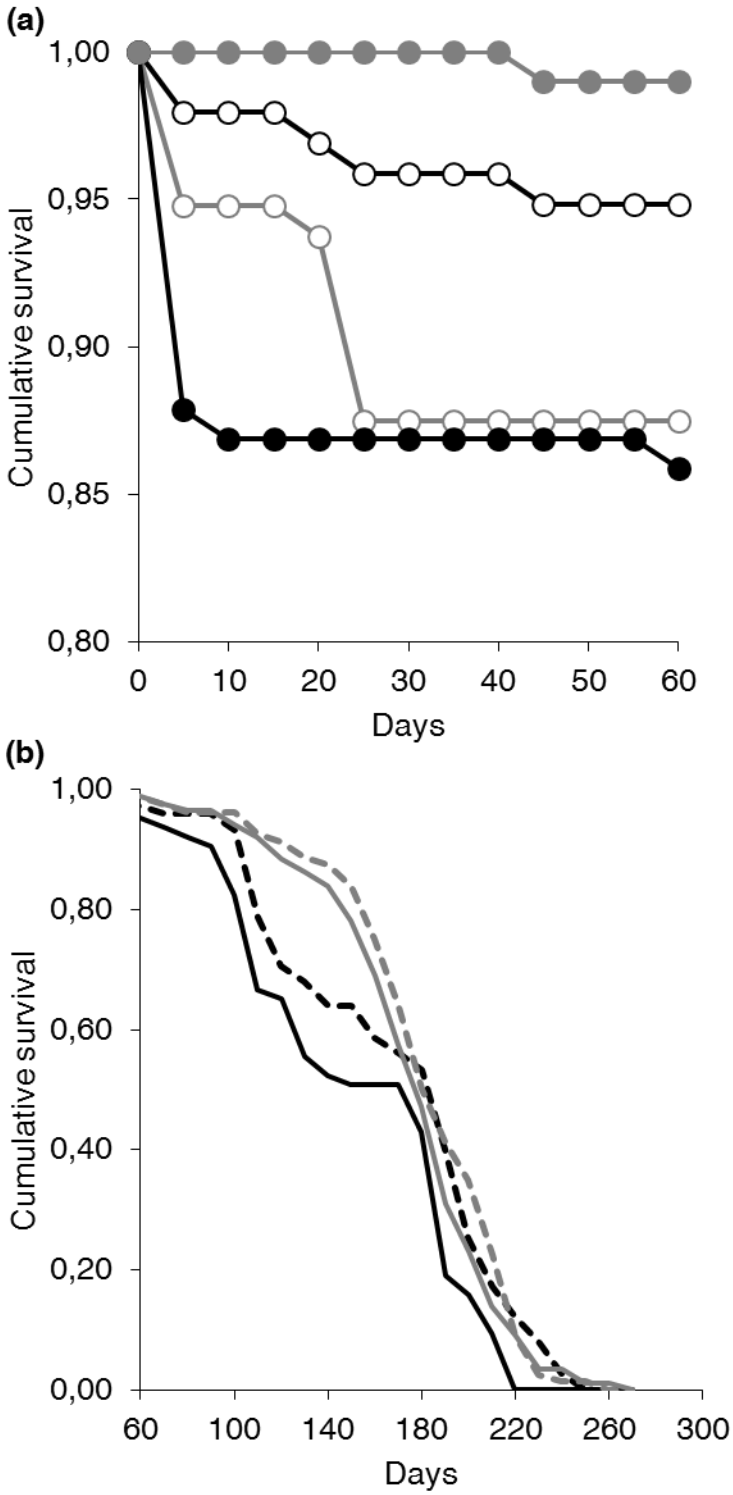

Figure 1. Larval survival (a) and whole insect longevity (b) of supplemented (grey lines) and non-supplemented (black lines) insects with astaxanthin after being exposed to an immune challenge by injection of a suspension of inactivated *Bacillus thuringiensis* (5µL, 10^8 cells.mL^{-1}). Larval survival is shown for larvae that were exposed (filled circles) or not (opened circles) to an immune challenge (a), whereas whole insect longevity is shown for males (dashed lines) and females (continuous lines) for which the immune challenge had no significant influence (b).

Larval developmental time of insects that reached the adult stage was prolonged by both the supplementation with astaxanthin and the immune challenge in an additive manner (Table 2; Figure 2). However, mass values of the resulting nymph and then adult were not affected by the dietary treatment or the immune challenge (Table 2).

Carotenoids and Immunity after an immune challenge

From the above experiment, we also measured levels of circulating carotenoids in the hemolymph and examined potential changes in immune defences resulting from both the dietary supplementation and the immune treatment of the larvae three days after their immune challenge occurred. Many of these variables were influenced by both the dietary and immune treatments, but not their interaction (Table 3). More specifically, dietary supplementation with astaxanthin significantly increased by 2 folds the circulating concentration of carotenoids in the hemolymph of *T. molitor* larvae after 3 weeks of food treatment (Table 3, Figure 3a). However, the immune challenge had no influence on the concentration of carotenoids (Table 3). All the immune parameters measured were influenced by the dietary treatment (Table 3). Overall, the supplemented larvae with astaxanthin exhibited lower hemocyte concentration (Table 3, Figure 3b), lower activity of the phenoloxidase system (Table 3, Figure 3c, d) and lower antibacterial activity (Table 3, Figure 3e) than non-supplemented larvae. Only antibacterial activity was found significantly affected by the immune challenge (Table 3). Indeed, unsurprisingly, the bacterial immune challenge resulted in an induced antibacterial response, which is known to last several days[41]. Hemocyte concentration tended to decrease after the immune challenge, but this effect was marginally significant (Table 3).

Survival to bacterial infection

Because of the apparent immune depressive effect of astaxanthin observed in the above experiment, we investigated whether the supplementation with astaxanthin affect the susceptibility of insect larvae to a bacterial infection using two known entomopathogenic bacterial pathogens of the mealworm beetle, *Bacillus cereus* and *B. thuringiensis*[42]. Dietary supplemented larvae with astaxanthin were more sensitive to the infection with *B. cereus* than non-supplemented larvae (Cox regression: W = 16.19, $p < 0.001$ Odd ratio = 2. 25, N = 200, Figure 4a). Similarly, dietary supplemented larvae with astaxanthin were slightly more sensitive to the infection with *B. thuringiensis* than non-supplemented ones, but this survival difference was only marginal (W = 2.91, p = 0.088, Odd ratio = 1. 79, N = 143, Figure 4b).

Table 2. Multivariate analysis of variance for development time, nymph mass and adult mass of mealworm beetle larvae (n = 335) as a function of food and immune treatments. Food and immune treatments had no interactive effect on the above parameters and were consequently removed from the statistical model. The multivariate test is shown first, followed by the respective univariate tests for development time, nymph mass and adult mass. Values $p \leq 0.05$ are given in bold.

Source of Variation		df	F	p
MANOVA (Pillai's trace)	Food	3, 330	3.70	**0.012**
	Challenge	3, 330	2.96	**0.033**
ANOVA development time	Global model	3, 332	7.74	**0.001**
	Food	1, 332	6.76	**0.010**
	Challenge	1, 332	7.37	**0.007**
ANOVA nymph mass	Global model	3, 332	0.77	0.463
	Food	1, 332	1.19	0.276
	Challenge	1, 332	0.25	0.618
ANOVA adult mass	Global model	3, 332	0.20	0.816
	Food	1, 332	0.01	0.931
	Challenge	1, 332	0.41	0.525

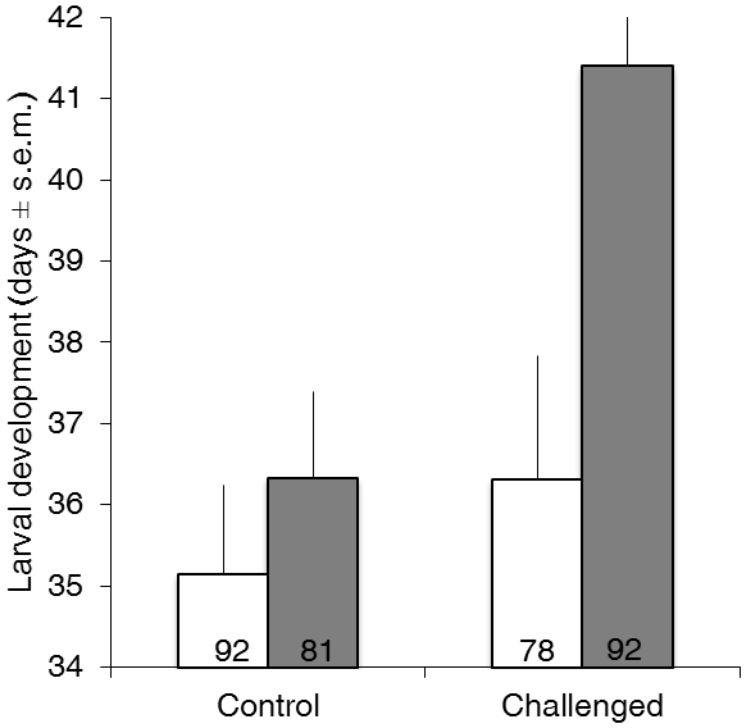

Figure 2. Larval development time in days of supplemented (grey bars) and non-supplemented (white bars) insects with astaxanthin after being exposed to an immune challenge by injection of a suspension of inactivated *Bacillus thuringiensis* (5µL, 10^8 cells.mL^{-1}). Numbers at the bottom of the bars refer to sample size.

Table 3. Multivariate analysis of variance for carotenoid concentration, hemocyte concentration, PO activity, total-PO activity and antibacterial activity of mealworm beetle larvae (n = 144) as a function of food treatment and immune treatment. Food and challenge treatments had no interactive effect on the above parameters and was consequently removed from the statistical model. The multivariate test is shown first, followed by the respective univariate tests for carotenoid concentration, hemocyte concentration, PO activity, total-PO activity and antibacterial activity. Values $p \leq 0.05$ are given in bold.

Source of Variation		df	F	p
MANOVA (Pillai's trace)	Food	5, 65	5.32	< **0.001**
	Challenge	5, 65	3.37	**0.009**
ANOVA Carotenoid	Global model	2, 69	3.54	**0.035**
	Food	1, 69	4.99	**0.029**
	Challenge	1, 69	2.08	0.154
ANOVA Hemocyte	Global model	2, 69	3.94	**0.024**
	Food	1, 69	4.32	**0.041**
	Challenge	1, 69	3.56	0.063
ANOVA PO activity	Global model	2, 69	4.88	**0.010**
	Food	1, 69	8.81	**0.004**
	Challenge	1, 69	0.96	0.331
ANOVA total-PO activity	Global model	2, 69	5.21	**0.008**
	Food	1, 69	10.00	**0.002**
	Challenge	1, 69	0.43	0.516
ANOVA antibacterial activity	Global model	2, 69	8.14	**0.001**
	Food	1, 69	9.31	**0.003**
	Challenge	1, 69	6.96	**0.010**

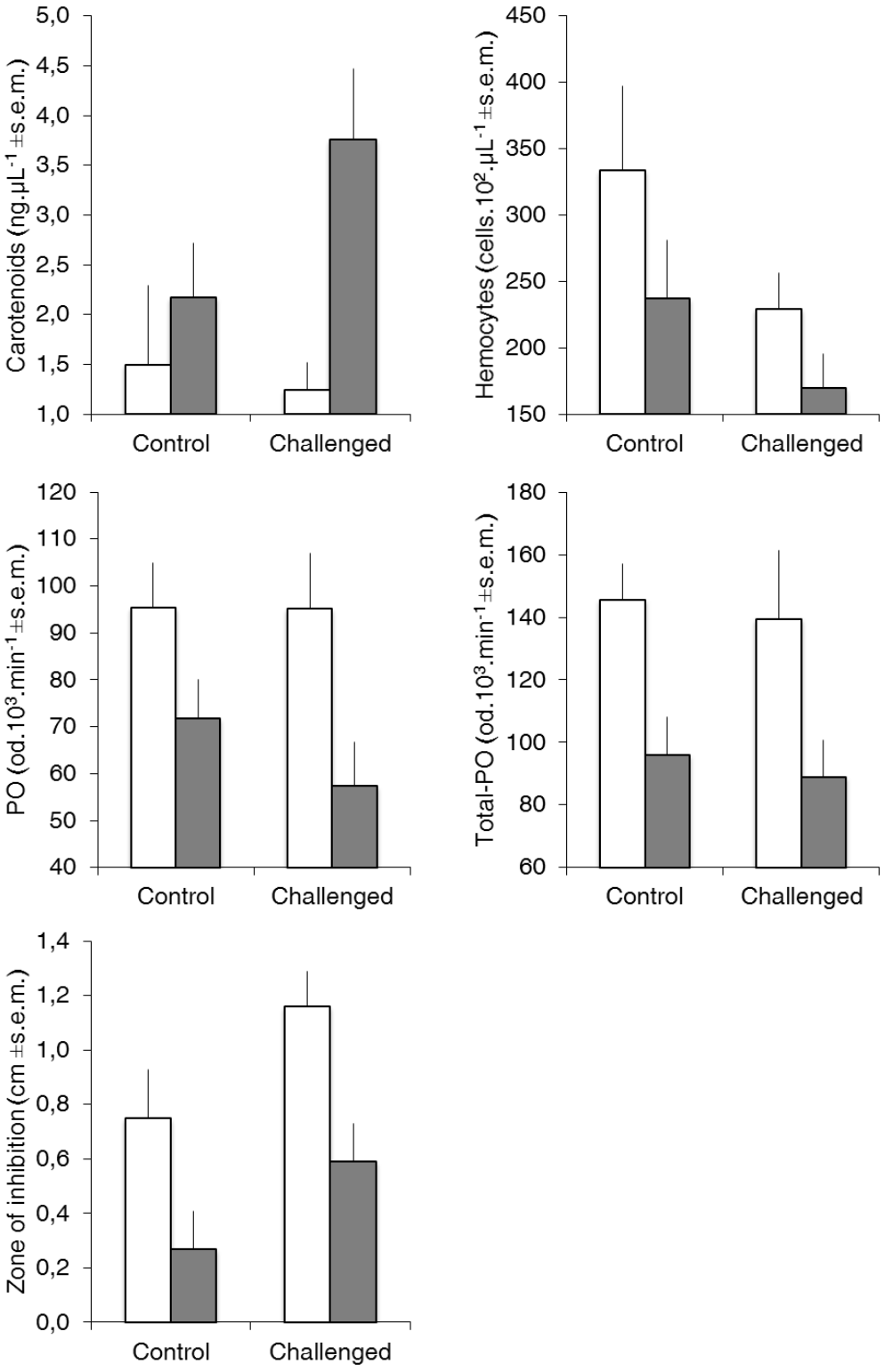

Figure 3. Concentration of carotenoids (a), concentration of hemocytes (b), PO activity (c), Total-PO activity (d) and antibacterial activity (e) in the hemolymph of supplemented (grey bars) and non-supplemented (white bars) larvae with astaxanthin after being exposed to an immune challenge by injection of a suspension of inactivated *Bacillus thuringiensis* (5µL, 10^8 cells.mL^{-1}). Numbers at the bottom of the bars refer to sample size.

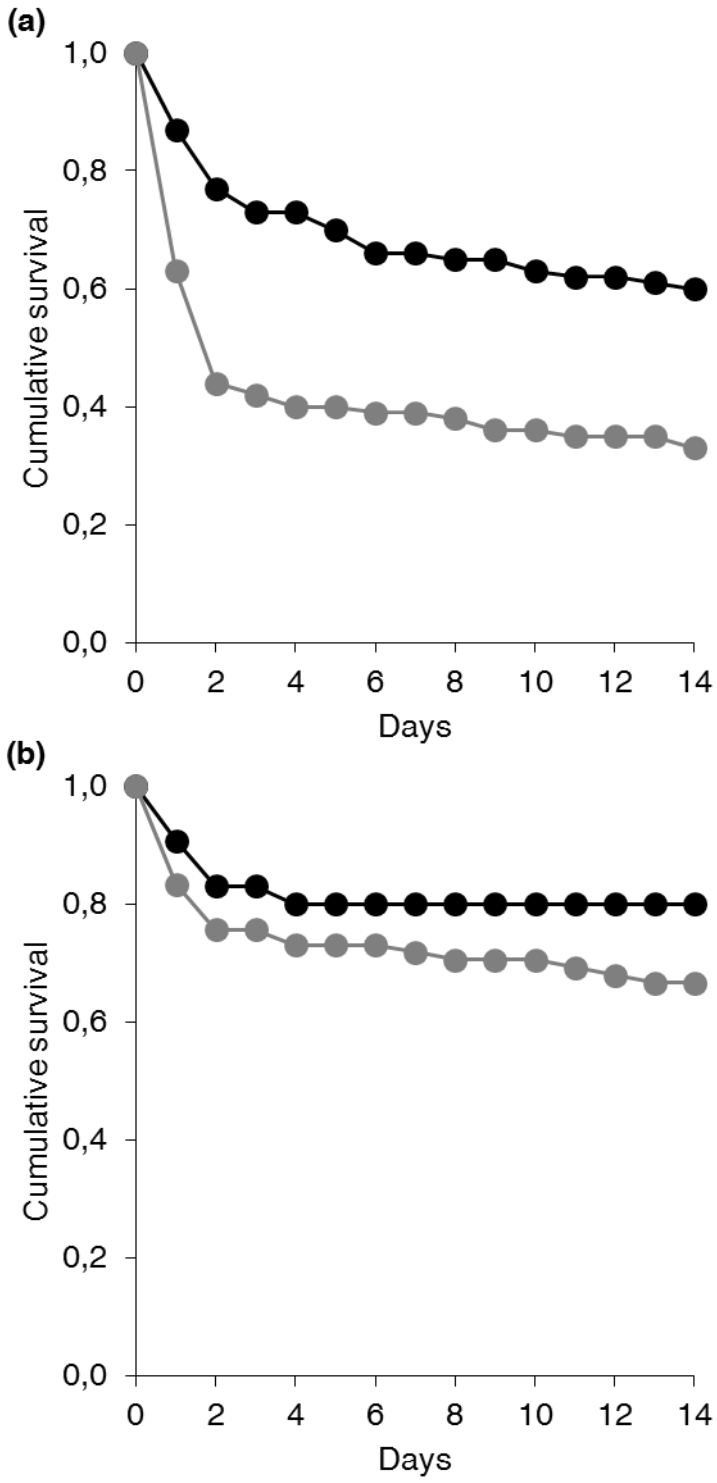

Figure 4. Survival of supplemented (grey lines) and non-supplemented (black lines) larvae with astaxanthin after being exposed to an infection with either (a) *Bacillus cereus* or (b) *Bacillus thuringiensis*.

Discussion

This study tested whether lifetime food supplementation with an important antioxidant can prevent short and long-term immunopathological consequences of early life inflammation in the mealworm beetle, *T. molitor*. Because astaxanthin is predicted to have a strong antioxidant activity[15, 20, 24, 26], lifetime food supplementation with that pigment was expected to improve larval survival and adult longevity of the insects exposed to an immune challenge at the larval stage. By contrast, since carotenoids, including xanthophylls, are also believed to induce context dependent detrimental effects[21-23], food supplementation with astanxanthin might also be associated to negative effects among non-immune-challenged insects.

Dietary supplementation with astaxanthin significantly increased by 2 folds the circulating concentration of carotenoids in the hemolymph of *T. molitor* after 3 weeks of food treatment. However, the amount of circulating carotenoids in the hemolymph was still relatively low compared to the amount of astaxanthin provided to supplemented insects. Furthermore, the circulating concentration of carotenoids in the hemolymph of supplemented *T. molitor* larvae with astaxanthin was about 400 times lower than the one found in the hemolymph of the freshwater crustacean, *G. pulex*, similarly supplemented in laboratory conditions[26]. In the present study, considering the bright red colour of the insect faeces, large amounts of the pigment were apparently not absorbed.

Food supplementation with astaxanthin induced contrasted results for survival under immune stimulation with inactivated bacteria (i.e. inducing an immune response without any pathogenic effect due to pathogen virulence). While supplementation with astaxanthin in larvae was associated with a survival benefit under immune stimulation, it was also associated with a survival reduction in absence of immune challenge. The survival benefit under immune challenge is consistent with the results of a previous study that tested the survival cost of an immune response produced by supplemented and non-supplemented gammarid crustaceans with a mix of astaxanthin and lutein[26]. The strong antioxidant property of astaxanthin enabling the capture of cytotoxic free radicals produced by immune activity[16-17] and its ability to stimulate enzymes of the endogenous antioxidant defence system[20] might have contributed to limit negative effects of the immune response by autoreactivity. However, an important result of this study is that supplementation with astaxanthin is also associated to a reduced immune activity, even upon an immune challenge with inactivated bacteria. Indeed, insects fed with astaxanthin exhibited reduced hemocyte concentration, lower levels of phenoloxidase activity and low synthesis of antibacterial activity upon a bacterial immune challenge. This further caused an increased susceptibility to infection with bacterial entomopathogens. These results clearly contrast with the general belief that carotenoids have immune stimulating properties. Indeed,

supplementation with astaxanthin was reported to stimulate some markers of immunity in vertebrates[15, 24] and invertebrates, mainly crustaceans[14, 20, 26, 43-45]. For instance, in the amphipod crustacean *Gammarus pulex*, experimental dietary supplementation with astaxanthin results in broad stimulation of gammarid innate immune defences, giving rise to increased resistance to microbial infection[26]. Similarly, astaxanthin dietary supplementation increases phenoloxidase activity and total hemocyte count in the giant freshwater prawn *Macrobrachium rosenbergii*[44]. In the same species, injection of astaxanthin increases total hemocyte count and survival in presence of the pathogenic bacterium *Lactococcus garvieae*[43]. Since crustaceans have evolved particular carotenoprotein complexes allowing the storage of large amount of astaxanthin in their tissues[45], this pigment might be of particular importance in their physiology, including immunity.

By contrast, *T. molitor* is not known to possess specialized features to store carotenoids and the supplementation of the food with astaxanthin is found here associated to a general down regulation of its innate immune system. Such a general immune depressive effect of astaxanthin may rely on the interaction of the pigment with the availability or production of nitric oxide (NO), which has been evidenced to be a major regulator of the insect immune response. Indeed, NO has been found to stimulate both cellular and humoral immunity of insects[46-49], and astaxanthin may affect the availability of NO in two ways. First, astaxanthin was reported to inhibit the activity of the nitric oxide synthase, the enzyme responsible of NO production from L-arginin[24]. Second, because of its strong antioxidant power, astaxanthin might also have interfered with NO cellular signalling, by scavenging a certain fraction of circulating NO, thus down regulating base levels of immune activity. Another but not exclusive explanation is that astaxanthin may also have regulatory effects on the host's metabolism[50-51]. Recently, astaxanthin was found to interact with nuclear receptors of the peroxisome proliferator-activated receptor superfamily, which regulates lipid and glucose metabolism in vertebrates[52]. Such an alteration of the host metabolism could reduce the allocation of energetic resource to the immune system. If these receptors are conserved among taxa, similar regulatory effects may occur as well in invertebrates. Whatever the mechanisms by which astaxanthin may down regulate the immune system of *T. molitor*, its immune depressive effect might have been a major cause of the reduced cost of the immune response to inactivated bacteria on larval survival. Furthermore, resources saved from reduced immune activity may also have contributed to the prolonged longevity of supplemented beetles, independently of the immune challenge at the larval stage.

The slight, but significant, survival cost in absence of immune challenge also confirms previous observations showing detrimental effects of carotenoid supplementation in non-stressed birds[22-23], although cautions should be taken about comparisons drawn from different taxa. In addition, the supplementation with astaxanthin was also associated to a prolonged larval development of the

beetles, although not affecting adult body size. Available data show that astaxanthin inhibits cell proliferation and induces enhanced apoptosis activity[53-54]. Apoptosis corresponds to an essential programed cell death occurring during the normal development of multicellular organisms[55]. Its activity is usually balanced with cell proliferation, ensuring normal growth and survival. By concomitantly promoting apoptosis and inhibiting cell proliferation, astaxanthin might have constrained the normal growth of the developing mealworm larvae, leading to the observed prolonged larval development in supplemented insects. This dual effect of astaxanthin may also have contributed to the slight increase of mortality among supplemented larvae that were not immune-challenged. The modulation of apoptosis by carotenoids appears to be variable according to several factors such as carotenoids concentration and antioxidant status[53]. The immune challenge of the larvae is believed to promote a pro-oxidant status by the release of oxidative free radicals[31-33]. The mobilization of astaxanthin for the detoxification of free radicals produced during the immune response may have relieve the negative impact of the pigment on the survival of the larvae, and could explain why immune-challenged larvae exhibited the highest survival among supplemented larvae. Further detailed analysis would be needed to test this hypothesis.

To summarize, we found that life-time supplementation of *T. molitor* with astaxanthin, an important dietary antioxidant, can prevent early and late immunopathology costs, which result in a better tolerance to immune cost at the larval stage and a prolonged longevity. However, while these beneficial effects might, to some extent, directly result from the strong antioxidant property of the dietary pigment, its strong down regulating effect on the insect immune system is likely to be a major cause. This immune depressive effect of astaxanthin has also the disadvantage of decreasing the insect resistance to bacterial infection. Other detrimental effects of the supplementation with astaxanthin could be revealed on survival of non-stressed larvae and on larval development of the insects. This study suggests that dietary carotenoids could be challenging for biological systems, at least for those that did not evolve specialized features to store them, and that beneficial and detrimental effects resulting from the supplementation with these pigments might be host specific and context dependent. While Dual effects of carotenoids will have to be considered for their use in the development of products promoting health.

Material and methods

Insect cultures

Experimental insects were produced in routine by allowing groups of 10 days old virgin adult beetles (10 males and 10 females) to reproduce 3 days in plastic boxes (L x 1 x H, 20 x l2 x 9.5 cm) supplied with 60 g of bran flour, a micro centrifuge tube of water in standard laboratory conditions (25°C, 70% RH; 24h dark). Parental insects where then removed and eggs produced in each box were allowed to develop. Six weeks after egg laying, the offspring larvae obtained in each box were counted and their number adjusted to 30 larvae per box, and provided with fresh bran flour. Half of the boxes were allocated to dietary supplementation with astaxanthin whereas the other half of the boxes was allocated to the control food treatment. Each box of supplemented insects was provided twice a week with micro 0.5 mL centrifuge tubes (3 per box) containing 500 µL of a solution of astaxanthin (Carophyll Pink® 10%, 30 mg per mL of distilled water in 1% mass/vol. of agar), corresponding to 1.5 mg of astaxanthin twice a week (or about 0.1 mg of astaxanthin per larvae and per week), whereas each control boxes were similarly provided with micro centrifuge tubes of distilled water in 1% agar only, for the whole duration of the experiments. Insects were used for the experiments three weeks after the start of the dietary supplementation.

The first experiment testing for larval survival, growth and insect longevity after an immune challenge used 8 boxes of each dietary treatment (total of 16 boxes), each containing 30 nine-weeks old larvae. Half of the boxes within each dietary treatment group were allocated to an immune challenge mimicking a bacterial infection, whereas the other half of the boxes was allocated to a control immune treatment. Challenged larvae were injected with a 5-µL suspension of inactivated *B. thuringiensis* (10^8 bacteria.mL^{-1}) in phosphate buffer saline (PBS 10 mM, pH 7.4) corresponding to a non deadly dosage previously used to characterize the immune response of the mealworm beetle[56-57]. Control larvae were treated in the same way, but without bacteria, as a procedural control. Three days later, five larvae per box were randomly taken to collect a 5 µL-sample of haemolymph to measure astaxanthin concentration, haemocyte concentration, antibacterial activity and the maintenance and use of the prophenoloxidase system. After sampling, these larvae were not returned into the experimental cultures. Starting from the immune treatment of the larvae, each box was checked twice a week to record larval survival, larval developmental time (duration in days from hatching to adult), nymph body mass, adult body mass and total longevity of the remaining insects. As soon as larvae reached the pupae stage, they were weighed and allowed achieving their live span isolated in grid boxes (boxes with 10 compartments; each compartment: L x 1 x H, 4.8 x 3.2 x 2.2 cm) supplied with bran flour and their respective dietary treatment.

The experiments testing the susceptibility of supplemented and non-supplemented larvae to the infection by *B. cereus* consisted of the inoculation of 100 supplemented larvae with astaxanthin and 100 non-supplemented ones with a fine sterilized needle dipped into a pellet of live bacteria. The

infection experiment using *B. thuringiensis* used exactly the same procedure, in which 78 supplemented larvae with astaxanthin and 65 non-supplemented larvae were inoculated. Larvae were kept individually in grid boxes supplied with bran flour and their respective dietary treatment. Survival to infection was recorded once a day for 14 days.

Bacterial culture for immune challenge and infections

The bacteria used in this study are known to be pathogens of *T. molitor*[42]. *B. thuringiensis* and *B. cereus* were obtained from the Pasteur institute: *B. thuringiensis* (CIP53.1); *B. cereus* (CIP69.12). Bacteria were grown overnight at 28°C in liquid Broth medium (10 g bacto-tryptone, 5 g yeast extract, 10 g NaCl in 1000 mL of distilled water, pH 7). Bacteria used to performed immune challenges were then inactivated in 0.5% formaldehyde prepared in PBS for 30 minutes, rinsed three times in PBS, and their concentration adjusted to 10^8 bacteria per mL using a Neubauer improved cell counting chamber[56-57]. The success of the inactivation was tested by plating a sample of the bacterial solution on sterile Broth medium with 1% of bacterial agar and incubated at 28°C for 24 hours. Aliquots were kept at -20°C until use. After being chilled on ice for 10 min for immobilization, insects were immune challenged by injection of 5µL of the bacterial suspension through the pleural membrane between the second and third abdominal segment using sterile glass capillaries that had been pulled out to a fine point with an electrode puller (Narashige PC-10). For bacteria used for insect infection, overnight bacterial cultures (20 mL) were centrifuged at 3500 g at 4°C for 30 min. The supernatant was discarded and the bacteria pellet was used for infection. After being chilled on ice for 10 min, insects were infected by dipping a sterilized 0.03 mm diameter needle (Fine Science Tools® n° 26000-25) into the bacteria pellet and pricking the animal through the pleural membrane between the second and third abdominal segment.

Hemolymph collection, astaxanthin dosage and immune parameters

Hemolymph was collected as described by Moret[58]. After being chilled on ice for 10 min for immobilization, each larvae provided 5 µL of hemolymph collected into a sterile pre-chilled 5 µL-graduated glass capillary (Ringcaps®, Hirshmann® Laborgerate, Germany) after wounding the insect between the second and the third abdominal segments with a sterile needle. The sample of hemolymph was immediately diluted in 30 µL of ice-cold PBS. A first 10-µL subsample was immediately used for the measurement of the concentration of hemocytes, using a Neubauer improved cell counting chamber under a phase-contrast microscope (magnification x 400). A second 10-µL subsample was frozen in liquid nitrogen and stored at -80°C for later estimation of the concentration of astaxanthin. Another 5-µL subsample was transferred into an N-phenylthiourea (Sigma-Aldrich, St Louis, MO, USA, P7629)-coated microcentrifuge tube, frozen in liquid nitrogen and stored at -80°C until

later examination for antibacterial activity. The remaining hemolymph solution was diluted with 10 µL of PBS, frozen in liquid nitrogen and stored à -80°C for later measurement of the phenoloxidase activity

Carotenoids were extracted and quantified following the method of Cornet and colleagues[25]. Briefly, pigments were extracted by adding the same volume of ethanol and washing pellets twice with 200 µL of methyl-*tert*-butyl ether (MTBE). Via a colorimetric assay, the concentration of pigments was determined at 470 nm in a microplate reader against a reference curve ranging from 0 to 50 ng/µL of a standard solution of astaxanthin in ethanol (standards obtained from Extrasynthèse, Genay, France). Values were corrected to obtain concentrations for 1 µL of pure hemolymph.

Antimicrobial activity in the hemolymph was measured using a standard zone of inhibition assay[58]. 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* from the Pasteur institute (CIP105365). An overnight culture of the bacterium was added to broth medium containing 1% agar to achieve a final concentration of 10^5 cells per mL. Six millilitres of this seeded medium was then poured into a Petri dish and allowed to solidify. Sample wells were made using a Pasteur pipette fitted with a ball pump. Two microlitres of sample solution were added to each well, and a positive control (Tetracycline: Sigma-Aldrich, St Louis, MO, USA, T3383; 2.5 $mg.mL^{-1}$ in absolute ethanol) was included on each plate[59]. Plates were then incubated overnight at 28 °C. Then, the diameter of inhibition zones was measured for each sample.

For each individual hemolymph sample, both the activity of naturally activated phenoloxidase (PO) enzymes only (PO activity), and the activity of the proenzymes (proPO) in addition to that of the PO (total-PO activity), were measured using a spectrophotometer[50]. The PO activity was quantified without further activation, while the total-PO activity required the activation of the proPO into PO with chymotrypsin. For this purpose, frozen hemolymph samples were thawed on ice and centrifuged (3500 g, 5 min, 4°C). Five microlitres 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-Aldrich, St Louis, MO, USA, C-7762, 0,07 mg.mL-1 of distilled water) to measure total-PO activity. Then 20 µL of L-Dopa solution (Sigma-Aldrich, St Louis, MO, USA, D-9628, 4 mg mL-1 of distilled water) was added to each well. The reaction was allowed to proceed at 30°C in a microplate reader (Versamax; Molecular Devices, Sunnyval, CA, USA) for 40 min. Readings were taken every 15 s at 490 nm and analysed using the software SOFT-Max Pro 4.0 (Molecular Devices, Sunnyval, CA, USA). Enzyme activity was measured as the slope (Vmax value: change in absorbance unit per min) of the reaction curve during the linear phase of the reaction and reported to the activity of 1 µL of pure hemolymph.

Statistics

Survival of larvae and the whole insect longevity with respect to dietary supplementation with astaxanthin and immune challenge were analysed using time-dependent Cox regression analyses because the proportional hazards assumption was not met (risk of mortality was not constant over time). Boxes in which the larvae were maintained did not explain survival within each treatment combination (non-supplemented and non-challenged larvae: W = 0.76, df = 3, p = 0.858; non-supplemented and challenged larvae: W = 3.01, df = 3, p = 0.390; supplemented and non-challenged larvae: W = 2.21, df = 3, p = 0.530; supplemented and challenged larvae: W = 2.41, df = 3, p = 0.492) and could therefore be ignored in further analysis. Our statistical models used a stepwise procedure and the reference survival functions were generated from the control data derived from the dietary (e.g. non-supplemented) and the immune treatment (e.g. control). Dietary and immune treatments were coded as categorical variables, and insect larvae that reached the adult stage during the survey were censored. Analysis of the whole insect longevity also used sex as categorical explanatory variable. A time-dependent covariate was specified and included in interaction with all explanatory variables to test for their time-dependent effect.

Larval development time, nymph and adult body mass were analysed using a MANOVA with dietary supplementation, immune treatment and sex as factors.

Carotenoid concentration, hemocyte concentration, PO activity, total-PO activity and antibacterial activity were analysed using a multivariate analysis of variance (MANOVA) with dietary and immune treatments as factors. Data on carotenoid concentration and antibacterial activity were natural log transformed whereas those on hemocyte concentration square root transformed to satisfy the requirements of parametric statistical tests.

Survival to bacterial infections with respect to dietary supplementation with astaxanthin was analysed using proportional hazards Cox regressions that used a stepwise procedure and the reference survival functions were generated from the control data derived from the dietary (e.g. non-supplemented).

All statistical analyses used IBM® SPSS® Statistics 19 for Macintosh.

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References

1. Graham, A. L., Allen, J. E. & Read, A. F. Evolutionary causes and consequences of immunopathology. *Annu. Rev. Ecol. Evol. Syst*. **36**, 373-397 (2005).
2. Sorci, G. & Faivre, B. Inflammation and oxidative stress in vertebrate host-parasite systems. *Phil. Trans. Roy. Soc. Lond. B*. **364**, 71-83 (2009).
3. Ashley, N. T., Weil, Z. M. & Nelson, R. J. Inflammation: mechanisms, costs, and natural variation. *Annu. Rev. Ecol. Evol. Syst*. **43**, 385-406 (2012).
4. Janeway, C. A., Jr. & Medzhitov, R. Innate immune recognition. *Ann. Rev. Immunol.* **20**, 197-216 (2002).
5. Stramer, B., Wood, W., Galko, M. J., Redd, M. J., Jacinto, A., Parkhurst, S. M. & Martin, P. Live imaging of wound inflammation in *Drosophila* embryos reveals key roles for small GTPases during in vivo cell migration. *J. Cell. Biol.* 168, 567-573.
6. Libert, S., Chao, Y. F., Chu, X. W. & Pletcher, S. D. Trade-offs between longevity and pathogen resistance in *Drosophila melanogaster* are mediated by NF kappa B signalling. *Aging Cell* **6**, 533-543.
7. Pursall, E. R. & Rolff, J. Immune responses accelerate ageing: proof-of-principle in an insect model. *PLoS ONE*. **6**, e19972 (2011).
8. Finch, C. E. & Crimmins, E. M. Inflammatory exposure and historical changes in human life-spans. *Science* **305**, 1736-1739 (2004).
9. Licastro, F. *et al*. Innate immunity and inflammation in aging: a key for understanding age-related diseases. *Immunity & Ageing*. **2**, 8-22 (2005).
10. Pawelec, G., Goldeck, D. & Derhovanessian, E. Inflammation, ageing and chronic disease. *Curr. Opin. Immunol*. **29**, 23-28 (2014).
11. Chew, B. P. & Park, J. S. Carotenoid action on the immune response. *J. Nutr.* **134**, 257S-261S (2004).
12. Felton, G. W. & Summers, C. B. Antioxidants systems in insects. *Arch Insect Biochem*. **29**, 187-197 (1995).
13. Catoni, C., Peters, A. & Schaefer, H. M. Life history trade-offs are influenced by the diversity, availability and interactions of dietary antioxidants. *Anim. Behav*. **76**, 1107-1119 (2008).

14. Flores, M., Diaz, F., Medina, R., Re, A. D. & Licea, A. Physiological, metabolic and haematological responses in white shrimp *Litopenaeus vannamei* (Boone) juveniles fed diets supplemented with astaxanthin acclimated to low-salinity. *Aquac. Res.* **38**, 740-747 (2007).
15. Park, J. S., Chyun, J. H., Kim, Y. K., Line, L. L. & Chew, B. P. Astaxanthin decreased oxidative stress and inflammation and enhanced immune response in humans. *Nutr. Metabol*. **7**, 1-18 (2010).
16. El-Agamey, A. *et al*. Carotenoid radical chemistry and antioxidant/pro-oxidant properties. *Arch. Biochem. Biophys*. **430**, 37-48 (2004).
17. Walrand, S. *et al*. In vivo and in vitro evidence that carotenoids could modulate the neutrophil respiratory burst during dietary manipulation. *Eur. J. Nutr.* **44**, 114-120 (2005).
18. Kim, J. H., Kim, Y. S., Song, G. G., Park, J. J. & Chang, H. I. Protective effect of astaxanthin on naproxen-induced gastric antral ulceration in rats. *Eur. J. Pharmacol*. **514**, 53-59 (2005).
19. Lee, D. H., Kim, C.S. & Lee, Y. I. Astaxanthin protects against MPTP/MPP plus-induced mitochondrial dysfunction and ROS production in vivo and in vitro. *Food Chem. Toxicol*. **49**, 271-280 (2011).
20. Babin, A. *et al*. Limiting immunopathology: interaction between carotenoids and enzymatic antioxidant defences. *Dev. Comp. Immunol.* **49**, 278-281 (2015).
21. Olson, V. A. & Owens, I. P. F. Costly sexual signals: are carotenoids rare, risky, or required. *Trends Ecol. Evol*. **13**, 510–515 (1998).
22. Huggins, K. A., Navara, K. J., Mendonça, M. T. & Geoffrey, E. H. Detrimental effects of carotenoid pigments: the dark side of bright coloration. *Naturwissenschaften* **97**, 637–644 (2010).
23. Simons, M. J. P., Briga, M., Leenknegt, B. & Verhulst, S. Context-dependent effects of carotenoid supplementation on reproduction in zebra finches. *Behav. Ecol*. **25**, 945-950 (2014).
24. Hussein, G., Sankawa, U., Goto, H., Matsumoto, K. & Watanabe, H. Astaxanthin, a Carotenoid with Potential in Human Health and Nutrition. *J. Nat. Prod*. **69**, 443-449 (2006).
25. Cornet, S., Biard, C. & Moret, Y. Is there a role for antioxidant carotenoids in limiting self-harming immune response in invertebrates? *Biol. Lett.* **3**, 284-288 (2007).
26. Babin, A., Biard, C. & Moret, Y. Dietary supplementation with carotenoids improves immunity without increasing its cost in a crustacean. *Am. Nat.* **176**, 234-241 (2010).
27. Fraser, P. D. & Bramley, P. M. The biosynthesis and nutritional uses of carotenoids. *Prog. Lipid. Res*. **43**, 228-265 (2004).
28. Grünwald, S., Adam, I. V., Gurmai, A. M., Bauer, L., Boll, M. & Wenzel, U. The red flour beetle *Tribolium castaneum* as a model to monitor food safety and functionality. *Adv. Biochem. Eng. Biotechnol.* **135**, 111-122 (2013).

29. Söderhäll, K. & Cerenius, L. Role of the prophenoloxidase-activating system in invertebrate immunity. *Curr. Opin. Immunol*. **10**, 23-28 (1998).
30. Siva-Jothy, M. T., Moret, Y. & Rolff, J. Insect immunity: an evolutionary ecology perspective. *Adv. Insect Physiol*. **32**, 1-48 (2005).
31. Nappi, A. J., Vass, E., Frey, F. & Carton, Y. Nitric oxide involvement in Drosophila immunity. *Nitric Oxide-Biol. Chem*. **4**, 423-430 (2000).
32. Nappi, A. J. & Ottaviani, E. Cytotoxicity and cytotoxic molecules in invertebrates. *BioEssays* **22**, 469-480 (2000).
33. Rivero, A. Nitric oxide: an antiparasitic molecule of invertebrates. *Trends Parasitol*. **22**, 219-225 (2006).
34. Luckhart, S., Vodovotz, Y., Cui, L. & Rosenberg, R. The mosquito *Anopheles stephensi* limits malaria parasite development with inducible synthesis of nitric oxide. *Proc. Natl. Acad. Sci. USA.* **95**, 5700-5705 (1998).
35. Whitten, M. M. A. & Ratcliffe, N. A. In vitro superoxide activity in the hemolymph of the West Indian leaf cockroach, *Blaberus discoidalis*. *J. Insect Physiol*. **45**, 667-675 (1999).
36. Sadd, B. M. & Siva-Jothy, M. T. Self-harm caused by an insect's innate immunity. *Proc. R. Soc. B-Biol. Sci.* **273**, 2571-2574 (2006).
37. Khan, I., Agashe, D. & Rolff, J. Early inflammation, immunopathology and aging. *Proc. R. Soc. B-Biol. Sci.* **284**, 20170125 (2017).
38. Howard, R. S. The biology of the grain beetle *Tenebrio molitor* with particular reference to its behaviour. *Ecology* **36**, 262-269 (1955).
39. Tognetti, C., Moliné, M., van Broock, M. & Libkind, D. Favored isolation and rapid identification of the astaxanthin-producing yeats Xanthophyllomyces dendrohous (Phaffia rhozyma) from environmental samples. J. Basic Microbiol. 53, 766-772 (2013).
40. Shah, M. M. R., Liang, Y. M., Cheng, J. J. & Daroch, M. Astaxanthin-producing green microalga *Haemotococcus pluvialis*: from single cell to high commercial products. *Front. Plant Sci*. **7**, 531 (2016).
41. Haine, E. R., Moret, Y., Siva-Jothy, M. T. & Rolff, J. Antimicrobial defense and persistent infection in insects. *Science* **322**, 1257-1259 (2008).
42. Jurat-Fuentes, J. L. & Jackson, T. Bacterial Entomopathogens in *Insect Pathology*, 2nd ed. (eds Kaya, H. & Vera, F.) 265-349. (Elsevier Academic Press, Cambridge, Mass 2012).
43. Angeles, I. P. Jr., Chien, Y-H. & Yambot, A. V. Effect of infected astaxanthin on survival, antioxidant capacity, and immune response of the giant freshwater prawn *Macrobrachium rosenbergii* (De Man, 1879) challenged with *Lactococcus garvieae*. *J. Shellfish Res.* **28**, 931-937 (2009).

44. Kumar, V., Pillai, B. R., Sahoo, P. K., Mohanty, J. & Mohanty, S. Effect of dietary astaxanthin on growth and immune response of Giant freshwater prawn *Macrobrachium rosenbergii* (de man). *Asian. Fish. Sci*. **22**, 61–69 (2009).
45. Wade, N. M., Gabaudan, J. & Glencross, B. D. A review of carotenoid utilisation and function in crustacean aquaculture. *Rev. Aquac*. **0**, 1-16 (2015).
46. Imamura, M., Yang, J. & Yamakawa, M. cDNA cloning, characterization and gene expression of nitric oxide synthase from the silkworm, *Bombyx mori*. *Insect Mol. Biol*. **11**, 257-265 (2002).
47. Kraaijeveld, A. R., Elrayes, N. P., Schuppe, H. & Newland, P. L. L-arginine enhances immunity to parasitoids in *Drosophila melanogaster* and increases NO production in lamellocytes. *Dev. Comp. Immunol*. **35**, 857-864 (2011).
48. Eleftherianos, I. *et al*. Nitric oxide levels regulate the immune response of *Drosophila melanogaster* reference laboratory strains to bacterial infections. *Infect. Immun*. **82**, 4169-4181 (2014).
49. Sanzhaeva, U., Vorontsova, Y., Glazachev, Y. & Slepneva, I. Dual effect of nitric oxide on phenoloxidase-mediated melanization. *J. Enzyme Inhib. Med. Chem*. **31**, 1063-1068 (2016).
50. Hussein, G. *et al*. Astaxanthin ameliorates features of metabolic syndrome in SHR/NDmcr-cp. *Life Sci.* **80**, 522-529 (2007).
51. Yang, Y. *et al*. Astaxanthin-rich extract from the green alga *Haematococcus pluvialis* lowers plasma lipid concentrations and enhances antioxidant defense in apolipoprotein E knockout mice. *J. Nutr*. **141**, 1611-1617 (2011).
52. Jia, Y. *et al*. Ursolic acid is a PPAR-α agonist that regulates hepatic lipid metabolism. *Bioorg. Med. Chem. Lett*. **21**, 5876-5880 (2011).
53. Palozza, P., Serini, S., Di Nicuolo, F. & Calviello, G. Modulation of apoptotic signalling by carotenoids in cancer cells. *Arch. Biochem. Biophys*. **430**, 104-109 (2004).
54. Shao, Y., Ni, Y., Yang, J., Lin, X., Li, J. & Zhang, L. Astaxanthin inhibits proliferation and induces apoptosis and cell cycle arrest of mice H22 hepatoma cells. *Med. Sci. Monit*. **22**, 2152-2160 (2016).
55. Bergmann, A. & Steller, H. Apoptosis, stem cells, and tissue regeneration. *Sci. Signal*. **3**, re8 (2010).
56. Dubuffet, A., Zanchi, C., Boutet, G., Moreau, J. Teixeira, M. & Moret, Y. Trans-generational immune priming protects the eggs only against Gram-positive bacteria in the mealworm beetle. *PLoS Pathog*. **11**, e1005178 (2015).
57. Dhinaut, J., Chogne, M. & Moret, Y. Immune priming specificity within and across generations reveals the range of pathogens affecting evolution of immunity in an insect. *J. Anim. Ecol.* doi: 10.1111/1365-2656 (2017).

58. Moret, Y. Trans-generational immune priming: specific enhancement of the antimicrobial immune response in the mealworm beetle, *Tenebrio molitor*. *Proc. R. Soc. B-Biol. Sci.* **273**, 1399-1405 (2006).
59. Zanchi, C., Troussard, J-P., Martinaud, G., Moreau, J. & Moret, Y. Differential expression and costs between maternally and paternally derived immune priming for offspring in an insect. *J. Anim. Ecol*. **80**, 1174-1183 (2011).