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Patrons de sénescence chez un insecte : interactions entre survie, reproduction et immunité

Charly Jehan

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**THESE DE DOCTORAT DE L'ETABLISSEMENT UNIVERSITE BOURGOGNE FRANCHE-
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Patrons de sénescence chez un insecte : interactions entre survie, reproduction et immunité

Par

Charly JEHAN

Thèse présentée et soutenue à Dijon, le 09/06/2020

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Titre : Patrons de senescence chez un insecte: interactions entre survie, reproduction et immunité

Mots clés : Cout de la reproduction, immunosenescence, biologie évolutive

Résumé : La sénescence est un processus quasiment universel qui se traduit par un déclin âge-dépendant de la reproduction, l'immunité et la survie des organismes. Parmi les théories avancées pour expliquer l'existence de ce phénomène, celle du « soma jetable » propose que la sénescence résulte d'un compromis d'allocation de ressources entre la reproduction et le maintien des fonctions somatiques. Ainsi, une forte allocation à la reproduction tôt dans la vie se réaliserait au détriment des défenses somatiques, notamment de l'immunité, et aboutirait à une sénescence reproductive et actuarielle accrue.

Cette thèse a étudié les relations entre reproduction, immunité et sénescence chez le ténébrion meunier (*Tenebrio molitor* : Coleoptera, Tenebrionidae) en se focalisant particulièrement sur les conséquences de l'investissement âge-spécifique à la reproduction et à l'immunité sur la sénescence.

Cette étude a permis de décrire les patrons intrinsèques de sénescence actuarielle, reproductive et immunitaire chez *T. molitor*. Elle montre aussi que l'allocation des femelles à la reproduction ou à l'immunité tôt dans la vie s'accompagne toujours d'une réduction de la reproduction tardive et parfois d'une longévité plus courte, suggérant ainsi que les femelles favorisent leur longévité au détriment de la reproduction. En outre, l'allocation à la reproduction ou à la fonction immunitaire tôt dans la vie a affecté aussi la fonction immunitaire, suggérant l'existence d'une immunosenescence qui se traduit aussi bien par le déclin de certains effecteurs immunitaires que par la dérégulation de l'activité d'autres effecteurs immunitaires. Ces résultats sont, de manière générale, en accord avec la théorie du soma jetable.

Title : Patterns of senescence in an insect: interactions between survival, reproduction and immunity

Keywords : ageing, cost of reproduction, immuno-senescence, evolutionary biology

Abstract : Senescence is an almost universal process in multi-cellular organisms corresponding to an age-dependent decline of reproductive, immune and survival performance. Among the theories explaining such a phenomenon, the disposable soma theory of ageing proposes that senescence is caused by a resource allocation trade-off between reproduction and somatic maintenance. Hence, high early-life allocation to reproduction should be achieved at the expense of somatic defences, including immunity, which would increase reproductive and actuarial senescence.

This thesis studied the relationships between reproduction, immunity and senescence in the mealworm beetle, *Tenebrio molitor* (Coleoptera, Tenebrionidae) by particularly focusing on the consequences of age-specific allocation in reproduction and immunity on senescence.

My results demonstrated clear evidence of patterns of intrinsic actuarial, reproductive and immune senescence in this insect. In addition, I have shown that early-life allocation to reproduction or to immunity always reduces late-life reproduction in females, while longevity is sometimes affected, thus, suggesting that *T. molitor* females favour longevity at the expense of reproduction. Furthermore, early-life allocation to reproduction or the immune function had contrasting consequences on immune effector systems, suggesting the occurrence of an immunosenescence revealed by both, the decline of some immune effectors and the deregulation of the activity of others. Together, these results are in agreement with the disposable soma theory of ageing.

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Introduction générale

Introduction générale

La théorie des traits d'histoire de vie (Life History Traits en Anglais) vise à expliquer la diversité des stratégies biodémographiques utilisées par les différents organismes (Stearns 1992). Elle cherche à comprendre comment la sélection naturelle a façonné l'expression de caractères, appelés « traits », liés à la fitness des organismes, c'est-à-dire à la capacité d'un individu d'un certain génotype à transmettre ses gènes et donc à produire une descendance viable et fertile. Les principaux traits liés à la fitness sont la taille à la naissance, la croissance, la taille et l'âge à maturité sexuelle, la fécondité et la longévité. Le fondement principal de cette théorie réside en l'existence de compromis évolutifs entre l'expression des différents traits d'histoire de vie. En effet, les ressources énergétique et temporelle étant limitées, un organisme ne peut pas maximiser tous ses traits à la fois. Ainsi, pour favoriser un trait, un individu doit le faire aux dépens d'un autre trait : c'est un compromis évolutif (trade-off en Anglais). Au cours de leur vie, les organismes vont donc devoir faire des compromis dans l'investissement entre différentes fonctions afin de maximiser leur fitness. Différents facteurs tels que la disponibilité des ressources, les conditions environnementales, la compétition ou la sénescence peuvent faire évoluer ces compromis évolutifs.

La sénescence est en elle-même un trait d'histoire de vie. Elle correspond au déclin progressif des performances des organismes avec l'âge, qui débute dès la fin de la phase de maturité et progresse de façon irréversible jusqu'à la mort (Hughes and Reynolds 2005). Elle se caractérise par une détérioration des capacités générales de l'organisme, principalement de la survie et du succès reproducteur. Cependant, la sénescence agit aussi sur d'autres traits tels que l'immunité, le comportement, la physiologie, avec comme conséquence globale un déclin de la fitness (Hughes and Reynolds 2005, Stanley 2012). C'est pourquoi la stratégie optimale d'investissement dans les différents traits d'histoire de vie doit évoluer en fonction de l'âge conjointement avec les échéances imposées par la sénescence de survie, appelée sénescence actuarielle, et la sénescence reproductive. En effet, les coûts et les bénéfices associés à l'investissement dans certaines fonctions sont susceptibles de changer au cours de la vie d'un organisme. Ceci est particulièrement vrai pour la fonction immunitaire, qui permet à l'organisme de protéger son soma afin d'améliorer ses chances de survie (faire face à des infections) et donc de pouvoir se reproduire dans le futur. Les juvéniles doivent, par exemple, impérativement maintenir une immunité relativement forte afin de pouvoir atteindre la maturité sexuelle et ainsi réaliser leur fitness.

De plus, l'efficacité de la fonction immunitaire varie souvent négativement avec l'effort de reproduction (Zera and Harshman 2001, Fedorka et al. 2004). Il est alors possible que la nature d'un tel compromis puisse varier avec l'âge, qui plus est, en interaction avec des facteurs tels que le sexe, l'accès à la reproduction et l'exposition au parasitisme.

1. Bref historique sur la sénescence

La sénescence est un phénomène quasiment universel chez les organismes pluricellulaires. Elle se traduit par un déclin progressif des fonctions de l'organisme avec l'âge, finissant par entraîner la mort (Kirkwood and Rose 1991). Longtemps, on a cru que la sénescence était limitée seulement aux humains et aux animaux captifs et d'élevage. En effet, il était considéré que, dans la nature, à cause de la prédation et du parasitisme, les animaux mouraient avant de pouvoir exprimer de la sénescence. Le phénomène de sénescence était donc considéré comme peu soumis à la sélection naturelle (Medawar 1952). L'idée selon laquelle la sénescence ne s'exprime pas en milieu naturel tenait surtout du fait qu'il n'existait, à l'époque, que très peu d'études de suivi de populations sur le long terme, et qu'il était considéré que la sénescence ne commençait que dans les stades très avancés de la vie (Nussey et al. 2013). Medawar (1952) précisait notamment qu'il n'observait presque jamais de sénilité chez les mammifères sauvages. C'est Williams en 1957, qui va le premier commencer à remettre en cause cette idée (Williams 1957). En effet, il jugea la théorie incorrecte en arguant, notamment, qu'il est inexact de réduire la sénescence à l'état de sénilité. Il la considéra plutôt comme un processus physiologique progressif de détérioration conduisant à une diminution de la fitness avec l'âge, et proposa qu'elle débute dès l'âge de la maturité sexuelle. De plus, il théorisa le concept de pléiotropie antagoniste qui stipule qu'un allèle ayant un effet positif sur la survie ou la reproduction tôt dans la vie peut être fortement sélectionné même s'il a des effets délétères sur la fitness tard dans la vie de l'organisme (Williams 1957). Par la suite, de nombreuses études ont montré que la sénescence est bien présente dans le milieu naturel (voir la synthèse de Nussey et al. 2013) et, de manière générale, semble affecter la reproduction, la survie (Kirkwood and Austad 2000), le comportement (Schumacher et al. 1997, Costantini et al. 2008, MacNulty et al. 2009) et l'immunité (DeVeale et al. 2004, Shanley et al. 2009).

2. Sénescence et stratégies d'investissement à la reproduction

La façon dont les organismes investissent dans la reproduction au cours de leur vie a des conséquences majeures sur leur fitness. Ceci est particulièrement vrai pour les organismes itéropares, qui ont la capacité de se reproduire plusieurs fois dans leur vie. En effet, les organismes dits sémelpares, qui, eux, n'ont la capacité de se reproduire qu'une seule fois dans leur vie, vont allouer le plus de ressources possibles dans cette unique épisode de reproduction afin de maximiser la production et les chances de survie de leurs jeunes (Williams 1966, Clutton-Brock 1984). A l'inverse, chez les organismes itéropares, l'investissement dans la reproduction peut varier au cours de la vie, et pourra être modulé en fonction de la survie et donc en fonction de l'investissement dans la maintenance du soma (Fritz et al. 1982). Afin de maximiser leur fitness, ces organismes doivent donc réaliser des compromis dans l'allocation des ressources disponibles à la survie (au travers la maintenance somatique) et à la reproduction.

Ces compromis peuvent évoluer au cours de la vie avec les échéances imposées par la sénescence. Deux hypothèses ont été proposées pour prédire comment les organismes itéropares devaient répartir leurs investissements à la reproduction en fonction de leur âge. La première est l'hypothèse de l'investissement terminal qui, en se basant sur la théorie des traits d'histoire vie, propose qu'avec l'âge, la valeur reproductive résiduelle (l'opportunité de future reproduction) d'un organisme diminue, et que donc l'investissement dans la reproduction courante devrait augmenter avec l'âge. Ainsi, sous cette hypothèse, les jeunes adultes devraient investir modérément dans la reproduction courante afin de ne pas compromettre les opportunités de reproduction future. Par contre à un âge avancé les individus devraient réaliser un « investissement terminal », c'est-à-dire investir toutes leurs ressources encore disponibles dans la reproduction, car les opportunités de reproduction future sont désormais faibles (Clutton-Brock 1984). Il est aussi attendu que cet investissement terminal s'accompagne d'une espérance de vie post-reproduction réduite car l'investissement des ressources restantes à la reproduction se réalise au détriment de la maintenance somatique. Une des clefs de cette hypothèse repose sur le fait que les organismes sont capables de prédire ou ressentir le moment où ils vont bientôt mourir afin de pouvoir déclencher cet investissement terminal. Par exemple, l'investissement terminal peut se déclencher lorsqu'un organisme perçoit directement ou indirectement une menace pour sa survie, comme lors d'une infection par un pathogène. On parle alors de signal d'investissement terminal. L'intensité de la menace nécessaire pour déclencher l'investissement terminal serait alors négativement corrélée avec l'âge de l'individu (Duffield

et al. 2017, 2018). Cette hypothèse a été testée à de nombreuses reprises chez différentes espèces, en présence ou en absence de signal déclencheur (voir Duffield et al. 2017 pour une synthèse). L'investissement terminal a ainsi été mis en évidence chez des mâles et des femelles de groupes taxonomiques différents : oiseaux, insectes et mammifères notamment (Duffield et al. 2017). Par exemple chez la souris sylvestre, *Peromyscus maniculatus*, qui lorsqu'elle est infecté par le parasite schistosome, *Schistosomatium douthitti*, produit des descendant plus lourd, qui une fois adultes auront une meilleur survie (Schwanz 2008). Cependant, cette stratégie ne semble pas être généralisable car chez certaines espèces, les données montrent une absence d'investissement terminal. Par exemple, chez la mouche tsé-tsé *Glossina morsitans*, la fécondité des femelles et la masse des descendants qu'elles produisent sont constantes durant leur vie. Les auteurs justifient cette absence d'investissement terminal comme résultant du fait que peu de femelles atteignent un âge avancé dans des conditions naturelles et qu'il n'y a donc pas de sélection pour une stratégie d'investissement terminal (Langley and Clutton-Brock 1998).

Par ailleurs il est important de noter que certains facteurs confondants peuvent altérer l'interprétation des résultats. D'une part, des patrons d'investissement à la reproduction pouvant être interprétés comme résultant d'un investissement terminal pourraient simplement être causés par la mortalité précoce d'individus d'une même cohorte qui sont aussi les moins performant pour la reproduction (Curio 1983, Forslund and Pärt 1995). D'autre part, les individus de certaines espèces (souvent des ongulés) peuvent voir leurs performances de reproduction s'améliorer avec l'âge par simple acquis d'expérience ou d'une position de dominance sociale (Coltman et al. 2002).

La deuxième théorie sur la répartition des investissements dans la reproduction est l'hypothèse de la reproduction restreinte. Elle prédit que pour maximiser leur fitness les organismes ont intérêt à réduire leurs investissements à la fonction de reproduction avec l'âge (Curio 1983, McNamara and Houston 1996, McNamara et al. 2009). Elle s'appuie sur le fait que la reproduction est coûteuse, qu'elle cause des dommages et entraîne donc une diminution de la survie. Ainsi, investir modérément dans la reproduction tard dans la vie devrait réduire les coûts de la reproduction et offrir aux organismes plus de temps pour réaliser des épisodes de futures reproductions, permettant de ce fait de maximiser leur fitness. Une étude sur les albatros hurleurs (*Diomedea exulans*) a montré que les mâles montraient une diminution des performances de reproduction et de recherche de nourriture avec l'âge, suggérant que les mâles plus âgés investissent moins dans les soins parentaux

que les jeunes. Les auteurs conclurent que les mâles albatros errants pourraient ainsi sacrifier leur investissement parental afin de maintenir un niveau d'aptitude physiologique élevé jusqu'à un âge avancé, conformément au concept de la reproduction restreinte (Lecomte et al. 2010). Les études qui ont testé l'hypothèse de la reproduction restreinte restent néanmoins très rares et il n'est souvent pas évident de déterminer si la réduction de l'effort de reproduction avec l'âge résulte d'une stratégie de reproduction restreinte ou simplement de l'incapacité progressive de l'animal à se reproduire en raison d'une déficience progressive de sa physiologie. Un examen de l'effort de reproduction et la mortalité âge-dépendants associés à des mesures de la protection du soma pourraient permettre de tester la validité de cette hypothèse.

3. Causes possibles de la sénescence et de son maintien

La sénescence diminue la performance des organismes avec l'âge. Se pose alors la question de comment un trait comme la sénescence a pu être sélectionné et se maintenir, alors qu'il est délétère pour la fitness des organismes. Différents mécanismes et théories ont été proposés pour expliquer ce maintien, qui peuvent être grossièrement divisés en deux catégories : les causes évolutives et les causes physiologiques. Ci-après sont présentées les principales théories de la sénescence, mais cet inventaire ne se veut pas exhaustif. Il est par ailleurs important de noter que ces différentes théories ne sont pas exclusives les unes des autres.

a. La théorie de l'accumulation des mutations

La théorie de l'accumulation des mutations est définie par Medawar dès 1952. Il se base sur le postulat qu'après la première reproduction, la pression de sélection à laquelle chaque organisme est soumis diminue, mais aussi qu'à cause de la mortalité extrinsèque (e.g. prédation ...), les individus âgés sont éliminés des populations et que, par conséquent, la force de sélection sur ces derniers est très faible. Il propose que la sélection naturelle ne puisse pas éliminer les mutations dont les effets délétères s'expriment après l'âge de reproduction. Ceci entraîne dans les populations une accumulation de mutations dont les effets délétères sur la fin de vie sont la cause de la sénescence (Medawar 1952).

b. La théorie de pléiotropie antagoniste

La théorie dite de la pléiotropie antagoniste (antagonistic pleiotropy hypothesis) est proposée par Williams (1957), à la suite des observations de Medawar. Il part du même postulat : l'intensité de la sélection naturelle diminue avec l'âge des organismes. Par contre, il propose que les allèles à effets positifs sur la fitness tôt dans la vie (c'est-à-dire sur la survie ou la reproduction) sont fortement sélectionnés même s'ils ont des effets délétères sur la fitness tard dans la vie (Figure 1). A l'inverse, un allèle bénéfique tard dans la vie mais délétère tôt dans la vie sera naturellement éliminé par la sélection naturelle. Cette théorie a depuis été étayée par des observations chez différentes espèces animales, notamment chez *Drosophila melanogaster* (Rose and Charlesworth 1980, Rose 1984, Sgrò and Partridge 1999). En effet, chez cet insecte, plusieurs études utilisant des lignées sélectionnées en laboratoire ou des mutants générés artificiellement ont montré que les femelles caractérisées par plus grande longévité étaient aussi celles qui avaient la plus faible fécondité tôt dans la vie adulte. Inversement, une forte reproduction tôt dans la vie était accompagnée d'une accélération de la mortalité tard dans la vie. D'autres études réalisées en milieux naturels sont parvenues aux mêmes conclusions. Notamment, les femelles du cerf *Cervus elaphus* ayant donné naissance à un plus grand nombre de jeunes tôt dans la vie montraient aussi des taux de sénescence reproductive plus importants que les autres (Nussey et al. 2013).

c. Théorie du soma jetable

On définit deux types de lignées cellulaires dans un organisme, qui se séparent très tôt au cours de l'embryogénèse. D'une part, les cellules de la lignée germinale (ou germen) sont celles dédiées à la reproduction, susceptibles de former les gamètes. Ces cellules transmettront ainsi les mutations génétiques qu'elles portent à la descendance. D'autre part, les cellules de la lignée somatique (ou soma) forment le reste du corps d'un organisme multicellulaire. Elles sont dédiées au maintien et au fonctionnement de l'organisme.

La théorie du soma jetable (« disposable soma theory of ageing » en Anglais) est basée sur la théorie des traits d'histoire de vie présentée plus haut mais distingue deux types de traits d'histoire de vie: ceux qui sont liés à la reproduction et ceux liés au maintien du soma de l'organisme. Les ressources étant limitées, un organisme doit faire des choix dans l'utilisation de ces dernières. La reproduction étant une fonction coûteuse, on peut prédire qu'un fort investissement à la reproduction tôt dans la vie entraîne une diminution

de l'investissement dans les traits liés à la maintenance du soma (réparation de l'ADN, immunité...). Ceci a pour conséquences d'entraîner une accumulation de dommages qui, à terme, causeront la sénescence chez les organismes (Kirkwood 1977, Kirkwood and Rose 1991, Kirkwood and Austad 2000). Cette théorie et celle de la pléiotropie antagoniste, proposent deux causes différentes (évolutive ou génétique) à des phénotypes observés comparables. Ces deux théories ne sont d'ailleurs pas exclusives. Par exemple, en compilant des études de suivi de populations de vertébrés, Lemaître et ses collaborateurs (2015) ont montré l'existence de compromis évolutifs entre le début et la fin de la vie. En effet, ils ont montré dans 21 des 26 études analysées, l'existence d'un tel compromis entre des traits d'histoire de vie mesurés tôt dans la vie (âge de la première reproduction par exemple) et des traits exprimés tard dans la vie (comme l'âge à la dernière reproduction ou la survie). Cette étude apporte donc un soutien solide à la théorie du soma jetable. Cependant, comme le précisent les auteurs, ces résultats sont aussi en accord avec la théorie de la pléiotropie antagoniste si les gènes impliqués dans les compromis observés sont identifiés (Lemaître et al. 2015).

d. Théorie développementale de la sénescence

Cette théorie se base sur des causes physiologiques pour expliquer la sénescence. Elle propose de relier le développement de l'organisme avec sa sénescence, considérant une fois encore que l'intensité de la sélection naturelle diminue avec l'âge des organismes. Cette théorie stipule que l'expression des gènes est mieux optimisée pour favoriser le développement et la reproduction tôt dans la vie que pour favoriser le maintien tard dans la vie. Elle postule ainsi que la sénescence est due à une physiologie sous-optimale à l'âge adulte plutôt que par le manque de ressources pour l'entretien somatique (évoqué dans le cas théorie du soma jetable) (de Magalhães and Church 2005, Maklakov and Chapman 2019). Cette théorie a reçu le support de différents travaux, notamment d'une étude du génome de *Caenorhabditis elegans* qui a mis en évidence 64 gènes répondant aux critères de la théorie développementale de la sénescence. En effet, ces gènes sont exprimés tout au long de la vie et ils sont indispensables au développement de *C. elegans*, leur inactivation durant le développement entraîne un arrêt développemental. Cependant, même si ils sont exprimé plus tard dans la vie, leur inactivation post-développementale entraîne une augmentation de la longévité de *C. elegans* (Curran and Ruvkun 2007).

e. Théorie radicalaire du vieillissement

Cette théorie est elle aussi basée sur des causes mécanistiques et physiologiques. Elle postule que la sénescence est due aux dommages cellulaires causés par les radicaux libres, plus particulièrement par les espèces réactives de l'oxygène (Harman 1956, 1981), qui sont des molécules possédant sur leur couche externe un électron non apparié, les rendant instables et très réactives. Ces molécules sont naturellement produites lors du métabolisme normal de la cellule, durant lequel 1 à 2% de l'oxygène utilisé dans la respiration mitochondriale est transformé en espèces réactives de l'oxygène, telles que l' O_2^- (Oliveira et al. 2010). Les radicaux libres ainsi générés vont créer un stress oxydant et s'attaquer aux protéines, aux lipides et à l'ADN, causant d'importants dommages aux cellules. L'accumulation au cours de la vie des organismes de tous ces dommages cellulaires et tissulaires causés par le stress oxydant serait alors à l'origine de la sénescence cellulaire. Cette théorie sera, par la suite validée, à plusieurs reprises avec différentes méthodes (voir Beckman and Ames 1998 pour une synthèse).

Un autre mécanisme entraînant l'apparition d'espèces réactives de l'oxygène est la réponse immunitaire. En effet, au cours de leur vie, les organismes peuvent faire face à des traumatismes physiques ou des infections parasitaires ou pathogéniques. Afin de s'en prémunir, les organismes doivent maintenir un système immunitaire fonctionnel, afin de tuer ou neutraliser les pathogènes. Une partie de la réponse immunitaire, la réponse inflammatoire, est désormais décrite comme un des facteurs responsables de la sénescence (Licastro et al. 2005, Khan et al. 2017). Elle consiste en effet en le relargage de composés cytotoxiques (comme des espèces réactives de l'oxygène) ayant pour but de tuer les pathogènes. Cependant les molécules relâchées ne ciblent pas spécifiquement les pathogènes, et vont donc aussi affecter les cellules et les tissus, causant un phénomène d'auto-immunité néfaste pour l'organisme.

4. Immunité

La fitness d'un organisme dépend grandement de son système immunitaire pour assurer une protection contre les parasites et les agents pathogènes. La réponse immunitaire apparaît cependant comme un facteur potentiel important pouvant moduler la sénescence. La structure des systèmes immunitaires est étonnamment complexe, cette complexité ayant très probablement été façonnée par l'écologie de l'organisme (Schulenburg et al 2009). Cette complexité est illustrée chez les vertébrés (Coico and Sunshine 2003, Zimmerman et

al. 2010), mais existe également chez les invertébrés et notamment les insectes (Schmid-Hempel 2005).

L'immunité des insectes peut être décomposée en trois principales composantes : l'immunité comportementale, l'immunité cuticulaire et l'immunité hémocoelique. Toutes ces composantes sont présentées et détaillées dans l'article Vigneron et al. (2019). Cet article, pour lequel je suis co-auteur, est présenté dans l'Annexe I. Il focalise sur *Tenebrio molitor*, le modèle biologique utilisé dans cette thèse, mais offre une bonne vue synthétique sur le système immunitaire des insectes en général. Un résumé en est présenté ci-après, la partie sur l'immunité hémocoelique étant la plus détaillée car c'est celle-ci qui sera étudiée dans les différents chapitres de cette thèse.

a. Immunité comportementale

L'immunité comportementale correspond à tous changements de comportements permettant à l'organisme d'éviter l'infection par des pathogènes ou de limiter leur croissance (De Roode and Lefèvre 2012, Vigneron et al. 2019). Ces comportements peuvent être classés en trois types. Le premier type se réfère à tous les comportements d'évitements (évitements spatiaux, temporels ou bien liés à l'évitement d'aliments infectés) et ceux liés à la consommation d'aliments prophylactiques. Le deuxième type de comportement intègre les comportements liés à l'élimination ou à la réduction de croissance parasitaire. On y retrouve par exemple le toilettage ou la « fièvre comportementale » (recherche d'endroits chauds qui augmentent la température corporelle à des niveaux nuisibles pour les parasites). Enfin, le troisième type de comportements correspond aux comportements dits « de tolérance », par lesquels l'hôte va réduire les effets négatifs de l'infection, permettant de maintenir un succès reproducteur correct. Par exemple, en réponse à l'infection parasitaire par le cestode *Hymenolepis diminuta*, les mâles de *T. molitor* produisent de meilleurs spermatophores, contenant un cadeau nuptial plus important, qui, une fois transmis à leur partenaire, leur permettront de pondre plus d'œufs (Carver et al. 1999).

b. Immunité cuticulaire

La cuticule ou l'exosquelette des insectes, est une barrière efficace qui empêche la plupart des parasites et des pathogènes de pénétrer dans l'hémocœle des insectes (Moret and Moreau 2012). Constituée majoritairement de chitine, la cuticule n'est pas une simple

barrière physique, car elle comprend aussi des éléments de défenses biochimiques et physiologiques, en partie liées à l'immunité hémocoelique (Moret and Moreau 2012, Vigneron et al. 2019).

c. Immunité hémocoelique

L'immunité hémocoelique est la somme de tous les processus mis en place pour localiser et éliminer ou limiter le développement des pathogènes, une fois qu'ils ont franchi la barrière cuticulaire.

Cette réponse immunitaire est activée lorsque l'organisme perçoit des motifs moléculaires associés aux pathogènes (Pathogen-Associated Molecular Patterns ou PAMPs) *via* les « récepteurs de reconnaissance de motifs moléculaires » (Pattern Recognition Receptors ou PRRs). Ces motifs sont des molécules spécifiques des pathogènes tels que les lipopolysaccharides (LPS), les peptidoglycanes bactériens ou les β -1,3 glucanes. La reconnaissance de ces molécules va entraîner l'activation de différentes voies immunitaires adaptées à répondre aux types de pathogènes associés grâce aux PAMPs détectés (Vigneron et al. 2019). Ces différentes réponses immunitaires sont aussi bien cellulaires (l'action des hémocytes) qu'humorales (production de peptides antimicrobiens et phénomène de mélanisation catalysé par la phénoloxydase).

Les hémocytes sont des cellules circulantes dans l'hémocœle, qui participent activement à la réponse immunitaire. Il en existe quatre types décrits chez *T. molitor*, chacun ayant différents rôles (Figure 1 -). Les prohémocytes représentent 10 à 15 % des hémocytes totaux. Ce sont de petites cellules ovales de moins de 10 μm avec un très grand noyau. Ce sont les précurseurs des autres hémocytes. Un deuxième type hémocytaire est représenté par les granulocytes qui sont les plus fréquents. Ils représentent 50 à 60% des hémocytes observés chez *T. molitor*. Ce sont des cellules ovales d'environ 10 μm contenant des granules visibles dans leur cytoplasme, et sont impliqués dans la phagocytose. Lors de ce phénomène de défense, les cellules vont internaliser et digérer des particules exogènes telles que des micro-organismes. Les plasmatoctes représentent quant à eux 23 à 28% des hémocytes totaux. Ce sont de grandes cellules allongées qui sont impliquées dans l'encapsulation des corps étrangers (dont les parasites ou pathogènes), les isolant du milieu interne de l'animal. Enfin, les œnocytoïdes représentent 1 à 2 % des hémocytes en circulation. Il s'agit de grosses cellules ovales à noyau central qui produisent les enzymes catalysant la mélanisation (production de mélanine dans l'hémocœle à la surface des pathogènes afin de les isoler). L'encapsulation et la mélanisation vont intervenir de concert

lorsqu'un objet exogène est trop gros pour être phagocyté. Au cours de ce processus d'encapsulation plusieurs plasmotocytes vont s'agglomérer autour du parasite pour le piéger. Ces hémocytes seront ensuite mélanisés afin de compléter la neutralisation du parasite en l'isolant complètement de l'hémocœle. Les granulocytes pourraient aussi être impliqués dans ce mécanisme d'encapsulation.

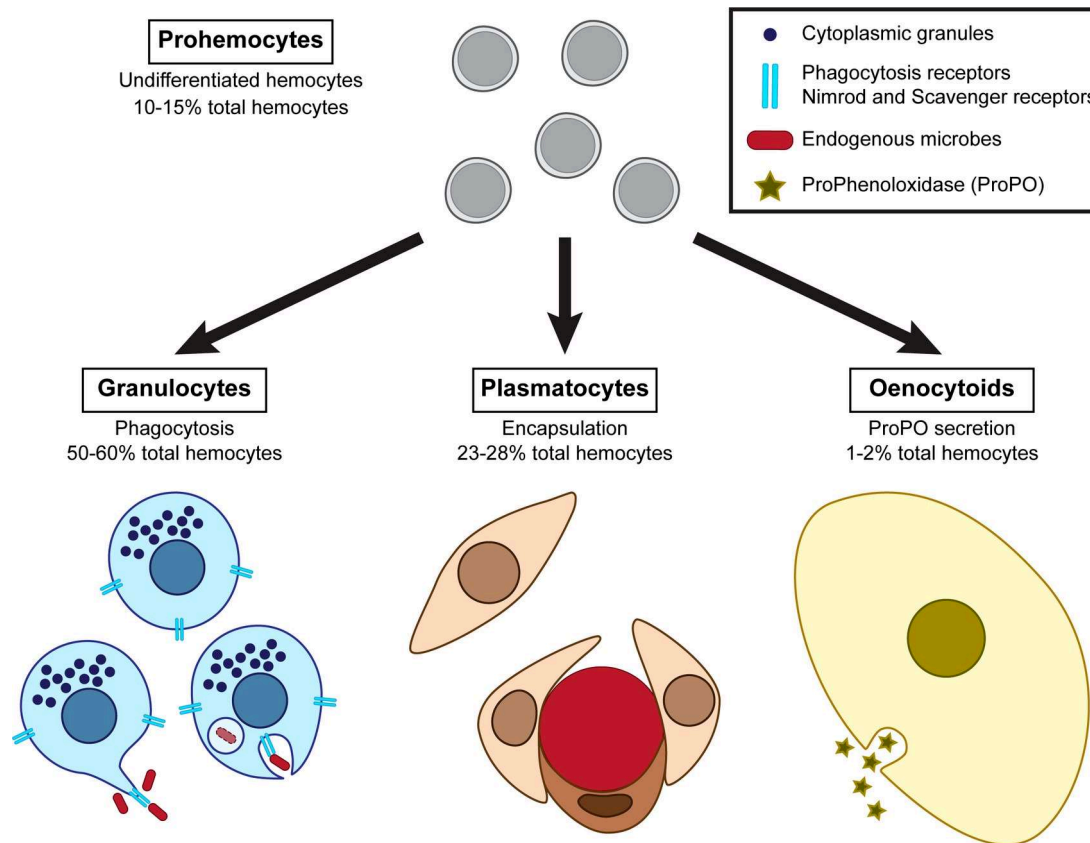


Figure 1 Différents type d'hémocytes et leurs fonctions associées intervenant dans la réponse immunitaire cellulaire de *T. molitor*. adapté de Vigneron et al., 2019 (annexe)

Le processus de mélanisation évoqué plus haut est aussi un des éléments majeurs de l'immunité des insectes. C'est une tyrosinase, la phénoloxydase (PO), qui catalyse la synthèse de mélanine. La production de PO à partir de son précurseur la prophénoloxydase (PPO) est induite lors que des PAMPs sont détectés. La mélanine ainsi produite peut encapsuler directement les pathogènes ou participer conjointement avec les hémocytes au processus d'encapsulation (Söderhäll and Cerenius 1998). Ainsi, l'activité de la PO est corrélée avec l'élimination et l'isolement des pathogènes. Cependant, cette enzyme est aussi décrite comme entraînant des coûts importants pour l'organisme. En effet, la cascade enzymatique conduisant à la mélanisation s'accompagne de la production de substances cytotoxiques, tels que les phénols, les quinones et des espèce réactives de l'oxygène (Nappi and Ottaviani 2000, Zhao et al. 2011). Ces molécules peuvent intervenir dans l'élimination

des pathogènes, mais elles ne ciblent pas spécifiquement ces derniers. Différentes études ont ainsi pu montrer qu'une activation de la PO peut entraîner de l'auto-immunité (le système immunitaire s'attaquant alors au soi aussi bien qu'au pathogène). Par exemple, il a été montré chez *T. molitor* qu'une activation de PO tôt dans la vie entraîne la mélanisation des tubes de Malpighi (Sadd and Siva-Jothy 2006, Khan et al. 2017), conduisant ainsi à une réduction de la longévité des insectes (Khan et al. 2017).

Les différents effecteurs immunitaires décrits précédemment sont mis en place rapidement après l'infection.

Le dernier élément majeur de l'immunité hémocœlique est la production de peptides antibactériens. Cette réponse immunitaire constitue une réponse plus spécifique mais plus lente (Siva-Jothy et al. 2005). Ces molécules sont produites dans les corps gras des insectes, suite à la détection de PAMPs. Leurs voies de synthèse, complexes, sont décrites dans Hoffmann (2003) et spécifiquement pour *T. molitor* dans Vigneron et al. (2019). Le pic de production de ces peptides antibactériens chez *T. molitor* se situe entre 24h à 48h après l'infection, contre quelques minutes pour la production de la réponse cellulaire. Cependant, contrairement à cette dernière, leur activité peut persister longtemps dans l'organisme, au moins durant 14 jours (Haine et al. 2008a).

5. Immunité et sénescence

Les liens entre immunité et sénescence sont particulièrement intéressants. D'une part la fonction immunitaire est décrite comme une des causes de la sénescence, alors que d'autre part l'immunité est aussi soumise à la sénescence. C'est la réponse dite inflammatoire qui est présentée comme principale cause du lien entre l'immunité et la sénescence (Vasto et al. 2007, Pursall and Rolff 2011a).

Comme évoqué plus haut, la réponse immunitaire provoque de l'auto-immunité chez les insectes (Sadd and Siva-Jothy 2006, Khan et al. 2017), l'activation précoce de la phénoloxydase entraîne des conséquences néfastes sur la physiologie de l'insecte. De plus, Khan et al. (2017) ont observé que lorsque des ténébrions meuniers, *Tenebrio molitor*, étaient exposés à un challenge immunitaire précoce, ils souffraient d'une diminution de leur longévité, reflétant ainsi un lien direct entre l'immunité et la sénescence. Cependant, dans ces travaux, les auteurs n'ont pas permis aux femelles de se reproduire, ne permettant pas d'étudier d'éventuels compromis entre ces deux traits au cours de la vie. Par ailleurs, Khan et al. (2017) ont maintenu leurs insectes dans des conditions de température plutôt supérieures à l'optimales (30°C ; la température optimale de *T. molitor* étant de 25-28°C,

(Punzo 1975, Ribeiro et al. 2018)). De tels effets négatifs d'une stimulation immunitaire précoce sur la survie n'est pas général, puisque Lippens et al. (2019), n'ont montré aucun effet d'une réponse inflammatoire précoce (induite par une infection par *Plasmodium yoelii* vecteur de la malaria murine) sur la sénescence reproductive ou actuarielle chez des souris.

Le phénomène de sénescence touche l'immunité elle-même, on parle alors d'immunosénescence (DeVeale et al. 2004, Nikolich-Žugich 2018). La mise en évidence de cette immunosénescence n'est cependant pas très claire. Certaines études décrivent des diminutions de paramètres immunitaires ou d'accumulation de mutations délétères dans les cellules de l'immunité (e.g l'immunité adaptative des vertébrés (Nikolich-Žugich 2018, Zhang et al. 2019)). Cependant, d'autres études ont montré des paramètres immunitaires en hausse chez les individus âgés, par exemple la production de peptides anti-microbiens chez les drosophiles, ou l'activité de phénoloxydase chez *Tribolium castaneum*, qui ne sont cependant pas corrélées avec une meilleure réponse immunitaire en cas d'infection (Zerofsky et al. 2005, Khan et al. 2016). De plus, des patrons différents d'immunosénescence peuvent être observés entre les sexes, complexifiant encore notre compréhension de ce phénomène (Metcalf et al. 2020).

Avec l'immunité, la reproduction est un trait important lié à la fitness et nous avons déjà noté que les ressources étant limitées, les organismes sont contraints de faire des compromis optimaux pour leurs investissements entre les traits du cycle de vie, afin de maximiser la fitness (Stearns 1992, Hughes and Reynolds 2005). Un tel compromis entre la reproduction et l'immunité a été décrit dans plusieurs espèces animales (Zera and Harshman 2001, Hosken 2001, Fedorka et al. 2004, Simmons and Roberts 2005, Stahlschmidt et al. 2013). Par exemple, chez la scatophage du fumier, *Scathophaga stercoraria*, la comparaison de lignées sélectionnées pour de la polyandrie et des lignées sélectionnées pour de la monogamie, a montré que les lignées polyandres investissaient davantage dans la reproduction du fait que les insectes de ces lignées présentaient notamment des glandes reproductrices plus grosses (Hosken 2001). Ces lignées polyandres étaient aussi caractérisées par un plus faible niveau d'activité de la phénoloxydase que les lignées monogames, suggérant ainsi l'existence d'un trade-off entre l'investissement de ressource dans la fonction de reproduction et l'immunité.

Cependant, l'effet de l'âge des individus sur ce compromis n'est pas connu. L'effet sur les taux de mortalité n'a pas non plus été abordé dans ces études. De plus, le timing et l'amplitude des compromis entre différents traits d'histoire de vie peuvent changer au cours

de la vie (Metcalf et al. 2020). À notre connaissance, cet aspect n'a jamais été abordé entre l'immunité, la survie et la reproduction au sein d'études uniques spécifiquement dédiées. Ainsi, il n'est pas connu, par exemple, si des individus jeunes stimulés immunitairement voient leur longévité plus intensément diminuée que des individus plus âgés, ou s'ils préfèrent, par exemple, « sacrifier » leur survie en faveur de leur reproduction. Ces questions ont fait l'objet d'une demande de plus de données expérimentales dans un article de synthèse récent (Metcalf et al. 2020).

6. Objectifs de la thèse

Cette thèse propose d'étudier les relations entre reproduction, immunité et sénescence chez l'insecte *Tenebrio molitor*. Les expériences réalisées nous ont d'abord permis de décrire les patrons de sénescence « intrinsèques » de *T. molitor*, patrons jusqu'ici peu ou pas décrits dans leur entièreté. Nous avons ensuite cherché à déterminer les conséquences d'une modulation précoce de l'investissement à la reproduction et à l'immunité sur le maintien de ces fonctions plus tard dans la vie ainsi que sur la longévité, afin de caractériser les stratégies d'investissement aux traits d'histoire de vie et les causes de la sénescence chez *T. molitor*. L'avantage de ce modèle biologique est que son immunité est bien connue (Vigneron et al. 2019), et que de nombreuses études en immuno-écologie sont disponibles, notamment celles ayant mis en évidence les compromis évolutifs entre immunité et différents traits de vie (ex : Haine et al. 2008 ; Zanchi et al. 2011, 2012 ; Khan et al. 2017). De plus, *T. molitor* est un insecte chez qui la phase adulte est relativement longue (quelques semaines). Le choix de ce modèle permet donc de suivre les individus adultes tout au long de leur vie, tout en réalisant des manipulations expérimentales (aussi bien sur l'immunité que la reproduction), ce qui n'est que rarement possible sur les modèles vertébrés, majoritairement utilisés pour les études écologiques sur la sénescence (Gaillard and Lemaître 2019).

Dans le Chapitre 1, nous avons étudié au niveau populationnel l'influence de l'intensité de la reproduction sur la sénescence des femelles et des mâles de *T. molitor*. Pour moduler l'intensité de la reproduction, nous avons manipulé le sex-ratio de populations artificielles de *T. molitor*. En effet, le sex-ratio d'une population influence l'effort reproducteur et la compétition intra-sexuelle des deux sexes. Par exemple, dans une population avec un sex-ratio biaisé en faveur des mâles, ces derniers sont soumis à une forte compétition intra-

sexuelle pour l'accès aux femelles mais peuvent également avoir un faible taux individuel d'accouplement. Les femelles sont quant à elles soumises à une faible compétition sexuelle mais à un fort taux d'accouplement et potentiellement subir un harcèlement plus intense de la part des mâles. En suivant trois populations artificielles avec des sex-ratios différents (75%/25% 50%/50% et 25%/75%), nous avons ainsi pu modifier l'investissement à la reproduction des mâles et des femelles simultanément. Nous avons suivi femelles et mâles de ces populations en mesurant leur reproduction, leur longévité, leur immunité et leur sénescence. Cependant, ce schéma expérimental ne nous donnant pas accès aux paramètres individuels de reproduction, par exemple, nous avons mené des expériences plus fines.

Dans le Chapitre 2, nous avons étudié les conséquences de la reproduction sur les sénescences immunitaire, reproductive et de survie chez les femelles de *T. molitor*. Pour ce faire, des femelles ont été amenées à effectuer deux épisodes de reproduction au cours de leur vie : un épisode de reproduction précoce (entre 10 et 30 jours post émergence) et un épisode de reproduction tardif (entre 40 et 60 jours post émergence), seuls ou en combinaison. La fécondité, la longévité et l'immunité de ces femelles ont été mesurées. Cette expérience a permis de tester la stratégie d'investissement à la reproduction des femelles de *T. molitor* selon deux hypothèses distinctes. La première hypothèse est celle de « l'investissement terminal » qui propose que les femelles devraient investir fortement à la reproduction en fin de vie, et cela aux dépens de la longévité et des défenses somatiques. La deuxième hypothèse est celle de la « reproduction restreinte » qui propose que les femelles devraient moduler leur effort de reproduction au cours de leur vie de manière à maintenir une meilleure longévité et assurer plus d'opportunités de reproductions futures. Cette expérience a également permis de mesurer précisément les patrons de sénescence reproductive et immunitaire des femelles ténébrions meuniers.

Le Chapitre 3 présente une étude qui est la « réciproque » de celle présentée dans le Chapitre 2, c'est-à-dire que nous avons stimulé le système immunitaire des femelles tôt et/ou tard dans leur vie pour en étudier les conséquences sur la sénescence de reproduction, d'immunité et de survie. Au cours de cette expérience, toutes les femelles ont eu l'opportunité de se reproduire tôt et tard dans leur vie adulte (voir le design précédent), mais chaque épisode de reproduction a été précédé ou non d'un challenge immunitaire. Nous avons mesuré l'influence de ces challenges immunitaires sur la fécondité, la longévité

et la fonction immunitaire des femelles de *T. molitor*. L'utilisation du challenge immunitaire permet aussi de mimer un statut infectieux pouvant signaler un plus fort risque de mortalité imminente, qui pourrait servir de déclencheur à un investissement terminal. Il permet enfin de mesurer les conséquences de l'immunopathologie induite par la réponse immunitaire sur la sénescence.

Dans chaque expérience (sauf besoins expérimentaux de prélèvements), les individus ont été suivis tout au long de leur vie.

Tenebrio molitor

Tenebrio molitor, le ténébrion meunier ou le ver de farine, est un insecte coléoptère polyphage de l'ordre des Tenebrionidae. *T. molitor* peut être à la fois considéré comme un insecte nuisible et un insecte bénéfique. En effet, historiquement, *T. molitor* est considéré comme un nuisible car il consomme ou altère la qualité de denrées stockées, particulièrement la farine ou les céréales. Cependant, désormais, les larves de ténébrion meunier sont utilisées comme nourriture pour les animaux domestiques. Elles offrent également une alternative prometteuse à l'alimentation animale riche en protéines et sont même recommandées comme source d'alimentation humaine. De plus, les larves de *T. molitor* peuvent être utiles en raison de leur capacité à dégrader efficacement le polystyrène et les déchets plastiques. On peut donc aussi le considérer comme un insecte bénéfique (Brandon et al. 2018).

Durant leur cycle de vie, les ténébrions meuniers passent par plusieurs stades larvaires, un stade nymphal et un stade adulte. Les stades larvaires sont de durées variables en fonction des conditions environnementales (Urs and Hopkins 1973, Punzo 1975), mais durent généralement de 3 à 6 mois. La phase adulte, quant à elle dure environ 16 semaines. Les mâles comme les femelles de *T. molitor* sont sexuellement matures à partir du cinquième jour suivant leur émergence de la nymphe. Ils sont itéropares et donc s'accouplent de multiples fois et avec différents partenaires au cours de leurs deux à cinq mois de vie adulte. Les femelles sont continuellement réceptives à l'accouplement à l'âge adulte et peuvent produire jusqu'à 30 œufs par jour, bien que la production d'œufs diminue généralement après trois semaines (Dick 1937, Drnevich et al. 2000, 2001). Bien qu'elles soient capables de stocker du sperme dans leur spermathèque, les femelles ont besoin de s'accoupler fréquemment pour maintenir une production d'œufs maximale (Drnevich et al. 2001). L'accouplement se déroule après une relativement brève parade nuptiale suivie par une intromission, durant laquelle les mâles transfèrent un spermatophore dans l'appareil génital des femelles. Le spermatophore contient un cadeau nuptial (contenant protéines et sucres) ainsi que les spermatozoïdes, qui ne seront libérés que sept à dix minutes après l'intromission (Gadzama and Happ 1974, Carver et al. 1999). C'est pourquoi les mâles peuvent effectuer un court gardiennage post-copulatoire, qui devient alors une composante de la compétition sexuelle inter-mâle. De plus, après la copulation, les mâles ont une courte

période réfractaire de 20 minutes au cours de laquelle ils ne peuvent pas s'accoupler (Drnevich 2003).

Au laboratoire, les ténébrions sont maintenus sous une température de 24 ± 2 ° C, sous 70% d'humidité relative et dans l'obscurité permanente. Ils vivent dans de la farine de son, dont ils se nourrissent aussi bien au stade larvaire qu'au stade adulte. Cette nourriture de base est complétée par de la pomme et de l'eau.

Chapitre 1

Chapitre 1 : Etude populationnelle de d'influence de l'effort reproducteur sur les patrons de sénescence de *Tenebrio molitor*

La théorie du soma jetable stipule que les organismes vieillissent en raison d'un compromis évolutif d'allocation de ressources entre la reproduction et le maintien des fonctions somatiques, telles que la réparation de l'ADN ou de l'immunité (Kirkwood and Rose 1991). Ainsi, une forte allocation à la reproduction précoce se réaliserait aux dépens des défenses somatiques et aboutirait à un déclin de la reproduction tardive et de la longévité. Par conséquent, les organismes sont contraints d'optimiser ce compromis tout au long de leur vie afin de maximiser leur fitness.

Les mâles et les femelles peuvent évoluer des stratégies d'histoire de vie différentes pour maximiser leur fitness. Notamment, il est souvent admis que les femelles obtiennent un gain de fitness en maximisant la longévité, tandis que les mâles maximiseraient leur fitness en augmentant leur succès d'accouplements, exposant ces derniers à une sélection intra-sexuelle intense (Trivers 1972, Rolff 2002). Ce dimorphisme sexuel d'allocation à la reproduction devrait alors favoriser l'évolution de patrons de senescence sexe-spécifiques

Dans ce chapitre, nous avons examiné ces possibilités en suivant des populations artificielles du ténébrion meunier, *Tenebrio molitor*, dans lesquelles nous avons manipulé le sex-ratio afin de générer des niveaux variables d'investissement dans l'effort reproductif et la compétition sexuelle chez les mâles et les femelles par rapport à des populations dont le sex-ratio était équilibré. Ainsi, dans les populations où le sex-ratio est biaisé en faveur des mâles (75% de mâles), les mâles sont soumis à une compétition intense pour l'accès aux femelles, qui elles devraient subir un taux d'accouplements élevé. Au contraire, dans les populations dont le sex-ratio est biaisé en faveur des femelles (25% de mâles), les mâles ont la possibilité de s'accoupler plus fréquemment tandis que les femelles ont moins d'opportunité d'accouplement. L'allocation âge-spécifique à la reproduction l'immunité et la survie des mâles et des femelles issue de chaque population a été estimée.

Comme attendu, la manipulation du sex-ratio a bien affecté l'effort de reproduction des mâles et des femelles, permettant de mettre en évidence des compromis sex-spécifiques d'allocation à la reproduction, l'immunité et la survie. En effet, chez les femelles,

un effort reproducteur élevé accélère le taux de mortalité, sans pour autant affecter leur immunité. Chez les mâles, la reproduction était coûteuse en termes de longévité et d'immunité. Cependant, nos résultats suggèrent que le coût de la reproduction chez les mâles ne dépend pas d'une compétition sexuelle intense mais du coût de la copulation. En effet, les mâles des populations où la compétition sexuelle est sensée être forte (75% de mâles) vivent plus longtemps que les mâles des autres populations.

Nos résultats suggèrent que les mâles de *T. molitor*, comme les femelles, augmentent leur fitness en maximisant leur longévité, ce qui expliquerait en partie leur longévité comparable à celle des femelles.

Sex-specific patterns of senescence in artificial insect populations varying in sex-ratio to manipulate reproductive effort

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1. Abstract

Background: The disposable soma theory of ageing assumes that organisms optimally trade-off limited resources between reproduction and longevity to maximize fitness. Early reproduction should especially trade-off against late reproduction and longevity because of reduced investment into somatic protection, including immunity. Moreover, as optimal reproductive strategies of males and females differ, sexually dimorphic patterns of senescence may evolve. In particular, as males gain fitness through mating success, sexual competition should be a major factor accelerating male senescence. In a single experiment, we examined these possibilities by establishing artificial populations of the mealworm beetle, *Tenebrio molitor*, in which we manipulated the sex-ratio to generate variable levels of investment into reproductive effort and sexual competition in males and females.

Results: As predicted, variation in sex-ratio affected male and female reproductive efforts, with contrasted sex-specific trade-offs between lifetime reproduction, survival and immunity. High effort of reproduction accelerated mortality in females, without affecting immunity, but high early reproductive success was observed only in balanced sex-ratio condition. Male reproduction was costly on longevity and immunity, mainly because of their investment into copulations rather than in sexual competition.

Conclusions: Our results suggest that *T. molitor* males, like females, maximize fitness through enhanced longevity, partly explaining their comparable longevity.

Key words: ageing, cost of reproduction, disposable soma theory, immunity, immuno-senescence, *Tenebrio molitor*

2. Background

Life history theory assumes that organisms are constrained to optimally trade-off limited energetic and time resources between reproduction and life span, to maximize fitness (Stearns 1992, Hughes and Reynolds 2005). This principle is at the core of the theory of ageing, which predicts that, as reproduction is resource demanding, current reproduction is traded-off against future reproduction and survival, caused by a reduced investment into somatic protection and maintenance (Williams 1966, Kirkwood and Rose 1991, Hughes and Reynolds 2005). However, recent studies have sometimes revealed patterns of actuarial (decline in survival rate with age) and reproductive (decline in reproductive success with age) senescence rather contrasted with this prediction (Jones et al. 2008, 2014, Rodríguez-Muñoz et al. 2019). Since individuals may differ in both resource acquisition and resource allocation between traits, depending on individual and environmental quality, the cost of reproduction can remain undetected at the population level (van Noordwijk and de Jong 1986, Bleu et al. 2016).

Studies that investigated cost of reproduction in terms of senescence mainly focused on females (Hamel et al. 2010, Lemaître et al. 2014). Those on males often referred to sexual selection theory and therefore on the cost of producing and maintaining sexual traits (Tidière et al. 2015). In males, cost of reproduction may result from resource demands for courtship, mating, struggling with female resistance, mate guarding, the production of sperm and accessory gland proteins (Walker 1980, Dewsbury 1982, Andersson 1994, Arnqvist and Rowe 2005). They may also engage into costly intra-sexual competition for females through pre- and post-copulatory contests with other males (Hosken 2001). In females, cost of reproduction may result from gamete production, offspring care, harassment by males, mating injuries, sexually transmitted diseases and damaging seminal substances (Stockley 1997, Jennions and Petrie 2000, Harshman and Zera 2007, Fortin et al. 2019). These differential costs may have contributed to the evolution of sexually dimorphic life-history strategies in many species through which males and females achieve maximal fitness. For instance, while males may maximize fitness by increasing mating success at the expense of longevity, females may maximize fitness through longevity because offspring production, although resource intensive, requires time too. The different reproductive costs may also contribute to different patterns of senescence between males and females, which may vary within and among populations, depending on their relative intensity. Strong investment into

reproduction early in life seems to contribute to accelerating reproductive and actuarial senescence (Lemaître et al. 2015). However, our understanding of the impacts of the costs of reproduction on senescence mainly relies on theoretical and correlative studies, whereas experimental investigations are still scarce.

Somatic protection partly depends on the immune system, whose competence may diminish with age. Such an immunosenescence causes enhanced sensitivity to infection and inflammatory diseases, increasing risk of morbidity and mortality with age (DeVeale et al. 2004, Shanley et al. 2009). Increased reproductive effort was found associated with enhanced susceptibility to parasitism and disease (Norris and Evans 2000) or decreased immune activity (Owens et al. 1999, Hosken 2001, Zera and Harshman 2001, Fedorka et al. 2004, Simmons and Roberts 2005). Trade-offs between reproductive and immune functions for limited resources, or negative pleiotropic effects of reproductive hormones on immune defence, have been proposed as proximate causes of the cost of reproduction (French et al. 2007). However, contradictory results are common as studies also failed to demonstrate such a cost (Drummond-Barbosa and Spradling 2001, Greenman et al. 2005, Schwenke et al. 2016). If investment into reproduction can induce a progressive decline in somatic functions, strong investment into early reproductive effort may generate accelerated immunosenescence and contribute to actuarial senescence.

Recent correlative evidence suggests that population structure, such as sex-ratio, affects individual reproductive effort with potential sex-specific consequences on senescence (Rodríguez-Muñoz et al. 2019, Tompkins and Anderson 2019). In particular, variation in sex-ratio is predicted to modulate the cost of mating, through the strength of sexual selection in males (Clutton-Brock and Parker 1992), influencing the putative trade-off between reproductive effort and somatic maintenance (Lemaître et al. 2014). Furthermore, cost of reproduction in females is also predicted to depend on population sex-ratio as it is expected to influence male competition for fertilization (Arnqvist and Rowe 2005). Hence, experimentally varying population sex-ratio appears to be a valuable tool to manipulate males and females reproductive effort and test its impacts on senescence at the population level.

Here, in a single experiment, we investigated the consequences of variable levels of investment in breeding effort on lifetime reproduction, survival and immunity of males and

females of the mealworm beetle, *Tenebrio molitor*, of which we have manipulated the sex-ratio in artificial populations. In this highly promiscuous insect, manipulating the sex-ratio of populations is expected to affect both the average intensity of intra sexual competition or sexual selection, and individual mating rate. In male-biased sex-ratio conditions, males should face fewer mating opportunities, whereas females should show high individual reproductive effort. By contrast, in female-biased sex-ratio condition, males should copulate more frequently, whereas females should have fewer opportunities to mate. This experimental design allowed us to test the cost of different key features of male and female reproduction in terms of senescence at the population level by examining their lifetime changes in survival, fertility, reproductive effort, body condition and immunity. Note, however, that manipulating sex-ratio may only affect the *opportunity* for sexual selection and not the *actual* sexual selection (Klug et al. 2010), and life-history particularities of biological models should be taken into account. For example, common wisdom is that male-biased sex-ratio conditions should accelerate male reduction of survival, reproduction and immunity because of intense pre- and post-copulatory intra sexual competition. However, in *T. molitor*, mating might be where the largest costs arise in reproduction for males (see below), and accelerated senescence is expected in populations with female-biased sex-ratio because males should produce higher reproductive effort. Indeed, direct observations of the mating behaviour of *T. molitor* suggest that males do not engage in costly physical contests to access females (Gage and Baker 1991, Carazo et al. 2007). Courtship and mating are relatively brief during which males transfer a spermatophore that does not release the sperm before 7-10 min post-copulation (Gadzama and Happ 1974). Males may then perform rather passive short post-copulatory mate guarding, consisting on staying within 1 cm of the female for more than 1 min in the presence of competitors (Carazo et al. 2007). However, males do not appear to have evolved specific post-copulatory mate-guarding behaviour like those observed in other insects (Drnevich 2003). The spermatophore transferred during copulation contains nutrient-rich substances that constitute a nuptial gift (Carver et al. 1999), whose cost may prevent males to copulate again for 20 min after the last copulation (Drnevich 2003). Hence, as mating is more costly than pre- and post-copulatory sexual competition, *T. molitor* males may best achieve fitness through longevity, just like females, which would ultimately prevent the evolution of divergent patterns of actuarial senescence between males and females. Females, for their part, may exhibit strong early reproductive effort in populations with male-biased sex-ratio, they also should exhibit accelerated decline in reproduction, and immunity or earlier immune dysregulation, correlating with reduced

survival with age. In populations with female-biased sex-ratio, females should survive, reproduce and maintain immunity at older age, as they might exhibit lower early life reproductive effort.

3. Methods

a. Mealworm beetles

Mealworm beetles are stored grain product pests that live several months in populations of variable density and at sex-ratio of about 50% ($\pm 20\%$). *T. molitor* males and females may initiate reproduction from the fifth day post emergence, although they reach their full sexual maturity from the eighth day post emergence. They can mate many times with several partners within their 2 to 5 months of adult life. Females are continuously receptive to mating during adulthood and may produce up to 30 eggs per day although egg production may decline after three weeks (Dick 1937). Although able to store sperm in their spermatheca, females need to mate frequently to maintain high egg production (Drnevich et al. 2001).

The immune system of *T. molitor* relies on both constitutive cellular (e.g. haemocytes) and enzymatic (e.g. prophenoloxidase system) components at the core of the inflammatory response (Vigneron et al. 2019). Their activity is cytotoxic (Nappi and Ottaviani 2000), causing self-damage (Sadd and Siva-Jothy 2006) and lifespan reduction (Pursall and Rolff 2011b, Daukšte et al. 2012, Krams et al. 2013, Khan et al. 2017). They were found to decrease after mating (Rolff and Siva-Jothy 2002) and either decline (Moret and Schmid-Hempel 2009) or increase (Khan et al. 2016) with age. In addition, the inducible production of antibacterial peptides in the haemolymph (Vigneron et al. 2019) is an energetically costly process that may reduce survival (Moret and Schmid-Hempel 2000). As selection on immune expression and immune regulation might be weaker after reproductive senescence, age-related decline of baseline levels of immunity might be observed and immune activation may occur at old age due to dysregulation (Licastro et al. 2005, Khan et al. 2016).

b. Artificial populations and experimental design

Virgin adult beetles of controlled age (10 ± 2 days post-emergence) were obtained from pupae haphazardly sampled from a stock culture maintained in laboratory conditions ($24 \pm 2^\circ\text{C}$, 70% RH in permanent darkness) at Dijon, France. Prior to the experiments, all these experimental insects were maintained separately in laboratory conditions, and supplied *ad libitum* with bran flour and water, supplemented by apple.

Fifteen artificial populations of 100 adult beetles were made according to three sex-ratio conditions. Five populations had a balanced sex-ratio, each comprising 50 males and 50 females (thereafter named the 50%_males condition), and were considered as the reference populations. Five populations had a male-biased sex-ratio, each comprising 75 males and 25 females (75%_males). Finally, five populations had a female-biased sex-ratio, each comprising 25 males and 75 females (25%_males). Each population was maintained in a plastic tank (L x 1 x H, 27 x 16.5 x 11.5 cm) containing bran flour, supplied once a week with apple and water. Every two weeks, each population was transferred into a clean tank supplied with fresh bran flour, thus avoiding the development of the progeny with the experimental adults.

c. Age specific reproductive assay

Reproductive capacity of females and males in each population was estimated weekly. To this purpose, 4 females haphazardly picked in each population were individually transferred into a plastic Petri dish (9 cm in diameter), containing bleached flour, a 2 mL centrifuge tube of water and a piece of apple. Each female was allowed to lay eggs in the Petri dish for 3 days, and was then returned to their initial population box. Two weeks later, the number of larvae was counted in each Petri dish to quantify female fertility, which is the number of viable larvae produced per female (Shenk 2015).

Concomitantly, four males haphazardly picked in each population were also individually transferred into Petri dishes, as above. Reproductive success of males was estimated through direct measures of their fertility (number of viable offspring per male (Shenk 2015)) instead of measuring spermatophores or counting the sperm, which are rough surrogates of male reproductive success. Each male was provided with a virgin female aged from 8 to 15 days for 24 hours and was then returned in its initial population. Each female was then allowed to lay eggs in the Petri dish for three additional days to estimate, as described above, male fertility. In *T. molitor*, males may affect female fecundity (number of potential eggs produced by the female) and therefore their fertility, according to the respective quality of spermatophores and sperm transferred during mating. Consequently, male's success was a measure of the *potential* reproductive effort, not the one realized within its experimental population.

While assayed for their reproduction, focal females and males were replaced by marked individuals of the same age and sex in all the populations, to keep sex-ratio and density constant. Substitutes were from the same cohort as the experimental insects, kept in a separate tank of mixed-sex population. They were marked by clipping a piece of one elytra. When focal insects assayed for their reproduction were returned into their initial population box, substitutes were removed and returned into their tank.

d. Estimation of male and female reproductive effort at the population level

Survival of the insects was checked weekly, and dead insects were replaced by marked substitutes of the same sex and about the same age to keep the population sex-ratio and density of individuals constant. No measurement was performed on these marked individuals.

As the experimental design does not allow gathering measurements of longevity and fertility for each individual of the population, we estimated male and female reproductive effort (RE) at the population level, from the above age-specific measures of fertility, for each of the five population replicates, within sex-ratio conditions. This estimate was calculated as the total number of viable larvae produced per female or male in each replicate (i.e. the cumulative number of larvae produced during the whole experiment in a given replicate divided by the number of females or males tested for this replicate), divided by their respective average lifespan in the population replicate (i.e. the average lifetime of females or males in each replicate). The equation is given as follow:

$$RE_r = \frac{l}{ML}$$

Where l is the total number of offspring (here viable larvae) produced per assayed females or males in the population replicate r , and ML is the recorded mean lifespan (in weeks) of males and females in the replicate r . RE values (as offspring per individuals and per mean weeks of survival in the population) of each sex and population replicate within each sex-ratio condition were used as data points for comparisons between modalities of sex-ratio.

Note that female RE values are likely representative of both female and male conditions resulting from the experiment, because the female reproductive performance resulted from mating with males from their respective population. By contrast, male RE values are representative of the male condition only, because male reproductive performance was standardized by pairing it with a virgin and age-controlled female that did not experience the experimental conditions. Therefore, male RE must be seen as a surrogate of male reproduction potential.

e. Body condition and haemolymph collection

At weeks 2, 4, 6 and 12 after the start of the experiment, 4 females and 4 males were picked at random in each population to estimate their body condition and immunity. The first three time points were chosen as being relevant of the time period during which most of the beetle reproduction is achieved and survival is still relatively high (Drnevich et al. 2001). It is also within this period of time that a potential decline in somatic protection, including immunity, is predicted. The last time point corresponds to a period of time when reproduction should have almost ceased and when few beetles remain alive. As immunity measurements was destructive sampling, sampled insects were replaced by marked substitutes as above, to keep sex-ratio and density constant. However, this substitution was definitive, as sampled individuals were not returned to their initial population box after being assayed. The below estimation of the insect body condition and immunity was done as described in (Moret 2006). Beetles were first sized by measuring the length of the right elytra with Mitutoyo digital callipers (precision 0.1 mm) and weighed to the nearest mg with an OHAUS balance (discovery series, DU114C). Body condition was then estimated by the residuals of the regression between body size and body mass. Then, beetles were chilled on ice for 10 min before the sampling of 5 μ L of haemolymph from a wound made in the beetle's neck and flushed in a microcentrifuge tube containing 25 μ L of phosphate-buffered saline (PBS 10mM, pH 7.4). A 10- μ L aliquot was immediately used to measure haemocyte count. Another 5- μ L aliquot was kept in an N-phenylthiourea-coated microcentrifuge tube (P7629, Sigma-Aldrich, St Louis, MO, USA) and stored at -80 °C for later examination of its antibacterial activity. The remaining haemolymph solution (15 μ L) was further diluted in 15 μ L of PBS and stored at -80 °C for later measurement of its phenoloxidase activity.

f. Immune parameters

Haemocyte count was measured using a Neubauer improved haemocytometer under a phase-contrast microscope (magnification x 400).

Antimicrobial activity of the haemolymph was measured using the inhibition zone assay described in (Moret 2006). Briefly, an overnight culture of the bacterium *Arthrobacter globiformis* from the Pasteur Institute (CIP105365) was added to a Broth medium containing 1 % agar to achieve a final concentration of 10^5 cells.mL⁻¹. Six millilitres of the medium was subsequently poured per Petri dish and, after solidification, 12 wells were made inside the agar plate in which 2 μ L of each haemolymph sample was deposited. Plates were then incubated overnight at 28 °C and the diameter of each zone of inhibition was measured.

For each haemolymph sample, both (i) the activity of naturally activated phenoloxidase (PO) enzyme only (hereafter PO activity) and (ii) the activity of PO plus that of proenzymes (proPO) (hereafter Total-PO activity) were measured using the spectrophotometric assay described in (Zanchi et al. 2011). Total-PO activity quantification required the activation of proPO into PO with chymotrypsin, whereas PO activity was measured directly from the sample. Frozen haemolymph samples were thawed on ice and centrifuged (3,500 g, 5 min, 4 °C). In a 96-well plate, 5 μ L of supernatant were diluted in 20 μ L of PBS and were added either 140 μ L of distilled water to measure PO activity or 140 μ L of 0.07 mg. mL⁻¹ chymotrypsin solution (Sigma-Aldrich, St Louis, MO, USA, C-7762) to measure Total-PO activity. Subsequently, 20 μ L of a 4 mg.mL⁻¹ L-Dopa solution (Sigma-Aldrich, St Louis, MO, USA, D-9628) were added to each well. The reaction proceeded for 40 min at 30 °C, in a microplate reader (Versamax, Molecular Devices, Sunnyval, CA, USA). Reads were taken every 15 s at 490 nm and analysed using the software SOFT-Max®Pro 4.0 (Molecular Devices, Sunnyvale, CA, USA). Enzymatic 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 μ L of pure haemolymph.

g. Statistics

Cox-regressions with a time-dependent covariate were used to analyse the difference in survival rates with respect to sex-ratio during the time (in weeks) from the start of the experiment and the death of all individuals. Sex-ratio was coded as categorical variables. The effect of sex ratio in the statistical model used the reference survival function generated from the data derived from the females or the males of the 50%-male sex-ratio condition.

Time (in weeks) was incremented as a covariate in interaction with sex-ratio in the model as hazard ratios when the survival functions were not constant over time (for more details, see (Norušis 2012)). The analyses of fertility (i.e. the number of larvae produced per female or male at each week) and immune parameters were performed using mixed models, either Linear or Generalized linear depending on the nature of the data (see table legends). Starting models included sex-ratio condition, week (continuous variable for fertility, ordinal variable for immunity), their interaction, body condition and replicates treated as a random factor (REML estimates of variance component). The models presented here are those minimizing the AICc, where $\Delta AICc > 2$ is usually considered to be good support (Burnham and Anderson 2004), after comparisons of all models including predictors and their interactions, in a stepwise fashion (see Table S1). The analyses of reproductive effort were made using ANOVA testing the effect of sex-ratio conditions. Analyses were made using IBM® SPSS® Statistics 19, JMP v. 10.0 and R version 3.3.2 (The R Foundation for Statistical Computing, Vienna, Austria, <http://www.r-project.org>).

4. Results

a. Demography: survival, fertility and reproductive effort

A first survival analysis comparing males and females of the 50%-male sex-ratio condition, which presumably corresponds to the sex-ratio condition in natural populations of *T. molitor*, showed no difference in longevity between males and females (Cox regression: Wald statistics = 0.004, d.f. = 1, $p = 0.947$, Figure 2). Survival of females and males was significantly affected by the sex-ratio condition (Table 1, Figure 3). In the 75%-male sex-ratio condition, females exhibited an accelerated mortality with time by a factor of 13 % per week compared to females of the 25% and 50%-male sex-ratio conditions (see odd ratio of Sex-ratio*Time-Cov in Table 1a). There was no significant difference in survival between females in the 25% and 50%-male sex-ratio conditions (Table 1a, Figure 3a). Contrasting with females, males in the 75%-male sex-ratio condition survived significantly longer than those in the 25% and 50%-male sex-ratio conditions (by 53% and 50%, respectively, see odd ratio in Table 1b, Figure 3b).

Whereas female fertility decreased with time, this pattern was dependent on the sex-ratio condition (Table 2a, Figure 4a, 5a). Indeed, female fertility in the 75% and 25%-male sex-ratio conditions was lower than that in the 50%-male sex-ratio condition during the first

two weeks, and became subsequently higher (Figure 5a). Male fertility decreased with time in all sex-ratio conditions, with no significant effect of sex-ratio condition (Table 2b, Figure 4b, 5b). As expected for both sexes, heavier females produced more larvae than lighter ones (Table 2).

Female's RE differed among sex-ratio conditions ($F_{2, 12} = 8.06$ $p = 0.006$) and was significantly higher in the 75%-male than in the 25%-male sex-ratio condition (Figure 6). Female RE in the 50%-male sex-ratio condition showed an intermediate value (Figure 6). Male RE significantly differed among sex-ratio conditions ($F_{2, 12} = 4.63$ $p = 0.032$). Male RE in the 75%-male sex-ratio condition was significantly lower than in the 25%-male sex-ratio condition (Figure 6). Like for females, male's RE from the balanced sex-ratio condition showed an intermediate value, which was not significantly different from the two other sex-ratio conditions (Figure 6).

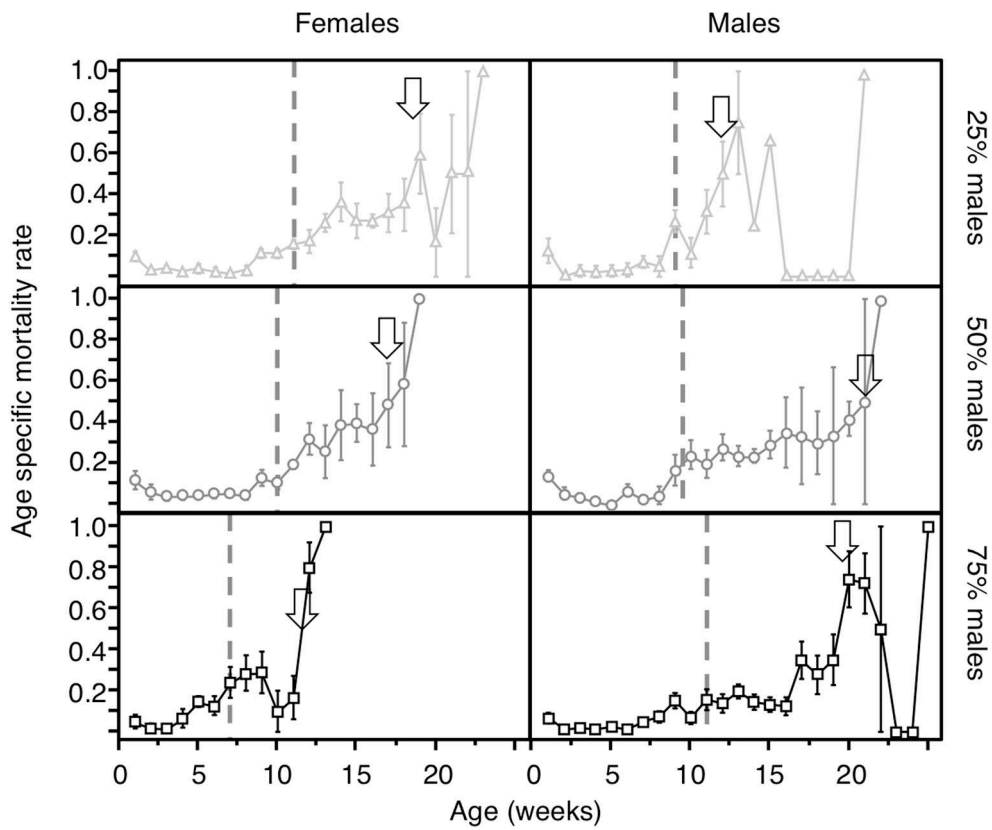


Figure 2 Male and female age specific mortality rate according to time for each sex-ratio condition. Arrow marks the time when the values of 50% of mortality rate is reach for the first time and dote line marks 50% of the population is dead. Values are means among replicates \pm s. e. m.

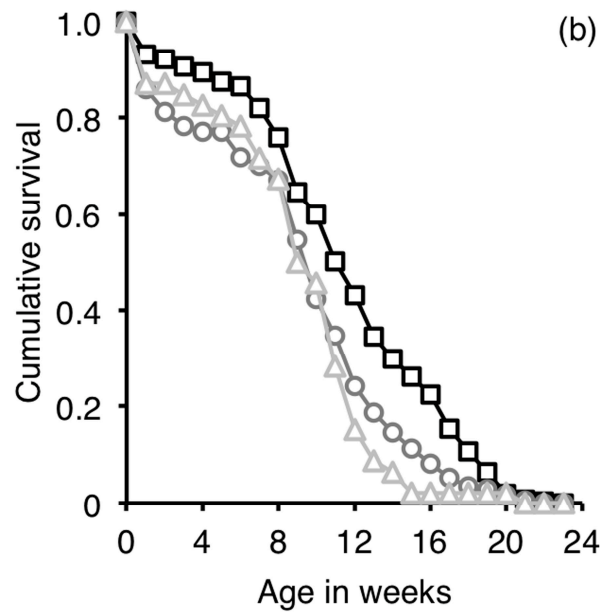
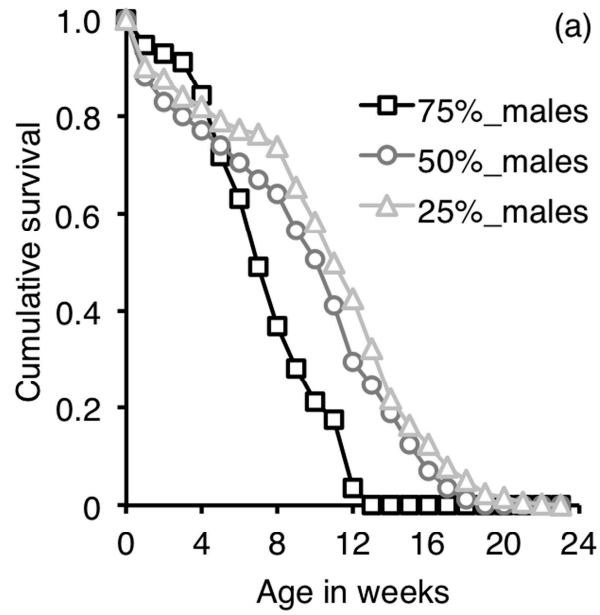


Figure 3 : Age-specific survival according to sex-ratio condition. **(a)** Females; **(b)** Males.

Table 1 : Survival of adult females (a) and males (b) of *Tenebrio molitor* according to sex-ratio condition (Sex-ratio). The “simple” contrast was used for Sex-ratio (survival of males in the 50 % of male condition was used as baseline). For females (a), a time-dependent procedure was used to account for the time-dependent effect of Sex-ratio on the risk of mortality ($T \times \text{Sex-ratio}$). This procedure was not necessary for males as the effect of Sex-ratio on the risk of mortality was constant over time (b).

A	Variables	b^a	s.e. ^b	Wald ^c	d.f.	p^d	Odds ^e
	Sex-ratio			1.61	2	0.744	
	$T \times \text{Sex-ratio}$			9.77	2	0.008	
	75% of males ^g vs 50% of males	0.12	0.04	9.28	1	0.002	1.13
	25% of males ^g vs 50% of males	0.00	0.02	0.05	1	0.831	1.00
	75% of males ^h vs 25% of males	0.12	0.04	9.03	1	0.003	1.12
B	Variables	b	s.e.	Wald	d.f.	p	Odds ratio
	Sex-ratio			21.34	2	< 0.001	
	75% of males vs 50% of males	-0.38	0.10	15.36	1	< 0.001	0.70
	25% of males vs 50% of males	0.15	0.17	0.82	1	0.366	1.16
	75% of males vs 25% of males	-0.53	0.16	10.98	1	0.001	0.59

^a b = regression coefficient of overall survival function for variables.

^b Standard error of regression coefficient.

^c Wald statistic for variable.

^d Significant level for Wald statistic. Values $p \leq 0.005$ are given in bold.

^e Odds ratio of survival for variable relative to control ($=\exp(b)$).

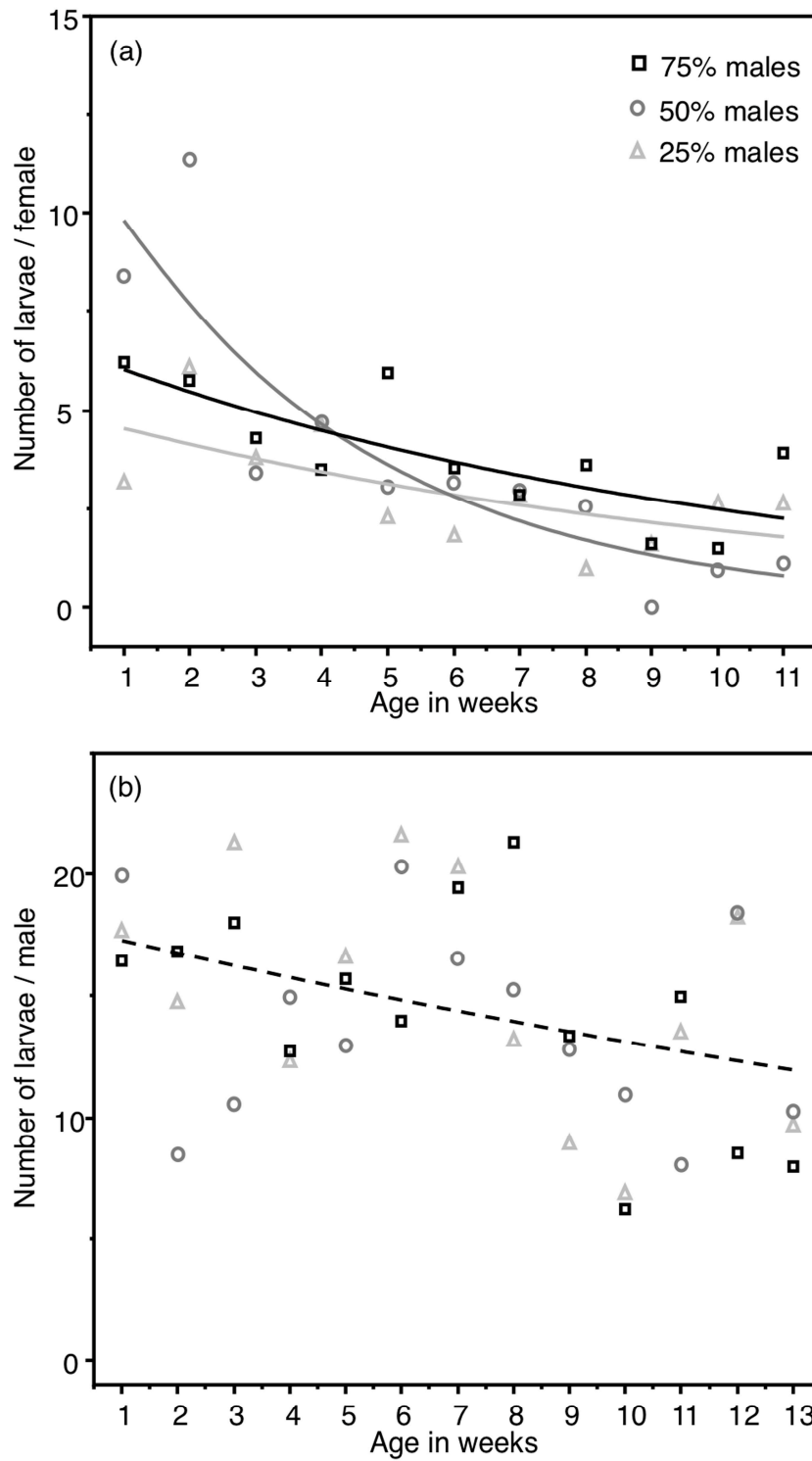


Figure 4 Age-specific fertility of females (a) and males (b) according to sex-ratio condition. (a) The tested females were those coming from the experimental tanks. (b) The tested females were virgin females mated with males coming from the experimental tanks. Dots are the means (for variation around the means see supplementary Fig. S2, Additional File 1) and lines are the predictions of the models.

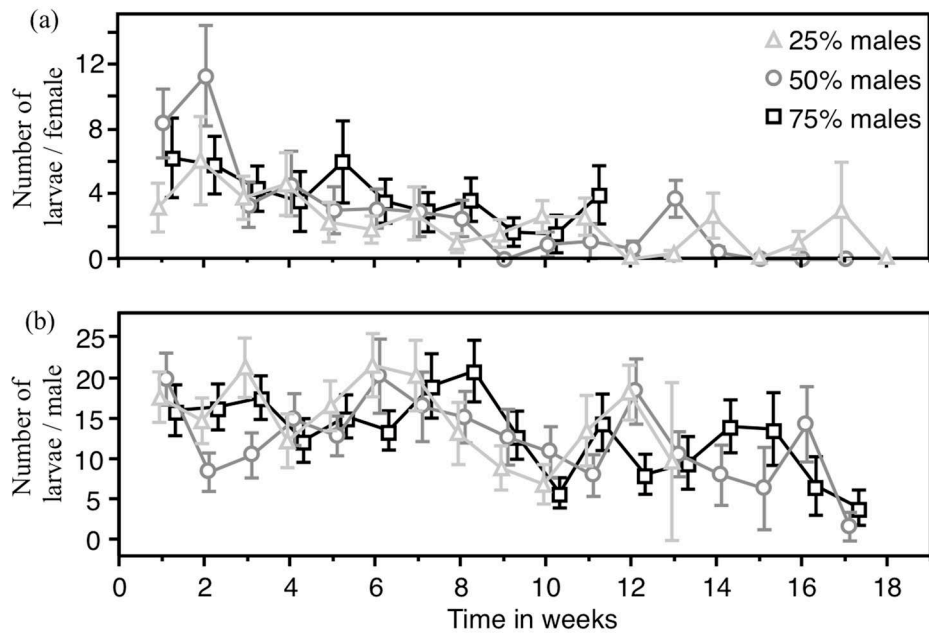


Figure 5: Details of variation in fertility in females (a) and males (b). Values are means among replicates \pm s. e. m

Table 2 Fertility: generalized linear mixt models (GLMM, Poisson distribution, Log link function) analysing the factors influencing the number of larvae produced by females (a), (n = 638) and males (b), (n = 737). Initial models included Sex-ratio condition, Age (in weeks as a continuous variable), their interaction, male and female body mass (Mass in mg) and replicates as a random factor. The models shown here are those minimizing the AICc, with $\delta AICc > 2$. Age was allowed to vary up to 11 and 13 weeks in females and males, respectively. Beyond these values no insects could be assayed anymore in some sex-ratio conditions (see supplementary Fig. S2, Additional File 1). Values where $p \leq 0.05$ are given in bold.

A				B			
Sources	<i>d.f.</i>	χ^2	<i>p</i>	Sources	<i>d.f.</i>	χ^2	<i>p</i>
Female mass	1	82.755	<0.001	Female mass	1	243.13	<0.001
Age	1	388.9	<0.001	Age	1	94.819	<0.001
Sex-ratio	2	0.7232	0.277				
Sex-ratio* Age	2	100.44	<0.001				

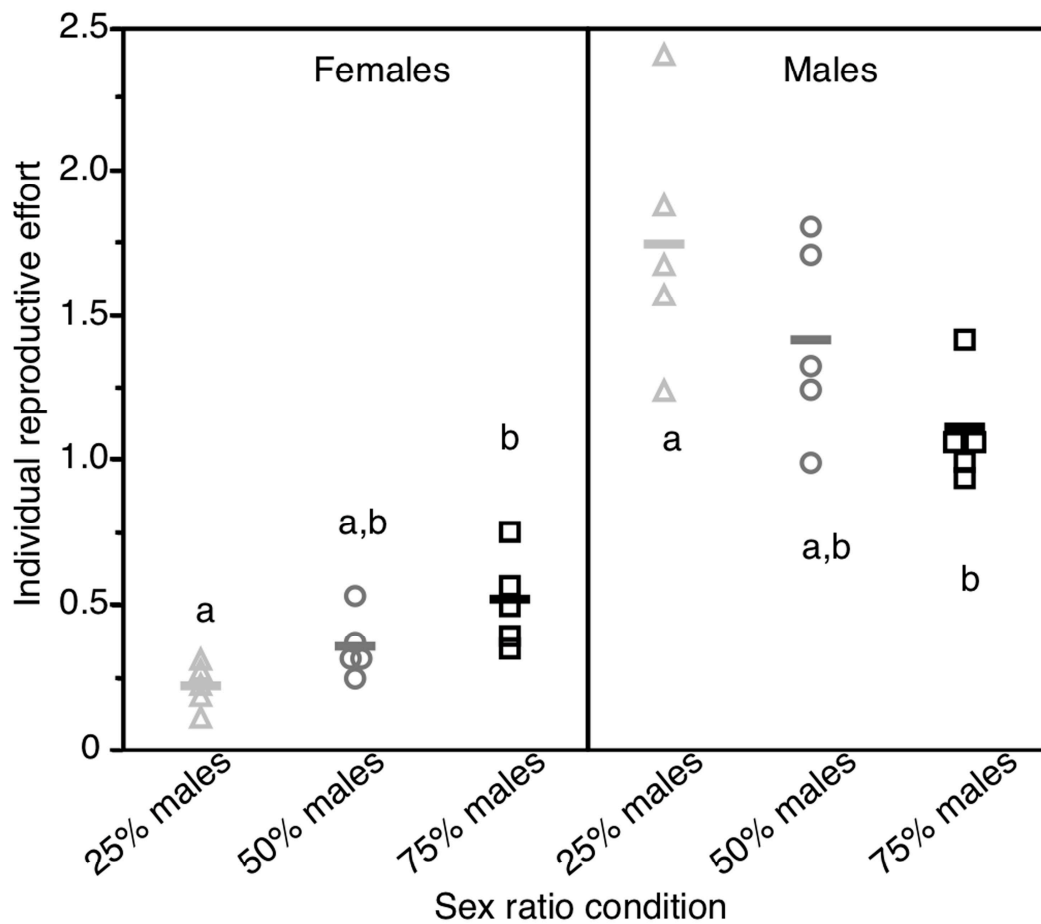


Figure 6 Estimated mean reproductive effort. Reproductive effort (RE - mean number of viable offspring produced per individual and per week of survival in the population) of females (left panel) and males (right panel) according to sex-ratio condition. Lines are means; dots are values of single replicates. Values surrounded by different letters were significantly different after Tukey-Kramer HSD post-hoc test ($\alpha = 0.05$).

b. Body condition and immunity

Male and female body condition, estimated by the residuals of the regression between body size and body mass, exhibited a similar decline with age, which was not affected by the sex-ratio condition (Table 3a). In females, immunological parameters were never affected by the sex-ratio condition (Table 3b-f). Both PO activity and Total-PO activity changed with age with lowest values at week 6 (Figure 7a), and were positively influenced by body condition (Table 3b, c). By contrast, anti-bacterial activity of the haemolymph increased with age (Table 3e, Figure 7b). Haemocyte counts of females only differed among population replicates (Table 3D). As opposed to females, some of the immunological parameters of males were affected by the sex-ratio condition (Table 3). Male PO activity was influenced by the interaction between time and sex-ratio (Table 3b). While PO activity of males in the 50%-male sex-ratio condition decreased during the first six weeks, PO activity of males in the other two sex-ratio conditions increased between week 2 and 4. In all sex-ratio conditions, PO activity increased again at week 12 (Figure 7c). In addition, PO activity of males in the 25%-male sex-ratio condition was overall lower than PO activity of males in the other sex-ratio conditions (Figure 7c). Total-PO activity only differed between population replicates (Table 3c). As in females, antibacterial activity in the haemolymph of males increased with age (Table 3e, Figure 7d). However, the size of the zones of inhibition of males exhibiting positive antibacterial activity (reflecting the intensity of this activity) was higher for males in the 50%-male sex-ratio condition than for males in the other sex-ratio conditions (Table 3f, Figure 7e). Finally, haemocyte counts of males in all sex-ratio conditions varied with time, mainly because of its high value at week 6 (Table 3d, Figure 7f).

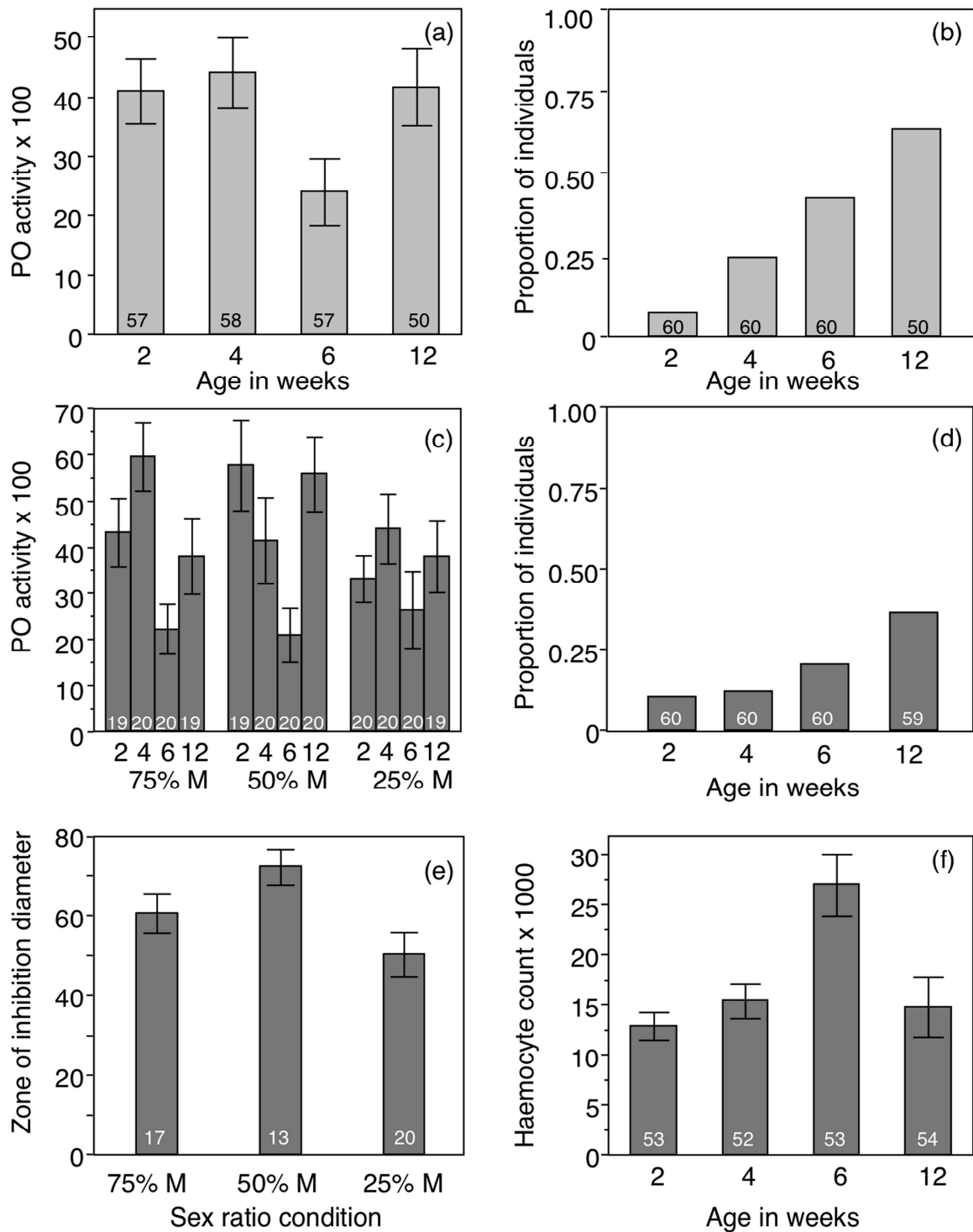


Figure 7 Immune parameters in females and males according to individual age and/or sex-ratio condition. **a** females PO activity; **b** proportion of females exhibiting antibacterial activity; **c** male PO activity; **d** proportion of males exhibiting antibacterial activity; **e** male intensity of antibacterial activity as the size (in mm) of zones of inhibition; **f** male haemocyte count. Values are means among replicates \pm s. e. m. Number in the bars are sample size.

Table 3 Body condition and immune parameters. Mixed linear models or generalized linear model analysing the factors influencing body condition (a), PO activity (b), Total PO activity (c), haemocyte count (d), the proportion of beetles exhibiting antibacterial activity in their haemolymph (e) and the intensity of this antibacterial activity as the size of the zone of inhibition (f) in both females (left) and males (right). Models included sex-ratio condition, Age in weeks (ordinal variable), their interaction, body condition, and replicates as a random factor. The models presented here are those minimizing the AICc, with $\Delta AICc > 2$. Values where $p \leq 0.05$ are given in bold. # Linear model, § GLM, distribution: binomial, link function: Logit, ¹ because a low number of animals showed antimicrobial activity in some conditions (Fig. 4b, 4d), data were not available for some replicates, therefore replicates were omitted for these models.

	Females				Males			
	<i>d.f.</i>	<i>d.f.</i> <i>den.</i>	<i>F</i> or χ^2	<i>P</i>	<i>d.f.</i>	<i>d.f.</i> <i>den.</i>	<i>F</i> or χ^2	<i>P</i>
A Body condition#								
Age	3	207.9	17.14	<0.001	3	215.1	6.64	<0.001
Sex-ratio	2	98.2	0.95	0.39	2	89.7	0.57	0.565
Age*Sex-ratio	6	207.6	0.72	0.637	6	215.1	0.23	0.964
B PO activity#								
Age	3	199.8	6.76	<0.001	3	211.9	8.20	<0.001
Sex-ratio	2	86.9	0.14	0.869	2	103.3	3.69	0.028
Age*Sex-ratio	6	198.3	0.75	0.606	6	211.3	2.21	0.043
Body condition	1	208.9	6.82	<0.001	1	222.9	2.25	0.135
C Total-PO activity#								
Age	3	189	10.90	<0.001	3	201.8	2.49	0.061
Sex-ratio	2	189	0.21	0.807	2	65.7	1.91	0.155
Age*Sex-ratio	6	189	0.40	0.877	6	201.2	1.25	0.282
Body condition	1	189	6.10	0.014	1	210.8	0.83	0.364
D Haemocyte#								
Age	3	193.8	1.28	0.281	3	208	6.90	<0.001
Sex ratio	2	11.4	0.04	0.963				
E Antibacterial activity (proportion)§								
Age	3		42.89	<0.001	3		12.879	<0.001
Body condition	1		1.1902	0.275	1		10.595	0.157
Age					3		14.391	0.002
Sex-ratio					2		2.2331	0.327
F Antibacterial activity (intensity)#¹								
Age	3		2.27	0.088				
Sex-ratio					2		4.27	0.019

5. Discussion

By manipulating the sex-ratio of artificial populations of mealworm beetles, *Tenebrio molitor*, we successfully affected the reproductive effort of both males and females. Note that for males, our estimations are rather relevant of their reproductive potential effort or maximal reproductive effort because they were tested using young virgin females. As predicted, female biased sex-ratio led females to exhibit a relatively low reproductive effort, whereas males' reproductive potential was the highest. By contrast, male biased sex-ratio increased the reproductive effort of females while that of males dropped. Males and females from populations with balanced sex-ratio exhibited intermediate reproductive effort. Interestingly, males and females of the 50% sex-ratio condition had similar longevity. This absence of divergent patterns of actuarial senescence between males and females may result from a relatively strong investment of males into mating activity rather than into sexual competition. Therefore, like in females, longevity appears to be an important criterion to maximize fitness in males of *T. molitor*.

While varying in their reproductive effort according to sex-ratio condition, females exhibited different patterns of actuarial and reproductive senescence. Females in the 75%-male sex-ratio condition (with the higher reproductive effort) suffered from an increased mortality compared to females in the other conditions. While we did not directly observed the mating behaviour in our experiments, frequent mating events and harassments by males may explain this accelerated mortality and is in line with previous observations from other insect species (Tatar et al. 1993, Adler and Bonduriansky 2011, Archer et al. 2012, Rodríguez-Muñoz et al. 2019).

Female fertility with time in the 50% sex-ratio condition contrasted to that of females in the other two sex-ratio conditions. They produced many offspring during the first two weeks of their adult life, then fewer to become almost null at 8 weeks onward. As previously reported in both vertebrates and invertebrates (Creighton et al. 2009, Lemaître et al. 2015), intense early reproductive activity is associated to earlier reproductive decline. Females in the other sex-ratio conditions (25% and 75% of males) produced relatively fewer offspring when young adults but kept this reproductive effort when becoming older. In the 25% sex-ratio condition, the low proportion of males may have constrained female access to mating, preventing them to reach their maximal early reproductive potential. Such a low early

reproductive investment, possibly accompanied by low male harassment, may have preserved female late reproduction. In the 75% male sex-ratio condition, high proportion of males was expected to increase interactions between males as well as enhancing mate guarding (Carazo et al. 2007). This may also have prevented young females to have optimal access to mating, while compensating by a higher probability of mating as they aged. However, female reproduction in this male-biased sex-ratio condition stopped earlier than in the others, because their survival had rapidly declined. Altogether, the data suggest that the reproductive effort of females in the 75% male sex-ratio condition was more costly than that of females in the other sex-ratio conditions, constraining them to trade-off their longevity against their reproduction.

Despite a more costly reproductive effort, females in the 75%-male sex-ratio condition did not exhibit any further functional decline compare to females in the other sex-ratio conditions. While body condition declined with female age, such a decline was similar in all sex-ratio conditions. Similarly, changes in female immune activity were never influenced by the sex-ratio. While haemocyte counts remained constant with female age, antibacterial activity increased. Similar results were reported in the bumblebee, *Bombus terrestris* (Moret and Schmid-Hempel 2009). With age, the probability of having been exposed to microbes increases. This may explain the higher proportion of older individuals exhibiting induced antibacterial activity in their haemolymph, as insects can produce prophylactic long lasting antibacterial responses after a single bacterial challenge (Haine et al. 2008a, Dhinaut et al. 2018a). PO activity declined at week 6 in females, which is consistent with the beginning of senescence, when reproduction started to end but survival is still relatively high (Fig. 1, see supplementary Fig. S2, Additional File 1). PO activity increased again at week 12, among the rare surviving individuals (Fig. 1). This higher late PO-activity may have two non-exclusive explanations. On the one hand, it may result from selection where individuals with the best somatic protection, involving high PO-activity, survived longer than those having poorer ones. On the other hand, high levels of PO activity at week 12 could also result from a deregulation of the host inflammatory response (Scherfer et al. 2008, Labaude et al. 2017). The impact of female reproductive effort on immunity seems limited, at least on the constitutive base levels of the immune parameters we have measured. We cannot exclude that female ability to produce an immune response upon challenge or others non-measured immune parameters could be affected. Nonetheless, our results show that increasing the

reproductive effort of *T. molitor* females affected demographic senescence, mainly through longevity reduction, but with apparently limited effect on immune senescence.

Changes in the reproductive effort (reproductive potential) of males through the manipulation of the sex-ratio also affected their survival. It is often assumed that most of the cost of reproduction in males involves sexual pre-copulatory competition. Thus males in the 75%-male sex-ratio condition could have been expected to engage in strong and costly intra-sexual competition for females, resulting in low reproductive success and shorter longevity compared to the other sex-ratio conditions, as previously shown in vertebrates (Promislow 1992, Clutton-Brock and Isvaran 2007, Lemaître et al. 2014) and invertebrates (Rodríguez-Muñoz et al. 2019). However, although male fertility slightly declined with age, it was not affected by the experimental sex-ratio condition, suggesting that males exhibited similar patterns of reproductive senescence, independently of their reproductive effort. Male reproductive senescence might also be revealed by the production of lower quality offspring with age (Carazo et al. 2011), which was not tested in this study. In addition, males from the 75%-sex-ratio condition showed longer longevity. This phenomenon may have two main explanations, consistent with predictions linked to the peculiar mating behaviour of *T. molitor*. On the one hand, competition for females in that sex-ratio condition was not very strong or costly. Under high risk of sexual competition, male reproductive success may depend on their investment into pre-copulatory (e.g., courtship and aggressive behaviours with other males) and/or post-copulatory (e.g., mate guarding) behaviours to limit sperm competition (Font and Desfilis 2003). So far, *T. molitor* males were never reported to engage in physical contest either before or after copulation (Carazo et al. 2007) and current evidence suggests that male-male competition is unlikely to bear strong costs (Happ 1969, Drnevich et al. 2000, Drnevich 2003, Carazo et al. 2007). Our experimental design nevertheless did not allowed us to verify these assumptions. On the other hand, each mating event is costly for *T. molitor* males, because nutrient-rich spermatophores are transferred to females in addition to the sperm (Carver et al. 1999). Since, on average, males in the 75%-male sex-ratio condition may have copulated less frequently than males in the other sex-ratio conditions, they may have saved resources that contributed to their longer survival. By contrast, males from the 25%-male sex-ratio condition likely performed more mating events than males in the other sex-ratio conditions, but this was apparently not costly enough to significantly impair their longevity compared to the 50%-male sex-ratio condition.

Our results suggest that males in this 25%-male sex-ratio condition had paid a functional cost for their higher reproductive effort, especially in terms of immunity. Indeed, males in the 25%-male sex-ratio condition showed a reduced immune activity possibly resulting from their higher reproductive effort. First, males in the 25%-male sex-ratio condition had reduced PO activity despite having a similar concentration of total phenoloxidase enzymes in their haemolymph than males of the other sex-ratio conditions. This lower PO activity was constant over time and contrasted with that of males of the two other sex-ratio conditions, for which the temporal pattern of PO activity resembled that of females (high levels in early weeks, decline in week 6, and re-increase in week 12). Since mating activity is known to transiently reduce PO activity in *T. molitor* (Rolff and Siva-Jothy 2002), such a down regulation of the PO activity in males of the 25%-male sex-ratio condition might be reflecting their higher mating activities. Higher secretion of juvenile hormone might be involved in mediating mating-induced PO activity depression (Rolff and Siva-Jothy 2002), which could contribute to reduce longevity (Herman and Tatar 2001). Juvenile hormone also prevents the release of cytotoxic substances by active PO enzymes that could reduce insect longevity by self-damaging host tissues and organs (Sadd and Siva-Jothy 2006, Daukšte et al. 2012, Krams et al. 2013, Zhao et al. 2011, Khan et al. 2017). These combined effects may have contributed to the observed absence of difference between the survival of males in the 25% and the 50%-male sex-ratio conditions. Second, as observed for females, the proportion of males exhibiting positive antibacterial activity in their haemolymph increased with age in all the sex-ratio conditions. As stated earlier, this was expected as the probability of having being challenged by opportunistic microbes increases with age. However, the size of the zones of inhibition observed from the haemolymph of males in the 25%-male sex-ratio condition was significantly smaller than that of males in the other sex-ratio conditions, suggesting that males in the 25%-male sex-ratio condition produced less antibacterial peptides than the other males. As mating activity, through the production and transfer of spermatophores, is particularly resource-demanding for males in terms of protein content (Carver et al. 1999), the higher mating activity of males in the 25%-male sex-ratio condition could have depleted the necessary protein resource to produce as much antibacterial peptides as in the other sex-ratio conditions. Mating may mediate such a trade-off through juvenile hormone secretion, which functions to switch on physiological processes associated with gametogenesis and spermatophore production (Wigglesworth 1972).

6. Conclusions

Manipulating sex-ratio of artificial populations of *T. molitor* had important impacts on reproductive effort of females and males, but resulted in contrasting sex-specific trade-offs on demographic and immune traits. Increasing female reproductive effort did not affect immunity but strongly reduced longevity. Not surprisingly, females may then maximize fitness by moderate early investment into reproduction and longevity. While decreasing male reproductive effort enhanced longevity, increasing it impairs immunity. Males may therefore favour reproduction at the expense of their immunity when given the opportunity to increase their reproductive effort. This is in line with the Bateman's principle applied to immunity, where males gain fitness by increasing reproductive effort at the expense of immunity (Rolff 2002). It is also consistent with the disposable soma theory of ageing, as reproduction compromises somatic protection (Williams 1966, Kirkwood and Rose 1991). Nevertheless, our results also suggest that sexual competition in *T. molitor* is not a strong modulator of the male reproductive strategy towards early mating opportunities (Bonduriansky et al. 2008). Basically, like in females, most of the cost of reproduction in males results from multiple copulations. This thus contrasts with the hypothesis that males should gain fitness by increasing mating success by investing in sexual competition at the expense of longevity (Vinogradov 1998, Carranza and Pérez-Barbería 2007). Since longevity is a key life history trait for both males and females of *T. molitor*, sex-specific patterns of actuarial senescence are not expected to evolve in this species. Accordingly, males and females showed similar patterns of survival with age in populations with balanced sex-ratio. Our results may further indirectly suggest that divergent actuarial senescence between males and females should evolve in species in which males strongly invest into sexual competition (Lemaître et al. 2014, 2015).

List of abbreviations

AICc: corrected Akaike Information Criterion; ANOVA: Analysis of Variance; *l*: number of larvae; ML: Mean Lifespan; PBS: Phosphate Buffer Saline; PO: Phenoloxidase; ProPO: ProPhenoloxidase; *r*: Replicate; RE: Reproductive Effort; REML: REstricted Maximum Likelihood.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets generated and analysed during the current study will be made available in the Dryad repository upon acceptance. All data files will be available from the Dryad Digital database at <https://doi.org/10.5061/dryad.cvdncjt11>.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

CJ, TR and YM conceived the ideas and designed methodology, CJ, MC and YM collected the data, CJ, TR and YM analysed the data, CJ, TR and YM led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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Chapitre 2

Chapitre 2 : stratégies d'investissement à la reproduction des femelles *Tenebrio molitor* : investissement terminal ou reproduction restreinte

Les résultats du chapitre 1 sont une première étape dans la compréhension des liens entre investissements dans la reproduction, l'immunité et la longévité chez *T. molitor*. Afin de les compléter nous avons réalisé cette fois une étude de suivi individuel de femelles de *T. molitor* afin d'appréhender plus précisément les conséquences de la reproduction sur la sénescence.

De plus, cette expérience permet de tester la stratégie d'investissement à la reproduction des femelles de *T. molitor* selon deux hypothèses distinctes. La première hypothèse est celle de « l'investissement terminal » qui propose que les femelles devraient investir fortement à la reproduction en fin de vie, et cela aux dépens de la longévité et des défenses somatiques (Clutton-Brock 1984). La deuxième hypothèse est celle de la « reproduction restreinte » qui propose que les femelles devraient moduler leur effort de reproduction au cours de la vie de manière à maintenir une forte longévité et assurer plus d'opportunités de reproductions futures (McNamara et al. 2009). Cette dernière stratégie devrait impliquer une reproduction modérée en fin de vie associée à un maintien des défenses somatiques. Un examen des relations potentielles entre fécondité tardive, survie et immunocompétence post-reproduction devrait permettre de trancher entre ces deux hypothèses.

Pour ce faire, des femelles ont été amenées à effectuer deux épisodes de reproduction facultatifs au cours de leur vie : un épisode de reproduction précoce (entre 10 et 30 jours post-émergence) et un épisode de reproduction tardif (entre 40 et 60 jours post-émergence). Les femelles ont été réparties selon quatre modalités d'investissement à la reproduction précoce ou/et tardive. La fécondité, l'immunité et la longévité de ces femelles ont été mesurées.

Cette expérience nous a permis de montrer que les femelles de *T. molitor* expriment une sénescence reproductive intrinsèque car leur fécondité est grandement diminuée avec l'âge, même si elles ne se sont pas reproduites tôt dans la vie. Nous avons aussi pu mettre en évidence un coût de la reproduction précoce sur la reproduction tardive car les femelles qui se sont reproduites tôt dans la vie pondent moins d'œufs au cours de leur reproduction tardive. Le fait de se reproduire tôt ou/et tard dans la vie n'a pas eu d'incidence sur la longévité.

En outre, si nous avons observé que les femelles âgées ont une plus faible capacité à survivre à une infection bactérienne que les femelles jeunes (suggérant l'existence d'une immunosénescence), la reproduction elle n'a pas d'effet sur la survie à l'infection.

Enfin, un résultat important de cette étude est que la fécondité tardive des femelles est associée positivement à leur durée de vie post-reproduction tardive. Les femelles qui se reproduisent le moins tardivement sont aussi celles qui meurent le plus tôt. Par ailleurs, si nous avons trouvé que certains paramètres immunitaires déclinaient fortement en fonction de l'âge et de la reproduction (notamment la concentration des prohéocytes), ce phénomène n'est pas généralisable à l'ensemble des paramètres immunitaires mesurés, suggérant que l'impact de l'âge et la reproduction sur une éventuelle sénescence immunitaire est complexe. Néanmoins ces résultats suggèrent que la stratégie de reproduction des femelles de *T. molitor* n'est pas conforme à l'hypothèse d'un investissement terminal.

Late life reproduction in an insect: terminal investment versus reproductive restraint

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1. Abstract

1. Theory predicts that reproduction changes as individuals reach the end of their lives by either increasing their allocation to reproduction in a terminal investment at the expense of their future survival or by restraining their reproductive effort to sustain future survival and gain more time for reproducing. Each strategy relies on the hypothesis that reproduction is costly and has contrasted implications in terms of late life post reproduction survival and somatic maintenance. Furthermore, the implementation of each strategy may also depend on early reproductive investment.

2. We tested the late life reproductive strategy of females of the mealworm beetle, *Tenebrio molitor*, an iteroparous and income breeder insect, by experimentally manipulating their age-specific allocation into reproduction and assessing its consequences of this manipulation on their late life fecundity, post reproduction survival and immunocompetence. We also tested the cost of early reproduction on female immunity, survival and late fecundity to apprehend its consequences on female late life reproductive strategy.

3. We found that females exhibit age-related decline in fecundity and that this reproductive senescence is accelerated by a significant cost of early reproduction. The cost of reproduction had no significant incidence on female longevity and their ability to survive a bacterial infection, despite that some cellular immune parameters could be depleted by reproduction. We found that female post-infection survival deteriorated with age, which could be partly explained by a decline of some immune parameters. Importantly, females did not increase their reproductive effort with age at the expense of their late life post reproduction survival.

4. Late life reproduction in *T. molitor* females is not coherent with a terminal investment. At the opposite, our results suggest that females allocate their resources according to priority scheme favouring longevity at the expense of reproduction, which is in line with the reproductive restraint hypothesis. Our study provides unconfounded support for the cost-of-reproduction hypothesis (i.e. current reproduction constrains future reproductive output) but not for the terminal-investment hypothesis, highlighting that alternative reproductive strategy could be adaptive.

Key words: ageing, cost of reproduction, innate immunity, life history, mealworm beetle, senescence, terminal investment.

2. Introduction

The way organisms schedule reproduction over their life times is assumed to have profound consequences on individual fitness, population dynamics and the evolution of life histories (Roff 1992, Stearns 1992). Whether organisms deteriorate with age and how reproductive effort depends on age are central questions in evolutionary ecology (Clutton-Brock 1984, Baudisch 2008, Monaghan et al. 2008). So far, theory predicts that organisms may optimise their age-related reproductive investment in two ways. First, organisms are predicted to increase their investment in reproduction as they approach the end of their life (Williams 1966, Clutton-Brock 1984, Forslund and Pärt 1995, Isaac and Johnson 2005). This hypothesis invokes that current reproduction is costly on future reproduction and/or survival (Reznick 1985, Alonso-Alvarez et al. 2004, Harshman and Zera 2007). Young individuals, with high reproductive residual value, are therefore expected to allocate less to current reproduction to ensure future reproductive opportunities, whereas older individuals, with low reproductive residual value, should allocate more to current reproduction. Under this assumption of age-dependent “terminal investment” (Clutton-Brock 1984), older individuals able to support higher costs of reproduction should be selected, because subsequent reproductive opportunities might be null. Empirical evidences for this prediction are inconclusive because differential survival of high and low performing reproducers could be confounding effects for this pattern (Forslund and Pärt 1995) as well as the fact that in some species, reproductive performance can be improved with age-dependent experience (Curio 1983). Second, and opposite to the terminal investment hypothesis, the “reproductive restraint” hypothesis predicts that organisms adaptively invest less in reproduction as they age (Curio 1983, McNamara et al. 2009). As reproductive activity may generate accumulation of damages, thus decreasing survival probability, organisms should benefit of reproducing less intensively late in life to defer death and therefore gain more time for reproducing (McNamara and Houston 1996, McNamara et al. 2009). However, reduced reproduction late in life could simply be a side effect of somatic deterioration (i.e., senescence), eventually associated with an age-dependent decline of somatic protection, including immunity, rather than adaptive changes in reproductive effort (Loison et al. 1999, Weladji et al. 2002). Therefore, assessing age-dependent reproductive effort as a function of survival after reproduction, as well as examining age-dependent changes in immune protection according to allocation in reproduction, should both provide valuable insights about late life reproductive strategies used by organisms (Coulson and Fairweather 2001, Reed et al. 2008, McNamara et al. 2009). The terminal investment hypothesis assumes that

as older organisms reach a state in which future reproduction is compromised, they should increase their allocation to current reproduction at the expense of their future survival. Therefore, late reproductive allocation should be inversely related to the survival and the immune protection after the last reproduction. By contrast, under the restraint reproductive hypothesis, late allocation in reproduction is not expected to compromise late post-reproduction survival, and immune protection should be maintained with age. However, as organisms invest in their early reproduction, immune protection and longevity are expected to decline rapidly.

Based on these hypotheses, we tested the lifetime reproductive strategy of females of the yellow mealworm beetle, *Tenebrio molitor* (Coleoptera: Tenebrionidae). The mealworm beetle is a cosmopolitan stored grain product pest that lives several months in populations of variable density. In this highly promiscuous insect, adult females are continuously receptive to reproduction from their fifth day post emergence and can mate many times with several males within their five months of adulthood. *T. molitor* is iteroparous and an income breeder insect (Jönsson 1997). It feeds at both the larval and the adult stages and females lay eggs in several reproductive bouts along its lifespan (Dick 1937, Hughes 2017). These features possibly allow females to adjust their lifetime reproductive effort according to their physiological state and environment (Kasumovic et al. 2009). Usually, females can produce up to 30 eggs per day but egg production often start to decline after three weeks (Dick 1937). Such an age-associated decline in female fecundity may reflect either intrinsic reproductive senescence or/and trade-off between early and late reproduction (Ricklefs and Wikelski 2002).

Here, we experimentally manipulated female age-specific allocation into reproduction at the individual level and assessed its consequences on female fecundity, late life post-reproduction survival and immunocompetence. The immune system of *T. molitor* relies on both constitutive and inducible innate mechanisms with cellular and humoral components (Vigneron et al. 2019). Haemocytes are circulating immune cells involved in parasite phagocytosis or encapsulation. *T. molitor* presents four main types of haemocytes that are morphologically and functionally distinct (Chung and Moon 2004, Urbański et al. 2018, Vigneron et al. 2019). Granulocytes and plasmatocytes are the two most abundant haemocytes circulating in *T. molitor* haemolymph that are involved in phagocytosis and encapsulation, respectively. Oenocytoids are relatively rare cells producing enzymes of the

melanization cascades, whereas prohemocytes, slightly more abundant than oenocytoids, may function as precursors of haemocytes. The activity of these immune cells is at the core of the inflammatory response, as they may generate cytotoxic compounds (Nappi and Ottaviani 2000), causing self-damage (Sadd and Siva-Jothy 2006) and lifespan reduction (Pursall and Rolff 2011b, Daukšte et al. 2012, Krams et al. 2013, Khan et al. 2017). Haemocyte counts were found to decrease after mating (McKean and Nunney 2001, Fedorka et al. 2004) and either decline (Horn et al. 2014) or remain unchanged (Adamo et al. 2001, Doums et al. 2002) with age, depending on the insect species studied. In addition, microbial challenge also induces the production of antibacterial peptides in the haemolymph (Vigneron et al. 2019), an energetically costly process that may reduce survival (Moret and Schmid-Hempel 2000). However, previous work found that variation in immune parameters, as a function of age and reproductive effort, does not always predict organism resistance or tolerance to an immune challenge by natural pathogens (Adamo et al. 2001, Khan et al. 2017). Therefore, a direct measure of immune protection, through a pathogenic exposure, is required in addition to the assessment of immune parameters for a comprehensive understanding of their variation. Hence, confronting putative up- or down-regulation of immune components with organism resistance as a function of age and reproductive allocation will help the interpretation of variation of immune parameters in terms of somatic protection or dysregulation (Licastro et al. 2005, Khan et al. 2016).

In this study, individual *T. molitor* females were allowed to reproduce either early or/and late in life to examine the effect of ageing and reproduction on fecundity, late post-reproduction survival and immunity. Immunity was assessed through the measurement of haemocytes counts, antibacterial activity and resistance to a bacterial infection. Because of ageing, female reproduction and immunity is expected to decline with age. Under the hypothesis that early reproduction is costly, we expect that female reproducing early would exhibit reduce late fecundity, immunity and longevity. If female late life reproductive strategy corresponds to a terminal investment, they are then expected to favour late reproductive investment at the expense of a reduced post-reproduction immunity and survival. By contrast, if females adopt a restraint reproductive strategy, late reproduction should not be associated to reduced late post-reproduction immunity and survival.

3. Material and methods

a. Insect culture and experimental design

Virgin adult beetles of controlled age (10 ± 2 days post-emergence) were obtained from pupae randomly sampled from a stock culture maintained in standard laboratory conditions at Dijon, France. Prior to the experiments, all the experimental insects were maintained separately, at $24 \pm 1^\circ\text{C}$, 70% RH in permanent darkness, supplied *ad libitum* with bran flour and water, and supplemented once a week with apple.

The present experiments had two objectives. First, we assessed the influence of age-specific reproduction and the intensity of the reproductive effort on patterns of actuarial, reproductive and immune senescence. To this purpose females were allowed to reproduce either early, late or both early and late in life and then assessed for their longevity, fecundity, resistance to a bacterial infection and variation in their immune parameters. Second, we assessed the immediate cost of one single reproductive event performed by young and old females, and immune defences with the aim of explaining the patterns of senescence previously observed.

To address these two questions, we conducted three successive experiments (Figure 8) because it was not possible to measure the three main parameters - namely longevity/reproduction, resistance to bacterial infection and measure immune parameters – in a single series.

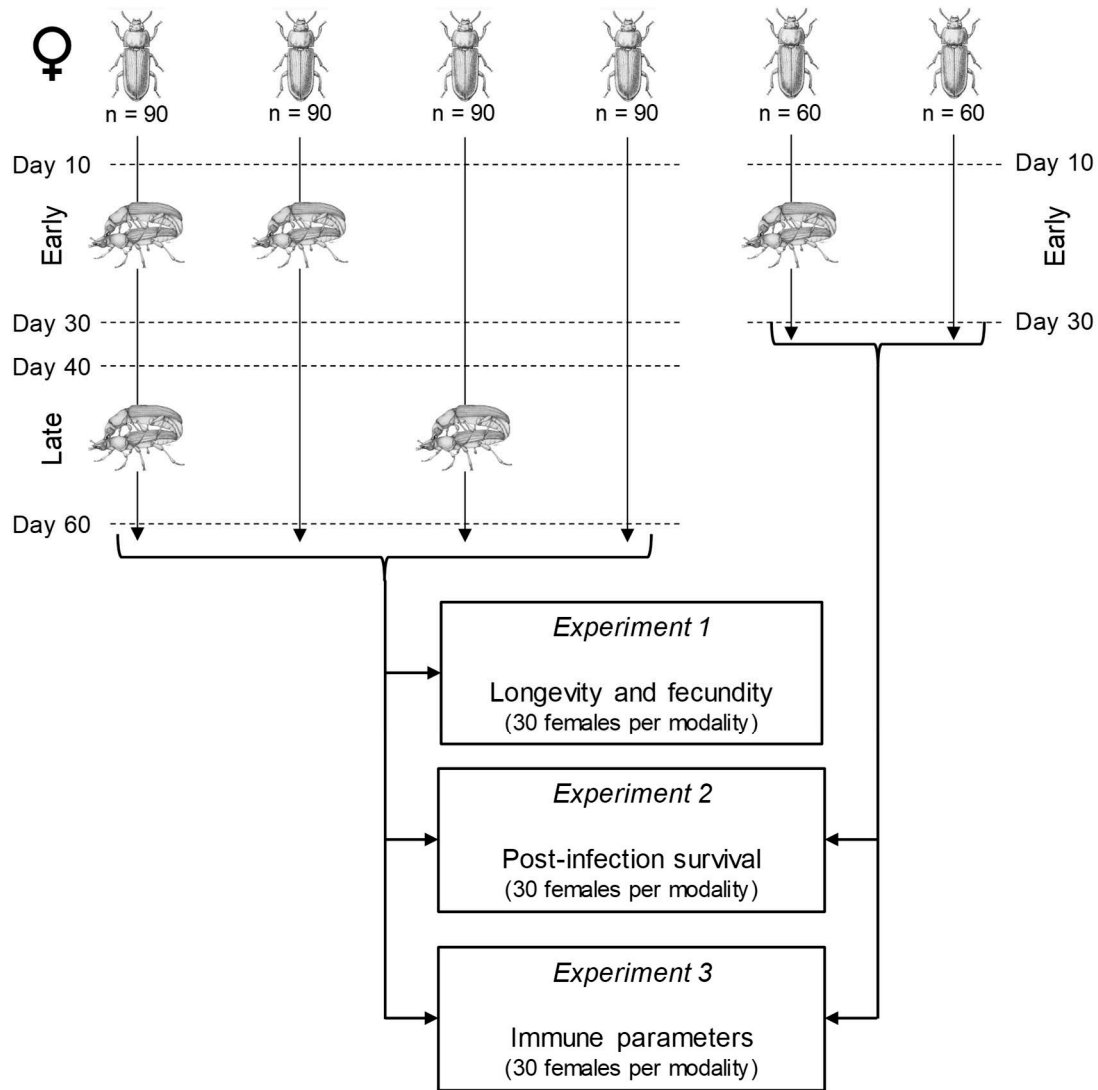


Figure 8 Common experimental design to the three experiments studying individual female fecundity, longevity (experiment 1), post-infection survival (experiment 2) and changes in immune parameters (experiment 3) as a function of their age-specific reproduction treatment. Females were either allowed to reproduce early in life (from day 10 to day 30) or /and late in life (from day 40 to day 60), as illustrated by copulating beetles, and then used for the respective measurements of the experiments they were allocated to. Note that for experiment 1, fecundity was assessed during the periods the females were allowed to reproduce and that longevity was recorded for the day the female had emerged. In addition some females were exposed to the early reproductive treatment only and then allocated to the experiment 2 or 3 for the assessment of their survival to bacterial infection and changes in their immune parameters, respectively.

b. Experiment 1: age-specific reproductive effort, fecundity and longevity

The influence of early or/and late reproduction on longevity and the cost of early reproduction on late reproduction were estimated from 4 groups of 30 females of *T. molitor* experimentally varying in their age-specific reproductive effort. In a first group, females were allowed to reproduce 20 days early in life only: between days 10 and 30 of their adulthood. Females of the second group were allowed to reproduce 20 days late in life only: between days 40 and 60 of their adulthood. Females in the third group reproduced 20 days early and 20 days late in life, whereas a last group of control females were never allowed to reproduce. For early and late episodes of reproduction, each female was paired with a 10 ± 2 days old virgin male for 10 days. Then, the male was replaced by a new 10 ± 2 days old virgin male, with which the female spent 10 additional days. Couples were maintained in a Petri dish containing bleached flour and provided *ad libitum* food and water under standard laboratory conditions. Female reproductive output was estimated by counting the number of eggs laid by the female every 3 or 4 days along the 20 days episode of reproduction. To this purpose, couples were transferred into a new Petri dish and eggs were counted by sieving the flour (mesh size $600 \mu\text{m}$) (Moreau et al. 2012). Body mass of the females was measured to the nearest 1 mg using an OHAUS balance (discovery series, DU114C) at the date of the first episode of reproduction. When females were not allowed to reproduce during the above specified reproductive episodes, they were treated as those allowed to reproduce, except they were paired with 10 ± 2 days old virgin females instead of males. From the end of each reproductive episode, females were maintained alone in a new Petri dish with *ad libitum* food and water, and checked twice a week until death.

c. Experiment 2: influence of age and reproduction on resistance to a bacterial infection

Potential changes in somatic protection according to female reproductive effort along life were examined in terms of resistance to a bacterial infection. To this purpose, groups of 30 females that did not reproduced, that reproduced early in life only, that reproduced late in life only and that reproduced both early and late in life, as described above, were exposed at the age of 60 days old (immediately after the late sequence of reproduction) to a systemic bacterial infection. The infection was done through wounding with a fine sterilized needle (0.03 mm, Fine Science Tools® n°26000-25) dipped into a pellet of live *Bacillus cereus*

(Dhinaut et al. 2018a). *B. cereus* is a natural pathogen of *T. molitor* (Du and Laing 2011, Jurat-Fuentes and Jackson 2012) known to stimulate its immune response and to induce significant mortality upon infection (Dhinaut et al. 2017). In addition to these groups of females that were infected late in life, two additional groups of 30 females were exposed to the infection at 30 days old (immediately after the early sequence of reproduction). These additional groups were kept virgin or allowed to reproduce early in life only, to assess the age-specific cost of reproduction on resistance to an infection when compared to the groups of old (60 days old) females that were kept virgin and those that reproduced late in life only. After exposure to the infection, survival was checked every hour during the first 12 hours post infection and then once a day for 14 days (Dhinaut et al. 2017, 2018a).

d. Experiment 3: influence of age and reproduction on the immune response

Potential variation in resistance to the infection by *B. cereus* was further examined by testing the effect of female age and reproductive effort on various immune effectors, before and after an immune challenge. To this purpose, we took a 5- μ L sample of haemolymph in females of 60 days old, coming from groups that varied in their reproduction (virgin, early reproduction only, late reproduction only and both early and late reproduction). This sample, therefore taken at the end of their reproductive treatment, was used to test for the concentration of the main haemocytes of *T. molitor* and its antibacterial activity while the females were unchallenged (corresponding to basal levels of these immune parameters). Immediately after this first sample of haemolymph, the females were immune challenged through a single injection of a 5- μ L suspension of inactivated *B. cereus* at a concentration of 10^8 cells per mL of phosphate buffer saline solution (PBS 10 mM, pH7.4). A second 5- μ L sample of haemolymph from each female was collected 3 days after this challenge, corresponding to the peak of the immune response (Haine et al. 2008a, b) for the concentration of the main haemocytes and the antibacterial activity of their haemolymph while they were immune challenged.

In addition to these groups of females that were assessed late in life, two additional groups of 15 to 25 females (kept virgin or allowed to reproduce early in life only) were assessed at 30 days old (immediately after the early sequence of reproduction). These additional groups were used to assess the age-specific effect of reproduction on immune parameters, when compared to the groups of old (60 days old) females that were kept virgin and those that reproduced late in life only.

e. Bacterial cultures for immune challenges and infections

The bacterium *Bacillus cereus* used in this study to perform immune challenges and infections was obtained from the Pasteur institute (CIP69.12). The bacterium was 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 perform immune challenges were inactivated in 0.5% formaldehyde prepared in PBS for 30 minutes, rinsed three times in PBS, and their concentration adjusted to 10⁸ 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. Immune challenges were performed by injection through the pleural membrane between the second and the 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, 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.

f. Haemolymph collection and immune parameters

Haemolymph collection was done as described by Dhinaut et al. (2018). Briefly, beetles were chilled on ice for 10 min before the sampling of 5 µL of haemolymph from a wound made in the beetle's neck. Samples were flushed in a microcentrifuge tube containing 25 µL of anticoagulant buffer (Lee et al. 1999). A 10-µL subsample was immediately used for haemocyte count using a Neubauer improved haemocytometer under a phase-contrast microscope (magnification x 400). The count of the main haemocyte types found in *T. molitor* haemolymph was further done using the description made by Urbański et al. (2018). We especially measured the concentration of granulocytes, plasmatocytes and prohemocytes. The observation of oenocytoïds was anecdotic in our study. Another 5-µL were transferred in an N-phenylthiourea-coated microcentrifuge tube (P7629; Sigma-Aldrich, St Louis, MO, USA) flash-frozen in liquid nitrogen and then stored at -80 °C for later examination of its antibacterial activity.

Antimicrobial activity of the haemolymph was measured using the inhibition zone assay described by Moret (2006). Briefly, haemolymph samples were thawed on ice, and 2 µL of the sample solution was used to measure antibacterial activity on zone 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.mL⁻¹. Six milliliters of this seeded medium were subsequently poured per Petri dish. After solidification, sample wells were made using a Pasteur pipette fitted with a ball pump. Two microliters of sample were deposited in each well. Plates were then incubated overnight at 28°C and the diameter of each zone of inhibition was measured.

g. Statistics

Cox regressions were used to analyse the effects of female age and reproductive treatment on longevity and resistance to a bacterial infection. Depending on the experiment and the questions asked, survival was analysed as a function of reproduction (occurring early or/and late in life), age and initial body mass (at 10 days old). The best statistical model was selected using a backward stepwise procedure from full models testing the main effect and two-way interactions between all categorical explanatory factors (e.g. early/late reproduction, presence/absence of bacterial infection and age), but the effect of continuous covariables, such as insect body mass was tested as a main effect only.

Depending on the question tested, individual variation in fecundity (number of eggs laid by females) was analysed as a function of reproduction event (early/late in life), age and female body mass as covariate using univariate analyses of variance (ANOVAs). In order to test whether the general state of females or the imminence of their death predicts late reproductive effort, late female fecundity was analysed in an ANOVA as a function of: (i) the occurrence or not of early reproduction (as a categorical fixed factors); (ii) the number of days from the late reproductive episode to female death; (iii) early and late female body mass as covariate. Finally, among female that reproduced twice (early and late in life) the potential correlation between the number of eggs laid early and that laid late in life was tested using a linear regression analysis. Individual variation in haemocyte counts and antibacterial activity, before and after an immune challenge were analysed as a function of reproductive event in life or/and age using analyses of variance for repeated measured. Data on haemocyte counts were standardized to z scores using the rank inverse-normal transformation procedure from (Templeton 2011), whereas data on antibacterial activity were log-transformed to satisfy the conditions of use of parametric tests. All the analyses used IBM® SPSS® statistics 19 for Macintosh.

4. Results

a. Age-specific reproductive effort and late life history traits

i. Fecundity

When considering females that reproduced only once in life, those that reproduced late in life produced almost twice less eggs than those that reproduced early (ANOVA global model $F_{2,56} = 28.25$ $P < 0.001$, Age $F_{1,56} = 52.24$ $P < 0.001$, Figure 10). Heavier females marginally laid more eggs than lighter ones (Body mass $F_{1,56} = 3.88$ $P = 0.054$).

Considering only the reproduction late in life, the number of eggs laid by females was lower when they had first reproduced early in life than when they had not (ANOVA global model $F_{4,52} = 5.17$ $P = 0.001$, early reproduction $F_{1,52} = 9.61$ $P = 0.003$, Figure 11). Neither early nor late body mass of females significantly explained late fecundity (early body mass $F_{1,52} = 0.14$ $P = 0.713$; late body mass $F_{1,52} = 0.34$ $P = 0.561$). However, late post reproduction survival (in days) was positively correlated with female late fecundity ($F_{1,52} = 6.02$ $P = 0.018$), demonstrating that females that had the shortest post reproductive survival were also those exhibiting the lowest fecundity late in life (Figure 11).

Finally, among the females that reproduced twice, there was a positive correlation between the number of eggs laid early and late in life ($F_{1,28} = 10.40$ $P = 0.002$, Figure 12).

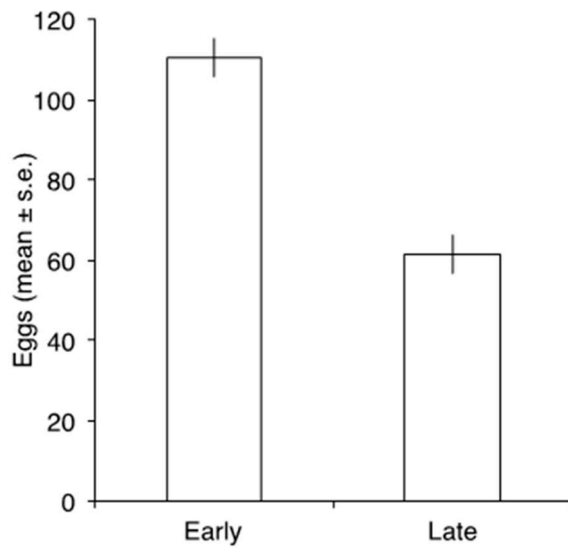


Figure 9 : Mean number of eggs (\pm s.e.) laid early and late in life by *Tenebrio molitor* females allowed to reproduce either early in life ('Early': from 10 to 30 days old) or late in life ('Late': from 40 to 60 days old) only.

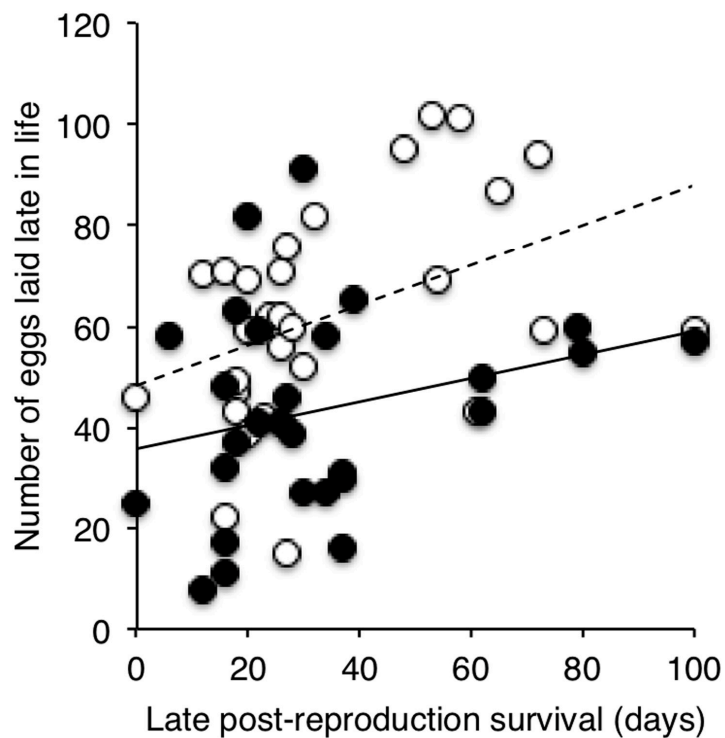


Figure 10 : Relationship between female late fecundity (number of eggs) and female post late reproduction survival (in days) as a function of whether they were allowed to reproduce early in life (filled circles and continuous line, $y = 0.23x$ post late reproduction survival days + 35.82, $r = 0.27$) or not (open circles and dashed line, $y = 0.40x$ post late reproduction survival days + 48.32, $r = 0.42$).

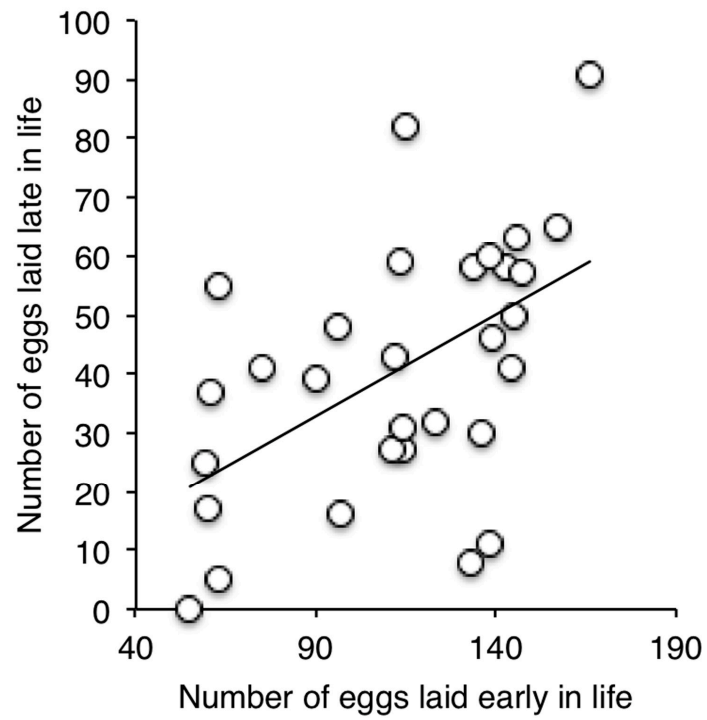


Figure 11 : Relationship between the number of eggs laid early in life (from 10 to 30 days old) and the number of eggs laid late in life (from 40 to 60 days old) by *Tenebrio molitor* females allowed to reproduce both early and late in life ($y = 0.34 \times \text{number of eggs laid early in life} + 1.88$, $r = 0.52$).

ii. Longevity

Early and late reproduction in life had no effect on female longevity either as main effect or in interaction (Cox Regression analysis: early reproduction Score = 0.001, df = 1, P= 0.946, late reproduction Score = 1.39, df = 1, P= 0.238, early × late reproduction Score = 1.00, df = 1, P= 0.752). Only the initial body mass of females explained variation in longevity, with the heaviest females surviving slightly longer than the lightest (Wald = 5.21, df = 1, P = 0.022, Odd ratio = 0.99).

iii. Post-infection survival

Female resistance to a bacterial infection occurring late in life (at 60 days old) was not significantly affected by reproduction either early in life (Score = 0.85 df =1 P = 0.356), late in life (Score = 2.08 df =1 P = 0.149) or their interaction (Score = 0.05 df =1 P = 0.821). Initial body mass of females was retained in the best model, but its effect was marginally significant, where heavier females tend to resist better the infection than lighter ones (Wald = 3.64, df = 1, P = 0.056, Odd ratio = 0.99).

iv. Immune parameters

Female total haemocyte count was similar whenever they had reproduced early or late in life (Table 4, Figure 13a). The immune challenge with the bacterium *B. cereus* resulted in a general increased of the total haemocyte count, irrespective of their age-specific reproductive effort (Table 4, Figure 13a).

Some of the haemocyte types constituting the total haemocyte count exhibited contrasted levels of variation according to the age-specific reproductive effort of the females and the immune challenge. Granulocyte represented about 60 to 70 % of the total haemocyte count, near to the range previously described in this insect species (Urbański et al. 2018). While early reproduction did not change significantly the granulocyte count, late reproduction resulted in a decline of this type of haemocyte (Table 4, Figure 13b). However, granulocyte count was insensitive to the immune challenge either as main effect or in interaction with early or late reproduction (Table 4, Figure 13b).

Plasmatocyte count, which varied between 29 and 69 % of the total haemocyte count, was significantly affected by the interaction between early and late reproduction (Table 4). While plasmatocyte count tended to increase when females had reproduced only once (early reproduction only or late reproduction only), it was at the lowest value in females that had reproduced both early and late in life (Figure 12c). The immune challenge significantly increased the plasmatocyte count irrespective of early or late reproduction (Table 4, Figure 13c).

The prohaemocyte count (about 0.5% of the total haemocyte count) was unaffected by early reproduction whereas it was significantly reduced by late reproduction (Table 1, Figure 13d). The immune challenge induced a decline in the count of this haemocyte type independently of early or late reproduction (Table 4, Figure 13d). Reproduction, either early or late, had no effect on the haemolymph antibacterial activity, which was stimulated by the immune challenge only (Table 4, Figure 13e).

Table 4 Summary of the analyses for repeated measures testing the effects of a bacterial immune challenge in 60 days old *Tenebrio molitor* females as function of their age-specific reproductive effort (early and late reproduction) in a full factorial design.

	Total Hemocytes			Granulocytes			Plasmatocytes			Prohemocytes			Antibacterial activity		
<i>Between subjects</i>															
	<i>d.f.</i>	<i>F</i>	<i>P</i>	<i>d.f.</i>	<i>F</i>	<i>P</i>	<i>d.f.</i>	<i>F</i>	<i>P</i>	<i>d.f.</i>	<i>F</i>	<i>P</i>	<i>d.f.</i>	<i>F</i>	<i>P</i>
Early reproduction	1	0.80	0.373	1	2.68	0.105	1	1.02	0.316	1	0.21	0.649	1	0.08	0.776
Late reproduction	1	2.49	0.118	1	4.78	0.032	1	0.00	0.997	1	7.40	0.008	1	0.06	0.816
Early*Late reproduction	N.R.			N.R.			1	5.79	0.018	N.R.			N.R.		
Error	84			84			83			85			88		
<i>Within subjects</i>															
	<i>d.f.</i>	<i>F</i>	<i>P</i>	<i>d.f.</i>	<i>F</i>	<i>P</i>	<i>d.f.</i>	<i>F</i>	<i>P</i>	<i>d.f.</i>	<i>F</i>	<i>P</i>	<i>d.f.</i>	<i>F</i>	<i>P</i>
Challenge	1	7.71	0.007	1	0.01	0.915	1	23.51	< 0.001	1	20.75	< 0.001	1	28.57	< 0.001
Challenge*Early reproduction	1	0.41	0.525	1	0.42	0.520	1	0.03	0.868	1	0.11	0.743	1	0.01	0.929
Challenge*Late reproduction	1	1.74	0.191	1	1.18	0.281	1	1.06	0.306	1	0.95	0.332	1	0.80	0.372
Challenge*Early*Late reproduction	N.R.			N.R.			1	2.09	0.152	N.R.			N.R.		
Error	84			84			83			85			88		

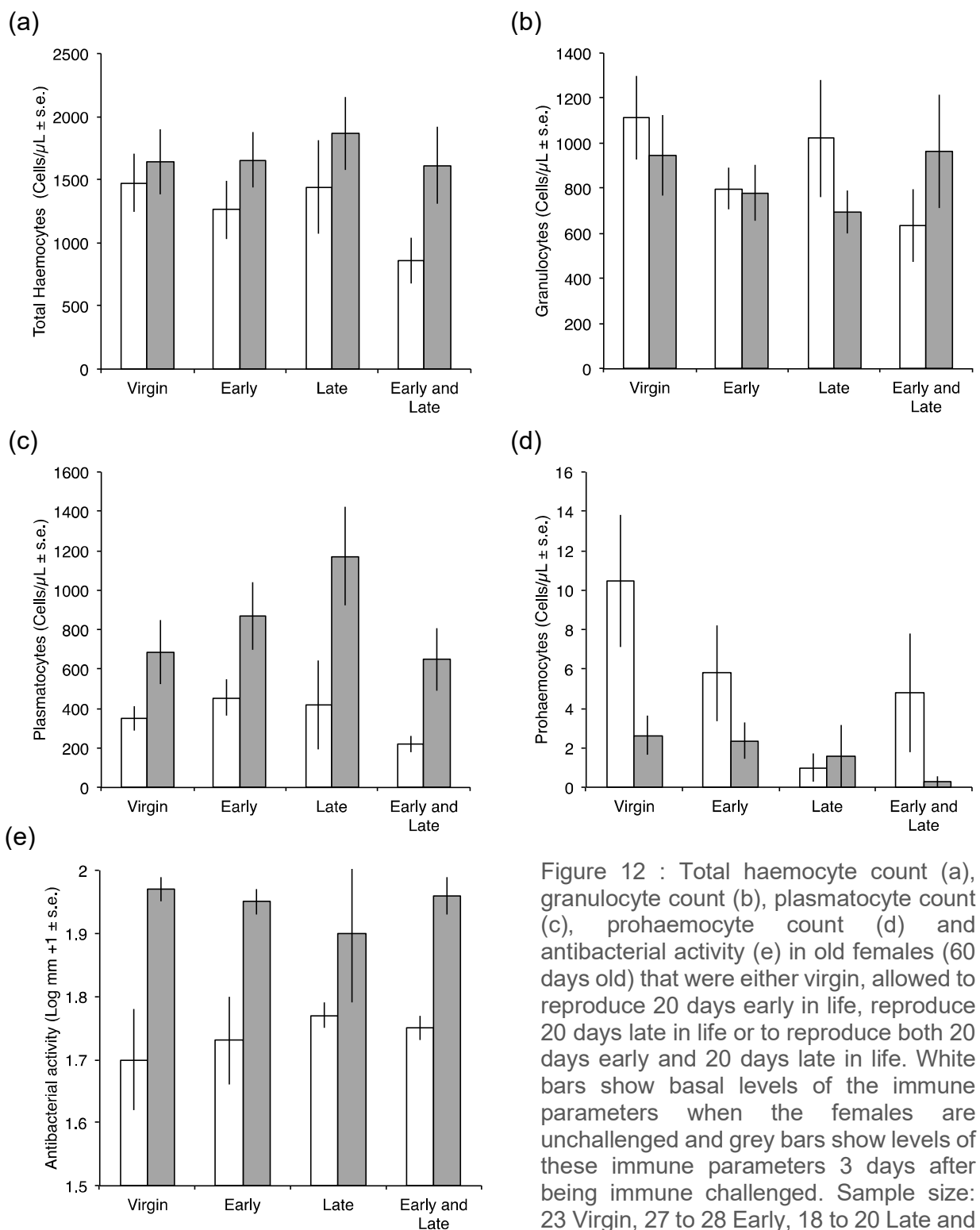


Figure 12 : Total haemocyte count (a), granulocyte count (b), plasmacyte count (c), prohaemocyte count (d) and antibacterial activity (e) in old females (60 days old) that were either virgin, allowed to reproduce 20 days early in life, reproduce 20 days late in life or to reproduce both 20 days early and 20 days late in life. White bars show basal levels of the immune parameters when the females are unchallenged and grey bars show levels of these immune parameters 3 days after being immune challenged. Sample size: 23 Virgin, 27 to 28 Early, 18 to 20 Late and 20 to 22 Early and Late.

b. Effect of age and reproduction on immunocompetence

i. *Pot-infection survival*

Young *T. molitor* females survived about 1.8 times longer the infection by *B. cereus* than old females (Cox Regression analysis: Wald = 7.67, df = 1, P= 0.006, Odd ratio = 1.84, Figure 14. Whether the females reproduced or not before being exposed to the infection had no effect on their resistance (Wald = 2.73, df = 1, P= 0.098). The interaction term between age and reproduction did not explain any variation of resistance to the infection (Score 0.03 df = 1 P = 0.870) as well as initial female body mass (Score =1.14 df = 1 P = 0.284).

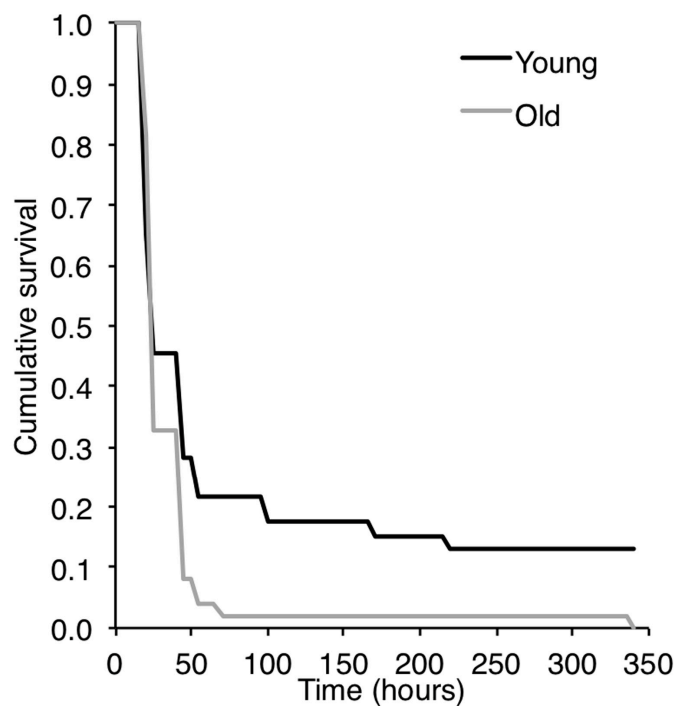


Figure 13 : Survival to an infection with the bacterium *Bacillus cereus* of young (30 days old) and old (60 days old) *Tenebrio molitor* females, independently of their reproduction treatment.

ii. Immune parameters

Female total haemocyte count was affected neither by age nor reproduction (Table 5, Figure 15a). As above, the immune challenge with *B. cereus* significantly raised the total haemocyte count, but this raise was much stronger in females that reproduced than in females that did not reproduce, as revealed by the significant Challenge × Reproduction interaction, independently of age (Table 5, Figure 15a).

The various types of haemocytes that constitute the total haemocyte count showed contrasted levels of variation according to age, reproduction and the immune challenge. Whereas the granulocyte count (here about 64% of the total haemocyte count), was unaffected by age, reproduction or the immune challenge (Table 5), plasmatocyte (about 37 % of the total haemocyte count) and prohaemocyte (about 1.2 % of the total haemocyte count) counts showed significant variation according to age and reproduction (Table 5). Plasmatocyte count was higher in old females than in young ones and was further increased by reproduction (Table 5, Figure 15b). The plasmatocyte count significantly increased upon the immune challenge, but this increase was much stronger in females that reproduced than in females that did not reproduce, irrespective of their age (Table 5, Figure 15b). Prohaemocyte count significantly declined with female age and as an effect of reproduction in an additive manner (Table 5, Figure 15c). Upon the immune challenge, prohaemocyte count decreased, and this decrease was much stronger in young females than in old females (Table 5), mostly because the basal levels of prohaemocytes decreased with female age (Figure 15c).

Antibacterial activity in the haemolymph of females was not affected by reproduction or age (Table 5). It only increased as an effect of the immune challenge with the bacterium *B. cereus*. (Table 5, Figure 15d).

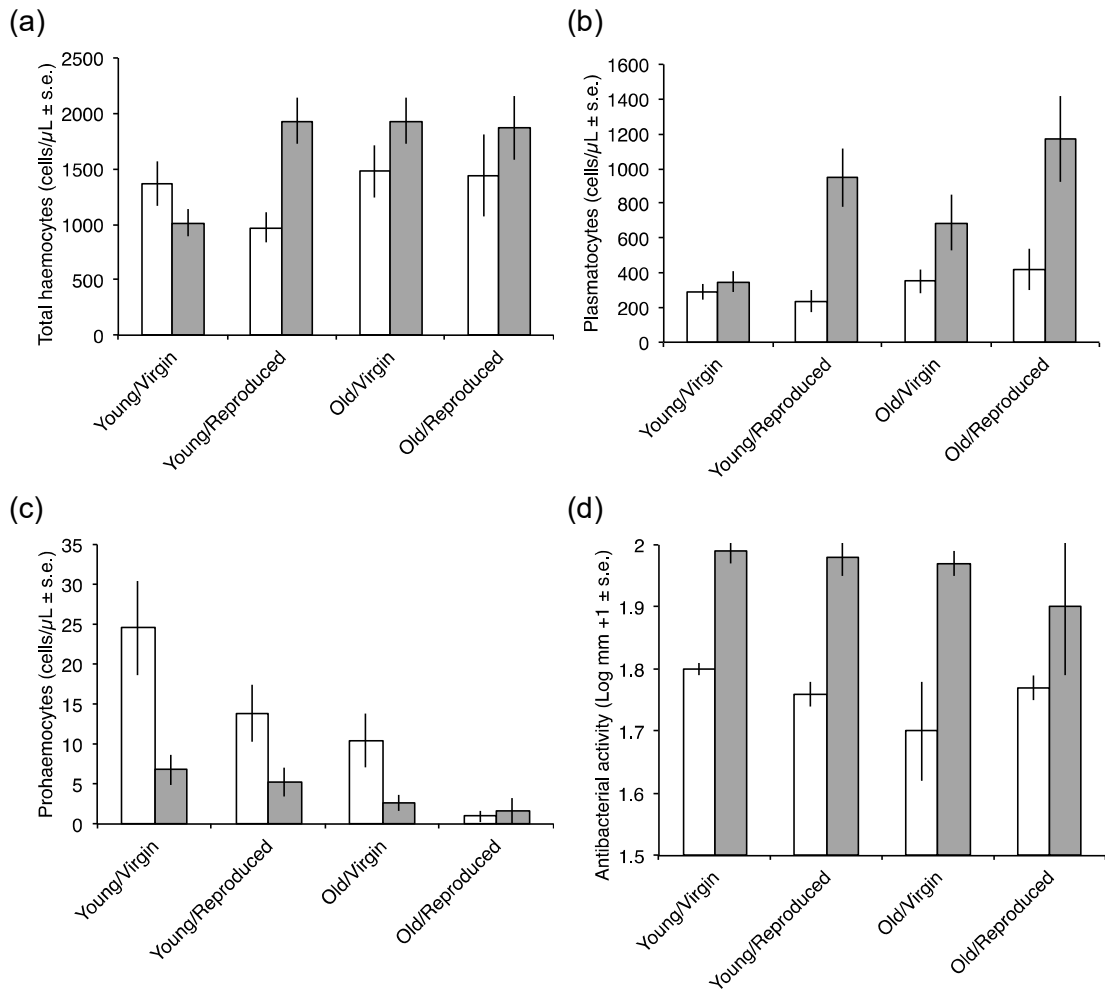


Figure 14 : Total haemocyte count (a), plasmacyte count (b), prohaemocyte count (c) and antibacterial activity (d) in young (30 days old) and old (60 days old) females that were either allowed ('Reproduced') or not ('Virgin') to reproduce 20 days prior to be assessed for their immune defences. White bars show basal levels of the immune parameters when the females are unchallenged and grey bars show levels of these immune parameters 3 days after being immune challenged. Sample size: 23 young/virgin, 14 to 16 young/reproduced, 23 old/virgin and 18 to 19 old/reproduced females.

Table 5 Summary of the analyses for repeated measures testing the effects of a bacterial immune challenge in 30 days old and 60 days old *Tenebrio molitor* females immediately after a 20 days reproductive period.

	Total Hemocytes			Granulocytes			Plasmatocytes			Prohemocytes			Antibacterial activity		
<i>Between subjects</i>															
	<i>d.f.</i>	<i>F</i>	<i>P</i>	<i>d.f.</i>	<i>F</i>	<i>P</i>	<i>d.f.</i>	<i>F</i>	<i>P</i>	<i>d.f.</i>	<i>F</i>	<i>P</i>	<i>d.f.</i>	<i>F</i>	<i>P</i>
Age	1	1.19	0.279	1	0.02	0.888	1	4.49	0.037	1	26.47	< 0.001	1	1.90	0.172
Reproduction	1	1.06	0.307	1	0.59	0.444	1	6.65	0.012	1	7.80	0.007	1	0.08	0.774
Error	77			76			76			77			76		
<i>Within subjects</i>															
	<i>d.f.</i>	<i>F</i>	<i>P</i>	<i>d.f.</i>	<i>F</i>	<i>P</i>	<i>d.f.</i>	<i>F</i>	<i>P</i>	<i>d.f.</i>	<i>F</i>	<i>P</i>	<i>d.f.</i>	<i>F</i>	<i>P</i>
Challenge	1	4.62	0.035	1	0.34	0.560	1	27.53	< 0.001	1	18.85	< 0.001	1	27.53	< 0.001
Challenge*Age	1	0.23	0.632	1	0.02	0.891	1	1.76	0.826	1	4.44	0.038	1	0.01	0.916
Challenge*Reproduction	1	8.36	0.005	1	2.63	0.109	1	11.07	0.001	1	2.59	0.112	1	0.72	0.398
Error	77			76			76			77			77		

5. Discussion

This study aimed at examining the effect of ageing and reproduction on late fecundity, post-reproduction survival and immunocompetence of *T. molitor* females.

T. molitor females are sexually mature from the fifth day post emergence. While they are continuously receptive during their adulthood, eggs production may decline from the third week onward of adult life (Dick 1937). Such an age-associated decline in female fecundity may reflect either intrinsic reproductive senescence or/and trade-off between early and late reproduction (Ricklefs and Wikelski 2002). In our study, the assessment of female age-specific fecundity demonstrated clear evidence for reproductive senescence in *T. molitor* females, as the number of eggs laid for the first time by older females (from 40 to 60 days old) is almost half less than that laid by younger females (from 10 to 30 days old), all else being equal. Hence, as previously found in other insect species (Moore and Sharma 2005, Churchill et al. 2019), *T. molitor* females are experiencing intrinsic reproductive senescence. In addition, older female fecundity is further reduced when they had reproduced early in life, demonstrating the occurrence of a trade-off between early and late reproduction, which accelerated reproductive senescence. However, our results failed to evidence such a trade-off at the individual level, as females with the highest fecundity early in life were also those with the highest fecundity late in life. Interestingly, body mass, which was found to be a good indicator of body condition and potential female fecundity (Zanchi et al. 2019), did not significantly explain variation in the female trade-off between early and late reproduction. This suggests that conditions of resource acquisition in our experiment were rather optimal. Note here that females were fed *ad libitum* and kept individually, preventing from competition for food. This condition likely prevented the observation of individual trade-off in resource allocation between early and late reproduction, as theorized by (van Noordwijk and de Jong 1986). This is especially relevant in an income breeder insect like *T. molitor*, who can replenish resources as an adult for reproduction (Jönsson 1997). Nevertheless, it is noteworthy that late fecundity was positively associated to post late reproduction survival time, which is opposite to the prediction from the terminal investment hypothesis. One could argue that, as we have imposed the late reproductive event for all the females at the same age, most females may have not reach the critical physiological state that would induce enhanced fecundity at the expense of late survival. However, numerous females had died soon after their late reproduction (within the first 30 days post late reproduction, Fig. 2), indicating they have reach this critical state. These females are

clearly not those that exhibited the highest fecundity, suggesting that the relationship we have found is rather strong. All together, these results demonstrate that *T. molitor* females are experiencing intrinsic reproductive senescence and that early reproduction accelerates the rate of reproductive senescence. In addition, the observed pattern of lifetime reproductive allocation does not support the occurrence of a terminal investment.

As the cost of early reproduction accelerated reproductive senescence, it was expected that actuarial senescence could have been affected too (Harshman and Zera 2007, Flatt 2011, Lemaître et al. 2015). However, our results do not support this view as females that reproduced early, late or both did not exhibit reduced longevity compare to females kept virgin. These results suggest the occurrence of an allocation priority scheme (Rigby and Moret 2000), where allocation of resources to reproduction is sacrificed before the allocation to survival. It therefore means that the fitness of *T. molitor* females is, to some extent, primarily achieved through longevity than through reproduction, which would potentially support the reproductive restraint hypothesis. In lines with this priority scheme hypothesis, we also found that female reproduction never jeopardized their survival to a bacterial infection, either early, late or both. Nonetheless, older females were less tolerant to the infection than younger ones. Hence, somatic protection is therefore intrinsically senescing in *T. molitor* females, but the cost of reproduction appeared insufficient to accelerate its pace, at least in our study. As explained above, it is possible that conditions were too favourable for females to exhibit any significant reproduction cost on resistance to infection. However, our result can be backup by a previous study using the ecologically and phylogenetically related species, *Tribolium castaneum*, which found a similar decline in post-infection survival with age and only a small negative effect of reproduction on female survival after infection (Khan et al. 2016).

Measured immune parameters do not fully explained the above pattern of resistance to a bacterial infection, although changes in some of them according to age, reproduction and the immune challenge are significant. Our results show that female post-infection survivorship is unlikely dependent on antibacterial activity, as its level was not found affected by age or reproduction prior to infection. Only the immune challenge raised its activity level in the haemolymph, and previous studies have demonstrated that it only provides a survival benefit to a bacterial infection when it has been previously induced (Moret and Siva-Jothy 2003, Dhinaut et al. 2018a).

Haemocyte counts, and especially variation of the different haemocyte types involved, may partly explain variation in female post-infection survivorship. Nonetheless, an important insight from our data is that measuring total-haemocyte count, without taking into account the variation of the different types of haemocytes, appears as insufficient to characterize female immunocompetence according to age and reproductive effort. Total-haemocyte count did not change with female age. As expected from previous studies (Zanchi et al. 2011, Dhinaut et al. 2018a), total-haemocyte count was slightly increased by the immune challenge, but this challenge-dependent raise of haemocytes was stronger in reproduced females than in virgin ones. However, reproduced females did not better survive the bacterial infection than virgin ones. The observed variation in total-haemocyte count could be partially explained by that of the plasmacytes. Plasmacytes are considered as the second most important haemocytes, after granulocytes, mainly involved in phagocytosis, encapsulation and wound healing (Theopold et al. 2004, Vigneron et al. 2019). Their recruitment was induced by the immune challenge and reproduction, putatively explaining variation in total-haemocyte count as a function of these factors. Such a reproduction-dependent increase of plasmacytes after an immune challenge suggests that females are likely wounded during mating or/and oviposition, which may have primed their immune system, resulting in an increased concentration of plasmacytes. Indeed, priming was previously found associated to enhanced circulation of haemocytes (Zanchi et al. 2011, Dhinaut et al. 2018a). It is not known whether increased of the plasmacyte count results from the synthesis of new cells (presumably from prohaemocytes) or/and the release of sessile haemocytes into circulation, which each process may involve different costs. Interestingly, plasmacyte count also increased with female age, which can be explained by a higher probability of having been wounded with age.

Old females that had cumulated an early and a late reproduction had less circulating plasmacytes than females that had reproduced only once, either early or late. This suggests that the two successive reproductive episodes were costly for the females, which were not able anymore to produce or release plasmacytes. Reproduction also had an apparent cost on granulocyte and prohemocytes counts, which were reduced in old females after a late reproductive effort. Nevertheless, these apparent costs of reproduction on circulating granulocytes and prohemocytes did not jeopardize female post-infection survivorship.

In contrast to plasmatocyte count, prohemocyte count was found to decline with female age, after reproduction and after an immune challenge. The decline of prohaemocytes after the immune challenge is smaller in old females than in young ones, because old females circulated the lowest number of this type of haemocytes. Together these results provide two important insights. On the one hand, prohemocytes contributed to the production of plasmatocytes as female grow in age, after reproduction and after the immune challenge. Considering the different proportion of each of these cell types in the haemolymph (plasmatocytes 37% and prohaemocytes 1.2 % of the total haemocyte count), prohaemocytes are unlikely contributing to the production of plasmatocytes at one for one ratio, although the dynamics of production of the prohaemocytes and their differentiation into other new immune cells is not known. On the other hand, there is a clear demonstration that prohemocyte count, which represents the ability of the insects to produce new immune cells, is senescing. Therefore, on the long term, age may limit the ability of the females to protect themselves from pathogens late in life, which is consistent with our finding that old females resisted less to the bacterial infection than young ones. Note that our study allowed assessing a limited number of immune parameters and it is likely that other unmeasured immune components, such as the activity of the prophenoloxidase system, lysozyme activity, could be helpful to better explain the pattern of longevity and post-infection survivorship of *T. molitor* females as a function of age and reproductive effort.

To summarize, we confirmed that *T. molitor* females exhibit age-related decline in fecundity and that this reproductive senescence could be accelerated by a significant cost of early reproduction. Females did not increase their reproductive effort with age at the expense of their late post-reproduction survival, which suggests that *T. molitor* female late life reproduction is following a restraint reproductive strategy rather than a terminal investment strategy. In our study, the cost of reproduction had no significant incidence on female longevity and late post-infection survival, despite that some cellular immune parameters could be depleted by reproduction. Conversely, reproduction appears to stimulate other immune parameters through an immune priming effect. We found that females become less able to survive a bacterial infection with age. This pattern of immunosenescence could not be conclusively explained by our measured immune parameters, even if the decreased in prohemocytes with age, which represents a decrease in the ability of the insects to produce new immune cells, may partly explain this pattern.

This suggests that the interpretation of immune measures in the context of immunosenescence is complex and may require the assessment of numerous parameters to better understand such a phenomenon. More generally, our study suggests the occurrence, in *T. molitor* females, of a life history priority scheme (Rigby and Moret 2000), that would favour longevity at the expense of reproduction, which is coherent with the restraint reproductive hypothesis. Such a priority scheme is likely frequent in income breeder insects such as *T. molitor*, as compare to capital breeder insects. Indeed, longevity may provide the opportunity to income breeder insects to optimise their reproductive effort overtime according to their resource access. This is supported by the fact that *T. molitor* females kept in rich food condition as adult can maintain higher fecundity over time than females kept in poor food conditions, irrespective of the food condition they were exposed as larvae (Zanchi et al. 2019). Further studies are needed to formally test this priority scheme hypothesis between longevity and reproduction in capital and income breeder insects, as it would highlight different life history strategies with regards of senescence.

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Authors' contributions

CJ, TR and YM conceived the ideas and designed methodology; CJ and CS collected the data; CJ and YM analysed the data; CJ, TR and YM led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Data accessibility

Data will be available from the Dryad Digital Repository.

Chapitre 3

Chapitre 3 : Etude des conséquences d'une stimulation âge-spécifique du système immunitaire sur les patrons de sénescence des femelles de *Tenebrio molitor*

La réponse immunitaire est un élément crucial de la physiologie des organismes, leur permettant de se protéger des infections parasitaires et ainsi de pouvoir maximiser leur fitness. Cependant, la réponse immunitaire est aussi décrite comme un des facteurs responsables de la sénescence. Plus particulièrement un élément du système immunitaire est mis en cause : la réponse inflammatoire. Celle-ci consiste en une libération de composés cytotoxiques (comme des espèces réactives de l'oxygène) qui permettent d'éliminer les pathogènes. Cependant, ces composés cytotoxiques affectent aussi les cellules et les tissus de l'hôte, générant ainsi des dommages auto-immuns.

Il a notamment été montré chez *T. molitor* qu'une activation précoce du système immunitaire et particulièrement de la phénoloxydase, entraînait des dommages auto-immuns et réduisait la longévité (Sadd and Siva-Jothy 2006, Khan et al. 2017). Néanmoins, dans ces études, les femelles n'ont pas eu l'opportunité de se reproduire.

Dans ce chapitre nous avons donc complété les résultats des études précédemment citées en étudiant les conséquences d'une stimulation du système immunitaire, sur les patrons de sénescence actuarielle, reproductive et immunitaire.

Au cours de cette expérience, toutes les femelles ont été amenées à se reproduire deux fois dans leur vie adulte (tôt : 10-30 jours post émergence ; et tard 40-60 jours post-émergence) mais chaque épisode de reproduction a été précédé ou non d'un challenge immunitaire. Nous avons mesuré l'influence de ces challenges immunitaires sur la fécondité, la longévité et la fonction immunitaire des femelles de *T. molitor*.

De plus, l'utilisation du challenge immunitaire permet aussi de mimer un statut infectieux pouvant signaler un risque imminent de mortalité plus fort qui pourrait servir de déclencheur à un investissement terminal. Cette expérience permet d'ainsi de tester la présence d'investissement terminal chez les femelles *T. molitor*, et ainsi de compléter les résultats du chapitre 2.

Nous avons observé que le challenge immunitaire, qu'il se produise tôt ou tard dans la vie, diminuait la fécondité des femelles de *T. molitor* mais qu'il n'avait aucune influence sur la longévité. De plus, comme pour le chapitre 2, nous avons observé une corrélation

positive entre la fécondité tardive et la longévité post-reproduction, quel que soit le statut immunitaire des femelles. Ces résultats confortent ceux obtenus dans l'étude du chapitre 2 et suggèrent l'absence d'un investissement terminal chez les femelles de *T. molitor*. Par ailleurs comme pour le chapitre 2, ces résultats semblent indiquer que les femelles *T. molitor* favorisent leur longévité aux dépens de leurs reproductions.

Impact of age-specific immune challenge on patterns of senescence

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1. Introduction

Ageing is defined as an age-dependent decline of organism performance and fitness (Rose 1991, Ricklefs 1998). It is characterized by a deterioration of the general capacities of the organism, especially survival and reproductive success. However, senescence also affects other functions such as immunity, behaviour, physiology, with the general consequence of reducing fitness (Kirkwood and Austad 2000). Ageing is an almost universal feature of multicellular organisms, and understanding why it occurs is a long-standing issue in biology (Hughes and Reynolds 2005). Number of theories has been developed to explain this phenomenon. First, Medawar (1952) hypothesized that accumulation of deleterious mutations that are expressed late in life, which is when the action of natural selection becomes too weak to purge them, could cause ageing. Moreover, it has been suggested that ageing could be the consequence of cellular damages caused by reactive oxygen species (ROS), naturally generated by the mitochondria or by the over-reactive activation of the immune system, called immunopathology (Harman 1956, Oliveira et al. 2010). Thereafter, it was proposed that antagonistic pleiotropy could be involved in ageing processes, as the strength of natural selection decreases with age, alleles having negative effects on late life performances could be selected if they have positive effects early in life (Williams 1957). Finally, the disposable soma theory of ageing proposes that reproduction is resource demanding, and that current reproduction is traded-off against future reproduction and survival, due to a reduced investment into somatic protection and maintenance (Kirkwood 1977, Kirkwood and Rose 1991).

Immunity and reproduction are important fitness-related traits that are believed to have key roles in ageing processes. Hence, studying links between those two functions can provide insights on organism life-history strategies and on causes of ageing. Life history theory assumes that as resources are limited, organisms are constrained to optimally trade-off their investment between life-history traits in order to maximise fitness (Stearns 1992, Hughes and Reynolds 2005). Such a trade-off between reproduction and immunity has been reported in several animal species (Zera and Harshman 2001, Hosken 2001, Fedorka et al. 2004, Simmons and Roberts 2005, Stahlschmidt et al. 2013). Nevertheless, schedule and magnitude of trade-offs between life-history traits can change during life. Indeed, fitness value of each life-history trait is supposed to change with individuals' age, as their residual reproductive value (that is the number of expected offspring after the current reproductive event) decreases with age (Williams 1966). A classical prediction is that reproductive

investment should increase with age, reaching its highest value as a terminal investment when future opportunities for reproduction become null (Hirshfield and Tinkle 1975, Clutton-Brock 1984). Such a terminal investment is expressed at the expense of longevity, which in any case would no longer provide fitness benefit (Clutton-Brock 1984). Terminal investment might be induced when individuals either reach the end of their expected lifespan or/and as a response to a survival threat such as a parasite or pathogen infection (Minchella 1985, Hochberg et al. 1992, Forbes 1993, Perrin et al. 1996, Agnew et al. 2000, Duffield et al. 2017).

Parasite and pathogen typically reduce host survival or fecundity (Hamilton and Zuk 1982). Host can reduce its loss of fitness by controlling the parasite through its immune system. Although this may keep infections at low levels, the immune response may come at a cost, by diverting resources from reproduction or/and survival (Moret and Schmid-Hempel 2000, Schwenke et al. 2016) and by inducing self-damages to host tissues and organs (Sadd and Siva-Jothy 2006, Khan et al. 2017). Alternatively, the host can make facultative adjustments of their reproductive effort towards a terminal reproductive investment (Minchella 1985, Hochberg et al. 1992, Forbes 1993, Perrin et al. 1996, Agnew et al. 2000, Duffield et al. 2017). While such a life history shift could result from parasite manipulation, similar outcomes were found from immune stimulations with non-living immune elicitors (Duffield et al. 2017). Triggering an immune response can mimic an infection predicting reduced residual reproductive value to the host either from the parasite it-self or from the cost of the immune response (Moret 2003). In the latter case, the induction of such a life history shift could be condition dependent as it was observed under stressful conditions affecting the individual general state (Moret and Schmid-Hempel 2004). Hence, induction of terminal investment in response to an immune challenge should be favoured as individuals become old, their body condition deteriorates and immunity declines, preventing them to overcome parasitic attacks late in life.

Here, we tested for this possibility by mimicking bacterial infections early or/and late in life of females of the mealworm beetle, *Tenebrio molitor*, and assessed their consequences on female fecundity, late life post-reproduction survival and immunity. *T. molitor* is a cosmopolitan stored grain product pest that lives several months in populations of variable density. In this highly promiscuous insect, adult females are continuously receptive to reproduction from their fifth day post emergence and can mate many times with

several males within their five months of adulthood. *T. molitor* is iteroparous and an income breeder insect (Jönsson 1997). It feeds at both the larval and the adult stages and females lay eggs in several reproductive bouts along its lifespan (Dick 1937, Hughes 2017). These features possibly allow females to adjust their lifetime reproductive effort according to their physiological state and environment (Kasumovic et al. 2009). Usually, females can produce up to 30 eggs per day but egg production often start to decline after three weeks (Dick 1937). We previously found that the late reproductive strategy of females is not consistent with the terminal investment hypothesis, as females tended to restrain reproduction late in life in favour of longevity (Chapter 2). However, this study did not involve immune challenges that would mimic pathogenic infections either early or late in life, which would be relevant cues for survival threat.

In this study, individual *T. molitor* females were therefore immune challenged either early or/and late in life to examine the effect of immune investment and ageing on fecundity, late post-reproduction survival and immunity. *T. molitor* immunity relies on both constitutive and inducible innate mechanisms with cellular and humoral components (Vigneron et al. 2019). Haemocytes are circulating immune cells involved in parasite phagocytosis or encapsulation. *T. molitor* presents four main types of haemocytes that are morphologically and functionally distinct (Chung and Moon 2004, Urbański et al. 2018, Vigneron et al. 2019). The two most abundant ones are granulocytes, which are involved in phagocytosis, and plasmatocytes, involved in encapsulation. Oenocytoids are relatively rare cells producing enzymes of the melanisation cascades (see below), whereas prohaemocytes, slightly more abundant than oenocytoids, may function as precursors of haemocytes. The activity of these immune cells is at the core of the inflammatory response, as they may generate cytotoxic compounds (Nappi and Ottaviani 2000), causing self-damage (Sadd and Siva-Jothy 2006) and lifespan reduction (Pursall and Rolff 2011b, Daukšte et al. 2012, Krams et al. 2013, Khan et al. 2017). Phenoloxidase (PO) cascade is also a major compound of beetles' immune system, it catalyses the production of melanin (Zhao et al. 2011, Vigneron et al. 2019), phenoloxidase is described as a mechanism for pathogen immobilization and killing. However, the enzymatic cascade leading to melanisation is accompanied by the production of cytotoxic intermediates, such as phenols, quinones, and reactive oxygen species (Zhao et al. 2011, Vigneron et al. 2019). Thus, phenoloxidase activity is known to induce immunopathology in *T. molitor*, where early PO activation causes reduced longevity (Sadd and Siva-Jothy 2006, Khan et al. 2017). Finally, *T. molitor* immune response also induces

the production of antibacterial peptides in the haemolymph (Vigneron et al. 2019), an energetically costly process that may reduce survival (Moret and Schmid-Hempel 2000). Immunity was therefore assessed through the measurement of haemocytes counts, antibacterial activity and phenoloxidase activity. Because of ageing, female reproduction and immunity are predicted to decline with age. Moreover, [since](#) young females still have high residual reproductive value, we expect that an immune challenge occurring early in life should not trigger a reproductive shift toward an immune challenge (Duffield et al. 2017). However, as the immune response is costly, we predict that early challenged females would still exhibit reduced fecundity (Moreau et al. 2012) and longevity (Khan et al. 2017). By contrast, late in life, female residual reproductive value becomes lower and a late immune challenge is expected to trigger a terminal investment (Duffield et al. 2017) that would favour late reproductive investment at the expense of a reduced late post-reproductive immunity and survival.

2. Material and Methods

a. Insect culture and experimental design

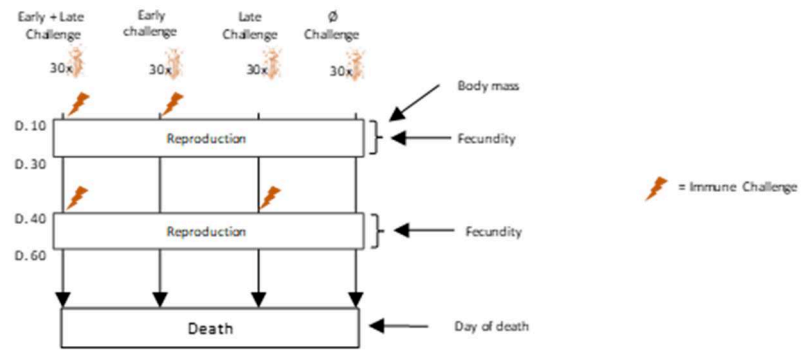
Virgin adult beetles of controlled age (10 ± 2 days post-emergence) were obtained from pupae, randomly sampled from a stock culture and maintained in standard laboratory conditions at Dijon, France. Prior to the experiments, all the experimental insects were maintained separately, at $24 \pm 1^\circ\text{C}$, 70% RH in permanent darkness, supplied with *ad libitum* bran flour and water, and supplemented once a week with apple.

The present experiments had two objectives. First, we assessed the influence of age-specific immune challenge on patterns of actuarial, reproductive and immune senescence. To this purpose, females were immune challenged either early (at 10 days old), late (at 40 days old) or both early and late in life and then assessed for their longevity, fecundity, and their immune parameters. Secondly, we assessed the immediate cost of a single immune challenge event on immune defences performed on young and old females, in order to explain the patterns of senescence previously observed. To address these two questions, we conducted two separated experiments, as it was impossible to measure together longevity, reproduction, and immune defences from the same individuals in a single experiment.

b. Experiment 1: effect of age-specific immune challenge on reproduction and longevity

The influence of early and/or late immune challenge on longevity and reproduction was estimated from four groups of 30 females of *T. molitor* experimentally immune challenged at different ages (Figure 15A). In a first group, females were immune challenged (see. 2.4) at 10 days post emergence. Females of the second group were immune challenged at 40 days post emergence. In the third group, females were immune challenged both at 10 and 40 days post emergence, while the last group of control females were never immune challenged. Females of all groups were allowed to reproduce during two periods of time in their life: between days 10 and 30 post emergence (early reproduction) and between days 40 and 60 post emergence (late reproduction). During each period of reproduction, isolated females were paired with a 10 ± 2 -day old virgin male for 10 days. Then, the male was replaced by a new 10 ± 2 -day old virgin male, with which the female spent 10 additional days. Couples were maintained in a Petri dish containing bleached flour and provided *ad libitum* food and water under standard laboratory conditions (see above). Female reproductive output was estimated by counting the number of eggs laid by the female every 3 or 4 days along the 20-day episode of reproduction. To this purpose, couples were transferred into a new Petri dish and eggs were searched by sieving the flour (600 μ m) and counted (Moreau et al. 2012). The body mass of females was measured to the nearest 1 mg using an OHAUS balance (discovery series, DU114C) prior they were paired with a new male. Outside the reproductive episodes, females were kept alone in a new Petri dish with *ab libitum* food and water. From the end of the specified late reproductive episode, females were checked twice a week until death.

A - Reproduction and longevity



B - Immune parameters

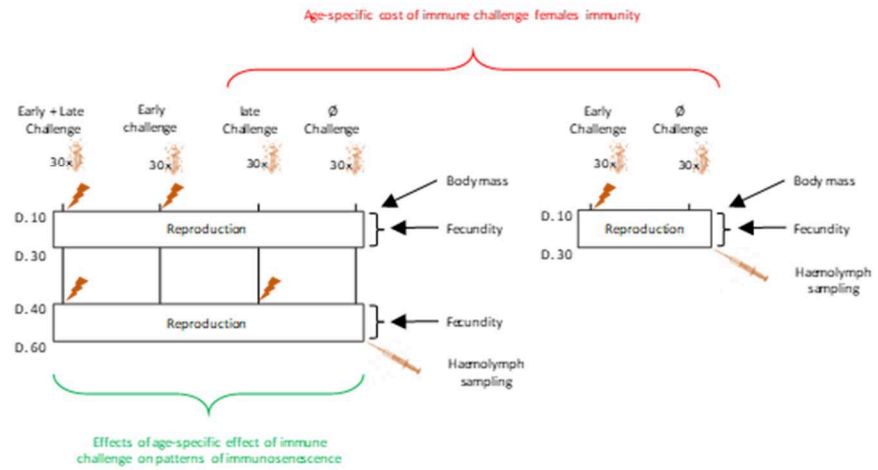


Figure 15 Experimental design to measure the effect of an immune challenge on (A) Reproduction and longevity, (B) Immune parameters

c. Experiment 2: Influence of age and immune challenge on immune parameters

The consequences of age and immune activity on *T. molitor* females' immunosenescence, was assessed on four groups of 20 females, treated as described above (immune-challenged early, late, both early and late, and not immune-challenged as control) (Figure 15B). Females were allowed to reproduce as previously described, and provided a sample of haemolymph immediately after their late reproduction episode (at 60 days post emergence), to estimate the concentration of the different types of circulating haemocytes in their haemolymph, the antibacterial activity and the activities of the prophenoloxidase system.

Two additional groups of 25 females were created, in which a sample of haemolymph at 30 days old was taken (that is immediately after their early reproductive episode). In one group, females were immune challenged early at 10 days post emergence, while in the second group they were not immune challenged. These two additional groups were created to test the effect of the immune challenge on immune parameters according to age when compared to the groups of old females that were immune-challenged late only (at 30 days post emergence) (Figure 15B).

d. Bacterial cultures for immune challenges

The bacterium *Bacillus cereus* used to perform the immune challenges was obtained from the Pasteur institute (CIP69.12). The bacteria were grown overnight at 28°C in liquid Broth medium (10 g/L bacto-tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7). Bacteria were then inactivated in 0.5% formaldehyde prepared in phosphate-buffered saline (PBS 10 mM, pH 7.4) for 30 minutes, rinsed three times in PBS, and their concentration adjusted to 10⁸ 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. Immune challenges were performed by injection through the pleural membrane between the second and the third abdominal tergites using sterile glass capillaries that had been pulled out to a fine point with an electrode puller (Narashige PC-10).

e. Haemolymph collection and immune parameters

Haemolymph collection was done as described by (Dhinaut et al. 2018a). Briefly, beetles were chilled on ice for 10 min before a first 3- μ L sample of haemolymph was collected from a wound made in the beetle's neck. The sample was flushed into a microcentrifuge tube containing 15 μ L of anticoagulant buffer (30 mM trisodium citrate, 26 mM citric acid, 15 mM sodium chloride, 20 mM EDTA, pH 4.6) (Lee et al. 1999). A 10- μ L subsample was rapidly used for haemocyte count using a Neubauer improved haemocytometer under a phase-contrast microscope (magnification x 400). The different haemocyte types found in *T. molitor* haemolymph were discriminated using the description made by Urbański et al. (2018). We especially measured the concentration of granulocytes, plasmatocytes and prohemocytes. The observation of oenocytoids was anecdotic in our study.

Immediately after the first sample of haemolymph, a second 3- μ L sample was collected from a new wound made in the female neck. This sample was flushed into a microcentrifuge tube containing 20 μ L of PBS. From this, 5- μ L were transferred in an N-phenylthiourea-coated microcentrifuge tube (P7629; Sigma-Aldrich, St Louis, MO, USA), flash-frozen in liquid nitrogen and then stored at -80 °C for later examination of its antibacterial activity. The remaining haemolymph solution (18 μ L) was also flash-frozen in liquid nitrogen and then stored at -80°C, waiting for the measurement of the activity of the prophenoloxidase system. Activity of the prophenoloxidase system was estimated through (i) the activity of naturally activated phenoloxidase (PO) enzyme only (hereafter PO activity) and (ii) the activity of PO plus that of the inactivated proenzymes (proPO) (hereafter total-PO activity) using the spectrophotometric assay described in (Zanchi et al. 2011). Total-PO activity quantification required the activation of proPO enzymes into active PO enzymes with chymotrypsin, whereas PO activity was measured directly from the sample. Frozen haemolymph samples were thawed on ice and centrifuged (3,500 g, 5 min, 4 °C). In a 96-well plate, 5 μ L of supernatant were diluted in 20 μ L of PBS and were added either 140 μ L of distilled water to measure PO activity or 140 μ L of 0.07 mg. mL⁻¹ chymotrypsin solution (Sigma-Aldrich, St Louis, MO, USA, C-7762) to measure Total-PO activity. Subsequently, 20 μ L of a 4 mg.mL⁻¹ L-Dopa solution (Sigma-Aldrich, St Louis, MO, USA, D-9628) were added to each well. The reaction proceeded for 40 min at 30 °C, in a microplate reader (Versamax; Molecular Devices, Sunnyval, CA, USA). Reads were taken every 15 s at 490 nm and analysed using the software SOFT-Max®Pro 4.0 (Molecular Devices, Sunnyvale, CA, USA). Enzymatic activity was measured as the slope (V_{max} value: change in absorbance unit per min) of the

reaction curve during the linear phase of the reaction and reported to the activity of 1 μL of pure haemolymph.

Antimicrobial activity of the haemolymph was measured using the inhibition zone assay described by Moret (2006). Briefly, haemolymph samples were thawed on ice, and 2 μL of the sample solution was used to measure antibacterial activity on zone 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.mL⁻¹. Six millilitres of this seeded medium were subsequently poured per Petri dish. After solidification, sample wells were made using a Pasteur pipette fitted with a ball pump. Two microliters of sample were deposited in each well. Plates were then incubated overnight at 28°C and the diameter of each zone of inhibition was measured.

f. Statistics

Longevity was analysed as a function of the immune challenge (occurring early or/and late in life) and initial body mass (at 10 days old) using Cox regressions. The best statistical model was selected using a backward stepwise procedure from full models testing the main effect and two-way interactions between all categorical explanatory factors but the effect of continuous covariables, such as insect body mass was tested as a main effect only.

Individual variation in fecundity (number of eggs laid by females) was analysed as a function of immune challenge (occurring early or/and late in life) and initial body mass using an analysis of variance for repeated measured.

Haemocyte concentration (global or population specific), PO activity, total PO activity and antibacterial activity of the haemolymph were analysed as function of immune challenge (occurring early or/and late in life), age and initial body mass using a multivariate analysis of variance (MANOVA). Data on haemocyte counts and activities of the prophenoloxidase system were standardized to z scores using the rank inverse-normal transformation procedure from (Templeton 2011), whereas data on antibacterial activity were log-transformed to satisfy the conditions of use of parametric tests. All the analyses used IBM® SPSS® statistics 19 for Macintosh.

3. Results

a. Reproduction

Females were immune-challenged with the bacterium *B. cereus* early in life, late, both early and late or not challenged at all. They were then allowed to reproduce twice: early (from 10 to 30 days old) and late (from 40 to 60 days old) in life. Females laid fewer eggs when they were immune-challenged early in life (Table 6, Figure 16). The late immune challenge never affected females fecundity, whether they were previously exposed to an early challenge or not (Table 6, Figure 16).

As previously observed (in Chapter 2), females laid fewer eggs late than early in life. However, fecundity variation from the early reproductive event to the late one depended on the early immune challenge (Table 6). This mainly results from the fact that early-challenged females laid fewer eggs during their early reproductive episode, which reduced the difference between early and late female fecundity (Figure 16).

Female late fecundity was positively correlated to late post reproduction survival (in days) (ANOVA Global model $F_{6, 58} = 5.17$ $P = 0.026$, late post reproduction survival $F_{1, 58} = 8.12$ $P = 0.006$), demonstrating that females that had the shortest post reproductive survival were also those exhibiting the lowest fecundity late in life (Figure 17). Besides, variation in female late fecundity could not be further explained by the immune challenges (early challenge $F_{1, 58} = 0.10$ $P = 0.757$, late challenge $F_{1, 58} = 2.73$ $P = 0.104$), female body mass (early body mass $F_{1, 58} = 1.98$ $P = 0.165$, late body mass $F_{1, 58} = 0.84$ $P = 0.362$) and female early fecundity ($F_{1, 58} = 2.10$ $P = 0.153$).

Table 6 Female production of eggs early and late in life (Reproductive event) as a function of their initial body mass as a continuous covariate and their experience of an immune challenge occurring early and/or late in life as fixed factors. Values $P \leq 0.05$ are given in bold.

Source	d.f.	F	<i>P</i>
<i>Between subjects</i>			
Early challenge	1	15.55	< 0.001
Late challenge	1	2.43	0.120
Error	237		
<i>Within subjects</i>			
Reproductive event	1	695.66	< 0.001
Reproductive event * Early challenge	1	27.21	< 0.001
Reproductive event * Late challenge	1	1.02	0.313
Error	237		

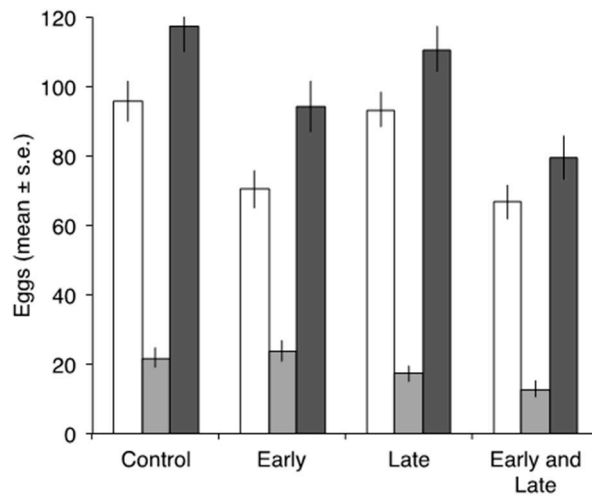


Figure 16 Mean number of eggs (\pm s.e.) produced by females early in life (white bars), late in life (light grey bars) and in total (dark grey bars) as a function of their immune treatment when not immune challenged (Control), immune challenged early in life only (Early), late in life only (Late) and both early and late in life (Early and Late) . Sample size is 60 females in each modality.

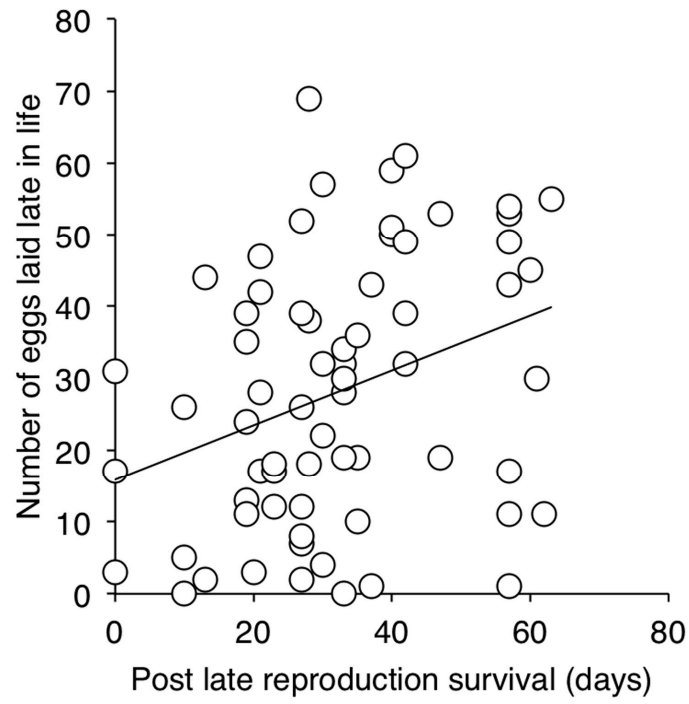


Figure 17 Relationship between female late fecundity (number of eggs) and female post late reproduction survival in days ($y = 0.38x + 15.83$, $r = 0.32$).

b. Longevity

Whether the immune challenge occurred early or late in life, it had no effect on female longevity either as a main effect or in interaction (Cox Regression analysis: early challenge Score = 0.10, df = 1, P= 0.749, late challenge Score = 0.18, df = 1, P= 0.676, early x late challenge Score = 0.67, df = 1, P= 0.413). Similarly female body mass could not explain any variation of longevity (Score = 0.53, df = 1, P= 0.468).

c. Immune parameters

i. Influence of age specific immune challenges on old female immunity

The multivariate analysis revealed that immune parameters of females assayed late in life (60 days old) were, in general, significantly affected by the late immune challenge as a main effect or in interaction with the early immune challenge (Table 7a). Such a variation was mainly driven by PO activity and antibacterial activity (Table 7). Indeed PO activity was dependent on the interaction between the early and the late immune challenge. Females that experienced a single immune challenge early in life exhibited the lowest PO activity, whereas those that experienced a single late immune challenge or both an early and a late one exhibited similar PO activity than control females (Figure 18a). Female antibacterial activity was marginally increased by the early immune challenge and significantly increased by the late immune challenge (Table 7g, Figure 18a).

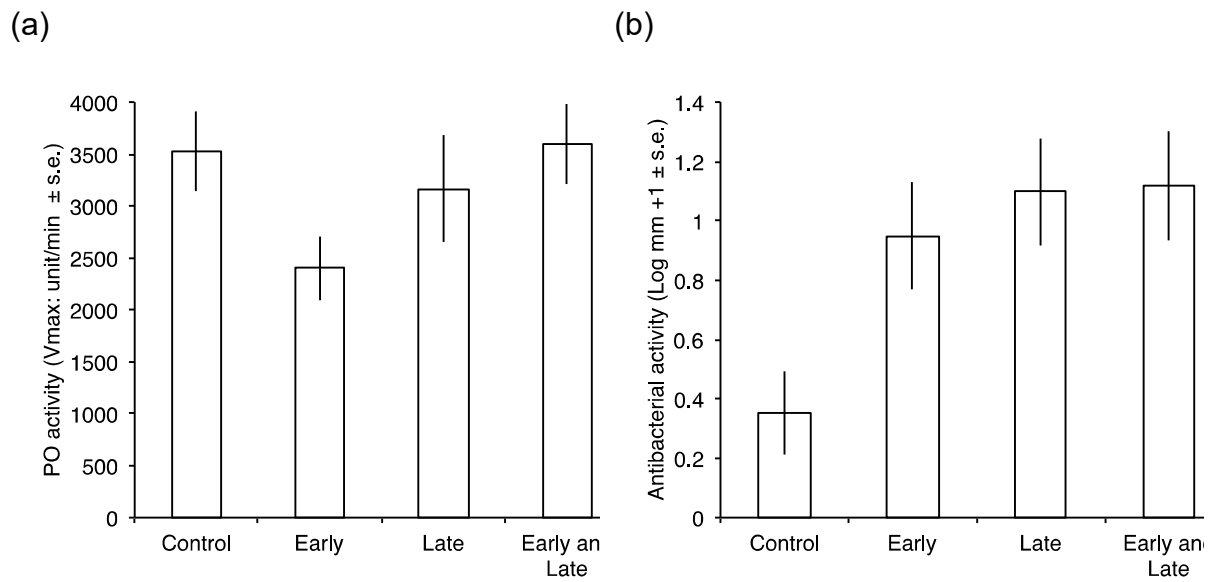


Figure 18 PO activity (a) and antibacterial activity (b) in old females (60 days old) that were either unchallenged (Control, N = 20) immune challenged early in life (Early, N =20) late in life (Late, N = 19) or both early and late in life (Early and Late, N = 17).

Table 7 Multivariate analysis of variance for immune parameters of *Tenebrio molitor* females assessed late in life (60 days old) as a function of their immune treatment early and late in life. The multivariate test is shown in (a) and the univariate tests for total haemocyte count, granulocyte count, plasmatocyte count, PO activity, Total-PO activity and antibacterial activity in (b), (c), (d), (e), (f) and (g), respectively. Values $P \leq 0.05$ are given in bold.

Source of variation	Factor d.f.	Error d.f.	F	P
(a) MANOVA (Pillai's trace)				
Early Challenge	6	61	0.68	0.668
Late Challenge	6	61	2.94	0.014
Early*Late Challenge	6	61	2.81	0.018
(b) ANOVA Total haemocyte				
Global Model	3	66	0.98	0.408
Early Challenge	1	66	0.08	0.785
Late Challenge	1	66	2.86	0.095
Early*Late Challenge	1	66	0.05	0.818
(c) ANOVA Granulocyte				
Global Model	3	66	0.66	0.579
Early Challenge	1	66	0.03	0.867
Late Challenge	1	66	1.93	0.169
Early*Late Challenge	1	66	0.004	0.949
(d) ANOVA Plasmatocyte				
Global Model	3	66	2.44	0.072
Early Challenge	1	66	0.43	0.514
Late Challenge	1	66	3.81	0.055
Early*Late Challenge	1	66	3.68	0.059
(e) ANOVA PO activity				
Global Model	3	66	3.11	0.032
Early Challenge	1	66	0.21	0.651
Late Challenge	1	66	0.58	0.449
Early*Late Challenge	1	66	8.95	0.004
(f) ANOVA Total PO activity				
Global Model	3	66	0.93	0.430
Early Challenge	1	66	0.13	0.723
Late Challenge	1	66	2.15	0.148
Early*Late Challenge	1	66	0.71	0.403
(g) ANOVA antibacterial activity				
Global Model	3	66	5.49	0.002
Early Challenge	1	66	3.62	0.062
Late Challenge	1	66	10.71	0.002
Early*Late Challenge	1	66	1.33	0.254

ii. Age-specific immunity

The multivariate analysis revealed that female immune parameters were overall affected by age and the immune challenge in an additive manner (Table 8a). Such a difference mainly resulted from variation in total haemocyte count, granulocyte count, PO activity and antibacterial activity (Table 8). Total haemocyte and granulocyte counts were found lower in old females than in young ones, but unaffected by the immune challenge (Table 8b, c; Figure 19a,b). As granulocytes account for the largest proportion of haemocytes in the total haemocyte count (Overall 87 %), their variation likely caused that of total haemocytes. By contrast to haemocyte count, PO activity was higher in old females than in young ones (Table 8f, Figure 19c). Overall, the immune challenge did not affect female PO activity (Table 8f). Antibacterial activity of the haemolymph was not affected by age but by the immune challenge, which significantly enhanced its activity in both young and old females (Table 8h, Figure 19d). Finally, in this experiment, too few prohemocytes could be counted in old females and it was therefore not possible to analyse how their count was affected by age and the immune challenges.

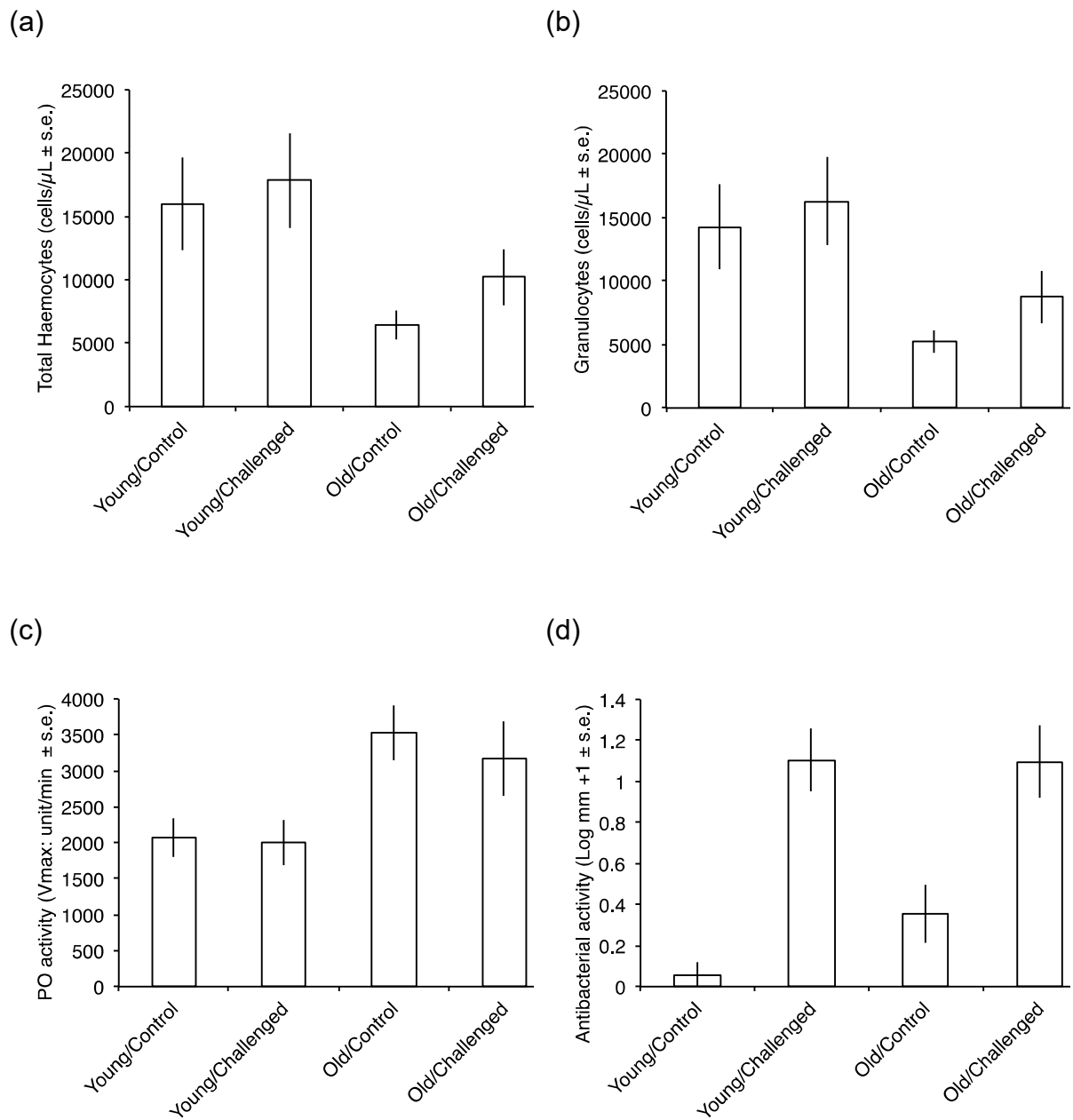


Figure 19 Total haemocyte counts (a), Granulocytes counts (b), PO activity (c) and antibacterial activity (d) in young (30 days old) and old (60 days old) females that were either unchallenged (Control, N = 20) or immune challenged (challenged) prior to their early or late reproductive period, respectively. Sample size: 24 young/control, 26 young/challenged, 20 old/control and 19 old/challenged females.

Table 8 Multivariate analysis of variance for immune parameters of *Tenebrio molitor* females as a function of their age and their age-specific immune treatment. The multivariate test is shown in (a) and the univariate tests for total haemocyte count, granulocyte count, plasmatocyte count, [prohaemocytes count], PO activity, Total-PO activity and antibacterial activity in (b), (c), (d), (e), (f), (g) and (h), respectively. Values $P \leq 0.05$ are given in bold.

Source of variation	Factor d.f.	Error d.f.	F	P
(a) MANOVA (Pillai's trace)				
Age	7	75	4.01	0.001
Challenge	7	75	7.46	< 0.001
(b) ANOVA Total haemocyte				
Global Model	2	81	3.94	0.023
Age	1	81	7.02	0.010
Challenge	1	81	0.63	0.429
(c) ANOVA Granulocyte				
Global Model	2	81	4.61	0.013
Age	1	81	8.24	0.005
Challenge	1	81	0.71	0.403
(d) ANOVA Plasmatocyte				
Global Model	2	81	0.07	0.931
Age	1	81	0.14	0.711
Challenge	1	81	0.003	0.956
(f) ANOVA PO activity				
Global Model	2	81	5.24	0.007
Age	1	81	8.38	0.005
Challenge	1	81	1.70	0.196
(g) ANOVA Total PO activity				
Global Model	2	81	1.85	0.163
Age	1	81	2.77	0.100
Challenge	1	81	1.11	0.296
(h) ANOVA antibacterial activity				
Global Model	2	81	21.03	< 0.001
Age	1	81	1.60	0.210
Challenge	1	81	41.18	< 0.001

4. Discussion

In this work, we have studied the effects of immune challenge and age on life history traits of *T. molitor* females. First, we have measured the consequences of age-specific immune challenge on reproduction and longevity. We were able to show that females' longevity was never affected by either early or late immune challenge. The immune function is costly for organisms (Moret 2003) and could cause self-damage (named immunopathology) (Sadd and Siva-Jothy 2006), which is expected to reduce longevity (Urabe et al. 1994, Pursall and Rolff 2011b). Previous studies found that the immune response causes self-damages in *T. molitor* (Sadd and Siva-Jothy 2006, Khan et al. 2017) and, in contrast to our study, showed that females exposed to an early immune challenge suffered from a reduced longevity (Khan et al. 2017). These contrasting results might have, at least, two main causes in addition of many other small differences. First, Khan et al. (2017) did not allow females to reproduce, whereas our experiment allowed all the females to reproduce early and late in life. We previously found that reproduction significantly stimulates immune activity in *T. molitor* females (Chapter 2), which may have buffered the effect of the immune challenge in our experiment. Second, Khan et al. (2017) kept their insects in a rather suboptimal temperature condition (30°C) compare to the optimum range of temperature of 25-28°C usually used to grow *T. molitor* (Punzo 1975, Ribeiro et al. 2018) and that we have used in the present study. Actually, in Khan et al. (2017) study, control (non-immune challenged) females of *T. molitor* showed a 90th percentile survival time of 83.1 days, whereas in our study it is 112 days. Such a difference suggests that in their study, females were maintained under stressful conditions, allowing the expression of the cost of an early immune activation on longevity in *T. molitor* females. In our study, rearing conditions were probably too favourable to highlight such a cost. Despite this, it also shows that under favourable conditions *T. molitor* females are able to compensate the cost of immunopathology on longevity.

However, even in our optimal conditions, the immune challenge we imposed to our animals had an effect on reproduction. In fact, early immune-challenged females exhibited a reduced fecundity early in life, while a late immune challenge had no impact on the following female's clutch size. In case of double immune challenge (both early and late), the slight decrease in fecundity compared to a single early immune challenge was not significant, highlighting the importance of the cost of the immune challenge early in life. *T. molitor* females are continuously sexually receptive during their adulthood, but their fecundity is known to reduce with age (see Chapter 2, Dick 1937, Drnevich et al. 2001).

Thus, late reproduction could be too weak to detect any effect of cumulated late immune challenges on late fecundity.

Beyond the effect of these immune challenges, female late reproduction was impacted by another factor. Females that exhibited the shortest post reproductive survival also had the lowest late fecundity, independently of their immune treatment. This result strongly suggests that females *T. molitor* did not perform terminal investment. Otherwise, females close to death would have produced the highest clutch-size, which is clearly not the case (Figure 3). In Chapter 2, we have observed the absence of such a terminal investment but in those conditions, there was no terminal investment trigger (Duffield et al. 2017). Here, we have used *B. cereus*, recognized as an important natural pathogen of *T. molitor* (Du and Laing 2011, Jurat-Fuentes and Jackson 2012), to challenge the females' immune system. Such an immune challenge was expected to mimic a systemic infection by the bacterium, representing a significant cues predicting imminent death. Chapter 2 showed that 90% of older females infected by *B. cereus* died in less than three days. Here, the use of *B. cereus* immune challenge did not trigger terminal investment in old females. Either this result confirms the absence of terminal investment, as observed in Chapter 2, or the way the immune challenge was performed was probably too artificial to accurately mimic a deadly infection by *B. cereus*. Indeed, the natural route of infection by bacilli is often oral and host immune responses can greatly vary according to the route of infection (Behrens et al. 2014), as host adaptation can be contingent to the infection route taken by pathogens (Martins et al. 2013). Future study may need to performed immune challenges with the appropriate route of infection to accurately mimic the potential trigger that would induce terminal investment.

The effect of an immune challenge at various ages on the immune parameters in old females showed only weak effects. We found no effect of these challenges on haemocyte numbers. However, females that experienced only an early challenge had a decreased PO activity, while those that experienced late challenges (with or without an early challenge) showed an increased antibacterial activity. Since all these old females experienced the same reproductive bouts (they reproduced twice), this factor cannot explain these results. The effect on PO activity is not straightforward to explain: how can an early challenge maintain a low PO activity for the whole female life, while the addition of a late immune challenge would reset its level to that of control females? Only the examination of variation

in PO activity between young and old females may help to provide an interpretation (see below). Variation in antimicrobial activity is much easier to explain: immune challenges, and particularly the late one, increase antimicrobial activity compared to control females. Such a phenomenon is compatible with an investment to somatic maintenance, confirming again the allocation priority scheme in favour of survival (Chapter 2). It should be noted, however, that the level of antimicrobial activity found in old females that experienced a single immune challenge early in life is relatively high. It means that these old females are still protected by the antibacterial activity resulting from a bacterial immune challenge occurring 50 days earlier, which is twice the time than what was previously observed by (Haine et al. 2008a) and is in line with some observations in dragonflies (Bulet et al. 1992).

Some of the immune parameters varied with age of the females. Indeed, independently of the immune challenge, haemocyte concentration of the female beetles was lower in old females compared to young ones. In Chapter 2, we showed that reproduction of *T. molitor* females is costly, especially when they reproduce twice, and that reproduction is traded off with immunity. Here, females exhibited a reduced number of haemocytes late in life, even those exposed to an immune challenge. In fact, the examination of figures 5a and 5b reveals that the slight increase of haemocyte and granulocyte counts observed in immune-challenged females was much weaker than the decrease of these immune parameters due to ageing, explaining the non-significant effect of the immune challenge. Similarly, the study of the phenoloxidase system also shows that immunity of *T. molitor* varies with age. PO activity was higher in 60-day-old females than in 30-day-old ones, whether they were immune challenged or not. PO can cause severe damages to host tissues and organs by producing reactive and cytotoxic intermediates (Sadd and Siva-Jothy 2006, Zhao et al. 2011, Khan et al. 2017). The high PO activity observed in older females may result from a deregulation of the immune function (Khan et al. 2016, 2017). This is in line with our previous observation, in Chapter 2, that older females were more susceptible to a bacterial infection than young ones. Conversely, antibacterial activity in *T. molitor* females' haemolymph was affected by the immune challenge, but not by age. Both old and young beetles exhibited a similar level of antibacterial activity 30 days after being challenged, whereas that of control females was very low. As observed in Chapter 2, antibacterial activity seems not affected by age, which could be explained by the fact that this defence does not cause immunopathology (Moret 2003) and is therefore less costly to maintain with age.

Such a difference in maintenance of different immune pathways with age may be an explanation for the low level of PO activity found in old females that have been immune challenged early in life (see above, Figure 4a). Immune challenge is known to activate both “constitutive” defences that rely on haemocytes and several rapidly activated enzyme cascades such as that of the phenoloxidase system, and “induced” defences, which consist of a slower production of a suite of antimicrobial peptides. This inducible production of antibacterial peptides takes several hours to reach peak levels and persists for weeks in a variety of insects (Haine et al. 2008). As mentioned above, PO activity causes immunopathology and is expected to be as reduced as possible after an acute immune response, to avoid the further accumulation of self-damages (Zhao et al. 2011). Young females appear to be able to down regulate PO activity to low levels, while maintaining high antibacterial activity. By contrast, old females seem unable to down regulate PO activity, even when unchallenged (Figure 5c). However, the exception is found in old females that experienced an early bacterial challenge. We propose that females can reduce costly PO-activity-based defences, leading to low levels of PO activity in old age, because they could maintain long-lasting antimicrobial defences as a result of the immune challenge early in life (Figure 4a). Such a shift between immune defences components, allowing to reduce the cost of the immune response (Moret 2003) has been often observed in other insects (eg., Rantala and Roff 2005, Moret and Schmid - Hempel 2009, Vogelweith et al. 2015). These results would therefore support the idea of an age-dependent trade-off between investment in immune function and prevention of its immunopathological costs.

Overall, our study therefore showed that a bacterial challenge early in life decreases female’s fecundity (mainly by decreasing the intensity of early reproduction), and may possibly induce a shift between immune pathways (allowing a decrease of the costly activity of the PO-based immune system). A bacterial challenge later in life has almost no effect. No effect at all was found on survival. We also confirmed that, beyond any immune challenge, age affects fecundity and several immune parameters (lower haemocyte counts, higher levels of the self-arming PO activity, etc.).

Altogether, our results on longevity and reproduction confirm the occurrence of an allocation priority scheme (Rigby and Moret 2000). Indeed, we showed that, while investing in immune function early in life, *T. molitor* females restrained their reproduction and maintained their longevity. Moreover, while being immune challenged late in life, instead of

a performing terminal investment, *T. molitor* females maintained their longevity, as observed in Chapter 2 females *T. molitor* life history strategy seems to rely on reproductive restraint hypothesis.

Discussion

DISCUSSION

Le but de cette thèse était d'étudier les relations entre reproduction, immunité et longévité dans le contexte de l'étude de l'évolution de la sénescence. Pour cela, nous avons utilisé le ténébrion meunier (*Tenebrio molitor*) comme modèle biologique car c'est un insecte relativement longévif avec une reproduction quasi continue au cours de la vie adulte, basée sur des accouplements multiples impliquant plusieurs partenaires sexuels (promiscuité sexuelle) et facilement quantifiable. Sa fertilité dépend principalement des ressources acquises au cours de la vie adulte (« income breeder »), contrairement aux insectes qui stockent les ressources nécessaires à la reproduction au cours des stades larvaires (« capital breeder ») (Tigreros and Davidowitz 2019). Ainsi, il est relativement aisé d'observer les conséquences des coûts de l'histoire de vie sur la reproduction. Par ailleurs, les aspects de son immunité ont été bien documentés que ce soit en rapport avec la reproduction (Rolff and Siva-Jothy 2002), la longévité (Krams et al. 2013, Khan et al. 2017) ou l'immunosénescence (Daukšte et al. 2012). Nous avons d'abord étudié l'influence de l'intensité de l'investissement dans la reproduction sur la sénescence chez cet insecte. Le chapitre 1 consistait en une étude au niveau populationnel portant sur les deux sexes, alors que le chapitre 2 a étudié seulement les femelles et à un niveau individuel. Le troisième chapitre a porté sur l'étude des conséquences d'un investissement âge-spécifique à la fonction immunitaire sur la sénescence des femelles *T. molitor*. Nous avons mis en évidence au sein de ces différentes études des patrons de sénescence actuarielle, reproductive et immunitaire chez *T. molitor*. Nous avons par ailleurs montré que, dans des conditions de vie favorables, les femelles allouent leurs ressources selon un schéma de priorité (« priority scheme ») qui favorise la longévité au détriment de la reproduction.

Dans un premier temps, je discuterai les patrons de sénescence observés sans manipulation particulière, c'est-à-dire dans les conditions « contrôles » de notre première expérience populationnelle. En effet, les patrons de sénescence intrinsèque de *T. molitor* n'avaient encore jamais été décrits. Dans un second temps, les résultats de nos manipulations sur les patrons de sénescence des femelles seront discutés, en commençant par l'étude des conséquences de l'investissement dans la reproduction, puis dans la fonction immunitaire, en mettant l'accent sur l'existence du schéma de priorité. Enfin, les conséquences de la reproduction sur la sénescence chez les mâles seront discutées.

1. Sénescence intrinsèque chez *Tenebrio molitor*

Dans nos travaux, la durée de vie adulte de *T. molitor* a varié entre 9 à 13 semaines. Elle était donc comparable à celle observée dans des travaux précédents (de 8,5 à 15 semaines environ) visant à améliorer les conditions de maintien de cette espèce (Urs and Hopkins 1973). Dans les conditions de maintien correspondant aux conditions qui seraient *a priori* les plus proches des conditions naturelles (chapitre 1, condition de sexe ratio équilibré), nous avons observé que la durée de vie des adultes de *T. molitor* est d'environ 9,5 semaines et que la longévité des mâles et des femelles était similaire. Chez de nombreuses espèces animales, la longévité des mâles et des femelles diffère (Austad and Fischer 2016). En général, une meilleure longévité est observée chez les femelles. Les mâles sont en théorie soumis plus fortement à la sélection intra-sexuelle que les femelles, ce qui expliquerait une mortalité adulte plus élevée et une sénescence plus marquée par rapport aux femelles (Trivers 1972, Promislow 1992). En effet, pour assurer leur succès reproducteur dans un environnement compétitif pour l'accès aux femelles, les mâles allouent souvent une grande partie de leur ressources énergétiques dans des caractères coûteux pouvant aller d'une masse corporelle ou capacité athlétique plus élevées, des caractères sexuels extravagants ou des comportements agressifs vis-à-vis des autres mâles (Trivers 1972, Clutton-Brock 2007). Chez *T. molitor*, le mâle présente une taille et des capacités athlétiques comparables à celles des femelles, il n'a apparemment pas développé de caractères sexuels extravagants et n'affiche pas un comportement très agressif vis-à-vis des autres mâles. Les mâles de *T. molitor* ne semblent également pas avoir développé un comportement spécifique de protection post-copulatoire des partenaires comme ceux observés chez d'autres insectes (Drnevich 2003). Le seul comportement en rapport avec la compétition sexuelle observé chez les mâles de *T. molitor* est un court gardiennage post-copulatoire plutôt passif, qui consiste pour un mâle à rester à moins de un cm de la femelle pendant plus d'une minute en présence de concurrents (Carazo et al. 2007). Par ailleurs, durant l'accouplement, les mâles transfèrent aux femelles un spermatophore qui contient un cadeau nuptial. La production de ce spermatophore est coûteux, sa production empêche les mâles de copuler pendant les vingt minutes qui suivent l'accouplement (Carver et al. 1999, Drnevich 2003). Le gardiennage post-copulatoire d'une minute suivant l'accouplement n'est donc pas très contraignant pour les mâles car il se déroule à un moment où ils ne sont pas aptes à se reproduire.

D'après les résultats du chapitre 1, les mâles ne semblent pas souffrir de sénescence reproductive, leur fécondité étant quasiment constante au cours de leur vie. Cette fécondité

constante est un argument de plus justifiant que les mâles maximisent la fitness *via* une meilleure longévité. Cependant, une autre étude montre que les mâles âgés de *T. molitor* produisent des descendants avec une masse à l'émergence plus faible et une proportion de descendants malformés plus importante (Carazo et al. 2011). Ceci suggère que même si les mâles âgés maintiennent leur fécondité en termes de quantité (le nombre de larves produites par les femelles qu'ils fécondent), ils souffrent d'une sénescence reproductive en termes de qualité de leur descendance. Ces paramètres n'ont pas été mesurés dans nos travaux, aussi cela paraît intéressant de les prendre en compte à l'avenir, car ils peuvent permettre de mettre en évidence une sénescence reproductive en termes de qualité de la descendance et non pas en termes de quantité.

Cependant, contrairement à ce qui est classiquement prédit chez les mâles, chez *T. molitor*, nous avons montré que c'est la copulation et non la compétition sexuelle qui semble être responsable des principaux coûts de la reproduction. Le coût de la copulation et la période réfractaire à laquelle ils sont ensuite contraints induisent que les mâles n'ont que peu d'intérêt à entrer en compétition pour maximiser leur taux d'accouplement. Ceci a pour conséquence que les mâles favorisent leur longévité afin de maximiser leur fitness, comme cela est observé chez les femelles. Par conséquent, cela empêche l'évolution de patrons divergents de sénescence actuarielle entre mâles et femelles.

Dans les différents chapitres de la thèse, nous avons observé que les femelles *T. molitor* souffraient de sénescence reproductive après trois semaines de vie adulte. Cette observation est en accord avec les données historiques sur le ténébrion meunier : les femelles commencent à se reproduire dès la maturité sexuelle et un déclin des performances reproductives se manifeste assez tôt (Dick 1937, Drnevich et al. 2001). Ce déclin de la fécondité des femelles avec l'âge peut être interprété soit comme du à une sénescence reproductive intrinsèque et/ou un compromis entre une reproduction précoce et tardive. Dans le Chapitre 2, nous avons observé que ce déclin de reproduction apparaissait même chez les femelles qui ne se reproduisent pas durant les trois premières semaines de leur vie adulte. Cette réduction de fécondité représente donc bien un phénomène de sénescence intrinsèque, indépendant du coût de la reproduction. De plus, cela suggère que les premières semaines de reproduction sont cruciales pour la fitness des femelles car au cours de cette période, elles ont une fécondité bien supérieure à celle du reste de leur vie. Il leur sera donc difficile de compenser cette perte de fécondité si elles ne produisent pas d'œufs à ce moment là. Ce type de patron de sénescence a déjà été observé

chez d'autres espèces d'insectes, tel que la blatte cendrée d'Afrique (*Nauphoeta cinerea*) ou le lépidoptère *Rhopobota naevana* (Moore and Moore 2001, Moore and Sharma 2005, Fitzpatrick 2006). La diminution de la fécondité avec l'âge serait due à la dégradation progressive de la qualité des ovocytes : en effet, avec l'âge, les cellules sexuelles entrent en apoptose et les femelles ne peuvent pas maintenir la même proportion d'ovocytes viables (Moore and Sharma 2005). Par conséquent, étudier la dynamique de la maturation des ovocytes des femelles de *T. molitor* et leur maintien au cours du vieillissement pourrait être intéressant afin d'essayer de comprendre le mécanisme sous-jacent à la sénescence reproductive que nous avons observé.

2. Reproduction, immunité et sénescence chez les femelles

a. Reproduction et sénescence

Dans les chapitres 1 et 2, nous avons modulé l'intensité ou le rythme de l'investissement des femelles à la reproduction, afin d'appréhender leurs conséquences sur la sénescence. Ces expériences nous ont notamment permis de tester la théorie du soma jetable chez *T. molitor*. Cette théorie postule que la reproduction est une fonction coûteuse et prédit qu'un fort investissement à la reproduction tôt dans la vie entraîne une diminution de l'investissement dans les traits liés à la maintenance du soma (réparation de l'ADN, immunité...) et que c'est ce manque de maintenance qui entrainerait la sénescence (Kirkwood and Rose 1991, Kirkwood and Austad 2000).

Ces deux études ont montré des résultats contrastés sur les liens entre reproduction et sénescence chez les femelles de *T. molitor*. Dans la première étude, nous avons modifié l'effort reproducteur des femelles et des mâles de *T. molitor* en manipulant le sex-ratio de populations artificielles. Ceci nous a permis de montrer qu'un effort reproducteur accru chez les femelles *T. molitor* entraîne une plus forte réduction de la fertilité avec l'âge mais surtout une accélération de la mortalité avec l'âge (sénescence actuarielle). Ces résultats sont donc en accord avec les prédictions de la théorie du soma jetable (Kirkwood and Rose 1991). Ils montrent que la reproduction est coûteuse et compromet la reproduction future ainsi que la longévité, comme observé chez les vertébrés (Lemaître et al. 2015) ou chez la drosophile (Sgrò and Partridge 1999, Salmon et al. 2001). Les résultats du chapitre 2 ne confortent cependant que partiellement cette observation. En effet, s'ils montrent bien qu'un investissement précoce à la reproduction des femelles entraîne une réduction de leur fertilité

future, ils ne montrent pas de réduction de la longévité. Le contraste entre ces deux études suggère que, dans les conditions expérimentales de l'étude du chapitre 2, soit la reproduction était moins coûteuse pour les femelles, soit les conditions expérimentales ont permis aux femelles de compenser ce coût (les deux n'étant pas exclusifs). D'une part, par rapport au maintien individuel des femelles dans l'étude du chapitre 2, le maintien des insectes en populations artificielles dans l'étude du chapitre 1 constituait des conditions de vie plus contraignantes (bien que plus naturelles), exposant ces derniers à la compétition pour l'eau, les ressources nutritives et les ressources reproductives (partenaires, sites de ponte etc.). D'autre part, le maintien des insectes en populations artificielles a certainement exposé les femelles, leur vie durant, à un plus fort taux de harcèlement par les mâles et d'accouplements multiples que dans les suivis individuels. Ceci a du représenter des coûts reproductifs supplémentaires pour les femelles, par rapport aux conditions de maintien individuel, dans lesquelles les femelles étaient exposées à un seul mâle à la fois et pendant des périodes relativement courtes. Si ces différences de protocoles expérimentaux expliquent probablement pourquoi les femelles de l'étude du chapitre 1 ont payé un tribut plus lourd à la reproduction que celles de l'étude du chapitre 2, elles permettent aussi de mettre en évidence une priorisation des traits d'histoire de vie affectés par le coût de la reproduction.

En effet, la comparaison des résultats de ces deux chapitres montre que dans des conditions favorables (Chapitre 2), les femelles *T. molitor* investissent fortement à la reproduction actuelle sacrifient d'abord la reproduction future plutôt que la longévité. Par contre, dans les conditions plus contraignantes de l'étude du chapitre 1, la reproduction future et la longévité se retrouvent toutes les deux contraintes. Ceci suggère l'existence d'un schéma de priorité dans lequel les femelles priorisent la longévité plutôt que la reproduction. Nous y reviendrons plus en détails ultérieurement.

Par ailleurs, l'accélération de la sénescence observée en cas de fort investissement à la reproduction tôt dans la vie fournit un bon appui à la théorie du soma jetable. En effet, cette théorie postule que le coût de la reproduction entraîne un compromis entre investissements à la reproduction tôt dans la vie et reproduction tardive ou longévité, et que ce compromis serait dû à une diminution de l'investissement dans les traits liés à la maintenance de l'organisme (Kirkwood and Rose 1991, Kirkwood and Austad 2000). Dans nos expériences, les femelles produisant l'effort reproducteur le plus important meurent plus rapidement que les autres (expérience populationnelle) ou voient leur fécondité diminuer plus intensément au cours du vieillissement (expériences populationnelles et individuelles). On peut donc

proposer que ces patrons de sénescence accrus sont dus aux dommages causés par le fort métabolisme induit par cet investissement important dans la reproduction. Pour compléter nos observations et aller plus loin dans la validation de cette théorie, il serait pertinent de mesurer les dommages oxydatifs accumulés par les femelles au cours de leur vie, en fonction de leur effort reproducteur.

b. Immunité et sénescence

Dans le chapitre 3 nous avons étudié les conséquences d'un investissement âge-spécifique dans la fonction immunitaire sur la sénescence des femelles *T. molitor*. La fonction immunitaire est connue pour être coûteuse pour les organismes, un investissement dans cette dernière pouvant engendrer des trade-offs avec d'autres traits d'histoire de vie tels que la longévité ou la reproduction (Moret and Schmid-Hempel 2000, Schwenke et al. 2016). De plus, certains processus mis en place lors de la réponse immunitaire entraînent des dommages à l'organisme lui-même (Urabe et al. 1994, Nappi and Ottaviani 2000, Zhao et al. 2011). Ces dommages auto-immuns ont été décrits chez différentes espèces et notamment chez *T. molitor* (Sadd and Siva-Jothy 2006, Khan et al. 2017). Ces études ont montré des réductions de la longévité consécutive à une activation du système immunitaire tôt dans la vie des insectes (principalement dues à l'effet de la phénoloxydase). L'étude réalisée dans le chapitre 3 avait pour objectif de compléter ces résultats, en ajoutant la reproduction comme composante étudiée. En effet, dans les études précédemment citées, les femelles étudiées étaient restées vierges toute leur vie. Notre étude a permis de confirmer qu'une activation du système immunitaire tôt dans la vie était coûteuse pour les femelles, mais seulement en termes de reproduction et non de longévité, contrairement aux études précédentes. Une nouvelle fois, on peut proposer que nos conditions expérimentales (à savoir : femelles isolées, nourries *ad libitum* et à température optimale 25 ± 1 °C) ont été très favorables pour les femelles et donc leur ont permis de compenser une partie du coût imposé par la stimulation du système immunitaire. Au contraire, dans les études précédentes, les conditions expérimentales présentaient une température d'élevage des insectes supérieure à la température optimale (29° C). C'est certainement cette température stressante qui avait permis de mettre en évidence le compromis physiologique entre immunité et longévité. Il serait intéressant de réaliser la même étude en conditions stressantes, mais en permettant aux femelles de se reproduire, afin d'observer si dans des conditions plus contraignantes les femelles favorisent toujours le maintien de leur longévité comme nous l'avons observé.

Les résultats des différents chapitres ont par ailleurs permis de caractériser l'immunosénescence chez les femelles *T. molitor*. Les femelles âgées meurent plus rapidement d'infections bactériennes que les jeunes femelles (Chapitre 2) et elles montrent un nombre global d'hémocytes plus faible. Ces résultats sont conformes à ceux observés chez la drosophile, chez qui les femelles deviennent plus sensibles aux infections parasitaires avec l'âge et dont la concentration hémocytaire diminue aussi avec l'âge (Ramsden et al. 2008, Mackenzie et al. 2011). De plus, nos résultats sont aussi en accord avec la diminution avec l'âge de la capacité d'encapsulation des femelles *T. molitor* (Daukšte et al. 2012), les hémocytes étant primordiaux dans ce processus (Vigneron et al. 2019).

Cependant, avec l'âge, l'activité basale de la phénoloxydase semble augmenter (Chapitres 1 et 3), de même que l'activité antibactérienne de l'hémolymphe. Ce type de résultats a été observé chez une espèce proche de *T. molitor* : le tribolion, *Tribolium castaneum* (Khan et al. 2016). Les tribolions présentent en effet une activité antibactérienne et une activité de phénoloxydase plus élevées chez les insectes âgés. De plus, ces auteurs ont également observé une plus grande susceptibilité à l'infection des insectes âgés. Concernant l'activité antibactérienne de l'hémolymphe, nous interprétons cette augmentation d'activité avec l'âge comme due au fait que la probabilité d'avoir rencontré un pathogène microbien est de plus en plus importante avec l'âge, comme cela a été observé chez le bourdon, *Bombus terrestris* (Moret and Schmid-Hempel 2009). Si on prend en compte le fait que l'activité antibactérienne de l'hémolymphe persiste de nombreux jours (voire des semaines) suite à des expositions, comme observé dans la littérature (Haine et al. 2008a, Dhinaut et al. 2018a) et dans le chapitre 3, il n'est pas surprenant d'observer ces résultats chez les individus âgés, qui sont aussi les plus susceptibles d'avoir rencontré des infections microbiennes au cours de leur longue vie.

Un aspect intéressant de nos résultats est l'évolution de l'activité phénoloxydase avec l'âge. Cette enzyme, de par son rôle de catalyseur de la mélanisation, est une des clefs du système immunitaire des insectes. Cependant, elle est aussi fortement reliée à l'immunopathologie car la réaction résultant de la synthèse de mélanine entraîne le relargage de composés cytotoxiques (quinones, espèces réactives de l'oxygène, etc.) (Zhao et al. 2011). De plus, la mélanisation ne cible pas spécifiquement le pathogène et peut donc s'attaquer aussi aux tissus et organes de l'hôte. Nous avons observé dans les chapitres 1 et 3 que l'activité basale de la phénoloxydase est plus élevée dans les stades avancés de la vie (expérience individuelle, chapitre 3), ou tout au moins égale à celle des stades les plus

jeunes (expérience populationnelle, chapitre 1). Une explication pourrait être que, de par son effet immunopathologique, la phénoloxydase devrait être mieux régulée au cours des stades précoces de la vie, qui sont cruciaux pour la fitness des insectes. En effet, comme décrit précédemment, les femelles *T. molitor* pondent une très grande partie de leurs œufs dans les premières semaines de leur vie adulte. Plus tard dans la vie, après que les organismes aient réalisé la majorité de leur reproduction, leur valeur reproductive résiduelle diminue fortement et donc la pression de sélection exercée sur ces stades avancés de la vie est plus faible. La régulation de l'activité de la phénoloxydase pourrait être moins efficace chez les insectes âgés. L'hypothèse de cette dérégulation avec l'âge est en accord avec les résultats observés dans le chapitre 1, où une augmentation de l'activité de la PO remonte chez les individus (mâles ou femelles) les plus âgés, et du chapitre 2, où les femelles plus âgées (ayant une activité de phénoloxydase plus élevée que les jeunes) meurent plus rapidement en cas d'infection bactérienne.

De plus, la quantité de prophénoloxydase (forme de réserve de la phénoloxydase) est la même chez les femelles jeunes et âgées, les femelles ont donc la même quantité de réserve de phénoloxydase, quel que soit leur âge. Le fait que seule la quantité de phénoloxydase libre varie, fournit un bon argument à l'hypothèse de la dérégulation de son activité avec l'âge. Une autre manière d'interpréter cette variation de l'activité de la phénoloxydase avec l'âge est de la voir comme un trade-off âge-spécifique dans l'investissement dans la fonction immunitaire, comme l'ont suggéré Khan et collaborateurs (2017). Les organismes moduleraient l'investissement dans l'immunité en fonction de leur âge de manière à être relativement protégés des coûts immunopathologiques lorsque leur valeur reproductive résiduelle est encore forte. Ainsi, alors que la quantité de prophénoloxydase est inchangée au cours de la vie, l'activité de la phénoloxydase libre serait maintenue à un niveau relativement faible tôt dans la vie, pour prévenir ses coûts immunopathologiques, tandis que plus tard dans la vie son activité serait plus élevée.

Par ailleurs, la phénoloxydase a été décrite comme étant impliquée dans la formation des œufs, plus particulièrement du chorion, chez différentes espèces par exemple : *Aedes aegypti* (Li and Christensen 1993, Li 1994) ou *Biomphalaria glabrata* (Bai et al. 1997). Ces observations permettent de proposer une hypothèse alternative aux variations de phénoloxydase au cours de la vie chez les femelles : la faible activité de phénoloxydase circulante observée chez les jeunes femelles pourrait être due au fait que ces femelles produisent beaucoup d'œufs et donc une partie la phénoloxydase pourrait être utilisée dans leur formation. Les femelles âgées, qui produisent moins d'œufs, ont donc activité de

phénoloxydase circulante plus importante car cette dernière ne serait pas accaparée par la formation des œufs. Si une telle hypothèse est vraie, nous pourrions nous attendre à observer une relation négative entre fécondité des femelles et activité de la phénoloxydase. Nos données ne montrent pas une telle relation, ce qui tendrait à ne pas valider cette hypothèse. Cependant, ces données n'étant que corrélationnelles il semblerait pertinent d'étudier plus profondément le lien entre production d'œufs et activité de la phénoloxydase.

Il serait par ailleurs intéressant dans des travaux futurs d'essayer de mieux caractériser l'expression de la phénoloxydase en fonction de l'âge et d'essayer de relier son activité à une efficacité de réponse immunitaire (survie à l'infection par exemple), afin de mieux comprendre ce paramètre immunitaire décrit comme primordial. En effet, il est à noter que, jusqu'à aujourd'hui, l'implication de l'activité de la phénoloxydase dans la résistance au parasitisme n'est pas clairement démontrée (Khan et al. 2016).

Globalement, ces observations suggèrent que l'immunosénescence ne correspond pas forcément à une simple diminution des paramètres immunitaires. Il se pourrait, par contre, que l'immunosénescence puisse résulter d'une déficience progressive de la régulation de l'activité de ces paramètres immunitaires. Ainsi, une forte activité des effecteurs immunitaires tard dans la vie pourrait bien correspondre à une dérégulation du système immunitaire. Il est donc important de confronter la mesure de ces paramètres immunitaires (notamment l'activité de la phénoloxydase) avec des mesures de résistance à l'infection, afin de caractériser réellement l'immunocompétence d'un organisme.

c. Schéma de priorité d'investissement des ressources des femelles de *Tenebrio molitor*

Nous avons testé la stratégie d'investissement à la reproduction des femelles *T. molitor* dans les chapitres 2 et 3. Nous n'avons jamais réussi à induire d'investissement terminal chez les femelles, que ce soit en mesurant la reproduction de femelles âgées (chapitre 2) ou en simulant une menace pour leur survie *via* l'injection de bactéries inactivées (chapitre 3). Pourtant de tels phénomènes ont été observés chez d'autres organismes, vertébrés ou invertébrés (Duffield et al. 2017). Par exemple, chez les invertébrés, une injection de cellules mortes de la bactérie *Serratia marcescens* chez la femelle du grillon, *Gryllus texensis* entraîne une augmentation du nombre d'œufs pondus (Shoemaker et al. 2006). Cependant, chez d'autres d'insectes tels que *Acyrtosiphon pisum* ou *Anopheles gambiae*, des études

ont constaté, comme nous, une absence d'investissement terminal (Ahmed et al. 2002, Barribeau et al. 2010).

Nous avons observé dans le chapitre 2 que les femelles *T. molitor* investissent dans la reproduction conformément à l'hypothèse de « reproduction restreinte » (Curio 1983, McNamara and Houston 1996, McNamara et al. 2009). En effet, elles investissent modérément dans la reproduction tard dans la vie, limitant ainsi les coûts de ce trait ce qui leur permet de maintenir leur longévité. Par ailleurs, comme nous l'avons précédemment décrit, lorsque les femelles investissent dans la fonction immunitaire, elles contraignent en priorité leur reproduction plutôt que leur longévité. Conjointement, ces résultats suggèrent l'existence, chez les femelles de *T. molitor*, d'un schéma de priorité (Rigby & Moret, 2000), qui favoriserait la longévité accompagnée d'une reproduction modérée, au détriment d'une forte reproduction.

Un tel schéma de priorité pourrait être fréquent chez les organismes à reproduction itéropare et notamment chez les insectes dont la reproduction dépend des ressources acquises au cours de la vie adulte (aussi appelés « income breeders » en anglais) dont *T. molitor* fait partie. En effet, favoriser la longévité peut donner l'occasion aux insectes « income breeders » d'optimiser leur effort de reproduction au fil du temps en fonction de l'accès aux ressources, à la nourriture ou aux partenaires sexuels. Cette hypothèse est renforcée par les observations de Zanchi et al. (2019), qui ont montré que les femelles de *T. molitor* placées dans des conditions alimentaires riches à l'âge adulte peuvent maintenir une fécondité plus élevée au fil du temps par rapport à des femelles placées dans des conditions alimentaires plus pauvres. D'autres études sont nécessaires pour tester plus en profondeur cette hypothèse de schéma de priorité entre la longévité et la reproduction chez les insectes « income breeders », particulièrement chez les espèces ne réalisant pas d'investissement terminal. Elles mettraient en évidence différentes stratégies de cycle de vie en ce qui concerne la sénescence.

3. Reproduction et sénescence chez les mâles

Dans le Chapitre 1, nous avons modulé le taux d'accouplement et l'opportunité de l'intensité de la compétition sexuelle des mâles *T. molitor*. Ceci nous a permis de mettre en évidence que le coût de la reproduction pour les mâles était principalement supporté par le taux d'accouplement, bien plus que par la compétition sexuelle. Nous avons observé que dans des populations où les mâles sont en surnombre (75% de mâles), leur longévité se retrouve augmentée. Ces résultats suggèrent que, comme nous l'avons observé pour les

femelles, les mâles de *T. molitor* semblent maximiser leur longévité au lieu d'investir fortement dans la reproduction. Cependant, nous n'avons pas observé directement le comportement des mâles dans les populations. Nous ne pouvons donc pas exclure la possibilité que seule une fraction des mâles se soit reproduite dans la population où ils étaient en excès. Les mâles exclus de la reproduction (donc ne payant pas le coût de la copulation) pourraient être responsables de la plus grande longévité observée dans ces populations. Toutefois, selon cette hypothèse, certains mâles auraient un taux d'accouplement très important dans la population, on s'attendrait donc à ce qu'ils meurent plus rapidement que ceux qui ne reproduisent pas. Ces mâles reproducteurs auraient alors une longévité comparable à celle observée dans les autres conditions de sex-ratio. Les courbes de survie devraient donc se chevaucher durant les premières semaines pour ne se séparer que plus tardivement. Or, la survie des mâles dans la condition de sex-ratio 75% de mâles 25% de femelles est supérieure dès les premières semaines, ce qui n'est alors pas en accord avec l'hypothèse selon laquelle seule une partie des mâles accède à la reproduction.

Selon nos observations, la stratégie d'investissement à la reproduction des mâles pourrait donc aussi être celle de la reproduction restreinte. Trois études ont cependant suggéré que les mâles de *T. molitor* réalisent des investissements terminaux (Sadd et al. 2006, Kivleniece et al. 2010, Krams et al. 2011). En effet, les mâles *T. molitor* produiraient plus de phéromones sexuelles lorsque leur immunité est stimulée. Cette production de phéromones augmenterait leur attractivité vis-à-vis des femelles : une femelle passe plus de temps sur un patch précédemment occupé par un mâle dont l'immunité a été stimulée. De plus, dans leur étude, Krams *et al.* (2011) montrent que les mâles les plus attractifs présentent aussi une activité de mélanisation plus faible que les mâles moins attractifs, suggérant que les mâles sacrifient leur immunité pour augmenter leur attractivité. Les données de ces différentes études suggèrent donc l'existence d'un investissement terminal chez les mâles *T. molitor*. Cependant, ces études n'ont pas été jusqu'au bout, en ce sens qu'elles n'ont pas été complétées par des données comparant la compétitivité et la fécondité des mâles dont l'immunité a été stimulée ou non. Il est donc difficile d'affirmer la véracité d'un investissement terminal chez les mâles de *T. molitor*. Il apparaît alors primordial de mener ce genre d'études par la suite, afin de voir si les données d'attractivité se traduisent bien par des données de compétitivité et de fécondité allant dans le même sens, ou bien si les mâles, comme les femelles, adoptent la stratégie de la reproduction restreinte.

4. Conclusions & perspectives

Les différents travaux menés au cours de cette thèse ont permis de confronter la sénescence avec les stratégies d'investissement dans la reproduction ou l'immunité des femelles de *T. molitor*, et de commencer à explorer ces phénomènes chez les mâles. Les principaux résultats de ce travail sont, d'une part, que les patrons de sénescence de *T. molitor* sont compatibles avec la théorie du « soma jetable », et, d'autre part, que les femelles de cette espèce semblent adopter une stratégie d'histoire de vie visant à maximiser leur longévité quand les conditions le permettent. Ainsi, leur stratégie d'investissement à la reproduction correspond à celle décrite par la théorie de reproduction restreinte, opposée à celle décrite dans des travaux chez les mâles *T. molitor*, la théorie de l'investissement terminal. Nos travaux ont aussi permis de caractériser l'immunosénescence de *T. molitor* en mettant notamment en évidence une diminution de l'efficacité du système immunitaire avec l'âge, associée cependant à l'augmentation en intensité de certains paramètres immunitaires en fonction de l'âge. Le cas de la régulation de la phénoloxydase avec l'âge et du lien entre l'activité de cette enzyme et l'efficacité de la réponse immunitaire devra faire l'objet de futures études pour approfondir nos observations.

Par ailleurs durant cette thèse, nous sommes concentrés sur le stade adulte de *T. molitor*, alors que les stades larvaires sont également très longs (il peut représenter plus de la moitié de la durée de vie totale d'un ténébrion) et que des événements se produisant durant ces stades larvaires peuvent avoir des conséquences bien plus tard dans la vie. Par exemple, Pursall et Rolff (2011) ont montré qu'une stimulation du système immunitaire durant le stade larvaire avait pour conséquence de réduire la longévité des adultes. Cependant, dans cette étude les adultes n'ont pas eu l'opportunité de se reproduire. Il serait donc intéressant de compléter ces résultats en étudiant la fitness d'adultes issus de larves dont l'immunité a été stimulée tôt dans la vie. De plus, il serait aussi pertinent de se pencher sur les liens entre temps de développement, longévité et sénescence chez *T. molitor*. En effet, la théorie du soma jetable propose aussi que la croissance est un trait coûteux susceptible d'impacter le taux de sénescence (Kirkwood 1977).

Enfin un aspect maintenant important de l'investissement à la fonction immunitaire et à la reproduction chez les animaux est le transfert trans-générationnel d'immunité (TTGI) (Little *et al.*, 2003). Le TTGI correspond à une modification de la fonction immunitaire de la descendance en fonction de l'expérience immunologique des parents, notamment de la mère (Zanchi *et al.* 2012, Dhinaut *et al.* 2018, Tetreau *et al.* 2019). Le TTGI serait adaptatif en permettant de doter la descendance d'une immunité plus forte quand le risque infectieux

auquel la mère est exposée est susceptible de persister à la génération suivante. Une conséquence importante du TTGI est de produire des descendants qui se caractérisent par un fort investissement à la fonction immunitaire tôt dans la vie. Les conséquences de ce fort investissement précoce dans la fonction immunitaire sur la sénescence de la descendance ne sont pas encore connues. Par ailleurs, l'influence de l'âge des parents et de leur statut reproducteur sur le TTGI n'a pour l'instant pas été étudiée. Les études réalisées à ce jour concernent des individus jeunes et vierges au moment où ils sont amenés à réaliser ce transfert trans-générationnel d'immunité, il serait intéressant de savoir si ce mécanisme est conservé tout au long de la vie des parents. Ces thématiques pourraient être pertinentes à développer à la suite des travaux présentés dans cette thèse, afin d'approfondir les connaissances sur *T. molitor* et son cycle de vie.

Références

Références

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Annexes

Annexes

Immune defenses of a beneficial pest: the mealworm beetle, *Tenebrio molitor*



Immune Defenses of a Beneficial Pest: The Mealworm Beetle, *Tenebrio molitor*

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The mealworm beetle, *Tenebrio molitor*, is currently considered as a pest when infesting stored grains or grain products. However, mealworms are now being promoted as a beneficial insect because their high nutrient content makes them a viable food source and because they are capable of degrading polystyrene and plastic waste. These attributes make *T. molitor* attractive for mass rearing, which may promote disease transmission within the insect colonies. Disease resistance is of paramount importance for both the control and the culture of mealworms, and several biotic and abiotic environmental factors affect the success of their anti-parasitic defenses, both positively and negatively. After providing a detailed description of *T. molitor*'s anti-parasitic defenses, we review the main biotic and abiotic environmental factors that alter their presentation, and we discuss their implications for the purpose of controlling the development and health of this insect.

Keywords: *Tenebrio molitor*, innate immunity, pest control, insect farming, ecoimmunology

INTRODUCTION

Insects conflict with human activities due to their ability to successfully colonize and adapt to the majority of terrestrial habitats. They are considered as pests when they damage crops and/or parasitize livestock, which decrease food production, or when they pose a health hazard to humans or other domesticated animals. By contrast, insects are deemed beneficial when they perform valuable services such as pollination and pest control, or when they are bred for being used in human activities. Such a distinction is rather subjective and only arises in light of desired outcomes from a human perspective. In this context, the mealworm beetle, *Tenebrio molitor*, could be seen as either a pest or a beneficial insect. On the one hand, *T. molitor* has generally been considered as a pest because they consume or degrade the quality of stored grains and grain products. On the other hand, mealworms are beneficial, as their larvae are often used as pet food. They also offer a promising alternative protein-rich animal feed and are recommended as a source of human nutrition. Furthermore, *T. molitor* larvae may be further useful due to their ability to efficiently degrade polystyrene and plastic waste (Brandon et al., 2018). For these reasons, *T. molitor* is currently being considered for production at an industrial scale.

T. molitor is the host of a wide range of pathogens and parasites such as entomopathogenic microbes, protozoa, and tapeworms, which reduce the mealworm survival or reproductive success. While some of these parasites might be used as biological insecticides to control unwanted

population development, they might also be a source of concern in mass rearing facilities. Like other insects, *T. molitor* possesses an arsenal of behavioral, physical, and physiological mechanisms that aim to prevent exogenic invasions or to lower the consequences of a successful infection. The efficacy of these lines of defense may independently or synergistically vary according to biotic and abiotic environmental factors including temperature, food, population density, and individual past interaction with pathogens and parasites. Characterization of such phenotypic plasticity may provide valuable insights for the purpose of improving control or protection of the insect populations. Here we review the prominent *T. molitor* anti-parasitic defense systems and the main environmental factors affecting their presentation. The impact of the environmental factors is considered from the phenotypic to the population level. The processes affecting mealworm anti-parasitic defenses are discussed in the context of controlling the development and health of the insect populations.

ANTI-PARASITIC DEFENSES IN THE MEALWORM BEETLE

Behavioral Immunity

Behavioral immunity refers to altered behaviors used by a host to avoid infection, reduce parasite growth, and/or alleviate disease symptoms. Such anti-parasitic behaviors are increasingly recognized in insects, including *T. molitor*. Behavioral immunity involves anti-parasitic behaviors categorized into three main infectious outcomes (de Roode and Lefèvre, 2012). First, behavioral immunity may provide qualitative resistance by avoiding contact with parasites and pathogens. Behavioral immunity comprises spatial and temporal avoidance of potentially infected places, individuals or food, implementation of hygienic behaviors such as grooming, and adapted social contacts, such as mate choice based on a partner's immunocompetence. This range of behavior was reported when *T. molitor* were exposed to the tapeworm *Hymenolepis diminuta*. *H. diminuta* is a rodent parasite that uses the mealworm beetle as an intermediate host. Beetles become infected by consuming eggs of the parasite when feeding on infectious rodent feces. Infection of the beetle is maximized by an increased attractiveness of infected rodents' feces compared to non-infected feces (Pappas et al., 1995). Infected male beetles, which pay a higher reproductive cost than do infected females (Hurd and Arme, 1986; Hurd and Parry, 1991; Worden et al., 2000), have developed an avoidance behavior for feces that harbor *H. diminuta*, thus decreasing their probability of coming into contact with the tapeworm (Shea, 2010). In addition, females developed qualitative resistance through mate choice, as they are able to evaluate male immunocompetence *via* pheromone signaling and then choose a more immunologically fit mate (Rantala et al., 2002). By choosing a male more refractory to pathogens, females reduce the probability of being infected by their mate and may transmit an enhanced level of immunocompetence to their offspring (Hamilton and Zuk, 1982).

Second, host behaviors may provide quantitative resistance by preventing parasite or pathogen replication. These behaviors

involve therapeutic medication, behavioral fever, and grooming, which are particularly beneficial in dense insect populations, where diseases can efficiently spread. No infection outbreak was reported from mealworm mass rearing, but alternatives to antibiotic use could be beneficial to avoid the rise of resistant pathogens. Particularly, adopting therapeutic behavioral medication would be of great interest for the prevention and control of diseases in large populations of beetles. Therapeutic medication can be defined "as a series of behaviors through which infected hosts exploit additional species or compounds to reduce or clear infections, whether mediated through defensive or nutritional properties" (de Roode and Lefèvre, 2012). So far, therapeutic medication has not been reported in the mealworm beetle. Hence, further investigations are needed on this aspect of the mealworm behavioral immunity.

Third, the host may tolerate infections by limiting the negative effects on their reproductive success. This limitation is mainly achieved through increasing their reproductive effort but often at the expense of their longevity. For instance, mealworm beetles tolerate a high number of cysticercoids of the parasite *H. diminuta* at the expense of their own fitness. Nevertheless, in response to parasite infection, males produce improved spermatophores that contain superior nuptial gifts that will be passed to their mating females. This increases female fecundity, and therefore, a higher number of eggs are fertilized by the male (Hurd and Ardin, 2003). Specifically, as beetle longevity is compromised by the parasite, infected males may gain a reproductive benefit, before dying from the infection, by increasing the total protein content of the spermatophores they transfer to females during mating (Carver et al., 1999; Hurd and Ardin, 2003). Males that are experiencing a non-infectious immune-challenge, e.g., a piece of nylon filament inserted into their hemocoel, present a similar increased reproductive effort. Indeed, the immune challenge may induce in males, the perception of a lower survival probability consequent to their simulated infection status, causing the insect to make a last attempt to achieve a maximized level of reproductive success. Consequently, females find those artificially challenged males more sexually attractive, probably due to an increased production of sexual pheromone consequent to the challenge (Sadd et al., 2006; Kivleniece et al., 2010; Krams et al., 2011). The underlying signaling may transit through juvenile hormone (JH), a hormone secreted by the *corpora allata* that is involved in the control of morphogenesis and reproduction in insects, as *T. molitor* males injected with JH are preferred by females (Rantala et al., 2003). So far, comparable adjustment of the reproductive effort upon infection has never been reported in females.

Cuticle Immunity

The insect integument forms a robust barrier that successfully prevents most parasites and pathogens from colonizing the hemocoel (Moret and Moreau, 2012). It usually constitutes the first barrier between an insect and endogenous invaders. The integument includes an outer layer, called the cuticle, which is produced by a monolayer of epidermal cells. This layer of cells, or epidermis, is separated from the underlying tissues by a thin matrix called the basal lamina. The basal lamina provides a defensive boundary

on the insect's surface due to the thickness of the cuticle and the degrees of sclerotization, or cross-linking, and melanization within cuticular layers. Melanization in the cuticle strengthens its property to act as a physical barrier against the penetration of parasites (Stleger et al., 1988; Hajek and Stleger, 1994). In addition, melanin is toxic to microorganisms and has potent antimicrobial activity (Soderhall and Ajaxon, 1982). Cuticular melanization naturally occurs during the process of molting in insect larvae and nymphs, and right after adult emergence from pupation (Vigneron et al., 2014). However, this process is also induced in response to a mechanical scratch to avoid loss of hemolymph (Benoit et al., 2017) or to microbial invasion (Golkar et al., 1993). In the mealworm beetle, the degree of cuticular melanization is a strong indicator of resistance to the entomopathogenic fungus, *Metarhizium anisopliae*. Indeed, darker beetles are more resistant than lighter ones (Barnes and Siva-Jothy, 2000). This could be explained by the thicker and less porous cuticle displayed by darker insects compared to lighter ones (Evison et al., 2017). A breach in the cuticle also triggers the production of antimicrobial peptides by the epidermal cells, such as cecropins, which are transported in the vicinity of a microbial challenge to abraded cuticle (Brey et al., 1993).

Insect growth and development involve a series of molts during which the old cuticle is partially digested, while a new cuticle is formed and the remnant is discarded. In addition to allowing insect growth, molting may serve as a defense mechanism by reducing the negative effects of a wound or a parasite invasion. For instance, molting quickly in response to a parasite exposure prevents parasites from remaining attached to the cuticle; subsequently, reducing the probability of a successful infection (Duneau and Ebert, 2012; Kim and Roberts, 2012). The benefit of such molting could be exploited by the host inducing precocious molts in response to parasite early attachment (Duneau and Ebert, 2012; Moret and Moreau, 2012). In *T. molitor*, whether wounding or parasite attachment can induce larvae to perform more molts is not known. However, larvae grow through a variable number of molts from 8 to 20. The variability in this number can be partially explained by the availability of resources, the quality of the diet, or the density of the population (Connat et al., 1991; Morales-Ramos et al., 2010). This suggests that the mealworm beetle can adjust its development in response to its environment. Hence, it would be highly relevant to investigate the capacity of the mealworm to molt subsequently to the pressure caused by a wound or a pathogen attempting to invade the insect.

The Hemocoelic Immune System

Once a parasite or a pathogen has breached the integumental defenses, the insect has to produce a rapid and effective response that localizes and neutralizes the growth and development of the microbe. Like in other insects, hemocoelic defenses of *T. molitor* rely on innate immune effector systems. These involve recognition by pattern recognition receptors (PRRs) that detect a range of conserved non-self microbe-associated molecular patterns (MAMPs), such as bacterial lipopolysaccharides (LPS) and peptidoglycans (PGN), fungal and bacterial β -1,3 glucans, and other sugar moieties. *T. molitor* possesses most known

insect PRRs (Johnston et al., 2014). Specifically, these include peptidoglycan recognition proteins (PGRPs) that bind bacterial PGN, Gram-negative binding proteins (GNBPs) that bind LPS, and together with glucan-binding proteins (GBPs), recognizing β -glucans (Figure 1; Zhang et al., 2003; Park et al., 2007; Kim et al., 2008; Lee et al., 2009; Johnston et al., 2014; Yang et al., 2017). Upon recognition, these PRRs trigger the action of various signaling pathways, including the prophenoloxidase cascade regulating melanization processes (Park et al., 2007), the Toll and immune deficiency (IMD) pathways leading to the synthesis of AMPs (Kim et al., 2008; Roh et al., 2009; Yu et al., 2010; Johnston et al., 2014), and hemocyte-driven phagocytosis (Zhu et al., 2013; Kim et al., 2017). Insect innate immune response relies mainly on those pathways, which, *via* their synergic actions, form efficient cellular and humoral responses.

Cellular defenses primarily involve the action of immune cells called hemocytes, which drive phagocytosis, nodulation, and encapsulation of endogenous organisms. Insects possess several types of circulating hemocytes that are morphologically and functionally distinct, and the prevalence of which is variable in the hemolymph. The mealworm beetle presents four main types of hemocytes: granulocytes, plasmatocytes, oenocytoids, and prohemocytes (Figure 2; Chung and Moon, 2004; Urbanski et al., 2018). Granulocytes account for 50–60% of the observed hemocytes. They are oval cells of about 10 μ m in size containing visible dense granules in their cytoplasm and are involved in phagocytosis. Plasmatocytes are the second most abundant hemocytes, representing 23–28% of the total hemocytes. Plasmatocytes are large elongated cells that are likely involved in encapsulation. Oenocytoids are the rarest type as they account for 1–2% of circulating hemocytes. They are large oval cells with a centrally located nucleus and presumably produce enzymes of the melanization cascades. Finally, prohemocytes represent 10–15% of the circulating hemocytes. They are small oval cells less than 10 μ m in size with a very large nucleus, probably functioning as precursors of hemocytes.

Hemocyte phagocytosis is achieved upon the recognition of microbes, either directly or after their opsonization by thioester proteins (TEPs), using Scavenger and Nimrod receptors or using the highly variable, alternatively spliced Dscam (Cherry and Silverman, 2006). Particularly, *T. molitor* Scavenger Receptor class C (SR-C) plays a crucial role in the ability of the insect to phagocytose fungi and bacteria (Kim et al., 2017). When an endogenous object is too big to be phagocytized, the cellular immune response also relies on melanization and encapsulation processes. Melanization corresponds to the production of melanin around foreign objects including bacteria, protozoan parasites, nematodes, or parasitoid eggs (Zhu et al., 2013). Upon wounding or recognition of a foreign object by GNBPs and PGRPs, prophenoloxidase (proPO), a zymogen present in some hemocytes and in the plasma, is cleaved through a cascade of serine proteases to liberate the active phenoloxidase (PO). This enzyme catalyzes the production of melanin. In arthropods, levels of melanin and circulating proPO enzymes are used to evaluate immune functions and status. For instance, larvae of the African armyworm, *Spodoptera exempta*, reared at high densities exhibit higher proPO levels in the hemolymph and higher resistance to

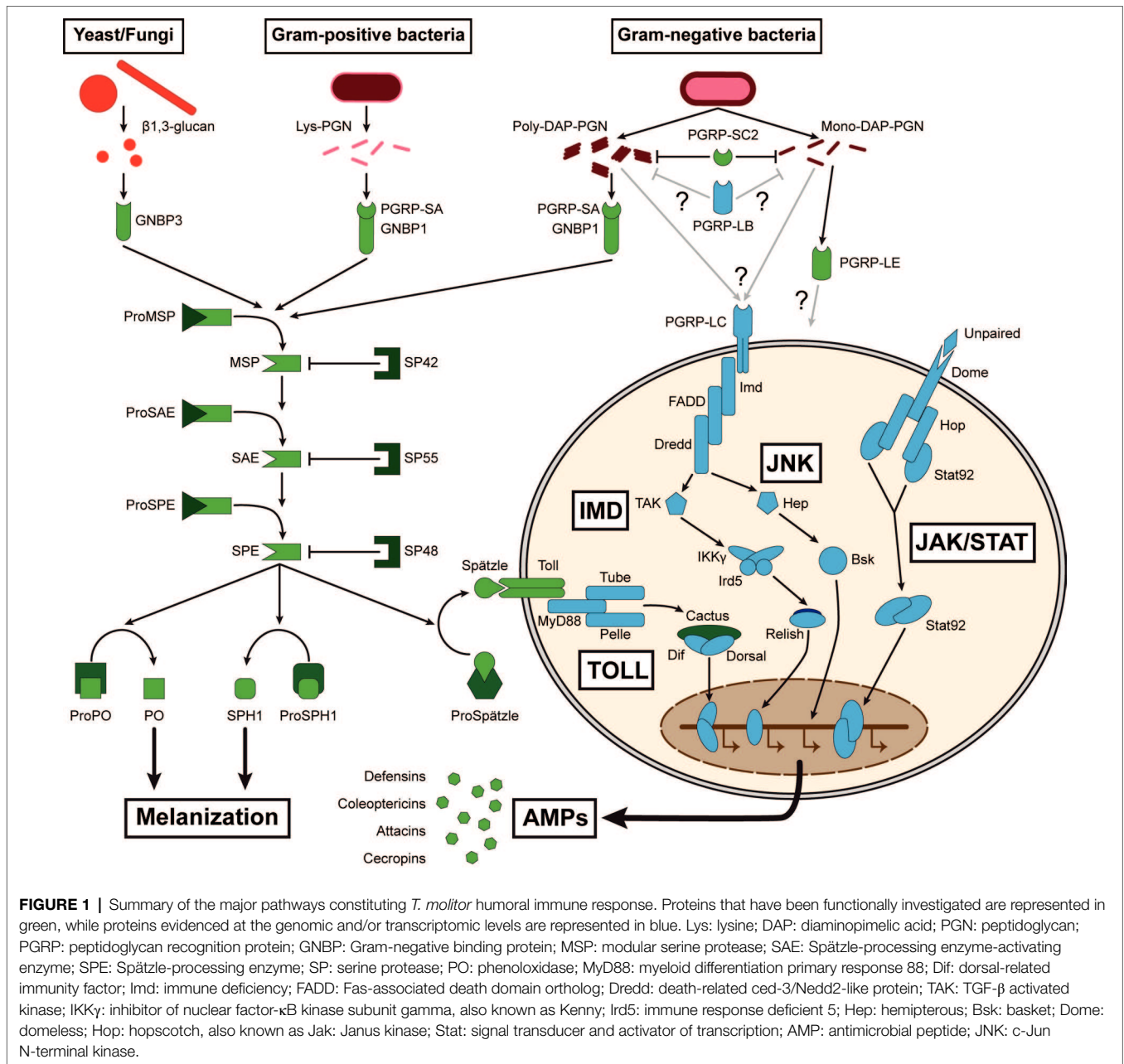
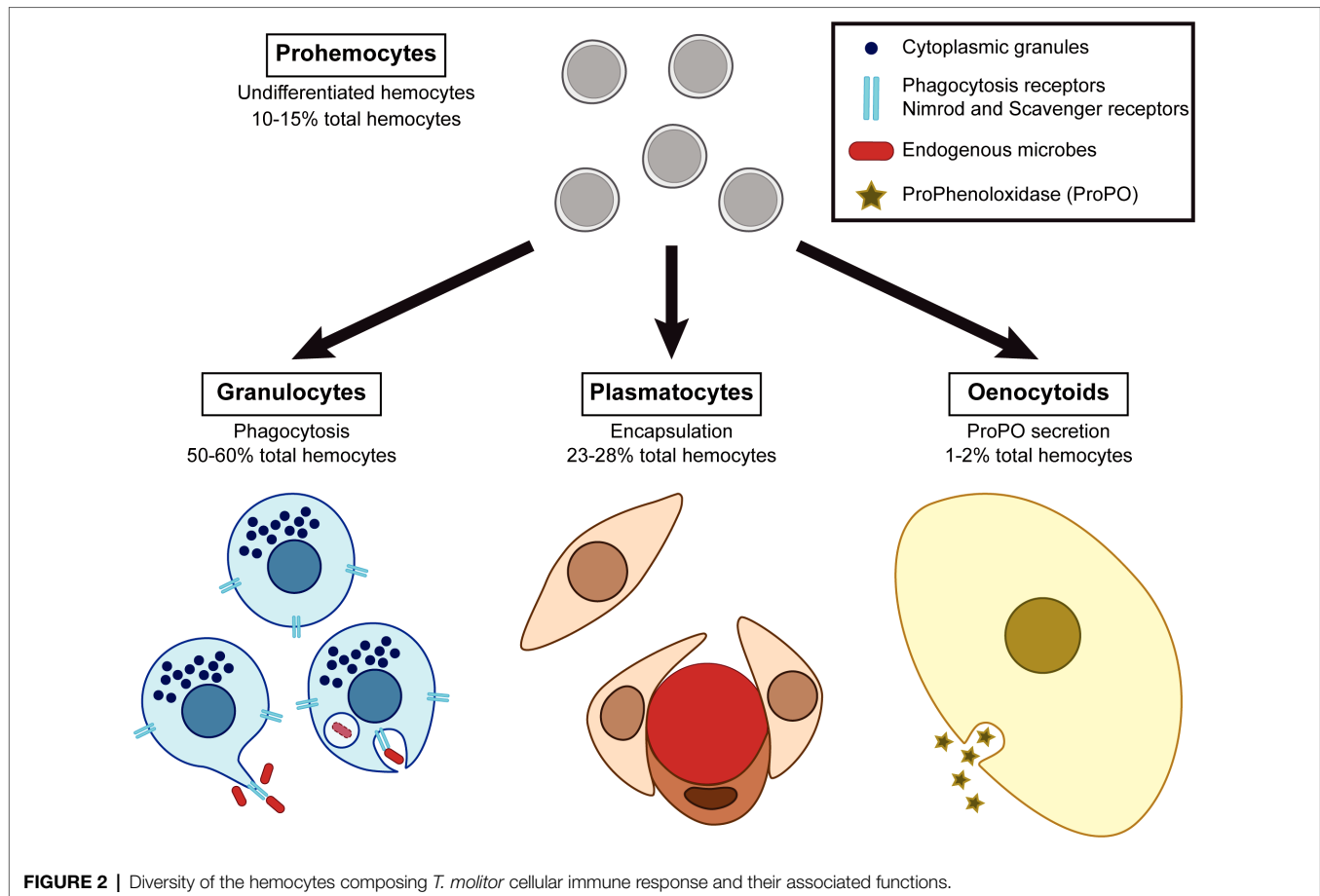


FIGURE 1 | Summary of the major pathways constituting *T. molitor* humoral immune response. Proteins that have been functionally investigated are represented in green, while proteins evidenced at the genomic and/or transcriptomic levels are represented in blue. Lys: lysine; DAP: diaminopimelic acid; PGN: peptidoglycan; PGRP: peptidoglycan recognition protein; GGBP: Gram-negative binding protein; MSP: modular serine protease; SAE: Spätzle-processing enzyme-activating enzyme; SPE: Spätzle-processing enzyme; SP: serine protease; PO: phenoloxidase; MyD88: myeloid differentiation primary response 88; Dif: dorsal-related immunity factor; Imd: immune deficiency; FADD: Fas-associated death domain ortholog; Dredd: death-related ced-3/Nedd2-like protein; TAK: TGF-β activated kinase; IKKγ: inhibitor of nuclear factor-κB kinase subunit gamma, also known as Kenny; Ird5: immune response deficient 5; Hep: hemipterous; Bsk: basket; Dome: domeless; Hop: hopscotch, also known as Jak: Janus kinase; Stat: signal transducer and activator of transcription; AMP: antimicrobial peptide; JNK: c-Jun N-terminal kinase.

nucleopolyhedroviruses than those reared solitarily (Reeson et al., 1998; Wilson et al., 2001). In addition, parasitized bumblebees (*Bombus terrestris*) exhibit twice as much PO activity in their hemolymph than do non-parasitized nest mates (Brown et al., 2003). *T. molitor* melanization processes play an important role in the ecology of the insect, as cuticle darkness polymorphisms correlate with PO activity, with a darker cuticle meaning a higher PO activity (Armitage and Siva-Jothy, 2005). The plasticity of those traits could indicate a higher cost for the insect to maintain a more efficient immune system (Barnes and Siva-Jothy, 2000). This could be due to the fact that the enzymatic cascade leading to melanization is accompanied by the production of cytotoxic intermediates, such as phenols, quinones, and reactive oxygen species (Nappi and Vass, 1993; Nappi and Ottaviani, 2000;

Sugumaran et al., 2000; Nappi and Christensen, 2005), which help to kill invading organisms, but at the cost of deleterious effects for the host (Sadd and Siva-Jothy, 2006). However, fecundity and lifespan of darker insects reared in laboratory conditions were not impacted (Krams et al., 2016). Unidentified trade-offs structuring insect life history traits may prevent the fixation of the darker cuticle phenotype in the wild.

Those intermediates are also part of a more systemic immune response as they are liberated in the insect hemolymph along with the inducible synthesis of AMPs produced by the fat body. This response constitutes the humoral immune defense, which is triggered upon microbe recognition *via* the Toll and IMD signal transduction cascades, complemented by c-Jun N-terminal kinase (JNK), and Janus kinase/Signal Transducer and Activator



of Transcription (JAK/STAT) pathways (Figure 1; Buchon et al., 2014). These pathways activate the NF- κ B transcription factors Relish, Dorsal, and Dif, which induce expression of antimicrobial peptides (Kounatidis and Ligoxygakis, 2012). These pathways are conserved in many insects including *T. molitor* (Johnston et al., 2014). Contrary to the transient and immediate induction of cellular immune effectors, *T. molitor* antimicrobial production is induced within 48 h following MAMP detection and last at least 14 days (Haine et al., 2008; Johnston et al., 2014). The mealworm beetle presents several genes potentially coding for antimicrobial peptides, including the Tenecins 1, 2, 3, and 4, for which the proteins have been isolated and assessed for their immune functions (Moon et al., 1994; Lee et al., 1996; Kim et al., 1998; Roh et al., 2009; Park et al., 2010b; Chae et al., 2012). Tenecins 1, 2, and 4 are inducible defensin, coleoptericin, and attacin, respectively, which are regulated by the Toll and IMD pathways, and mainly display antibacterial activities (Kim et al., 1998; Kim et al., 2001; Roh et al., 2009; Park et al., 2010b; Chae et al., 2012). Tenecin 3 is a constitutively expressed Thaumatin, the role of which is suggested to prevent fungal infections (Chae et al., 2012; Maistrout et al., 2018). Interestingly, AMPs are induced in *T. molitor* eggs in response to a septic injury (Jacobs et al., 2017). Moreover, the levels of immune gene expression in the eggs reach comparable levels to the expression in adults. This suggests that eggs contain immunocompetent cells in addition to maternal effects to defend themselves against potential invaders. Investigation of egg immunity

in *Tribolium castaneum* has shown that most of the immune response originates from the serosa, an extra layer of cells that envelops the yolk and the developing embryo (Jacobs et al., 2014). It is not known if *T. molitor* serosa is capable of such immune response, but this is likely due to their close relation to *T. castaneum* and the similar environment they exploit.

SOURCE OF VARIATION IN ANTI-PARASITIC DEFENSES

Density

The risk for an individual to be infected with pathogens and parasites increases when it lives in a population with higher density. Hence, it would be beneficial for individuals developing in such conditions to invest more in their defense mechanisms than individuals experiencing a low-density environment. This hypothesis was introduced as “density-dependent prophylaxis” (DDP) and predicts that individuals developing in high-density conditions will exhibit a more efficient immune response against parasites and pathogens (Wilson and Reeson, 1998). This concept was first defined investigating the noctuid moth *Spodoptera exempta*, which presents a higher resistance to baculovirus when developing in high-density conditions (Wilson and Reeson, 1998). These density-dependent effects are associated with elevated antibacterial activity and higher numbers of circulating hemocytes

in the hemolymph (Reeson et al., 1998; Wilson and Reeson, 1998). DDP is observed in several insects, including both holometabolous insects (Cotter et al., 2004a,b) such as *T. molitor* (Barnes and Siva-Jothy, 2000) and hemimetabolous insects (Wilson et al., 2002), suggesting that it is widely conserved among distant species and even in other invertebrates (Mills, 2012).

Interestingly, DDP usually correlates with melanism polyphenism, for which insects living in high-density population are darker (Figure 3; Barnes and Siva-Jothy, 2000; Wilson et al., 2001; Wilson et al., 2002; Cotter et al., 2004a). This degree of melanization is the main factor correlating with higher resistance to microbial invaders (Barnes and Siva-Jothy, 2000; Cotter et al., 2004a). Hence, it can be argued that the DDP-driven improved resistance to pathogens comes from the higher number of darker individuals generated in high-density conditions rather than being a shared trend among insect reared in high-density conditions, independently of their color (Barnes and Siva-Jothy, 2000; Cotter et al., 2004a). This is especially supported in *T. molitor* as darker individuals, compared to paler insects, present a thicker and less porous exocuticle

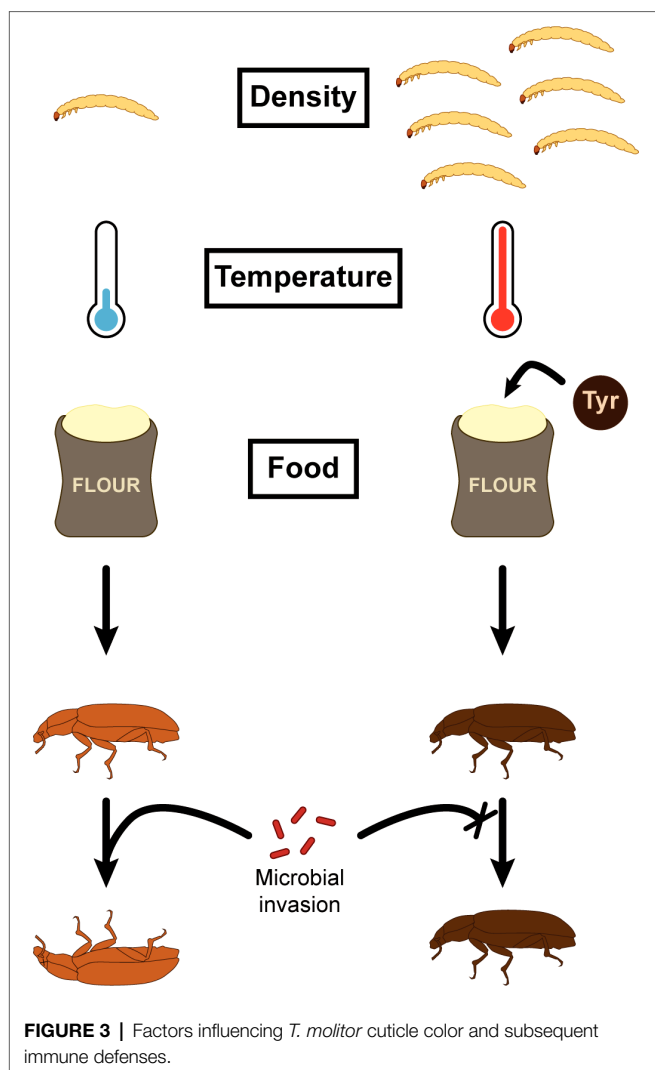
(Silva et al., 2016) and an increased PO activity (Evison et al., 2017) and do not suffer the same deleterious effects following a mock hemolymph infection (Krams et al., 2016) independently of the population density. Nevertheless, the plasticity of the melanization phenotypes in response to population density added to the absence of predominance of darker individuals among *T. molitor* populations suggests underlying trade-offs preventing the fixation of the darker phenotype.

While population density influences insect melanization, the underlying molecular mechanisms governing DDP are still unclear. Nevertheless, DDP demonstrates that interaction between individuals can structure their development, including the immune defenses. Horizontally transferred immunity from insects exposed to an infection to naïve insects is another aspect of individual interaction shaping the efficiency of immune defenses. Social and behavioral immunity have been mainly described in eusocial insects (Traniello et al., 2002; Ugelvig and Cremer, 2007) and may be relevant in highly interacted insect populations (Elliot and Hart, 2010). A recent study investigated whether *T. molitor* displays social immunity through exposing *Staphylococcus aureus*-infected insects to naïve individuals (Gallagher et al., 2018). The authors did not report any evidence leading to the conclusion that social immunization exists in the mealworm beetle. However, they observed an improved tolerance to bacterial infection in naïve insects that were interacting with insects injected with heat-killed bacteria (Gallagher et al., 2018). This suggests that *T. molitor* can sense and respond to immune-related signals originating from pathogen-exposed individual.

Density- and infection-sensing can also modulate resistance mechanisms across generations, as shown by the water flea, *Daphnia magna* (Michel and Hall, 2016), and the cotton leafworm, *Spodoptera littoralis* (Wilson and Graham, 2015). In those models, crowding the parental generation conditioned the immune response of the offspring associated to increased levels of parasite resistance. No such result has been yet demonstrated in *T. molitor*. This insect can protect the egg through the transfer of maternal immune effectors and the induction of egg immune genes in response to a parental microbial challenge (Dhinaut et al., 2018b). Hence, because the mealworm displays DDP, it would be highly relevant to investigate whether high-density conditions could lead to improve egg immunity.

Temperature

With the rising concern for climate change, a growing number of studies have focused on how temperature fluctuation impacts insect biology. One recent aspect of this field of investigations relates to the influence of temperature on insect immunity. Temperature stress triggers the production of heat-shock protein (HSP) that helps organisms to sustain said stress. Interestingly, those proteins are also expressed in *T. castaneum* following a microbial infection (Altincicek et al., 2008). Another example comes from *Musca domestica* that is expressing Hsp70 in response to a bacterial stimulation, and for which the knockdown via RNA interference (RNAi) leads to a decrease resistance to infection (Tang et al., 2012). Altogether, these results show that temperature-related genes are intimately intricate with immune pathways, suggesting a potential influence of temperature on immune defenses.



Several recent studies have investigated the effects of temperature on life history traits of insects and conclusions vary depending on the model studied (Prokkola et al., 2013; Kaunisto et al., 2015; Silva and Elliot, 2016; Yin et al., 2016; Laughton et al., 2017). For example, when reared in a higher set of temperatures, the Indian meal moth, *Plodia punctella*, presents more hemocytes (Laughton et al., 2017). Conversely, the velvet bean caterpillar, *Anticarsia gemmatalis*, presents fewer hemocytes when developing at a higher temperature (Silva and Elliot, 2016). As organisms evolve and build trade-offs between their life history traits influenced by their environment, it is expected that mechanisms underlying resistance or tolerance to stress correlate differently to the impacted life history traits. It is especially relevant for insects, which, because they are ectothermic, must resist the variations of their environmental temperature. In *T. molitor*, investigations have shown that encapsulation and cuticle darkness negatively and positively correlate with temperature, respectively (Figure 3; Prokkola et al., 2013). Moreover, while developing in warmer temperature conditions, the mealworm beetle experiences a shortened larval development and presents longer elytra. It shows that the insect biology in its whole is impacted by the temperature, which imposes trade-offs between different life history traits of an individual.

While temperature stress impacts insect immune defenses, little is known about how and whether it also affects the immune system of offspring. This inquiry has been explored in *T. castaneum*, where the offspring resistance to a bacterial challenge was found to be improved when both parents received a cold shock, while no effects were observed when the parents received a heat shock (Eggert et al., 2015). In addition, a cold shock experienced by the mother or both parents induced higher PO activity in the offspring, while a heat shock of either parent, or both, reduced the PO activity of their offspring. In conclusion, temperature is an important parameter that can significantly influence the biology of individuals, including their immune system. Hence, it would be beneficial to further investigate how temperature relates to *T. molitor* in the aim to better promote or regulate its population.

Food

Food quality and quantity are critical to immune defenses against parasites and pathogens. While leveraging food amount and quality for restricting or controlling pest populations is difficult, we may use nutrients that directly or indirectly improve the immune system of insects that we would like to maintain or mass rear.

As immune functions require metabolic resources, food restriction can impair immune activity. For instance, adult *T. molitor* PO activity can be reduced by half during short-term food privation, but it returns rapidly to initial levels when given access to food again (Siva-Jothy and Thompson, 2002). Furthermore, following an immune challenge, *T. molitor* larvae can eat five times more food per day than usual to compensate for the caloric expense of the immune response (Catalan et al., 2011). Hence, unsurprisingly, food supply is important to keep insects healthy. While the amount of food available matters, its nutritional composition is also important, especially with regard to its protein to carbohydrate ratio (Ponton et al., 2011). For instance, infected caterpillars select food containing higher protein to carbohydrate ratio, which improves their resistance

to viral or bacterial infection (Lee et al., 2006; Povey et al., 2009). The same observation has been made for *T. molitor*, whose healthy larvae usually prefer diets with lower protein to carbohydrate ratio but shift toward food with higher protein contents after immune challenge with bacteria (Catalan et al., 2011). As a consequence of this diet shift, hemocyte circulation and antibacterial activity are enhanced in the hemolymph, which presumably maximizes resistance against bacterial infection. However, PO activity is not affected by this shift in diet-choice, suggesting that either PO activity is likely less sensitive to protein intake, or it is limited by a trade-off consequent to the upregulation of antibacterial activity (Moret and Schmid-Hempel, 2001; Cotter et al., 2004b; Moret and Schmid-Hempel, 2009). In fact, diet effects on PO activity appear controversial in insects, as it has been found to be slightly enhanced by diets rich in either proteins (Lee et al., 2006; Povey et al., 2009) or carbohydrates (Koella and Sorensen, 2002; Cotter et al., 2011). Nevertheless, PO activity appears less variable with respect to diet than other immune traits (Catalan et al., 2011; Cotter et al., 2011; Evison et al., 2017). On the one hand, excess levels of PO activity could be dangerous, as uncontrolled activation of PO in the hemocoel would result in the production of toxic quinones and reactive oxygen species, which could harm self-tissues and organs (Nappi and Vass, 1993; Sadd and Siva-Jothy, 2006). Therefore, preventing excessive diet-mediated upregulation of PO activity might be required. On the other hand, PO is also involved in a large set of physiological functions independent of immunity (Hiruma and Riddiford, 1988). Thus, preventing its excessive downregulation may help to maintain homeostasis of those other physiological functions.

Interestingly, immune-challenged *T. molitor* larvae exhibit significant weight loss when fed either protein- or carbohydrate-rich diets, while their weight remains stable when they were given both protein- and carbohydrate-rich diets (Catalan et al., 2011). This suggests that no single blend of ingested nutrients can optimize all the physiological needs, and that the composition of the diets ingested by the insects represents a trade-off between optimizing different traits (Cotter et al., 2011). Hence, a relevant way to maximize growth and immunity of mealworm beetles would be to supply the insect with nutrient adjusted according to their physiological needs.

While diet composition appears to exert a moderate effect on hemocoelic PO activity, it may nevertheless influence cuticle melanization and sclerotization, which subsequently impacts cuticular color (Reeson et al., 1998; Wilson et al., 2001; Cotter et al., 2004a) and resistance to important entomopathogens (Wilson and Reeson, 1998; Barnes and Siva-Jothy, 2000; Wilson et al., 2001; Dubovskiy et al., 2013). PO enzymes mediate melanization and sclerotization of the cuticle (Andersen, 2010), and central to these latter processes is the production of 3, 4-dihydroxyphenylalanine (DOPA) from the hydroxylation of the semi-essential amino acid tyrosine (Vavricka et al., 2010). *T. molitor* exhibits plastic variation in cuticle color, and darker individuals are often more resistant to fungal diseases (Barnes and Siva-Jothy, 2000). Darker individuals exhibit alteration in the physical and chemical properties of the cuticle such as thickening and a higher degree of melanization (Silva et al.,

2016; Evison et al., 2017). The experimental supplementation of *T. molitor* larvae with the amino acid Tyrosine led to the development of adults with a darker cuticle (Figure 3; Evison et al., 2017). Interestingly, the cuticle was also thicker, but only in females, suggesting that males and females are allocating their tyrosine resources differently. In addition, while tyrosine supplementation resulted in improved cuticle defenses, it did not affect hemocoelic melanin-mediated defenses (Evison et al., 2017), suggesting that the regulation of both lines of defense could be uncoupled despite their similar precursor.

Insect immune activities are associated with the production and release of cytotoxic compounds such as reactive oxygen and nitrogen species (ROS and RNS, respectively) (Nappi and Vass, 1993). While these toxic substances help to kill invading organisms, they also cause self-damage in *T. molitor* (Sadd and Siva-Jothy, 2006), which results in a significant lifespan reduction (Pursall and Rolff, 2011; Khan et al., 2017). While insects rely on endogenous antioxidants to scavenge these free radicals, this process might be supported by dietary sources of antioxidants (Chew and Park, 2004). For instance, carotenoids have the ability to scavenge free radicals produced by immune activities (El-Agamey et al., 2004) and the potential to interact with endogenous antioxidant enzymes (Lee et al., 2011; Babin et al., 2015). Importantly, in addition to their potent antioxidant property, carotenoids stimulate the immune system of both vertebrates (Blount et al., 2003; Park et al., 2010a) and invertebrates (Flores et al., 2007; Babin et al., 2010; Babin et al., 2015). Contrary to these general observations, life-time dietary supplementation of *T. molitor* with astaxanthin, a carotenoid with strong antioxidant activity (Chew and Park, 2004), strongly depressed the insect immune system and decreased its resistance to bacterial infection (Dhinaut et al., 2017). Investigations pointed that this could result from the interaction between the pigment and nitric oxide (NO), which stimulates both cellular and humoral immunity of insects (Imamura et al., 2002; Kraaijeveld et al., 2011; Eleftherianos et al., 2014; Sanzhaeva et al., 2016). Indeed, astaxanthin may either inhibit the activity of the nitric oxide synthase, the enzyme responsible of NO production from L-Arginine (Hussein et al., 2006), or interfere with NO cellular signaling by scavenging a fraction of circulating NO, and consequently, downregulating base levels of immune activities. In addition, astaxanthin may also have regulatory effects on the host's metabolism, which would collaterally impair immune activities (Hussein et al., 2007; Yang et al., 2011). Astaxanthin interacts with nuclear receptors of the peroxisome proliferator-activated receptor superfamily, which regulates lipid and glucose metabolism in vertebrate (Jia et al., 2011). Such an alteration of the host metabolism may reduce the allocation of energetic resources to the immune system. If these receptors are conserved among taxa, similar regulatory effects may also occur in insects. These results suggest that supplying *T. molitor* with carotenoids, especially with astaxanthin, is rather detrimental. Hence, the use of this carotenoid might not be adequate when rearing this insect. By contrast, its immune-depressive effect could be used to improve the success of microbial insecticides, where the insect is detrimental (Figure 4).

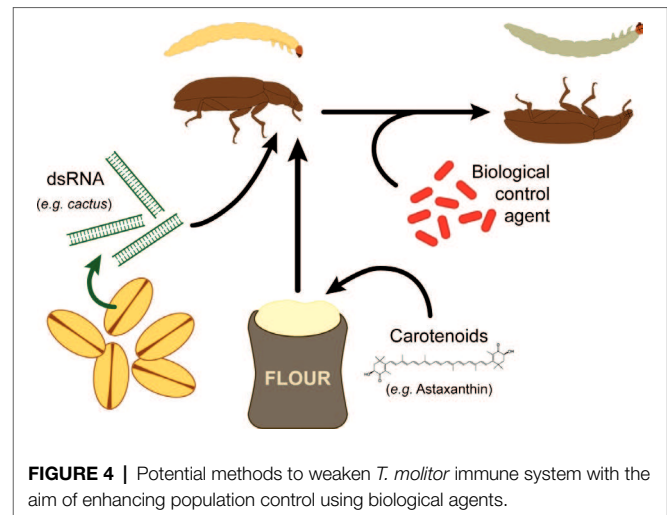


FIGURE 4 | Potential methods to weaken *T. molitor* immune system with the aim of enhancing population control using biological agents.

Previous Experience of Pathogens: Immune Priming

Like other invertebrates, *T. molitor* lacks immune effectors that are responsible for the acquired immune response of vertebrates. However, the invertebrate immune system is capable of functional modulation similar to the acquired immune response of vertebrates (Moret and Siva-Jothy, 2003; Moret, 2006). This form of innate immune memory in an invertebrate is termed “immune priming,” which is broadly defined as increased protection to a pathogen following previous exposure (Little and Kraaijeveld, 2004). Immune priming may exhibit a variable degree of specificity, from cross-reactive (non-specific) (Moret and Siva-Jothy, 2003) to highly specific (more effective against the pathogen encountered during the primary challenge), especially when cellular processes are involved for the latter (Pham et al., 2007; Roth and Kurtz, 2009). Priming response can also be obtained from a challenge with an inert immune elicitor such as a nylon implant, which can provide immune protection against a subsequent fungal infection (Krams et al., 2013). This suggests that immune priming originates, at least partially, from activation of immune defenses rather than solely from the presence of MAMPs.

Functionally, individual immune priming may rely on three types of responses (Coustau et al., 2016). First, it may involve a sustained response, corresponding to the long-lasting upregulation of the same immune effectors after the initial immune challenge. Second, a recalled response results in a faster and stronger response after a secondary infection in a way that is reminiscent of the vertebrate acquired immune response. Third, priming may induce an immune shift, involving different immune effector systems during the primary and the secondary immune responses. Current evidence suggests that individual immune priming in *T. molitor* is achieved through a sustained antibacterial activity, which can be active for at least 20 days after a primary immune challenge by injection of a suspension of killed Gram-positive bacteria (Makarova et al., 2016; Dhinaut et al., 2018a). Hemocyte concentration also increased two-fold upon the secondary challenge with

the bacteria (Dhinaut et al., 2018a), which might be consistent with a hemocyte-mediated recall response. However, while this enhanced hemocyte concentration could be involved in the priming response, this relationship is speculative because of the persistent antibacterial activity in the hemolymph resulting from the primary challenge (Dhinaut et al., 2018a). No such long-lasting antibacterial activity and change in hemocyte concentration was found in primed insects with Gram-negative bacteria, and consistently, the priming with Gram-positive bacteria provided the most effective protection against microbial reinfection (Dhinaut et al., 2018a). Therefore, individual priming responses induced by Gram-positive bacteria are stronger and more protective than those induced by Gram-negative bacteria in *T. molitor*, possibly because Gram-positive bacteria have played an important evolutionary role in shaping the immune system of this insect.

An individual may not only gain immune protection from its own immunological experience, but it can also benefit from that of its parents through “trans-generational immune priming” (TGIP). TGIP allows immune-challenged parents to produce more resistant offspring (Figure 5; Moret, 2006). In *T. molitor*, TGIP effects were revealed through enhanced immune activity in primed eggs (Moreau et al., 2012; Zanchi et al., 2012; Dubuffet et al., 2015; Dhinaut et al., 2018b), larvae (Moret, 2006), and adult offspring (Zanchi et al., 2011; Dhinaut et al., 2018b). Furthermore, enhanced immunity in the offspring may result either from the immune challenge of fathers or mothers, although paternal and maternal TGIP are associated with the enhancement of different immune effectors in the offspring (Zanchi et al., 2011). Similar to individual immune priming, TGIP of offspring does not appear to be pathogen-specific (Dhinaut et al., 2018b). For example, the offspring of mothers primed with the Gram-negative bacteria, *Serratia entomophila*, and those primed with the Gram-positive bacteria, *Bacillus thuringiensis*, had a similar enhanced survival to bacterial infection (Dhinaut et al., 2018b). However, while the maternal challenge with *S. entomophila* had no apparent effect on base levels of cellular or humoral immune defenses of the offspring, the maternal challenge with *B. thuringiensis* slightly enhanced PO activity of the offspring (Dhinaut et al., 2018a). In addition, the offspring of mothers immunized with purified LPS from bacterial cell wall displayed increased base levels of hemocyte concentration (Zanchi et al., 2011). Hence, offspring immunity is affected differently through TGIP depending on the nature of the maternal challenge.

Although TGIP occurs in several invertebrate species, investigations on its molecular mechanisms have just begun. In *T. castaneum*, bacterial components cross the midgut epithelium and are stored in eggs (Knorr et al., 2015), probably via a vitellogenin-mediated transfer. Vitellogenin, the major egg storage resource for embryo nutrition, also displays multiple immune functions by acting as a multivalent pattern recognition receptor with opsonin and antibacterial activity (Singh et al., 2013). Vitellogenin can recognize bacteria by specifically binding to MAMPs, such as PGN and LPS, and mediate the translocation of bacterial proteins to the eggs (Salmela et al., 2015). This transfer was associated with an increased expression of immune

genes in the eggs (Knorr et al., 2015; Salmela et al., 2015). Interestingly, *T. molitor* females provide enhanced antibacterial activity to eggs that are produced from the second to the eighth day after the maternal challenge only (Zanchi et al., 2012). Offspring resulting from eggs laid after this restricted period of time are not protected by enhanced antibacterial activity, but they still exhibit a higher concentration of hemocytes in their hemolymph at the adult stage (Zanchi et al., 2011). These results suggest that mechanisms regulating protection of eggs and adult offspring are probably different. Hence, TGIP likely involves independent mechanisms that are acting simultaneously or sequentially over the development of the insect.

How and where the signal of a primary infection is “memorized” appears of paramount importance in the understanding of within and trans-generational immune priming. While hemocytes may play a role in immune memory, evidence is still scarce. For instance, during the antiviral immune response of *Drosophila*, infected cells generate double-stranded RNA (dsRNA) to inhibit viral molecule expression via RNAi (Tassetto et al., 2017). Part of the produced dsRNA is taken up by hemocytes, which then produce virus-derived complementary DNAs (vDNA) used as templates for *de novo* synthesis of small interference RNAs (siRNAs) targeting viral sequences (Tassetto et al., 2017). These siRNAs, secreted in exosome-like vesicles of immune cells, may represent the source of information storage. Furthermore, as TGIP requires the transfer of the information from the primary challenge to the offspring, the integration of viral elements into the genome through recombination with specific classes of retrotransposons and their organization into large loci of endogenous viral elements (EVEs) may represent a reservoir of immune memory in *Aedes aegypti* (Whitfield et al., 2017). Similar processes are unknown in *T. molitor*, which was also not reported to exhibit immune priming against virus infection.

Epigenetic reprogramming was also proposed as an important process to support within and trans-generational immune priming (Ottaviani, 2015). Epigenetic reprogramming of immune cells could be achieved through remodeling of DNA methylation patterns, changes in histone marks, modifications of chromatin structure, or changes in miRNA or lncRNA expression patterns. While indications of transcriptomic changes involving enzymes that control DNA methylation and histone acetylation in *Galleria mellonella* have been documented (Heitmüller et al., 2017), experimental evidence failed to link epigenetic differences to immune priming, both within and across generations (Eggert et al., 2014; Norouzitallab et al., 2015). A recent study has investigated the occurrence of methylation from total DNA and RNA extraction in *T. molitor* subjected to either individual or trans-generational immune priming by the fungus *Metarhizium anisopliae* or the bacteria *Micrococcus lysodeikticus* (Castro-Vargas et al., 2017). No global changes in DNA methylation resulting from either within or across generation immune priming were detected. However, whether DNA methylation was affecting smaller relevant portions of the genomic DNA, for instance, targeting the regulatory regions of a restricted number of genes was not investigated. In addition, a low proportion of RNA methylation results from individual immune

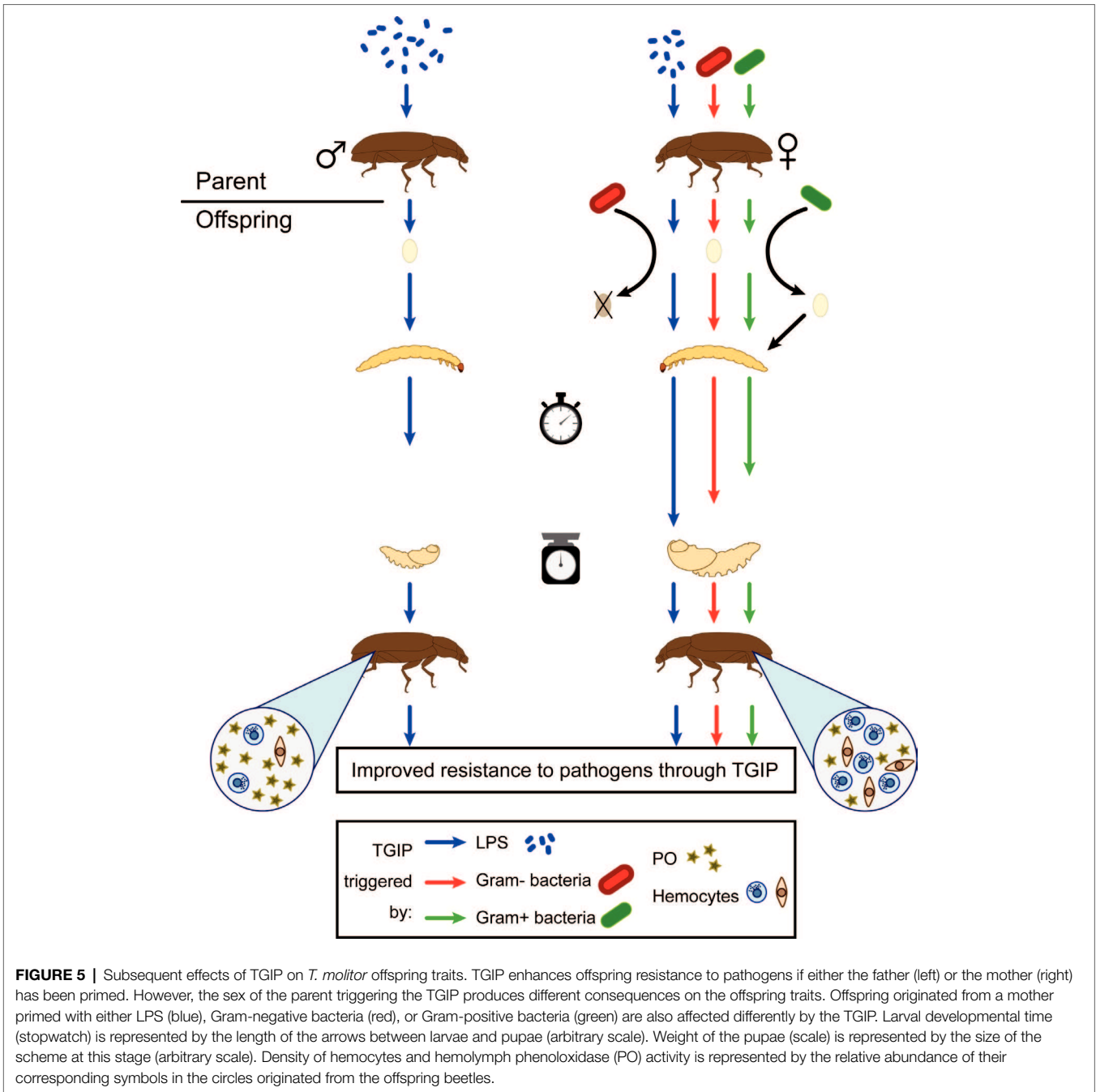


FIGURE 5 | Subsequent effects of TGIP on *T. molitor* offspring traits. TGIP enhances offspring resistance to pathogens if either the father (left) or the mother (right) has been primed. However, the sex of the parent triggering the TGIP produces different consequences on the offspring traits. Offspring originated from a mother primed with either LPS (blue), Gram-negative bacteria (red), or Gram-positive bacteria (green) are also affected differently by the TGIP. Larval developmental time (stopwatch) is represented by the length of the arrows between larvae and pupae (arbitrary scale). Weight of the pupae (scale) is represented by the size of the scheme at this stage (arbitrary scale). Density of hemocytes and hemolymph phenoloxidase (PO) activity is represented by the relative abundance of their corresponding symbols in the circles originated from the offspring beetles.

priming, indicating that RNA methylation could be involved in the process. However, no such change results from TGIP. Further study is needed to identify the types of RNA involved in methylation and their implication in the individual priming process.

The involvement of microRNA, lncRNA, and changes in chromatin structure has not been investigated in TGIP, despite their involvement in invertebrate immunity and host-pathogen interactions (Asgari, 2013). In *G. mellonella*, experimental selection for resistance to *Bacillus thuringiensis* resulted in trans-generational modification of acetylation of specific histones,

DNA methylation, and transcription of genes encoding the enzymatic writers and erasers of these epigenetic mechanisms (Mukherjee et al., 2017). Hence, considering the prominent role of epigenetics in many trans-generational adaptation processes in animals and its implication in the modulation of several immune response pathways, its involvement in immune priming, especially in TGIP, might be a promising avenue to explore in greater depth.

Immune priming, either within or across generation, is beneficial by enhancing individual immune protection against repeated infections. However, this process likely exerts

energy-related costs that would constrain the expression of other important functions. These costs might be bearable upon high risks of repeated infection but could be heavy when re-infection is unlikely (Tate, 2017). The cost of individual immune priming includes the cost of the initial immune response, upon a primary contact with the pathogen, and the additional cost of keeping the immune system upregulated for an extended period of time (*i.e.* memory), which are almost impossible to discriminate. The cost of TGIP is likely shared by both parents and offspring. On the one hand, parents, especially mothers, may support part of the cost of TGIP by producing and transferring immune substances to their eggs in addition to paying the usual immune activation costs resulting from the priming infection (Moret and Schmid-Hempel, 2000). For instance, bacterially immune-challenged females of *T. molitor* transiently endow a variable proportion of their eggs with antibacterial activity, which negatively correlates with female fecundity (Zanchi et al., 2012). Furthermore, the level of antibacterial activity found in eggs correlates negatively to that of their mother's hemolymph, suggesting that mothers trade-off their own immunity against that of their eggs (Moreau et al., 2012). On the other hand, enhanced immunity in the offspring may compromise other important functions. TGIP enhances immunity in the offspring of *T. castaneum* and *T. molitor* at the expense of a prolonged larval development time (Roth et al., 2010; Zanchi et al., 2011; Dhinaut et al., 2018a). A prolonged larval development time increases the probability of mortality (Bell, 1980), especially in tenebrionid beetles, which exhibits cannibalism on juveniles (Ichikawa and Kurauchi, 2009). However, such a cost in *T. molitor* depends on the bacterial pathogen to which mothers were previously exposed, as larval development time of maternally primed offspring with Gram-positive bacteria was much shorter than maternally primed offspring with Gram-negative bacteria (Dhinaut et al., 2018a). As pathogens may vary in the selective pressure they impose on hosts, *T. molitor* may have evolved an optimal immune priming against the most pervasive and threatening range of pathogens it encounters. In other insects, primed offspring exhibit reduced fecundity at the adult stage (Trauer and Hilker, 2013) and reduced resistance to a different parasite type to which the mother was exposed (Sadd and Schmid-Hempel, 2009). Further investigations are needed to reveal whether *T. molitor* primed offspring are paying comparable costs to TGIP. These negative effects associated to TGIP may result from the offspring trading-off their immunity against other functions. Alternatively, they may be the consequence of a reduced parental investment per offspring resulting from the cost of the parental immune challenge. In this case, reduced parental investment into their progeny should be observed early in the offspring's life. However, recent evidence showed that immune-challenged *T. molitor* females produced eggs with a stronger hatching success and that the resulting young larvae show enhanced survival to starvation within the first month post hatching (Dhinaut et al., 2018b), although they are known to exhibit a prolonged developmental time later on (Zanchi et al., 2011; Dhinaut et al., 2018a). This suggests that TGIP cost is likely to arise from offspring trade-offs and not from a reduced parental investment.

Microbiota

The importance of characterizing the mealworm's microbial community is proportional to the increased interest in this insect as a food source. Indeed, understanding the microbiota of insects that are used for consumption is an essential for identifying potential spoilage bacteria and food pathogens. The bacterial community from living, processed, and laboratory-reared mealworms reared for consumption indicated that their microbiota was dominated by Tenericutes, Firmicutes, and Proteobacteria (Jung et al., 2014; Stoops et al., 2016; Garofalo et al., 2017; Osimani et al., 2018). The gut-associated microbiota is an important mediator of host development and growth. For instance, microbiota-free mosquito larvae (axenic individuals) developmentally arrest due to the lack of bacteria-mediated hypoxia (Coon et al., 2014; Coon et al., 2017). Another striking example is the contribution of the gut-associated bacteria *Lactobacillus plantarum* to *Drosophila* growth (Storelli et al., 2011). The bacterium promotes protein assimilation from *Drosophila*'s diet, optimizing diet-derived amino acid levels in the hemolymph. This activates the target of rapamycin (TOR) signaling pathway, which triggers the insulin-like and ecdysone pathways that promote growth rate and reduce growth duration, respectively (Storelli et al., 2011). While no such intimate interaction has yet been described between *T. molitor* and its microbiota, axenic *T. molitor* experiences a change in digestive enzyme expression, supporting the hypothesis that associated microbes are involved in the insect's physiological homeostasis. Especially, the microbiota may help the mealworm to defend against the detrimental effects of food-derived toxic compounds such plant-derived glucoside salicin (Genta et al., 2006). In addition, microbe-free *T. molitor* does not produce pentadecene (Genta et al., 2006), a volatile that functions as a defensive secretion against predators in *T. castaneum* (Arnaud et al., 2002).

As previously mentioned, another growing interest for *T. molitor* concerns its ability to digest polystyrene foam. While polystyrene foam decreases *T. molitor* fecundity (Nukmal et al., 2018), the insect can fully develop using the plastic as its primary source of food. This makes the insect a relevant alternative to recycle polystyrene. Interestingly, when the mealworm's microbiota is disrupted following an antibiotic treatment, the insect loses its ability to digest polystyrene, indicating that its associated microbes play a crucial role in the digestion process (Yang et al., 2015). Especially, the bacterium *Exiguobacterium* sp. (Firmicutes) was isolated from the midgut of mealworms and was demonstrated to degrade the polystyrene *in vitro* (Yang et al., 2015). This shows that specific members of the microbial community confer the mealworm its ability to digest polystyrene. Hence, targeting the microbial community of *T. molitor* could help to boost its polystyrene digestion efficiency. This could be achieved *via* isolation of bacteria originating from the mealworm microbiota that would be genetically engineered to produce polystyrene-degrading enzymes.

In addition to supporting insect growth, indigenous microbes can mediate the development and function of their host immune system. In tsetse flies, microbe-free adults exhibit a severely compromised immune system that is characterized by a significantly depleted population of hemocytes (Weiss et al., 2011). Interestingly, larval *Drosophila*'s indigenous microbiota

regulates orthologous hematopoietic pathways in their host (Benoit et al., 2017). These examples demonstrate the intricate impact that the microbiota plays on host immune development. Such an association between *T. molitor* and its microbiota has yet to be investigated. Nevertheless, the enhanced immune response conferred by oral priming and TGIP demonstrates that immune mechanisms are adjustable according to *T. molitor* interaction with microbes. Also, it was suggested that microbiota influences oral priming in the red flour beetle *T. castaneum* (Futo et al., 2016). Hence, exploring whether *T. molitor*'s microbiota could influence TGIP would be a major milestone to understand the molecular mechanisms underlying this process.

The microbiota of *T. molitor* includes microbial taxa that may be pathogenic for human and animals, such as *Enterobacteriaceae*, *Streptococcaceae*, and *Enterococcaceae* (Jung et al., 2014; Stoops et al., 2016; Garofalo et al., 2017; Osimani et al., 2018). Methods used to process mealworm larvae for food, such as pulverization, could allow microbes to be released from the insect gut, which, if not eliminated, may be hazardous to livestock and human consumers (Klunder et al., 2012; Megido et al., 2016; Stoops et al., 2016; Garofalo et al., 2017; Grau et al., 2017). Thus, the presence of pathogens in mealworms would serve as an impediment to the use of this insect as a source of human and animal food. How the immune system could cope with controlling pathogens or modifying the microbiota structure is still an open question. However, the activation of the immune system through the production of antimicrobial peptides before processing the larvae could help to prevent an unwanted microbial community growing within insect-processed products. Indeed, insect antimicrobial peptides are active against a large range of microbes (Bulet et al., 1999). Immune priming could be used to trigger the long-lasting production of antimicrobial peptides in the hemolymph of the insect (Dhinaut et al., 2018a) prior to processing mealworm larvae. In consequence, primed insects would incorporate antimicrobial peptides that prevent unwanted microbes from contaminating the mealworm-derived food and feed.

PROMOTING AND CONTROLLING *T. MOLITOR* POPULATIONS

T. molitor is increasing being recognized as an alternative source of protein-rich food with a low ecological footprint (Grau et al., 2017). Hence, insect farms have started to mass produce mealworms despite the risk of an infection outbreak. Each group of insect pathogens presents unique biological characteristics for which a clear understanding of the interaction with the host is required to implement efficient control (Eilenberg et al., 2015). Nevertheless, controlling disease involves improving the insect's first line of defense, with the aim being to reduce the probability of infection. In *T. molitor*, one promising approach involves enhancing the integrity of the cuticle so as to render adult insects more resistant (Figure 3). Indeed, darker beetles present a thicker cuticle that is less likely to be circumvented by external pathogens (Silva et al., 2016; Evison et al., 2017), and they present enhanced immune parameters, such as the

PO activity and hemocyte concentration (Armitage and Siva-Jothy, 2005). As *T. molitor* population density positively correlates with cuticle darkness, we can expect that mass rearing conditions are already optimized for this parameter. Temperature and food can also influence the quality of the cuticle, as higher temperature and better access to the aromatic amino acid tyrosine increase the darkness of the cuticle (Prokkola et al., 2013; Evison et al., 2017). While supplementation of tyrosine could be easily achieved, increasing the rearing temperature would cause some deleterious effects on the insect immune system, which makes it a versatile parameter to account for.

Immune priming could be an asset for the mass production of healthy insects while keeping in mind the deleterious effects on other traits of the offspring. However, all the studies investigating immune priming in *T. molitor* used systemic injection of immune elicitors to obtain primed insects. This may prove difficult in mass-reared insects due to their large number. If *T. molitor* could be orally primed like *T. castaneum* (Knorr et al., 2015), the provision of inactivated bacterial materials in the food should be valuable to prevent disease outbreaks in the insect cultures (Grau et al., 2017). Alternatively, managing individually the parental line to enhance offspring immune parameters would be an easier task than trying to apply a method to each mass-produced insect. TGIP or a cold shock experienced by the parents could potentially improve the immunity of the offspring (Eggert et al., 2015). Hence, applying such stress only to insects destined for reproduction could enhance the overall immunity of the colony (Figure 5).

Immune priming may also have strong implications for the control of populations of pest insects, such as *T. molitor*, using microbial bio-insecticides. Indeed, when failing to kill the insects, biocontrol agents may subsequently enhance the insect resistance or tolerance, rendering their use less efficient over time. The control of unwanted populations might be even further complicated when, like in *T. molitor*, the priming response provides cross-specific protection that would impair the efficiency of control strategies using different microbial pathogens. Furthermore, insects may not necessarily need to suffer from the infection by the pathogen to become primed, as the consumption of dead bacteria in the food could be sufficient to prime them, as shown for *T. castaneum* (Knorr et al., 2015). Such an infection-free priming process may keep the insects vigorous enough to maintain prolific reproduction while becoming more immunocompetent.

Optimizing the production of insects such as *T. molitor* would allow other uses, such as producing pharmaceuticals or using them for de-pollution purposes. Interest in using insect AMPs as an alternative to antibiotics in livestock production has been growing over the past decade (Li et al., 2014; Wang et al., 2016). Given the broad spectrum activity exhibited by insect AMPs, their production in heterologous systems can reveal difficult. However, *T. molitor*'s large mass, in conjunction with the ability to mass produce the insect, makes it useful for generating AMPs *via* immune stimulation during the rearing process. Because the purification of AMPs could prove difficult, the use of a whole insect extract could be a viable alternative.

T. molitor is especially relevant for its ability to digest polystyrene foam. It would be highly beneficial to optimize production of the insect, in combination with improving its digestive capacity via microbiota manipulation, as an alternative to conventional polystyrene recycling schemes. Fundamentally, *T. molitor* and its associated microbes are highly suited candidates for investigating microbe-driven impacts on insect development, with a special focus on the immune system maturation. Understanding the molecular dialog between the insect and its microbiota opens up the possibility for exploiting this interaction as a target for pest control strategies to undermine the insect defenses, or, on the contrary, for enhancing the insect's defenses to optimize its application as a beneficial resource.

Recently, the insect immune system has been studied in the context of developing new tools for insect pest control. A proposed approach consists on using RNA interference (RNAi) to target genes that are crucial for the survival of insects (Baum et al., 2007). RNAi uses homologous double-stranded RNA (dsRNA) to downregulate specific mRNAs, which, for the purpose of pest control, would target genes leading to a severe decrease in fitness. This method was demonstrated successful on insects that feed on plants expressing hairpin dsRNA constructs, or on crops sprayed with dsRNA (Baum et al., 2007; Mao et al., 2007; Whyard et al., 2009; Huvenne and Smagghe, 2010). Recently, this method was applied to downregulate the expression of genes involved in *T. castaneum* Toll signaling pathway, especially focusing the pathway regulator *cactus* and its interacting genes (Bingsohn et al., 2017). RNAi-driven knockdown of *cactus* leads to the death of the insect, validating the relevance of targeting this gene as a novel control method. However, as *cactus*, and more globally the Toll pathway, is required for dorsoventral patterning in *Drosophila melanogaster* (Belvin and Anderson, 1996), the observed effect on *T. castaneum*

may be due to a disruption in developmental processes rather than immune-related functions. Using such methods also brings the issue of collateral damage due to an unspecific effect of the RNAi, which could target conserved genes across different species that reside in the target insect's environment. As developmental genes are usually highly conserved across taxa, targeting genes specifically involved in the immune system of an organism under infection could prevent such an effect. Knockdown of immune genes could be combined with the use of *T. molitor* parasites such as the Apicomplexan gregarines (Harry, 1967; Rodriguez et al., 2007) or the ectoparasitoid *Scleroderma guani* (Zhu et al., 2013) to successfully control the pest (Figure 4).

AUTHOR CONTRIBUTIONS

AV and YM conceived the ideas. AV, CJ, TR, and YM performed the literature search and contributed to the writing of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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