

ÉCO-IMMUNOLOGIE DE L'HIRONDELLE BICOLORE
(*TACHYCINETA BICOLOR*) EN MILIEU AGRICOLE

par

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mémoire présenté au Département de biologie en vue
de l'obtention du grade de maître ès sciences (M. Sc.)

FACULTÉ DES SCIENCES
UNIVERSITÉ DE SHERBROOKE

Sherbrooke, Québec, Canada, novembre 2012



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ISBN: 978-0-494-91686-5

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ISBN: 978-0-494-91686-5

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Le 14 novembre 2012

*le jury a accepté le mémoire de Monsieur Gabriel Pigeon
dans sa version finale.*

Membres du jury

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Directrice de recherche
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Professeur Marc Bélisle
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Département de biologie

Professeur Dany Garant
Président rapporteur
Département de biologie

À mes parents

SOMMAIRE

Si la tendance se maintient, d'ici 50 ans, l'impact de l'expansion et de l'intensification agricole pourrait rivaliser, en termes d'impact sur la biodiversité, avec les changements climatiques. Les oiseaux insectivores aériens et des prairies sont fortement affectés par l'intensification agricole, ce qui pourrait expliquer le déclin des populations en Amérique du Nord. Un des mécanismes possibles de ce déclin est un affaiblissement du système immunitaire dû à une réduction de la qualité de l'habitat. Hors, l'importance du contexte écologique sur le système immunitaire est encore assez mal compris

L'objectif de mon projet de recherche était de déterminer l'effet de l'intensification agricole sur le potentiel immunitaire des oisillons et des femelles d'une population d'Hirondelle bicolor (*Tachycineta bicolor*) dans le Sud du Québec. Pour ce faire, j'ai participé à un suivi individuel à long terme initié en 2004 par Marc Bélisle. Ce vaste système de 400 nichoirs sur 40 fermes est réparti sur une superficie d'environ 10200 km². Les nichoirs sont installés le long d'un gradient d'intensification des pratiques agricoles, ce qui permet d'étudier l'impact de ce changement anthropique majeur. De plus, tous les nichoirs sont suivis aux 2 jours durant la reproduction, tous les individus sont bagués avec une bague officielle du gouvernement, les mesures morphologiques de tous les individus sont prises avec précision et le sexe des individus est déterminé génétiquement. Ce système fournit donc une excellente opportunité de répondre à des questions écologiques d'intérêt.

Dans le premier chapitre de mon mémoire, visant l'impact de l'hétérogénéité environnementale (intensité agricole et température) sur la réponse immunitaire chez les oisillons et les femelles hirondelles en reproduction, j'ai utilisé une mesure de la capacité pro-inflammatoire. J'ai donc

étudié l'impact de l'environnement sur cette réponse immunitaire chez les oisillons et chez les femelles adultes. J'ai considéré deux variables environnementales, soit l'intensité des pratiques agricoles et la température. Mes résultats montrent que les conditions environnementales affectent la réponse à la phytohémagglutinine (PHA) aussi bien chez les femelles que chez les oisillons. La réponse à la PHA des adultes était significativement plus élevée en milieu agricole extensif qu'en milieu intensif. La température avait aussi un impact important; des températures plus élevées menant à de plus fortes réponses chez les adultes et de plus faibles réponses chez les oisillons. Mes résultats suggèrent ainsi que des individus soumis à des conditions difficiles pourraient faire un compromis entre leur immunité et d'autres fonctions importantes, telles que prodiguer des soins parentaux. De plus, il semble que les adultes soient en grande partie capables de tamponner l'effet négatif d'un habitat de moindre qualité sur les oisillons.

Le second chapitre de mon mémoire est consacré à l'impact de l'hétérogénéité environnementale sur les corrélations entre les différentes mesures immunitaires chez les oisillons et sur l'effet d'un indice immunitaire intégrateur sur la valeur adaptative chez les oisillons. Pour ce faire, j'ai mesuré sept marqueurs immunitaires, ce qui permet d'avoir une image plus global du système immunitaire des individus. Des études récentes ont montré que les corrélations entre mesures immunitaires varient selon l'espèce et selon la population. J'ai donc comparé les relations entre les mesures immunitaires des oisillons selon la qualité de l'habitat (intensif ou extensif) et l'année. Mes résultats montrent que les relations entre mesures immunitaires varient selon l'environnement aussi bien spatial que temporel. Les mesures fonctionnellement reliées sont positivement corrélées tel que prévu, mais pas de façon constante. J'ai également tenté de relier un indice global d'immunité (les deux premières composantes principales) à plusieurs indicateurs de performance individuelle. Mes résultats suggèrent que l'immunité des oisillons Hirondelle bicolor influence la masse à l'envol ainsi que la charge parasitaire, mais seulement dans certains environnements.

En conclusion, mes recherches montrent le rôle clé de l'environnement sur le système immunitaire. Que le système immunitaire soit observé à l'aide d'un seul ou de plusieurs marqueurs immunitaires, les réponses immunitaires sont influencées de façon significative. La qualité de l'habitat, la température, les variations annuelles semblent tous avoir un impact sur la capacité immunitaire de l'Hirondelle bicolore. Mon mémoire met donc en évidence la complexité et la variabilité du système immunitaire ainsi que l'attention qu'il faut porter à son étude.

REMERCIEMENTS

J'aimerais tout d'abord remercier ma directrice, la Pre Fanie Pelletier, pour tout son soutien et son mentorat. Elle a suscité un grand enthousiasme pour les résultats que nous trouvions et m'a poussé à me dépasser. De plus, elle m'a soutenu dans mon perfectionnement dans le monde de R. Merci aux membres de mon comité, Dany Garant et Marc Bélisle, pour leurs conseils et leur aide pour la rédaction de mes articles. Merci à Alan Cohen pour son point de vue d'écophysiologiste qui fut fort utile pour mon second article. Je voudrais aussi remercier Hélène, qui me voyait plus souvent que son chum l'été, pour son aide infinie. Je n'aurais jamais eu des CV aussi bas sans elle. Merci aussi à tous les participants du projet hirondelles, qu'ils soient étudiants gradués (passés et présents), assistants de terrain ou stagiaires; Merci à tous. Merci à Renaud Baeta d'avoir débuté les mesures immunitaires des hirondelles et de m'avoir donné ses deux années de données. Merci à toute la gang du labo pour l'aide et les discussions (sérieuses ou non). J'aimerais aussi remercier mes parents qui m'ont poussé dans le derrière quand j'étais dans des creux.

Mon projet de recherche a été rendu possible grâce au soutien financier du Conseil de recherches en sciences naturelles et en génie du Canada, du Fonds de recherche Québec – Nature et technologies, du Centre de la science de la biodiversité, de la Fondation canadienne pour l'innovation, de la Chaire de Recherche du Canada en démographie évolutive et conservation et du Fond Anne Vallée, puis grâce aux fermiers qui participent au projet hirondelle et sans qui on aurait juste quelques nichoirs sur le campus.

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« It's complicated »

CHAPITRE 1

INTRODUCTION

1.1 L'importance de l'éco-immunologie

Des mécanismes de défense contre l'invasion du soi par d'autres organismes sont présents chez tous les êtres vivants. Les bactéries ont des mécanismes de défense contre les phages (Labrie *et al.*, 2010). Les plantes ont également des mécanismes de défense contre les pathogènes (Glazebrook, 2005). Les animaux ne font pas exception ; tous les animaux ont un système immunitaire. Le niveau de complexité de celui-ci diffère cependant. Tous les animaux ont des mécanismes immunitaires innés, i.e. : macrophage, lysosome, système de complément, etc. Les mécanismes de défense plus complexes de l'immunité sont apparus plus tardivement dans l'évolution (chez les vertébrés) (Cooper et Herrin, 2010). Tous les vertébrés à mâchoire (*Gnathostomata*) ont un système immunitaire basé sur les mêmes composantes: un système de complément, des interférons, des lysozymes et des globules blancs dont les lymphocytes B qui produisent des anticorps (Figure 1.1).

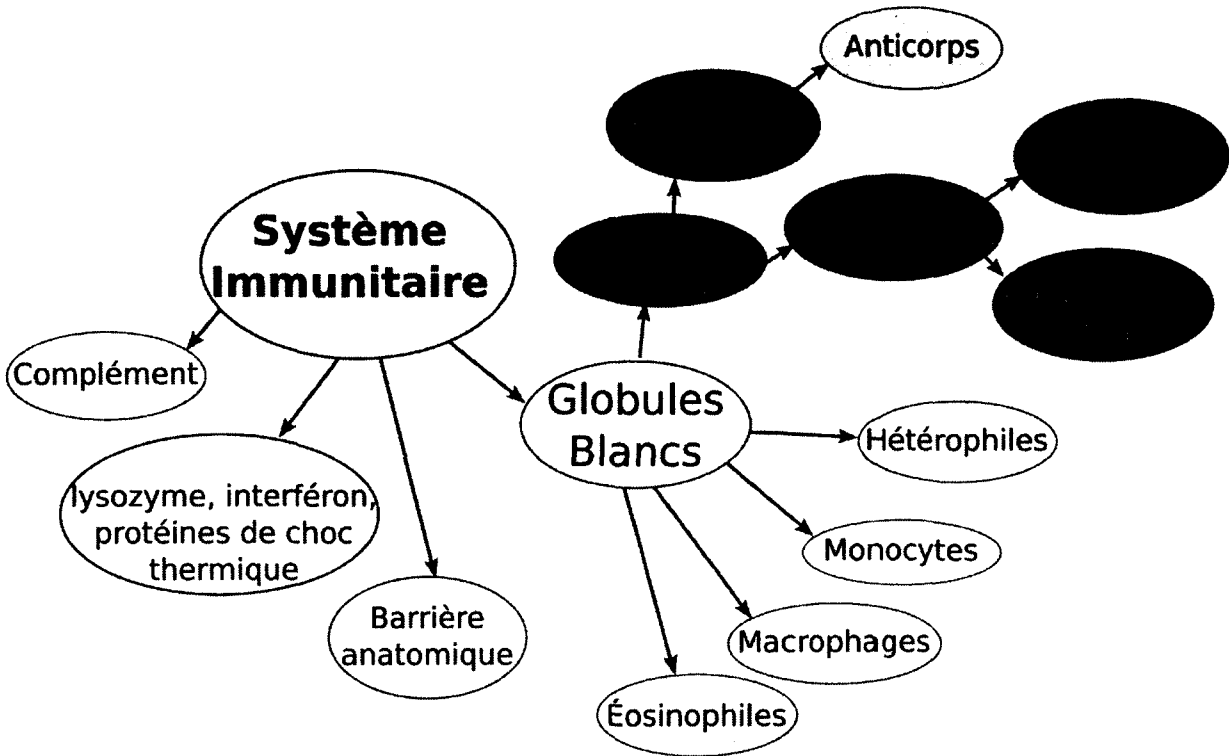


Figure 1.1. Différentes composantes du système immunitaire des mammifères et des oiseaux. Les composantes innées sont en blanc alors que les composantes adaptatives sont grises. Les anticorps, pouvant faire partie des deux sont en gris pale.

Malgré une composition de base similaire, il y a énormément de variation interspécifique et interindividuel dans le système immunitaire. Étant donné que le système immunitaire est composé de plusieurs composantes autonomes, il est possible de le développer et de le réguler de façon modulaire, ce qui mène à différents indices immunitaires qui ne sont pas forcément corrélés. Par exemple, certaines espèces ont un système immunitaire dont les composantes innées sont plus développées que les composantes adaptatives (Previtali *et al.*, 2012). Il est aussi possible d'investir moins dans les mécanismes immunitaires et de tolérer les pathogènes (Ayres et Schneider, 2012; Baucom et de Roode, 2011). Cette flexibilité laisse place à une large gamme de variations pouvant agir à trois échelles. Premièrement, les espèces diffèrent entre elles. Lee (2006) a proposé que les variations interspécifiques puissent être liées aux

stratégies d'histoire de vie. Certaines espèces suivent une stratégie de vie rapide, comme la souris domestique (*Mus musculus*) : taux de reproduction rapide, courte vie. D'autres suivent une stratégie lente, comme l'éléphant (*Loxodonta Africana*) : taux de reproduction faible, longue vie (Promislow et Harvey, 1990). Les espèces à stratégie rapide, qui ont habituellement un système immunitaire adaptatif moins important, préfèrent miser sur la reproduction actuelle plutôt que la survie à long terme. Chez les oiseaux, la réponse la réponse immunitaire suit les prédictions avec les espèces plus grosses, plus longévives et au développement plus lent ayant une réponse immunitaire plus forte (Tella *et al.*, 2002). Deuxièmement, au sein d'une même espèce, les populations diffèrent dans leurs réponses immunitaires. Un exemple commun est une différence immunitaire selon la latitude (Martin II *et al.*, 2004). Les espèces nordiques ont un risque d'infection parasitaire plus faible et subissent des variations saisonnières de l'environnement. Ces différences sont reflétées au niveau du système immunitaire. Par exemple, une étude a montré que chez le Moineau domestique (*Passer domesticus*), la réponse à la PHA de la populations du Panama est plus faible et plus stable que celle de la population du New Jersey, USA (Martin II *et al.*, 2004). Troisièmement, il y a de la variation individuelle. Cette variation peut avoir une multitude de causes : génétique, différence des sexes, effets maternels, environnement, histoire immunologique, âge, etc.

L'éco-immunologie ou l'étude du système immunitaire dans un contexte écologique est une discipline qui a été développé récemment. Les premiers articles en traitant datent des années 1990 (Martin *et al.*, 2011). L'éco-immunologie fait le lien entre les écologistes, qui jusqu'alors traitaient l'immunité comme une boîte noire, et les immunologistes, qui étudiaient le système immunitaire aux niveaux cellulaire et moléculaire, hors de tout contexte écologique. Heureusement, des avancées récentes au niveau méthodologique permettent maintenant l'étude du système immunitaire d'individus sauvages (Salvante, 2006). Le but de l'éco-immunologie est d'expliquer comment et pourquoi des facteurs biotiques et abiotiques influencent les variations au niveau du système immunitaire. La capacité immunitaire d'un individu a un impact direct sur sa survie, donc sur sa valeur adaptative (Møller et Saino, 2004). De plus,

certaines composantes de la capacité immunitaire sont hautement héritable. Par exemple, la production d'interleukine IL-1 β suite à une stimulation par des lipopolysaccharides a une hérabilité de 0,86 (de Craen *et al.*, 2005), ce qui suggère que ces composantes peuvent évoluer. Deux grands courants de pensée sont présents en écologie évolutive en ce qui a trait au système immunitaire. D'une part, sa spécificité ou comment la réponse à certains pathogènes a évoluée. Certains auteurs ont suggéré la présence de coévolution et d'une course aux armements entre hôtes et pathogènes (Sasaki et Godfray, 1999; Thompson, 2005). D'autre part, le système immunitaire est coûteux et doit protéger l'individu contre une grande variété de pathogènes. L'étude du système immunitaire s'inscrit donc bien dans le cadre de la théorie de l'histoire de vie (Lee, 2006). Une bonne compréhension de l'influence des facteurs biotiques et abiotiques sur les variations en réponses immunitaires est nécessaire pour interpréter de façon appropriée l'impact des variations observées d'un point de vue évolutif. L'éco-immunologie a aussi des impacts plus appliqués comme dans le cas de l'épidémiologie. Des variations individuelles en réponses immunitaires pourraient, entre autres, expliquer le phénomène des individus hyper-infecteurs (« super-spreader »; Hawley et Altizer, 2011). Une meilleure compréhension de ce phénomène et des variations immunitaires saisonnières sont deux exemples qui pourraient mener à l'élaboration de meilleurs modèles de propagation des pathogènes. Par exemple, des études ont récemment mis en évidence la coévolution entre différents systèmes de moustiques et de *Plasmodium*, ce qui pourrait aider dans la lutte contre la malaria (Tripet *et al.*, 2008). L'éco-immunologie est aussi importante pour la conservation. Les perturbations anthropiques peuvent avoir un impact important sur le système immunitaire des individus et donc sur la viabilité de la population. Les perturbations peuvent mener à un stress chronique dont une des conséquences est une immunosuppression. Par exemple, chez l'iguane marin des Galapagos (*Amblyrhynchus cristatus*), la présence d'activité touristique cause une diminution de plusieurs paramètres immunitaires dont la capacité bactéricide et l'activité du complément (French *et al.*, 2010). De plus, les changements globaux de température peuvent avoir des conséquences imprévisibles. Par exemple, l'augmentation de la température des océans est corrélée à une augmentation des épidémies chez les coraux (Bruno

et al., 2007). L'exposition à des polluants peut aussi causer des diminutions de la capacité immunitaire (Dunier et Siwicki, 1993). Ainsi, une meilleure compréhension des facteurs écologiques influençant le système immunitaire pourra mener à la formation de meilleurs plans de gestion.

1.2 Fondements théoriques

1.2.1 Les changements environnementaux

La population humaine est en augmentation constante, ce qui a des répercussions importantes sur l'environnement, telles que la destruction d'habitats et la perte de biodiversité. Un des impacts importants de l'homme est lié à l'agriculture. L'augmentation des besoins alimentaires a modifié les pratiques agricoles. En effet, la superficie agricole totale au Canada n'a augmenté que de 1 % entre 1971 à 1991 (Agriculture and Agri-Food Canada, 1997). L'augmentation de la production agricole est mieux expliquée par le changement des pratiques agricoles d'un mode familial à un mode commercial. Les agriculteurs de la région des basses terres du Saint-Laurent se tournent de plus en plus vers la monoculture de céréales (Bélanger et Grenier, 2002). Les changements du paysage agricole peuvent être décrits comme un passage d'un mode de culture extensif à un mode de culture intensif. Les milieux extensifs sont caractérisés par la forte présence de fourrages et de pâturages. Les milieux intensifs, quant à eux, sont caractérisés par des monocultures de blé ou de soya, l'augmentation de l'utilisation de produits chimiques (fertilisants et pesticides), l'augmentation de l'utilisation de machinerie lourde, des récoltes plus précoces et fréquentes, la perte d'habitats marginaux, la diminution de champs en jachère (27 % entre 1971 et 1991; Agriculture and Agri-Food Canada, 1997), et l'augmentation de la densité de plantation (Bélanger et Grenier, 2002; Donald *et al.*, 2006a; Newton, 2004). Le changement d'un mode de culture extensif à un mode

intensif a comme avantage de maximiser le rendement.

Cette quête d'une plus grande productivité n'est pas sans conséquence pour la biodiversité. Ces changements peuvent cependant avoir un effet déplorable sur les individus et la population de certaines espèces (Stoate *et al.*, 2001). Il est difficile d'évaluer l'importance de chacun des facteurs anthropiques (machinerie, transformation du paysage, etc.) sur la persistance des espèces puisqu'ils apparaissent simultanément et sont fortement corrélés (Chamberlain *et al.*, 2000; Murphy, 2003; Newton, 2004). En effet, il a été montré que l'abondance moyenne d'espèces d'oiseaux diminue significativement avec l'homogénéité du paysage (Jobin, 1996). Si la tendance se maintient, l'impact de l'expansion agricole sur la biodiversité pourrait rivaliser avec celui des changements climatiques d'ici 50 ans (Teysseire et Couvet, 2007; Tilman *et al.*, 2001). Sur 58 espèces d'oiseaux champêtres étudiés, les populations de 41 espèces montraient un déclin d'effectif entre 1990 et 2000 (Donald *et al.*, 2006a). Au Canada, les populations d'oiseaux insectivores aériens ont diminuées constamment depuis 1970 (Initiative de conservation des oiseaux de l'Amérique du Nord, 2012). L'effet de l'agriculture sur les oiseaux est un sujet important en écologie : 10 % des publications récentes (1998-2000) dans le *Journal of applied ecology* portent sur ce sujet (Ormerod et Watkinson, 2000). Chamberlain a montré que l'état des populations d'oiseaux champêtres d'Angleterre suivait les changements de pratiques agricoles (Figure 1.2). La relation entre l'intensification agricole et la perte de biodiversité et d'abondance a été montrée à maintes reprises (Donald *et al.*, 2001; Donald *et al.*, 2006b; Geiger *et al.*, 2010; Murphy, 2003; Robinson et Sutherland, 2002).

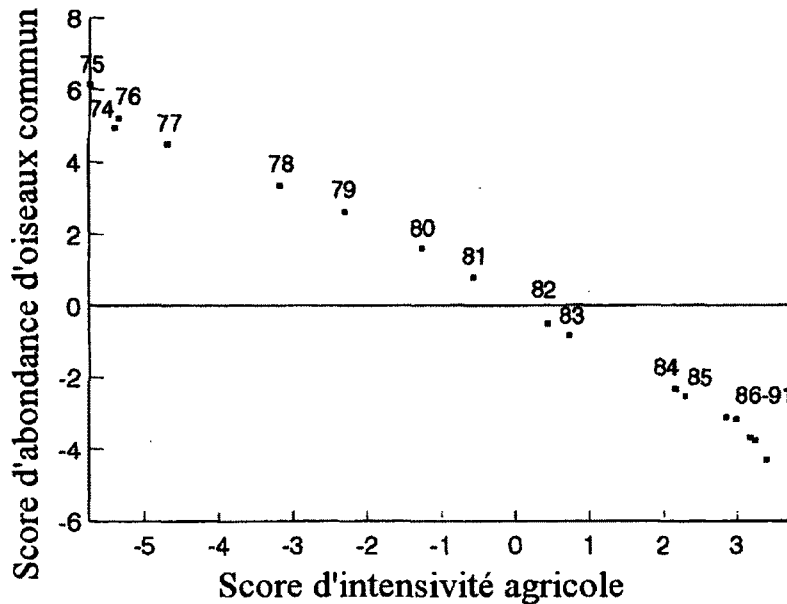


Figure 1.2. Index de population en fonction du niveau d'intensivité agricole. L'axe des Y représente l'axe principal dérivé d'une analyse de composante principale des index de population du recensement d'oiseaux commun (Commun bird census) tandis que l'axe des X représente l'axe principal dérivé d'une analyse de composante principale des variations environnementales. Les années correspondant aux scores d'abondance d'oiseaux sont données sur la figure (tirée de Chamberlain *et al.*, 2000).

Les oiseaux insectivores semblent être énormément touchés par la transformation du paysage (Blancher *et al.*, 2009; Collins et Downes, 2009; Evans *et al.*, 2007). Ce déclin a été associé à une diminution de la qualité de la diète (Boatman *et al.*, 2004; Nebel *et al.*, 2010b). En effet, il a été observé que la densité d'insectes volants est moins élevée dans les habitats agricoles simplifiés tels que les champs intensifs (abondance d'insectes volants 3,5 fois plus élevée dans des pâturages que dans des champs céréaliers) et que les pâturages accueillent deux fois plus d'Hirondelle rustique (*Hirundo rustica*) que les champs céréaliers (Evans *et al.*, 2007). De même, dans un système d'étude de l'Hirondelle bicolor (*Tachycineta bicolor*), Rioux Paquette

et al. (2012) ont montré que les différences d'abondance d'insectes entre cultures intensives et extensives étaient faibles en début de saison mais s'intensifiaient avec le temps. De plus, les oisillons de Tarier des prés (*Saxicola rubetra*) recevaient 30% plus de biomasse d'insectes en milieu extensif qu'en milieu intensif, résultant en un taux d'envol plus grand (Britschgi *et al.*, 2006). Il est donc possible que l'intensification agricole ait un effet indirect sur les oiseaux champêtres via son effet sur les ressources alimentaires.

Il peut être supposé qu'un déclin en richesse spécifique et en abondance tel qu'observés chez les passereaux insectivores soient dus à une diminution de la valeur adaptative ou à la performance des individus. Plusieurs études ont observé un lien entre l'intensification agricole et des éléments de la valeur adaptative. Par exemple, chez l'Hirondelle rustique, une grande quantité de monticules de fumier où abondent les insectes (associés au milieu non-intensif) est significativement reliée à une augmentation de taux de survie des oisillons (Gruebler *et al.*, 2008). Chez cette même espèce, il a été observé que le succès reproducteur annuel sur des fermes laitières était plus élevé que pour les fermes intensives (Møller, 2001). La même étude a montré que les oisillons provenant de fermes intensives étaient en moins bonne condition que ceux des fermes laitières, plus petits et dotés d'une immunocompétence réduite. Une telle diminution de la condition des oisillons pourrait expliquer les différences en succès d'envol entre les types d'agriculture malgré les tailles de couvée similaires observées chez l'Hirondelle bicoloré par Ghilain et Bélisle (2008).

L'effet de l'intensification agricole ne se limite pas à une diminution des ressources disponibles. En effet, elle pourrait aussi diminuer la valeur adaptative en augmentant le niveau de stress physiologique des oiseaux, ce qui pourrait mener à un stress chronique causant une diminution de la reproduction, une diminution de la capacité immunitaire et une perte de masse. L'utilisation accrue de pesticides associés à l'intensification agricole a aussi un impact important. En plus de réduire l'abondance de proie (Boatman *et al.*, 2004; Morris *et al.*, 2005;

Poulin *et al.*, 2010; Rioux-Paquette *et al.*, 2012), les pesticides peuvent avoir des impacts physiologiques important chez les oiseaux. En effet, l'exposition à des pesticides peut modifier la réponse immunitaire (Bishop *et al.*, 1998; Galloway et Handy, 2003). Une incapacité à produire une réponse immunitaire appropriée pourrait ensuite nuire à la défense contre les pathogènes et donc la valeur adaptative. Mon projet apportera ainsi des éléments nouveaux permettant de mieux comprendre les causes du déclin de cette population.

1.2.2 L'immunité

Le système immunitaire est notre principale ligne de défense contre les pathogènes, il est donc crucial pour la survie des organismes. Les animaux sont continuellement exposés à une panoplie de pathogènes, p.ex. : virus, bactéries, parasites. Ces pathogènes ont le potentiel de réduire radicalement la valeur adaptative d'un individu (revue par Møller et Saino, 2004), et donc un système immunitaire efficace apporte un avantage marqué. Chez les moutons de Soay (*Ovis aries*), la longévité des femelles est positivement corrélée à la concentration d'anticorps antinucléaires, un indice de la sensibilité immunitaire adaptative (Graham *et al.*, 2010). Chez la Mésange bleue (*Cyanistes caeruleus*), la réponse à une injection de PHA, une mesure d'immunité cellulaire, est un bon indice de la probabilité de recrutement des oisillons (Cichon et Dubiec, 2005). De plus, les Faucon crécerelle (*Falco tinnunculus*) avec un niveau plus élevé d'anticorps naturels avaient une charge parasitaire plus faible (Parejo et Silva, 2009). Le système immunitaire étant si important pour la survie d'un individu, nous pourrions nous attendre à ce que son efficacité soit maximisée chez tous les individus. Ce n'est pas le cas.

Comme pour toutes adaptations, il y a des coûts. Des études ont montré qu'une réponse immunitaire pourrait avoir un coût énergétique élevé. Par exemple, Chez les Moineau domestique, le taux métabolique au repos augmente pendant une réponse immunitaire (Martin

II *et al.*, 2003). Puisque l'énergie est limitante, le système immunitaire pourrait donc aussi être en compétition avec d'autres fonctions d'un organisme, comme la reproduction et la croissance, menant à des compromis entre ces différents traits d'histoire de vie (Ardia, 2005). Des compromis entre l'immunité et d'autres fonctions biologiques ont été observés à maintes reprises (Deerenberg *et al.*, 1997; Lee, 2006; Martin II *et al.*, 2003; Mauck *et al.*, 2005). Par contre, plusieurs études ont montré des résultats plutôt contre-intuitifs. Par exemple, une étude n'a trouvé aucune évidence de compromis entre le niveau d'immunoglobuline G (un indicateur de la capacité immunitaire acquise) et la reproduction (taux de croissance des oisillons et taille de couvée) chez la Sterne pierregarin (*Sterna hirundo*) (Apanius et Nisbet, 2006). Des corrélations positives entre les traits d'histoire de vie pourraient être expliquées par des ressources non limitantes, par des ressources nécessaires différentes ou par des différences dans la capacité d'acquisition de l'énergie entre les individus (van Noordwijk et de Jong, 1986).

Une hypothèse alternative pour expliquer des associations inverses entre l'immunité et la valeur adaptative est qu'un système immunitaire trop fort augmente les risques de problème d'auto-immunité, menant à une diminution de la valeur adaptative (Bergstrom et Antia, 2006). Dans ce cas, un système immunitaire maximum n'est donc pas forcément optimal puisqu'il peut aussi avoir des coûts en endommageant l'organisme (Sadd et Siva-Jothy, 2006). Par exemple, les Mésanges bleue avec une haute ou une basse réactivité des anticorps ont une probabilité de survie inférieure à celle montrant des niveaux intermédiaires (Raberg et Stjernman, 2003). Finalement, la complexité du système immunitaire pourrait aussi expliquer en partie ces résultats contradictoires : il peut y avoir un compromis, non pas entre le système immunitaire et un autre trait d'histoire de vie, mais entre deux aspects du système immunitaire. Ainsi, un individu pourrait réduire sa réponse immunitaire acquise en faveur de la réponse immunitaire innée. Étant donnée la présence de coûts et de différences environnementales (menant à des avantages adaptatifs différents pour chaque composante du système immunitaire), la réponse optimale va être variable. Cette optimisation locale de

l'immunité va mener différentes populations, ou individus, à avoir une réponse immunitaire différente, ce qui est au cœur de l'éco-immunologie (Sadd et Schmid-Hempel, 2008).

Comme tous les systèmes de l'organisme, le système immunitaire est un système complexe composé de plusieurs éléments capables d'interagir entre eux, organisés en réseau et agissant en commun pour la défense de l'organisme (Matson *et al.*, 2006a). Le système immunitaire peut être séparé en deux grands axes, le système immunitaire inné et le système immunitaire acquis. Le système immunitaire inné aviaire est constitué de macrophages, de monocytes, d'hétérophiles, d'éosinophiles, de basophiles et du complément alors que le système immunitaire adaptatif est constitué de lymphocytes B et T et des anticorps. Le système immunitaire adaptatif ou acquis est spécifique, car cette immunité apparaît au cours de la vie d'un individu suite à l'exposition à un pathogène et ne protège que contre ce pathogène en particulier. La majorité du temps, cette protection immune est acquise pour la durée de la vie. Une espèce à longue espérance de vie ayant plus de chance de se reproduire dans le futur aura donc plus d'avantage à investir dans son système immunitaire adaptatif (Lee, 2006). L'immunité innée, quant à elle, ne requiert pas d'exposition préalable et est capable de protéger contre un grand nombre de pathogènes (Janeway *et al.*, 2003). Le système immunitaire peut aussi être divisé en immunité cellulaire et humorale. L'immunité cellulaire est médiée par les cellules alors que l'immunité humorale est assurée par des molécules solubles (p. ex. : anticorps et complément). Une autre distinction possible est celle entre les mécanismes induits (qui nécessitent la détection du pathogène pour être déclenché) et les mécanismes constitutif (qui sont toujours exprimé). Bien entendu, ces composantes du système immunitaire sont intimement liées. Le système immunitaire acquis requiert des signaux produits par le système inné pour avoir de l'information sur l'origine de l'antigène et le type de réponse à produire (Medzhitov et Janeway, 1997).

Plusieurs auteurs suggèrent l'utilisation d'un grand nombre d'indice de la capacité

immunitaire de façon à pouvoir différencier les compromis entre les divers composants du système immunitaire et mesurer la capacité globale de celui-ci (Ardia, 2007; Boughton *et al.*, 2011; Norris, 2000). Certains auteurs suggèrent d'utiliser au moins trois mesures de façon à mesurer les 3 grands axes immunitaires qu'ils ont observés suite à une analyse multivariée (Salvante, 2006). Il existe plusieurs mesures disponibles qui sont réalisables sur des populations sauvages. Les mesures utilisées durant ma maîtrise seront présentées en détails dans les prochaines sections (Figure 1.3).

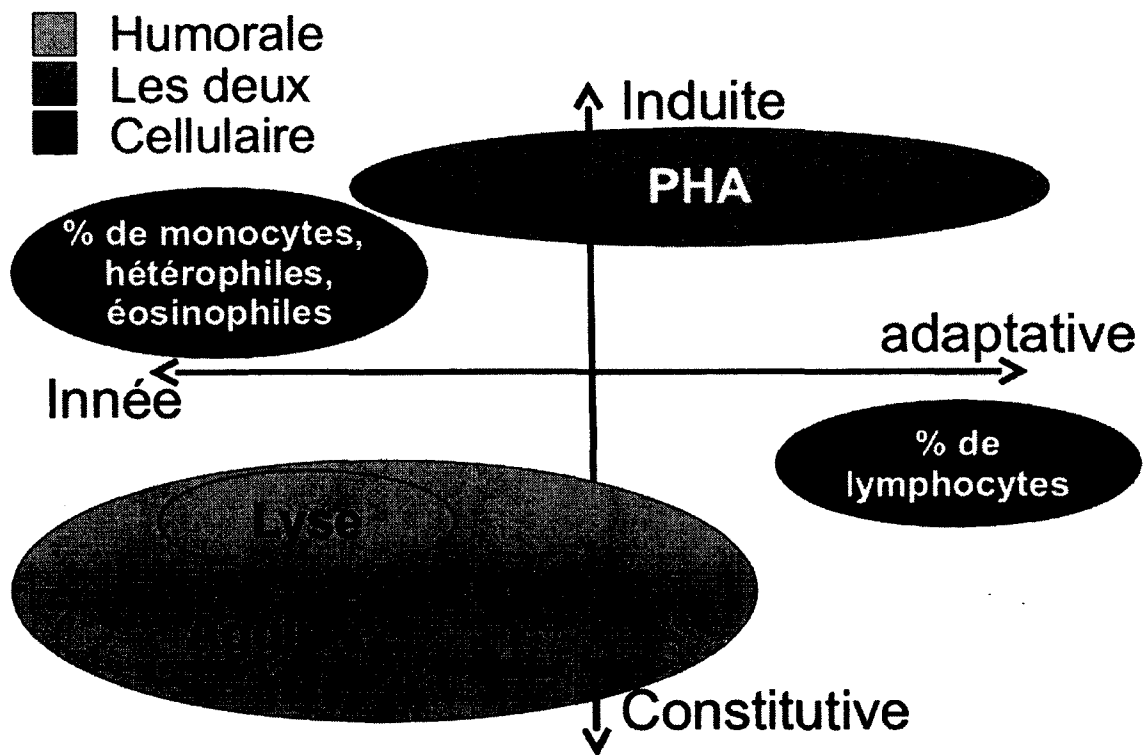


Figure 1.3. Caractérisation des différents indices de la capacité immunitaire utilisés dans cette étude selon les trois axes de caractérisation du système immunitaire. Acronymes : lyse, hémolyse; Agglu, Hémagglutination; CBP, capacité bactéricide du plasma;

PHA, réponse à la PHA.

1.2.3 Décompte des leucocytes

Une des mesures les plus classiques de l'immunologie est le décompte des globules blancs (WBC) à partir d'un frottis. Cette mesure de l'immunité cellulaire suppose qu'un individu avec beaucoup de WBC sera capable d'en maintenir une grande quantité en prévention à une réponse immunitaire. Un décompte différentiel peut aussi informer de façon plus précise sur la capacité d'un individu à produire certaines réponses immunitaires (Lebigre *et al.*, 2011). Les lymphocytes sont associés à l'immunité adaptative et ont une longue durée de vie, ils sont donc un investissement immunitaire à long terme. Les monocytes, les hétérophiles et les éosinophiles sont associés à l'immunité innée (Beldomenico *et al.*, 2008). Les monocytes et les hétérophiles jouent un rôle important dans la lutte contre les infections bactériennes tandis que les éosinophiles visent les gros parasites et les virus (Venge, 1990). Les décomptes leucocytaires sont pourtant critiqués : le décompte varie de l'ordre de trois fois selon le manipulateur (Walberg, 2001). Aussi, une grande quantité de WBC pourrait tout aussi bien indiquer un système immunitaire sain ou un individu en pleine lutte immunitaire contre un pathogène. De plus, il y a des évidences que le nombre de leucocytes n'est pas forcément corrélé avec l'activité leucocytaire (Salvante, 2006). Le ratio hétérophile/lymphocyte est aussi de plus en plus utilisé. Ce ratio serait un bon indicateur du niveau de stress d'un individu chez les oiseaux, étant donné que le stress cause une réduction des lymphocytes et une augmentation des neutrophiles (Davis *et al.*, 2008; Gross et Siegel, 1983). Cependant, cette mesure serait sujette à une variation saisonnière, avec un ratio plus élevé durant la migration printanière qu'automnale (Owen et Moore, 2006).

1.2.4 Réponse à la PHA

Le test de PHA consiste en une mesure de l'inflammation causée par l'injection de phytohémagglutinine (PHA). La PHA est une molécule dérivée de plante qui déclenche la division des lymphocytes T et ainsi une inflammation (Goto *et al.*, 1978). L'intensité de l'inflammation est alors considérée comme un indicateur de l'immunité cellulaire acquise. Cette mesure a plusieurs avantages. Premièrement, c'est un challenge immunitaire nouveau, ce qui permet d'éviter le problème d'expositions antérieures de l'oiseau. Deuxièmement, cela déclenche une réponse forte et donc facilement mesurable. Cependant, cette mesure est parfois critiquée. La PHA active directement la division des lymphocytes, contrairement à une réponse naturelle où une cascade d'interactions cellulaires et chimiques, incluant la reconnaissance et la présentation d'un antigène, mène à l'activation sélective des lymphocytes. Certaines études ont donc été suggérées que la réaction à la PHA ne reflète pas nécessairement la vraie réponse immunitaire (Owen *et al.*, 2010). Ce test est cependant largement utilisé et considéré comme fiable par plusieurs tant que l'interprétation est faite avec soin (Tella *et al.*, 2008; Vinkler *et al.*, 2010). Une étude a montré que chez les oisillons d'Hirondelle bicolor, la réaction à une injection de PHA dépendait de la température et de l'abondance de nourriture (Lifjeld *et al.*, 2002). Chez cette même espèce, une augmentation de la réaction inflammatoire a été observée chez les oisillons élevés en présence de pesticides, possiblement à cause d'une hypersensibilité ou en raison d'une suppression de la corticostérone (un immunosuppresseur) (Bishop *et al.*, 1998).

1.2.5 Test d'hémagglutination

Le test d'hémagglutination-hémolyse est une mesure d'immunocompétence relativement récente (Matson *et al.*, 2005). Ce test permet de mesurer l'immunité humorale innée. Il

consiste à mettre en contact des érythrocytes de lapin avec différentes concentrations de plasma de l'espèce d'intérêt. Plus précisément, il permet de mesurer deux choses, la quantité d'anticorps naturels (NABs) et l'efficacité de l'interaction NABs/complément. Les anticorps sont souvent vus comme des éléments de l'immunité acquise, mais les anticorps naturels sont présents même sans exposition antérieure à un antigène particulier. Les NABs sont produits par une sous-population de lymphocytes B-1 CD5+ et sont capables de réagir avec une grande variété d'antigènes provenant de globules rouges étrangers, de virus, de bactéries et de toxines. L'expression des NABs est principalement génotype dépendant et n'est donc pas influencée par les différentes expositions des individus (Baumgarth *et al.*, 2005). Ce sont les NABs qui causent l'agglutination des globules rouges de lapin dans ce test. Suite à cette agglutination, la cascade enzymatique du complément se déclenche, menant à la lyse de l'envahisseur (Carroll et Prodeus, 1998). Un score élevé d'agglutination est donc un signe d'un grand nombre de NABs et donc d'une capacité immunitaire élevée. Un score élevé de lyse démontre une capacité élevée à détruire un corps étranger et donc une capacité immunitaire élevée. Chez les femelles Hirondelle bicolore adultes, un niveau plus élevé de corticostérone a été associé à un score plus faible d'agglutination, mais pas de lyse (Palacios *et al.*, 2007). L'influence possible de l'environnement sur cette mesure la rend particulièrement intéressante dans le cadre de l'éco-immunologie.

1.2.6 Activité bactéricide du plasma

Ce test intégrateur est très avantageux en raison de sa facilité d'interprétation. En effet, tuer des bactéries est une fonction principale du système immunitaire. Cette mesure de l'immunité innée intègre les composantes cytologiques aussi bien que sérologiques (Matson *et al.*, 2006c). La phagocytose par les hétérophiles et les macrophages est particulièrement importante pour la défense contre les bactéries. Dans le plasma, les NABs et le complément sont aussi très importants. Le lysozyme, une autre composante du plasma, est aussi capable de bactéricide par

une digestion enzymatique des parois cellulaires des bactéries. De plus, certaines protéines de phase aigüe peuvent aussi augmenter la capacité bactéricide du sang (Matson *et al.*, 2006c). Un des défauts de cette mesure est qu'elle nécessite plusieurs jours de manipulation et un décompte de colonies sur un pétri (Liebl et Martin II, 2009). De plus, la réponse peut être plus forte pour certaines espèces de bactérie que pour d'autres. De plus, le stress a été associé à une diminution significative de la capacité bactéricide, cependant l'importance de cette réduction variait selon l'espèce rendant l'interprétation de cette mesure complexe (Matson *et al.*, 2006c). Néanmoins, cet essai est fort utile car il s'approche d'une situation de réponse immunitaire pouvant avoir lieu dans un contexte naturel.

1.3 Objectifs et importance de mon projet

L'objectif général de mon projet de maîtrise est d'évaluer l'effet de l'intensification agricole sur le potentiel immunitaire d'une population sauvage d'Hirondelle bicoloré dans le Sud du Québec. Les trois objectifs spécifiques de mon projet de maîtrise sont :

- 1) Déterminer l'impact de la qualité des conditions environnementales (intensité agricole et température) sur la réponse à la PHA chez les oisillons et les femelles reproductives.
- 2) Déterminer l'impact de l'hétérogénéité environnementale sur les corrélations entre les différentes mesures immunitaires chez les oisillons.
- 3) Mesurer l'effet d'un indice immunitaire intégrateur sur la valeur adaptative des oisillons.

Pour ce faire, j'ai participé au suivi d'une population nichant en milieu agricole dans deux environnements de qualité différente. Environ 200 oisillons et 100 femelles ont été capturés chaque année entre 2008 et 2011 dans un vaste réseau de nichoirs répartis sur plus de 10200 km² au sud du Québec. Les mesures morphométriques de chaque individu ont été prises ainsi que sept mesures de capacité immunitaire. Il s'agit d'un contexte idéal pour étudier l'impact de l'habitat sur des mécanismes physiologiques. De plus, tous les individus de l'étude sont rigoureusement suivis. J'ai ainsi pu considérer, en plus de multiples variables

immunitaires et environnementales, des indices de condition et de performance.

Considérant le phénomène de déclin de l'hirondelle, l'étude de l'effet de l'intensification agricole sur le système immunitaire prend tout son sens. Mon projet pourrait ainsi aider à mieux comprendre le déclin de la population. Mon projet a aussi un aspect plus fondamental qui consiste à approfondir nos connaissances sur l'éco-immunologie de l'hirondelle bicolor. L'éco-immunologie est un domaine évoluant rapidement. Les immunologistes issus de laboratoires sous-estiment souvent l'importance des variations environnementales. À l'opposé, les écologistes se lançant en immunologie ont tendance à sous-estimer la complexité du système immunitaire. Mon projet tente de faire le lien entre les deux. Mon étude est parmi les premières à utiliser un grand nombre de mesures immunitaires. C'est la première à couvrir plusieurs années et plusieurs habitats de qualités différentes pour une même population. Évaluer l'impact de l'hétérogénéité environnementale sur la structure des différentes fonctions immunitaires est crucial, car ignorer l'effet de l'environnement pourrait mener à des conclusions erronées ou non généralisables.

1.4 Méthodologie générale

1.4.1 Espèce à l'étude

L'espèce modèle de mon projet de maîtrise sera l'hirondelle bicolor. Cet oiseau migrateur, qui passe l'hiver dans la région du Mexique et de l'Amérique centrale, se retrouve couramment en Amérique du Nord pendant la période de reproduction. Cet oiseau est principalement retrouvé en milieu découvert à proximité de l'eau, de marais, de ruisseaux, de lacs, de fossés, de prés (Winkler *et al.*, 2011). C'est un oiseau socialement monogame qui se reproduit pendant l'été (mi-mai à fin juin). La ponte se fait vers la fin mai suivie d'une incubation d'environ 14

jours. Durant cette période, l'incubation des œufs est assurée par la femelle. Une fois les œufs éclos, les deux parents nourrissent les oisillons en alternance jusqu'à l'envol qui est en moyenne 18 jours après l'éclosion. L'Hirondelle bicolor se nourrit principalement d'insectes volants qu'elle attrape en vol. C'est un nicheur de cavité secondaire qui s'établit couramment dans les nichoirs. Ceci est fort utile puisque ça rend leurs captures relativement aisées. L'Hirondelle bicolor pèse en moyenne 20 g à l'âge adulte et 23 g à l'envol. Sa petite taille est un des facteurs qui la rend facile à manipuler. Les hirondelles peuvent supporter la manipulation sans abandonner leur nichée, ce qui est un préalable crucial à un projet de ce type (Jones, 2003).

Plusieurs études suggèrent que l'intensification des pratiques agricoles affecterait les oiseaux insectivores agricoles (Tariel des prés (Britschgi *et al.*, 2006); Hirondelle rustique : (Ambrosini *et al.*, 2002; Møller, 2001); Hirondelle bleue (*Hirundo atrocaerulea*) : (Wakelin et Hill, 2007); Hirondelle bicolor : (Ghilain et Bélisle, 2008). Les populations d'Hirondelle bicolor au Canada diminuent d'environ 4,6 % chaque année depuis les années 1970 (Nebel *et al.*, 2010a; Shutler *et al.*, 2012). Cette diminution pourrait être causée par une perturbation de l'environnement. Ces effets pourraient être dus à la diète (Evans *et al.*, 2007; Rioux-Paquette *et al.*, 2012) ou être dus à la pollution (Bishop *et al.*, 1995; McCarty et Secord, 1999). Cette sensibilité aux perturbations causées par l'intensification agricole, combinée au fait que la biologie de cette espèce est bien connue, fait de l'Hirondelle bicolor une espèce modèle idéale pour mon projet.

1.4.2 Aire d'étude

L'aire d'étude est définie par un réseau de 400 nichoirs établi en 2004 répartis sur 40 fermes et couvrant une superficie d'environ 10200 km² entre Sorel, Beloeil et Sherbrooke (Ghilain et

Bélisle, 2008). Le système de nichoirs est caractérisé par un gradient est-ouest d'intensification agricole allant de petites fermes extensives à d'immenses monocultures intensives. L'aire d'étude couvre donc une large portion des combinaisons possibles entre la superficie relative recouverte par des cultures intensives et extensives à l'intérieur d'un rayon de 5 km. Ce rayon a été utilisé puisqu'il s'agit de l'aire d'alimentation maximale durant la période de nourrissage des oisillons (Robertson *et al.*, 1992). Une étude a montré que l'échelle spatiale influençait peu la relation entre l'intensité agricole et le succès reproducteur durant la ponte, mais que le succès d'envol diminuait significativement avec une intensification des pratiques agricoles dans un rayon de 5 km (Ghilain et Bélisle, 2008). Un sous-échantillon de 10 fermes représentant les deux extrêmes du gradient environnemental a été utilisé pour les mesures immunologiques (Figure 2.1). À chaque ferme, 10 nichoirs ont été disposés à intervalles de 50 mètres le long de fossés ou de clôtures en bordure des champs. Les nichoirs ont été installés sur des poteaux de 1,5 mètre avec l'ouverture vers le sud-est.

CHAPITRE 2
EFFECTS OF AGRICULTURAL INTENSIFICATION AND TEMPERATURE ON
IMMUNE RESPONSE TO PHYTOHEMAGGLUTININ IN TREE SWALLOWS
(TACHYGINETA BICOLOR)

Introduction de l'article

L'article «Effects of agricultural intensification and temperature on immune response to phytohemagglutinin in tree swallows (*Tachycineta bicolor*)» traite de l'effet de l'environnement sur la réponse immunitaire chez l'Hirondelle bicolor. Il existe une certaine variation individuelle dans la capacité à produire une forte réponse immunitaire. Il est attendu que cette capacité soit influencée par des effets génétiques, de condition corporelle et par des facteurs environnementaux. Dans cet article, j'ai utilisé des modèles linéaires mixtes pour quantifier l'effet de facteurs environnementaux (intensité agricole et température), individuels (masse, âge, sexe), de couvée (taille de couvée, charge parasitaire, date de ponte, sexe ratio) et de qualité maternelle (âge et masse de la mère) sur la réponse à la PHA chez les oisillons et les femelles nichantes. De plus, étant donné que l'effet de la température est variable temporellement (surtout pour les oisillons), j'ai fait une analyse de « sliding window » pour déterminer les périodes où la température avait le plus fort effet sur la réponse à la PHA.

Les résultats présentés dans cet article sont importants pour une meilleure compréhension des variations immunologiques individuelles et de l'impact des facteurs environnementaux sur la réponse immunitaire. Cette population est en déclin depuis les années 1970 (4.6 % par année) et ce déclin est probablement causé par les modifications anthropiques du milieu, soit l'intensification agricole. Cet article apporte des informations supplémentaires à notre

compréhension du déclin des oiseaux insectivores en milieu agricole.

Ma contribution à cet article a été substantielle puisque j'ai pris part activement à la prises de données, fait les analyses statistiques, écrit l'article et coordonné le processus de publication. J'ai été encadré lors de ce projet par la Pre Fanie Pelletier. De plus, plusieurs versions de cet article ont été révisées par Renaud Baeta (qui a supervisé la prise de données en 2008 et 2009 et qui a initié le projet), la Pre Fanie Pelletier, le Pr Marc Bélisle et le Pr Dany Garant. Cet article est en révision majeur à *Canadian Journal of Zoology* (24 octobre 2012)

Effects of agricultural intensification and temperature on immune response to phytohemagglutinin in tree swallows (*Tachycineta bicolor*)

par

G. Pigeon, R. Baeta, M. Bélisle, D. Garant and F. Pelletier

Abstract

Lower immune response usually translates into lower fitness. Environmental quality can play a key role in shaping immune responses in the wild as it influences both resource availability and costly maintenance functions. The aim of this study is to evaluate the effect of habitat quality on pro-inflammatory response to phytohemagglutinin (PHA) in tree swallows under contrasting agricultural practices. Specifically, we tested the hypothesis that lower quality habitat (intensive agricultural practices and low temperature) negatively impairs immune responses. To do so, we compared the response to PHA of adult female and nestling tree swallows *Tachycineta bicolor* (Vieillot, 1808) nesting in intensive vs non-intensive agricultural landscapes over three years (2008-2010). In accordance with our predictions, we found that habitat quality affected adult female responses to PHA. The response of adult females was significantly higher in non-intensive agricultural areas than in intensive ones. The level of agricultural intensity had however no influence on the response to PHA of nestlings. Our results suggest that female adults can buffer the negative impact of a low quality habitat on their nestlings. Furthermore, our results suggest that under limiting conditions, individuals may have to trade between immunity and other functions such as parental effort.

Introduction

Immunity has wide implications for animal life history as it represents the main line of defence against pathogens, and is thus intimately linked to fitness (Saino *et al.* 1997a; Christie *et al.* 2001; Cichon and Dubiec 2005; Moreno *et al.* 2008). Understanding how a species deals with trade-offs involving the immune system is crucial in order to integrate knowledge of physiological processes into our comprehension of the evolution of life-history strategies. The immune response can be energetically costly and, consequently, the energy devoted to immunity is not available for other physiological processes resulting in trade-offs (reviewed by Lochmiller and Deerenberg 2000). For example, Cai *et al.* (2009) reported that antibody production increased metabolic rate and reduced reproductive physiology in male Brandt's voles (*Lasiopodomys brandtii*). In the wild, environmental conditions play an important role in shaping immune responses (Avtalion and Clem 1981; Arriero 2009). Trade-offs between immunity and other costly functions might be easier to detect in poor environmental conditions, when individuals face lower resource abundance and where the cost of maintenance is higher. For example, in tree lizards (*Urosaurus ornatus*), increased immune activity diminishes and even suppresses reproductive investment when resources are limited (French *et al.* 2007). Similarly, the growth of sand martin (*Riparia riparia*) nestlings is negatively correlated to the immune response to phytohemagglutinin (PHA) injection when food is scarce, but no trade-off is found when resources are abundant (Brzek and Konarzewski 2007). Another environmental factor which may be important for the development, maintenance and expression of an immune response is temperature, because in colder environments, animals will have to devote a greater part of their energy budget to thermoregulation. For example, previous studies have shown that adult tree swallows, *Tachycineta bicolor* (Vieillot, 1808) have a lower response to PHA in colder weather than in warmer conditions (Liffield *et al.* 2002). Considering the environmental context is therefore critical to our understanding of the immune response outside lab settings.

Given the influence of environmental conditions on the development and maintenance of immune functions, human-driven changes also have the potential to affect immunity (French *et al.* 2010; Martin *et al.* 2010). Agricultural intensification has been associated to declines in farmland bird populations in many countries (Chamberlain *et al.* 2000; Murphy 2003). Insectivorous birds, especially aerial ones, appear to be affected to a greater extent than other bird groups (Blancher *et al.* 2009; Collins and Downes 2009). Agricultural intensification is characterized by many highly correlated environmental changes such as the homogenisation of landscape and habitat structure and the augmented use of pesticides. These changes are hypothesized to lower insect prey abundance, reduce habitat quality, and have a negative effect on birds (Benton *et al.* 2002). Changes in farming practices that lead to lower food abundance may result in a weaker immune response to PHA (Møller 2001). For instance, food supplementation in barn swallow (*Hirundo rustica*) nestlings caused an increase in their response to PHA (Saino *et al.* 1997b). However, investigations of how habitat quality interacts with other factors to modulate an individual immune response in wild populations are still scarce and often yield contradictory results (Ambrosini *et al.* 2006; Neve *et al.* 2007; Harms *et al.* 2010).

The immune system is very complex and immune responses can be measured in many ways (reviewed in Salvante 2006). In birds, one of the most commonly used measures is the skin swelling response caused by an injection of PHA (Goto *et al.* 1978). The PHA test was developed as a measure of T-cell mediated immunity (Smits *et al.* 1999), but whether or not it actually measures T-cell response is subject to controversy because the exact mechanism is complex and involves lymphocytes, heterophils, basophils, and eosinophils (Martin *et al.* 2006; Tella *et al.* 2008). Although more studies are required to elucidate the physiological basis of the immune response to PHA, this test is still a robust tool for characterizing an individual's pro-inflammatory response (Vinkler *et al.* 2010; hereafter referred to as response to PHA). Previous studies in birds have shown that the response to PHA increases the resting metabolic rate (Martin *et al.* 2003) and is positively correlated with survival (Gonzalez *et al.*

1999; Soler 1999; Cichon and Dubiec 2005). Given the strong correlation between these variables and fitness, variation in response to PHA may have an important impact for wild populations in habitats suffering from degradation.

The aim of this study was to evaluate the effect of environmental quality on the immune response of tree swallows by comparing the pro-inflammatory response to PHA of breeding adult females and nestlings within intensive and non-intensive agricultural landscapes, as well as under variable temperature conditions, over three years in southern Québec, Canada. . We hypothesized that (i) the pro-inflammatory response of adult females and nestlings would differ between good quality (non-intensive agriculture) and low quality (intensive agriculture) habitats and (ii) the pro-inflammatory response of adult females and nestlings would be greater in warmer temperatures. Given the above, we predicted that response to PHA in tree swallows would be lower in intensively cultivated landscapes than in non-intensive habitats. However, we expected that the effects of habitat quality could be smaller for nestlings since parents could compensate for lower habitat quality by providing food at a higher rate (Tremblay *et al.* 2004). We also expected to find a positive correlation between ambient temperature and response to PHA given that low temperature can lead to lower resource availability (Gruebler *et al.* 2008) and to increased thermoregulatory costs. However, we expected that the strength of this correlation would vary according to the temperature period selected. Notably, we expected that the early growth period would be particularly important for nestlings given their rapid development and lack of thermoregulatory capacity at that time. Additional variables were also considered in order to control for their additional sources of variance (see methods for details).

Materials and methods

Study species and area

The tree swallow is a small passerine bird (adult mean mass in our sample: 21.9 g, SD = 1.5g) that migrates from Mexico and Central America to North America in the summer to breed. This species is an obligate secondary cavity nester that is mostly found in open habitats near water or agricultural fields and pastures. The tree swallow is a good model species for studying immune response in the field because they use artificial nest boxes in which they can be caught easily during reproduction. Also, this species is not overly disturbed by manipulation, reducing the chance of abandonment caused by regular monitoring of young in the nest (Jones 2003). The study was conducted using a nest box network established in 2004 in southern Québec covering 10 200 km² (Figure 2.1). The landscape of this region is characterized by an east-west gradient of agricultural intensification (see Ghilain and Bélisle 2008; Porlier *et al.* 2009 for details; Figure 2.1). This anthropogenic modification of the landscape allowed us to evaluate the effect of landscape composition on response to PHA.

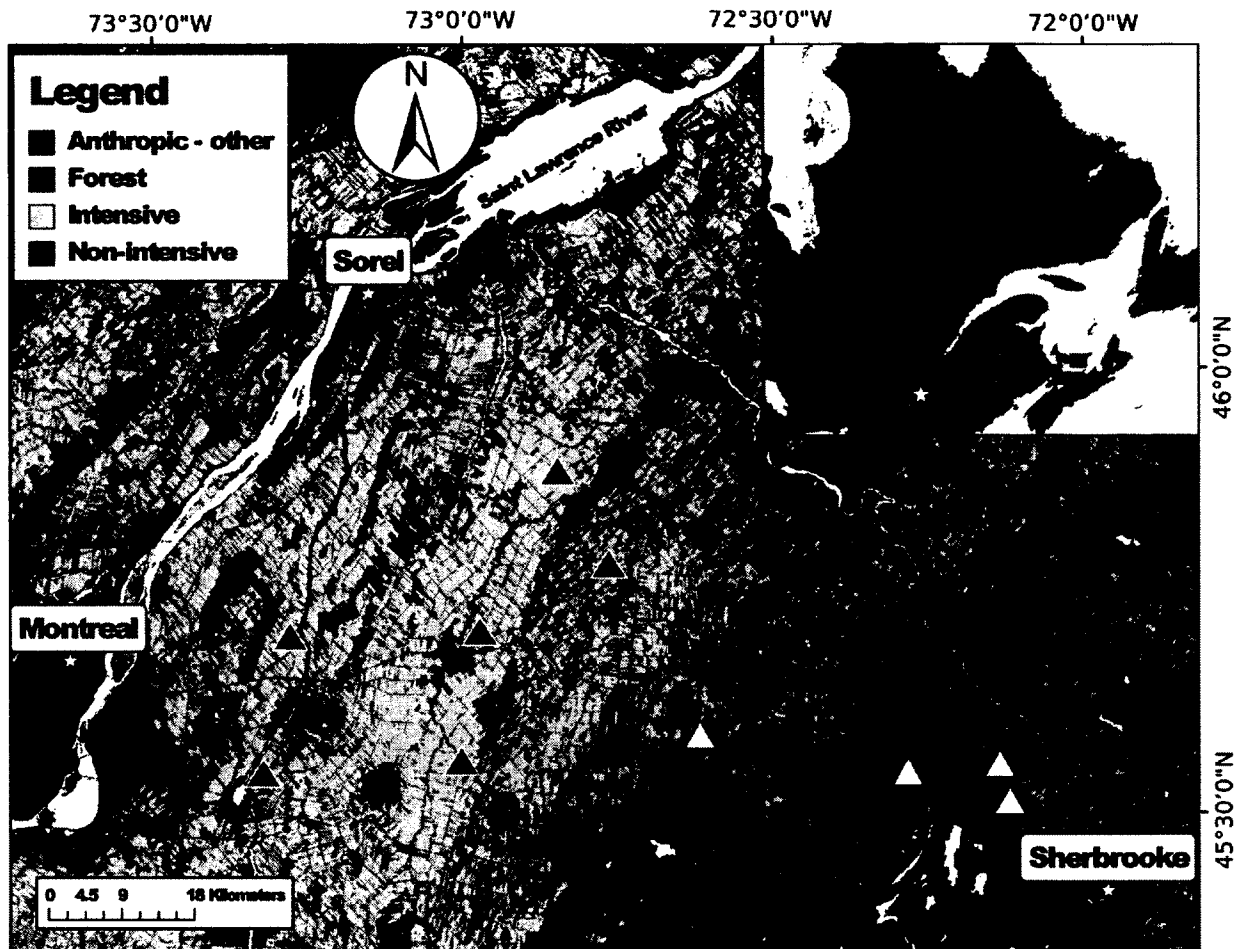


Figure 2.1. Distribution of the 10 farms used in this study of tree swallows (*Tachycineta bicolor*) along a gradient of agricultural intensification in southern Québec, Canada, 2008–2010. The location of the study area at larger scale is represented by a star on the map in the top right corner. Land cover types (represented by the shades of grey) are based on a mosaic of classified LANDSAT-TM satellite images (Canadian Wildlife Service, 2004). Dark triangles indicate intensive farm locations and white triangles indicate non-intensive farm locations.

Landscape characterization

We monitored 100 nest boxes distributed equally among 10 farms at each extreme of the agricultural intensification gradient from 2008 to 2010. Agriculture type was assessed based on the mean proportions of intensive cultures (e.g., corn, soybean, and other cereals) and non-intensive cultures (e.g., hayfields, pastures, and fallows) surrounding nest boxes both at fine and large geographical scales (proportion of intensive cultures in a radius of 500 m and 5 km around each nest box respectively). Fledging success declined significantly with intensification in a radius of 5 km (Ghilain and Bélisle 2008). Hence, this two-scale gradient can be used as a proxy for habitat quality around the nest. Land-use was estimated at the 5 km scale based on a mosaic of geo-referenced and classified Landsat-7 satellite images taken between August 1999 and May 2003 (pixel resolution 1/4 25 3 25 m; Canadian Wildlife Service 2004). Land-use at the 500 m scale was assessed visually in the field each year and recorded on orthophotos (1:40 000). Relative cover of intensive and non-intensive land was then calculated at both scales using ArcView GIS Spatial Analyst 2.0a (ESRI 2005). Each farm at the extreme of the gradient of agricultural intensification was then classified as being located in an intensive or non-intensive agricultural area. Although land-use varied slightly from one year to the next, the relative cover varied little and farms remained in the same habitat class over the entire study period. We monitored a higher number of farms within intensive areas to account for lower nest box occupancy in those habitats and ensure sufficient sample size in both environments (4 farms in the non-intensive area and 6 farms in the intensive area). Mean proportion of intensive agricultural landscape strongly differed between farm groups at both the 500 m and the 5 km scales (see supplementary material, Table A1.1).

Nest monitoring

Nests were monitored every two days during the breeding season (from early May to mid-July) to record laying, incubation and hatching dates. Females were captured in nest boxes during incubation. At first capture, adult females were marked individually and classified as 'second year' (SY) or as 'after second year' (ASY) according to plumage coloration (Hussell 1983). Nestlings were marked individually by nail clipping until their 12th day of life, and, thereafter, were marked with an official aluminium ring. Finally, blood samples of adults and nestlings were collected from the brachial vein to confirm the sex and the identity of the mother of each individual (for details on genotyping and sexing methods see Porlier *et al.* 2009). Out of the 100 nest boxes monitored each year, 47 to 55 nest boxes contained nestlings. Occupancy varied greatly by farm, ranging from 1 to all 10 nest boxes being occupied. Nests contained on average 4.57 nestlings (SD = 1.23).

Phenotypic measurements

Morphometric measurements were taken on all adult females captured during incubation. Birds were weighed (± 0.01 g) with a platform scale equipped with a contention device (MXX-412, Denver instrument, Bohemia, NY, USA). The parasitic load was measured as the number of *Protocalliphora* pupae found in the nest material after the breeding season. *Protocalliphora*, blood-sucking ectoparasites, have been reported to reduce physiological performance and recruitment probability (Thomas *et al.* 2007), and to affect response to PHA (Dawson *et al.* 2005a). Nestlings were weighed (± 0.01 g) 12 days after hatching. The length of their left tarsus was recorded (± 0.01 mm) as a measure of their size.

Immune response measurements

Responses to PHA were measured following Martin *et al.* (2006). For adult females, the injection was made on day 4 (i.e., 4 days after the eggs hatched) in the years 2009 and 2010. Nestlings of 2008 to 2010 were tested on day 14 as they were too small before this age. Each bird was injected in the left wing patagium with 0.1 ml of PHA (L8754-50MG, Sigma Aldrich, St Louis, MO, USA) diluted in phosphate-buffered saline (1mg/ml, no. 811-010-CL, Wisent, St-Bruno, Canada). The response to PHA was measured as the difference in thickness of the patagium before and after PHA injection, as measured (± 0.001 mm) with a digimatic micrometer (293 MDC-Lite, Mitutoyo Corporation, Montréal, Canada). Given the size of the study area and the logistic difficulty of recapturing adults several times, it was impossible for us to take the second measurement at a fixed time interval. Second measurements of the patagium were thus taken either 24 h or 48 h ± 0.67 following injection. The exact time interval was initially included in all analyses as a control, but was removed during model selection (see supplementary material Table A1.2 and A1.3 for model simplification). Response to PHA was not affected by the delay between measurements (Supplementary material Figure A1.1). All measurements of the patagium were made by the same observer in each year. We measured the repeatability of our PHA measurement according to Wolak *et al.* (2012). The same observer always took three measurements of the same bird, and repeatability between measurements was very high (ranging from 0.986 to 1.00 depending on the year, $p < 0.001$). All procedures described in this study were approved by the University of Sherbrooke's Animal Care Committee (protocol number FP2009-01) and comply with current Canadian laws regarding animal research.

Temperature monitoring

Ambient temperature data (± 0.5 °C) were obtained hourly from May 1st until August 15th of each year using a temperature data logger (Thermochron iButton DS1922L, Dallas Semiconductor, Dallas, TX, USA) fixed under a nest box at each farm.

Statistical analysis

We applied a square root transformation to the inflammatory response to PHA in order to normalize the data which were slightly skewed. Given that previous studies had mixed results concerning the period during which temperature affects the response to PHA (Ambrosini et al 2006), we first used a sliding window approach to identify the period during which ambient temperature had the greatest effect on this variable (Brommer et al 2008). To do so, we calculated the mean temperature during an interval ranging from 1 to 45 days before the second measurement of the patagium ("window"), and repeated this calculation for each individual. We then repeated this approach for every possible position of every time interval in the 45 days before the skin swelling assay ("sliding"), thus obtaining 1 080 different periods during which the mean temperature was measured for each individual. We finally quantified the correlation coefficients between the response to PHA and the mean ambient temperature across individuals, for each of the 1 080 time periods, using Pearson's moment correlations. We analysed nestlings and adult females separately because of the potential difference in explanatory variables affecting response to PHA between these stages. Yet, we found no significant difference in response to PHA between adult females and nestlings (mean = 0.90 and 0.89 mm, SE = 0.06 and 0.02 respectively, $P = 0.86$).

A linear mixed effects model was used to analyze the response to PHA of adult females

($n = 54$) because of the potential for non-independence of data (nest boxes nested within farms). Farm identity was thus initially included as a random effect. Female identity was not included as a random variable since only 6 measurements (out of the 54) represented females that were measured in different years. Explanatory variables included agriculture type (as a two categories factor, intensive or non-intensive) and temperature. Additional variables reported to affect PHA were also included in the initial model as control variables. These variables included individual characteristics, such as mass (Navarro *et al.* 2003), age (Palacios *et al.* 2007), laying date (Hasselquist *et al.* 2001; Ardia 2005a), brood size (Pap and Márkus 2003), and *Protocalliphora* burden. As mass might not always be representative of physical condition (Johnson *et al.* 1985), we also tested a model including the ratio between mass and tarsus length. However, this model had lower support than the model including only mass (AICc = 71.74 and 78.33 respectively), and thus all our subsequent analyses only included mass. The interactions between agriculture type and all other explanatory variables were included in the initial model. As we had measurements taken over multiple years, we included "year" as a factor to account for potential inter-annual differences. We also included the exact number of hours between injection and measurement (measurement delay) in all models to account for the effect of time on PHA measurements.

The response to PHA of nestlings ($n = 408$) was also analyzed using a linear mixed effects model because of the potential for non-independence of data (nestlings nested in nest boxes nested in farms). Explanatory variables included environmental effects (agriculture type, temperature) as well as control variables for brood and individual nestling characteristics, such as mass at 12 days after hatching (Ardia 2005a), sex, laying date (Møller *et al.* 2009), brood size (Ardia 2005b) and *Protocalliphora* burden. Female age and mass were also taken into account (Potti 2008). The interactions between agriculture type and all other explanatory variables were also included in the initial model. Year and number of hours between injection and measurement of PHA were also included in the models.

For both adult females and nestlings, we fitted a complete model with all fixed effects and interactions previously described and then selected the best model (out of all possible nested sub-models) according to second order Akaike Information Criteria (AICc) using the MuMIn package (Bartoń 2012). Variables contained in significant interactions were kept in the final model even if not significant independently. All statistical analyses were performed using the R statistical environment (R Development Core Team 2010; version 2.11.0; package nlme version 3.1-77). Details on model selection can be found in Table A1.2 and A1.3 in supplementary material.

Results

Identification of temperature periods

Adult females: The highest correlation between adult response to PHA and mean temperature occurred using the temperature window spanning from 1 to 2 days before the second patagium measurement (Pearson correlation coefficient, $r = 0.29$, $P = 0.03$). We thus used this temperature window in subsequent analyses of adult response to PHA. Nestlings: The strength and direction of the correlation between temperature and response to PHA varied greatly depending on the length and position of the temperature window, with coefficients ranging from -0.34 to 0.23. This variation nevertheless showed a temporal pattern (supplementary material, Figure A1.1). The most negatively correlated window spanned from 5 to 16 days before the second patagium measurement ($r = -0.34$, $P < 0.001$), a time period which corresponded approximately to the first 11 days after hatching (further referred to as 'growth temperature'). Thereafter, r -values increased up to a maximum of 0.23 corresponding to a window spanning 28 to 31 days before the second patagium measurement (further referred to as 'laying temperature'). We included both growth and laying temperatures when modelling

the response to PHA of nestlings. The correlation between these two temperatures was 0.38; hence, auto-correlation was not a concern.

Response to PHA of adult females

The swelling response to PHA injection of adult females was significantly lower in intensive agricultural areas than in non-intensive areas with means of 0.769 mm (SD = 0.495, $n = 21$) and 0.985 mm (SD = 0.336, $n = 33$) respectively (Figure 2.2). Our model suggests that this effect was modulated by female body mass (Table 2.1a): while light birds had a lower response to PHA than heavy birds in intensive areas, body mass had no effect on the response to PHA in non-intensive areas (Figure 2.3). Laying date and parasitic load did not have significant effects on the amount of swelling caused by PHA injection and were removed from the model (supplementary material, Table A1.2). Females in their first year of reproduction (SY) had a weaker PHA response (mean = 0.893 mm, SD = 0.421, $n = 8$) than older females (mean = 0.948 mm, SD = 0.401, $n = 46$) (Table 2.1a). Clutch size was kept in the model following AICc selection although its effect was only marginally non-significant ($P = 0.057$). Our model also revealed that temperature affected response to PHA, with females in colder environments having smaller response to PHA (Table 2.1a). The amount of swelling increased by 0.088 mm for every 1°C increase, ranging from 0.314 mm in colder environments (10°C) to 1.906 mm in warmer environments (27°C).

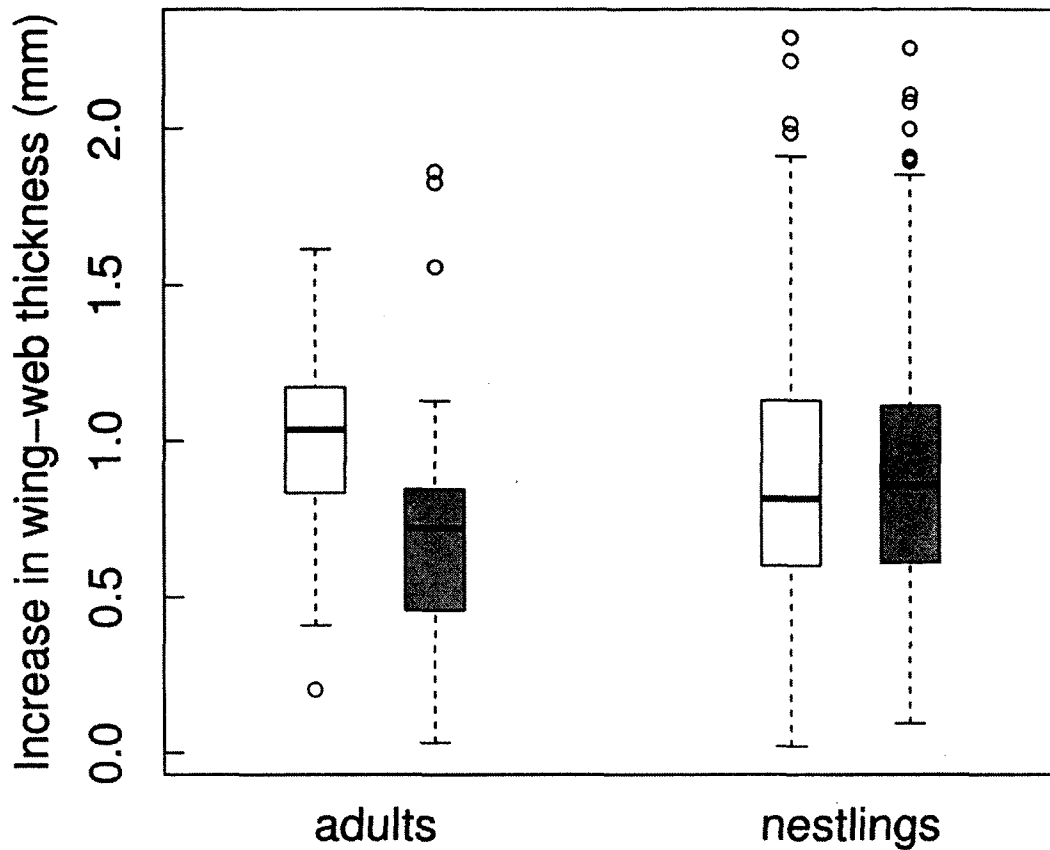


Figure 2.2. Box-plot of raw data for the response to PHA of adult females (2009-2010) and nestlings (2008-2010) depending on their habitat quality (highly intensive agricultural landscape vs. non-intensive landscape) for tree swallows monitored on 10 farms in southern Québec, Canada. The box-plot represents the 25th, median and 75th percentiles of the raw data. Open boxes represent non-intensive agricultural landscape while filled boxes represent intensive agricultural landscape. Sample sizes are 33 and 21 for adult females and 277 and 131 for nestlings in non-intensive and intensive farms respectively.

Table 2.1. Estimates from the a) linear mixed effects model for response to PHA of adult females for 2009 and 2010 ($n = 54$) and b) linear mixed effects model for response to PHA of nestlings from 2008 to 2010 ($n = 408$) monitored on 10 farms in southern Québec, Canada. Random effects of adult model include farm identity (18 % of variance explained). Random effects of nestling model include farm identity (8 % of variance explained) and nest box identity (10 % of variance explained). Details on model selection using second-order Akaike Information Criteria are presented in Tables S2 and S3 in supplementary material. R^2 refers to the proportion of variance in the response variable explained by fixed effects.

Age group	Variables	Coefficients	SE	<i>t</i> -value	<i>P</i> -value
a) Model for adult females $R^2 = 0.335$	Intercept	0.928	1.006	0.923	0.316
	Agriculture type (intensive)	-4.761	1.378	-3.456	0.009
	Mass	-0.076	0.042	-1.790	0.0815
	Year (2010)	-0.349	0.153	-2.283	0.028
	Mean temperature	0.088	0.018	4.959	<0.001
	Brood size	0.106	0.054	1.965	0.057
	Age (second year)	-0.423	0.142	-2.976	0.005
	Agriculture type (intensive) x mass	0.161	0.062	3.199	0.003
b) Model for nestlings $R^2 = 0.208$	Intercept	1.282	0.197	6.502	<0.001
	Year (2009)	0.114	0.047	2.434	0.017
	Year (2010)	0.113	0.039	2.930	0.004
	Mean temperature during growth	-0.024	0.009	-2.451	0.016

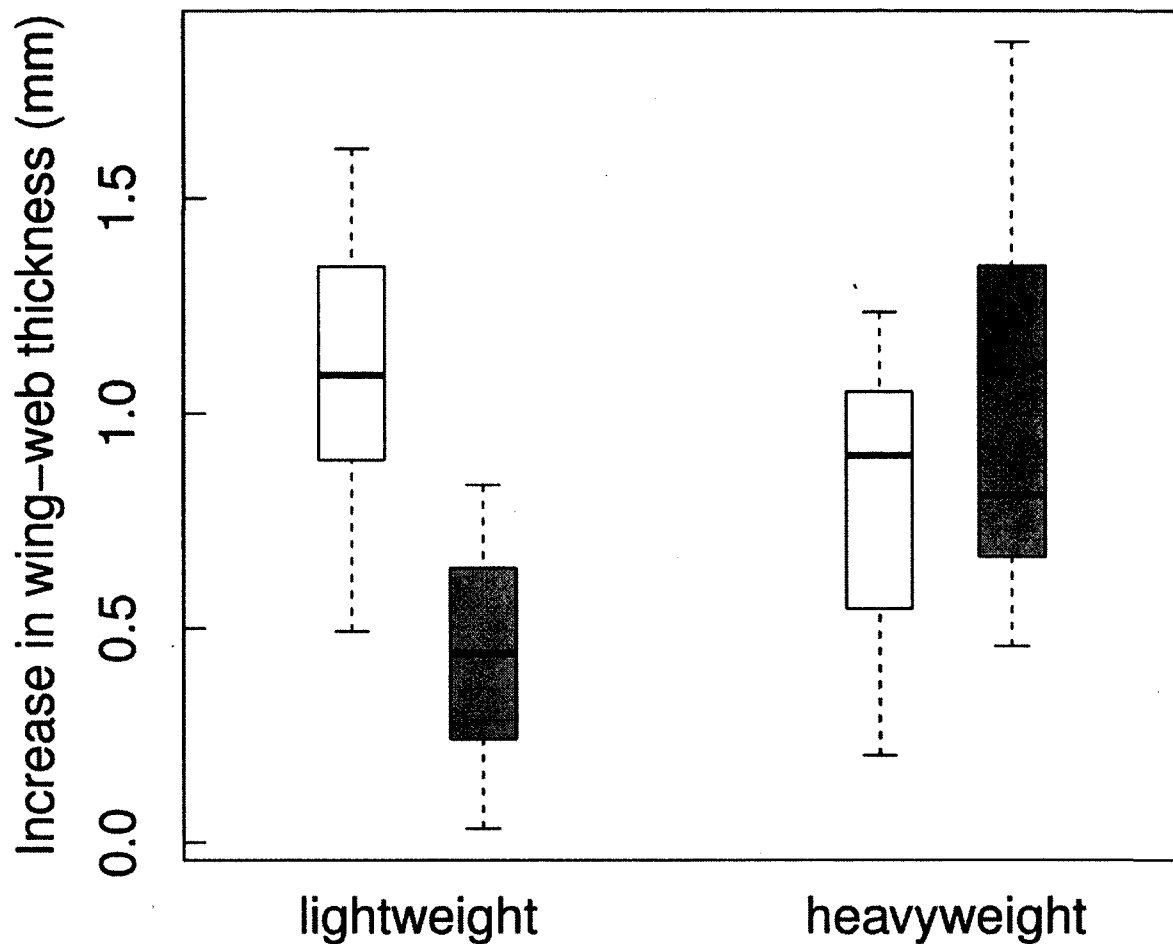


Figure 2.3. Box-plot of the raw data for the response to PHA of adult females according to their body mass (lighter or heavier than mean mass) and their breeding environment in 2009 and 2010 on 10 farms in southern Québec, Canada. The box-plot represents the 25th, median and 75th percentiles. Open boxes represent non-intensive agricultural landscape ($n = 32$) while filled box represent intensive agricultural landscape ($n = 21$).

Response to PHA of nestlings

Variables selected in our final model for nestlings included only year and growth temperature (Table 2.1b). Contrary to adult females, agricultural intensity did not affect nestling responses to PHA (Figure 2.2). Temperature during growth was negatively correlated with the response to PHA (Table 2.1b).

Discussion

We found that habitat quality affects the immune response of both adult female and nestling tree swallows. Adult females breeding in intensively managed agricultural landscapes exhibited a smaller pro-inflammatory response to PHA than adult females found in non-intensive areas. Interestingly, the environmental effect on female response to PHA was mediated by body mass: lighter adult females' response to PHA was lower in intensively managed areas, whereas heavier females displayed the same response in both agriculture types. The response to PHA of adult females was also affected positively by temperature, age and brood size. The response to PHA of nestlings was affected by temperature as well, but the effect was opposite to the one found in adults. Our results suggest that temperature during the first 11 days after hatching was important, as nestlings had a lower pro-inflammatory response to PHA in high temperature environments. Surprisingly, neither parasitic load nor laying date influenced adults' or nestlings' response to PHA.

Response to PHA of adult females

In accordance to our predictions, habitat quality affected the immune responsiveness of adult

female tree swallows. Response to PHA was lower in intensive than in non-intensive agriculture areas, yet this effect decreased with increasing body mass. The effect of habitat quality was thus more important for small birds than heavy birds. Although the exact link between agricultural intensification and response to PHA is unknown, it is likely to represent an energetic constraint that birds face in low quality habitats. Faced with an immune challenge, heavy birds could use part of their larger reserves to mount an immune response even resources are limited. A bird's mass could also be an index of its efficiency at gathering resources; heavier birds would then be better able to gather resources to mount an immune response. A study by Pap and Márkus (2003) showed that breeding female barn swallows nesting with artificially increased broods responded by reducing their energetically expensive response to PHA. Also, providing food for nestlings is probably more demanding in low quality habitats than in habitats with abundant food resources, such as non-intensive farmland (Poulin *et al.* 2010). Evans *et al.* (2007) reported that flying insect abundance was 3.5 times lower in cereal fields than in pastures. Furthermore, studies in our system suggest that aerial insect abundance declines at a faster rate as the season progresses in highly intensive landscapes than in non-intensive landscapes (Rioux Paquette *et al.* In press). The ensuing increase in parental effort could lead to a reduced immune response of parents (Pap and Márkus 2003). Unfortunately, information on insect abundance and biomass is unavailable for 2009 and 2010 which limits our ability to explore the link between food abundance and PHA. Although the fitness consequences in the wild of a lower immune response are still being questioned (Graham *et al.* 2011), such differences in response to PHA between habitats of different quality might lead to lower survival in low quality habitats and explain part of the observed population decline in tree swallows (Shutler *et al.* 2012). Indeed, observed survival probability until PHA measurement in our study was 0.61 in non-intensive farmland and 0.52 in intensive farmland.

The response to PHA of adult females was positively correlated with temperature during our test. This result is concordant with Lifjeld *et al.*'s results (2002), which showed a strong

positive effect of temperature on response to PHA of adult tree swallows. Cold weather reduces the abundance of flying insects (Gruebler *et al.* 2008), which is the only source of energy for tree swallows. It has also been shown that warmer temperatures reduce the energy needed for thermoregulation, thus permitting its reallocation to the immune response (Bryant 1997). Consequently, in cold weather, breeding female tree swallows, already under the energetic stress caused by egg production, incubation and the feeding of nestlings, could be forced to reduce the amount of energy allocated to an immune response such as the one involved in the skin swelling assay.

Control variables that were retained in the final model included age and clutch size. Females in their first year of reproduction had lower PHA responses than older females. A lower PHA response could be caused by a lower quality diet for young females due to lower foraging capacity. Incidentally, lower foraging success has been observed in young female European blackbirds (*Turdus merula*) and American robins (*Turdus migratorius*) (Gochfeld and Burger 1984; Desrochers 1992). Clutch size had a positive effect on the response to PHA of adult females. We were expecting that parents would suffer a greater cost of having larger clutches and that it should have resulted in a trade-off between immunity and reproduction (Lifjeld *et al.* 2002; Ardia 2005b, c). This result suggests that clutch size may be more representative of interindividual heterogeneity than of the reproductive cost incurred by females.

Response to PHA of nestlings

Contrary to adult, the difference in response to PHA of nestlings between intensive and non-intensive areas was not significant. We suggest that this outcome might be due to the buffering effect of parental effort. Several studies have shown that parents increase their parental effort in low quality habitats (Bishop *et al.* 2000; Bruun and Smith 2003; Stauss *et al.* 2005). On the

other hand, a previous study showed that farmland-breeding pallid swifts (*Apus pallidus*) did not alter their feeding rate between intensive and non-intensive agricultural sites despite lower food abundance in intensively-cultivated areas (Cucco and Malacarne 1996). If nestlings receive similar amounts of resources across habitat types, the cost of reduced habitat quality would then be mostly buffered by adults, which is concordant with our results.

Temperature affected nestlings' response to PHA. Results reported on the effect of temperature on nestlings' response to PHA are equivocal (Dawson et al. 2005b; Whittingham et al. 2007). For instance, Ambrosini et al. (2006) found that the response to PHA was not correlated to the temperature that prevailed on the day the response was induced, but to the temperature that occurred 5 days before induction. However, in our case, the most influential temperatures were experienced several days before the assays and only growth temperature (first 11 days after hatching) was retained in our final model. Rapid development of nestlings during the first 12 days after hatching is very energetically demanding (Ricklefs 1981). Also, nestlings are not yet able to thermoregulate, which makes them more vulnerable to temperature variations (Marsh 1980). Our final model showed that temperature during early growth had a significant negative effect on response to PHA. Most studies to date have only reported a positive correlation between response to PHA and temperature (Ardia 2005a; Butler et al. 2009). One possible explanation for our result is a reduction of immune response due to heat stress, which has previously been reported in chickens (*Gallus gallus domesticus*) (Mashaly et al. 2004; Gehad et al. 2007). However, the growth temperatures experienced by nestlings ranged from 13 to 22°C, which are below the point at which Lifjeld et al. (2002) observed the beginning of a decline in response to PHA in tree swallows. The lack of a relationship between nestling response to PHA and temperature at the moment of the PHA assay could yet again reflect the buffering capability of adults. By this time, nestlings can thermoregulate and the indirect effects of low temperature via insect abundance could be compensated for by parental effort.

Conclusion

Our study, conducted on individually marked birds breeding in contrasting agricultural habitats, emphasizes the importance of habitat quality on the immune response of wild birds and shows that trade-offs between immunity and other biological functions may be context-dependent. Our results confirm that both habitat quality and temperature can affect the response to PHA of tree swallows. These results suggest that one needs to be careful when making generalizations about the causes and consequences of pro-inflammatory response to PHA across study areas, since local environments could strongly modify expected relationships. Although the use of a single immune measure is insufficient to conclude on individual global immune responsiveness, the pro-inflammatory response is an important component of the overall immune response (Cichon and Dubiec 2005). Our results also suggest that agricultural intensification in southern Québec is severe enough to cause the reduction in response to PHA of an aerial insectivore. Several studies have reported negative effects of agricultural intensification on reproduction and survival of farmland birds (Møller 2001; Gruebler *et al.* 2010). Our study provides additional information contributing to our understanding of the potential mechanisms underlying the reduction in farmland bird performance within intensive agricultural environments.

Acknowledgements

This work was supported by funding from the Natural Sciences and Engineering Research Council (NSERC) of Canada through Discovery grants to M.B., D.G. and F.P., the Canadian foundation for innovation (CFI) grants to M.B. D.G. and F.P., the Fonds Québécois de la Recherche sur la Nature et les Technologies (FQRNT) new researcher grant to F.P., and the Canada Research Chair program (CRC) to F.P. and M.B.. We are very grateful to H. P.-Gauvin

and C. Gayet for help in the field, and to several research assistants that have contributed to the long-term tree swallow data collection operations. We also thank two anonymous reviewers for their constructive comments on previous version and Jennifer Chambers for English proofing of this article. We also thank all the farmers who graciously let us conduct our research on their properties.

CHAPITRE 3
ECOLOGICAL IMMUNOLOGY IN A FLUCTUATING ENVIRONMENT: AN
INTEGRATIVE ANALYSIS OF TREE SWALLOW NESTLING IMMUNE
RESPONSES

Introduction de l'article

Il existe plusieurs façons de mesurer l'état et la réponse du système immunitaire. Les éco-immunologistes ont récemment atteint le consensus qu'il est nécessaire d'utiliser plusieurs mesures pour décrire adéquatement le système immunitaire et ses réponses. Peu d'études à ce jour ont suivi ces recommandations. Les quelques études qui l'ont fait ont surtout mis en évidence la complexité du système immunitaire. Matson *et al.* (2006a) ont montré que les relations entre de multiples mesures immunologiques étaient différentes d'une espèce à l'autre. Par la suite, Ardia (2007) a montré que même au sein d'une espèce, les relations entre mesures immunitaires pouvaient varier d'une population à l'autre. J'ai poursuivi la réflexion dans cet article en tentant de savoir si pour une même espèce, au sein d'une même population, les relations entre mesures immunitaires pouvaient varier selon l'environnement. J'ai observé l'impact de deux niveaux de variation environnementale (spatiale et temporelle) sur les relations entre 7 mesures immunitaires différentes. Cet article traite aussi du lien entre l'immunocompétence (ou l'immunité globale) des oisillons hirondelles et la masse à l'envol, la croissance et la charge parasitaire, trois mesures de performance individuelle fortement liées à leur valeur adaptative. Cet article met en évidence la complexité du système immunitaire. Il y avait peu de corrélations très fortes entre les 7 mesures immunitaires utilisées. L'utilisation d'une seule mesure immunitaire donne donc une image incomplète. Certains auteurs ont suggéré l'utilisation de 3 mesures représentant 3 grands axes immunitaires (innée, cellulaire et humorale), mais nos résultats montrent que même à l'intérieur d'un même axe, les corrélations

entre mesures peuvent varier. Mes résultats montrent aussi l'importance du contexte écologique ; les corrélations entre mesures immunitaires variaient selon la qualité de l'habitat et l'environnement. Il est donc crucial de ne pas tirer de conclusions hâtives lors d'études de petites échelles, car elles risquent de ne pas être généralisables.

Ma contribution à cet article a été substantielle puisque j'ai effectué la prise de toutes les données immunologiques, fait les analyses statistiques, écrit l'article et coordonné le processus de publication. J'ai été encadré lors de ce projet par la Pre Fanie Pelletier. De plus, plusieurs versions de cet article ont été révisées par la Pre Fanie Pelletier, par le Pr Alan Cohen, par le Pr Marc Bélisle et par le Pr Dany Garant. Cet article a été soumis à *Ecology and Evolution* le 17 octobre.

Ecological immunology in a fluctuating environment: an integrative analysis of tree swallow nestling immune responses

par

Gabriel Pigeon, Marc Bélisle, Dany Garant, Alan Cohen and Fanie Pelletier

Abstract

Evolutionary ecologists have long been interested by the link between immune responses and fitness. Given the importance of a proper immune response for survival in the wild, it is important to understand how its numerous components are affected by environmental heterogeneity. Previous studies targeting this question have rarely considered more than two immune markers. In this study, we measured seven immune markers in tree swallow (*Tachycineta bicolor*) nestlings raised in two types of agro-ecosystems of contrasted quality and over two years. First, we assessed the effect of environmental heterogeneity (spatial and temporal) on the relationships between different immune measures. Second, we investigated the effect of an immune score integrating information from several immune markers on individual performance (including growth, size and parasite burden). Both a multivariate and a pair-wise approach showed variation in relationships between immune measures across years and habitats. We also found a positive association between our integrated immune response and individual performance under certain environmental conditions. We conclude that the ecological context can strongly affect the interpretation of immune responses in the wild. Given that spatiotemporal and environmental variation is likely to affect individual immune responses, further studies on multiple immune measures are critically needed to quantify the importance of environmental variation on immune responses.

Introduction

Wild organisms are continuously exposed to pathogens such as viruses, bacteria or macroparasites. An individual's immune capacity is thus closely linked to its fitness (Cichon and Dubiec, 2005, Saino *et al.*, 2004). Several studies have shown that environmental conditions can affect an organism's immune responses (Neve *et al.*, 2007, Arriero, 2009). Understanding why and how biotic and abiotic factors contribute to variation in immunity in free-living organisms is therefore critical to understanding the evolution of physiological systems (Martin *et al.*, 2011). The fact that environmental conditions can interact with an organism's immune responses has prompted the emergence of a new field often referred to as ecological immunology. Given that immune responses are costly to develop, maintain, and use (reviewed in Lochmiller and Deerenberg, 2000), individuals are expected to trade off limited resources between immunity and other costly functions such as growth and reproduction. Maximisation of an organism's immune responses is also limited due to the risk of an over-responsive immune system, which can lead to autoimmune pathology (Graham *et al.*, 2005, Sadd and Siva-Jothy, 2006). Immune responses are thus expected to be under stabilizing selection. For example, in blue tits (*Parus caeruleus*), individuals at both extremes of antibody responsiveness have lower survival probability (Raberg and Stjernman, 2003). The optimal level of response, however, is not fixed because different environments have different pathogen pressures, which can lead to local optima (Viney *et al.*, 2005). Relating immunity and fitness is therefore not a trivial task because the environment and immune system are involved in complex interactions.

An organism's immune responses are the result of a complex network system composed of many effectors or functions that deal with infections originating from diverse pathogens (Segel and Cohen, 2001). This complexity causes important challenges for researchers interested in studying the immune system in an ecological context. In recent years, eco-immunologists have

come to the consensus that multiple immune indices should be used to quantify the immune responses of an individual (Salvante, 2006, Boughton *et al.*, 2011). Few studies have however simultaneously considered more than two immune indices yet. Matson *et al.* (2006a) measured 13 immune markers and detected positive correlations between functionally related immune markers, such as hemolysis and hemagglutination, within waterfowl (Anseriformes) species. However, those correlations were no longer significant when compared across species, suggesting different interspecific immune constraints. Correlations among immune markers could also vary within a species if environmental constraints vary across its distribution range. For example, the correlation between phytohemagglutinin (PHA) induced inflammation and humoral immune response differs between tree swallows (*Tachycineta bicolor*) living in Tennessee and those living in New York or Alaska (Ardia, 2007). The rearing environment is also known to modify the correlation between PHA response and immunoglobulin level in blue tit nestlings (Arriero, 2009). Although few studies to date have investigated within-population differences, based on patterns observed across populations and species, different environmental conditions within populations are also expected to affect the strength and direction of correlations among immune indices. For example, low quality habitat could force trade-offs between different components of the immune responses leading to negative correlations.

The aim of this study was to assess the effects of environmental quality and annual variation on correlations among five immune markers in tree swallow nestlings raised in contrasted agro-ecosystems of southern Québec, Canada. More specifically, we asked: (i) if correlations among different immune measures changed consistently across habitat and year, and (ii) if an integrated immune score correlated with individual performance. We combined a multivariate and a simpler pair-wise correlation approach (as suggested in Buehler *et al.*, 2011) to assess how environmental heterogeneity affects the consistency of relationships among immune measures and to evaluate the effect of immunity on three proxies of individual performance (fledgling mass, nestling growth, and parasite burden).

Materials and methods

Study area and population

Our study was conducted using a nest-box study system established in 2004 in southern Québec, Canada, over a 10 200 km² area (Figure 2.1). We monitored tree swallow nestlings from 100 nest-boxes equally distributed on 10 farms at each extreme of a gradient of agricultural intensification in 2010 and 2011. Agricultural intensification is characterised by a homogenisation of the landscape, a shift to higher productivity cultivars, a reduction of marginal habitats and increased use of pesticides and fertilizers (Donald *et al.*, 2006). Nest-boxes were located in either intensive (low-quality habitat) or extensive (higher-quality habitat) agricultural landscapes according to the relative cover of intensive and extensive cultures within a radius of 500 m and 5 km. At the 500 m scale, the type of cultures was measured visually in the field each year and reported on orthophotos (1:40 000). The relative cover was then calculated using ArcView GIS Spatial Analyst 2.0a (ESRI 2005). The relative cover at the 5 km scale was measured based on a mosaic of geo-referenced and classified Landsat-7 satellite images taken between August 1999 and May 2003 (pixel resolution 1/4 25 3 25 m; Canadian Wildlife Service 2004). Measurements were obtained with ArcView. We defined intensive agricultural landscapes as those mostly composed of annual crops, such as corn, soybean, and other cereals, and extensive ones as those mostly composed of hayfields, pastures and fallows (for more details on landscape characterization see Ghilain and Bélisle, 2008). The intensification of agricultural practices covered by our study has been associated with a decline of farmland bird populations in many countries (Chamberlain *et al.*, 2000, Murphy, 2003) and to a greater extent, aerial insectivorous birds (Blancher *et al.*, 2009, Nebel *et al.*, 2010). In addition to homogenisation of landscape and habitat structure, the negative effects agricultural intensification on birds may also originate from pesticide use (Poulin *et al.*, 2010); all these factors are hypothesized to lower insect prey abundance and are highly

correlated (Benton *et al.*, 2002).

Blood sampling

We collected blood samples of nestlings on day 8 after hatching from the left brachial vein using heparinised capillaries. The first 5 μ l were used for leukocyte counts. An additional 30 μ l was dried on filter paper to determine sex based on DNA (for details, see Porlier *et al.*, 2009). All remaining blood was immediately put on ice and centrifuged within 45 minutes. Capillaries were centrifuged 10 minutes at 15,000 rpm (using a Clinical 200 centrifuge, VWR, Quebec, Canada) before freezing. Plasma was kept frozen until used for agglutination and bacteria-killing assays (see below). If the total plasma volume collected was inferior to 50 μ l, nestlings were re-sampled two days later.

Immunity measurements

PHA response: We measured pro-inflammatory response by measuring the inflammatory responses to an injection of phytohemagglutinin (PHA) following Martin *et al.* (2006). Nestlings were tested on day 14 after hatching. Each bird was injected with 0.1ml of PHA (L8754-50MG, Sigma Aldrich, St Louis, MO, USA) diluted in phosphate-buffered saline (1mg/ml, no. 811-010-CL, Wisent, St-Bruno, Canada) in the left wing patagium. The PHA response was measured as the difference in patagium thickness before and after PHA injection as determined with a screw micrometer (293 MDC-Lite, Mitutoyo Corporation, Montreal, Canada, ± 0.001 mm). The second patagium measurements were taken 48 hours following injection (mean = 47.92, SD = 0.49). All patagium measurements were made by the same observer. Repeatability (proportion of the total variation that can be attributed to variation among individuals vs. variation among measurements within individuals) of PHA

measurements was very high ($r = 0.97$, $n = 408$ nestlings, 3 repeated measures; as in Wolak *et al.*, 2012).

Agglutination and lysis: We measured natural antibodies and complement effectiveness using a hemolysis-hemagglutination assay following the protocol of Matson *et al.* (2005) modified by Palacios *et al.* (2009). Briefly, 10 μ l of plasma was used for a serial dilution (1:2) in phosphate buffered saline in a 96-well u-bottom plate (3797, Corning Inc., Corning NY, USA) to obtain 11 plasma concentrations with a negative control for each sample. We then added 10 μ l of a 2% rabbit red blood cell solution (R309-0050, Rockland Immunochemicals, Gilbertsville, PA) to each sample and incubated them 90 minutes at 37 °C, then 20 minutes at 20 °C inclined at 45° before scanning the samples at 300 dpi resolution (HP CM1312nfi mfp). Agglutination and lysis were scored from the scan from 1 to 12 according to the last plasma dilution exhibiting agglutination and lysis. We tested individuals in duplicate when sufficient plasma was available ($n=175$ out of 210) and used mean score in statistical analyses.

Bacteria killing assay: We measured the bactericidal capacity of plasma following the bacteria-killing assay protocol used by Morrison *et al.* (2009) (modified from Matson *et al.*, 2006b). *E. coli* pellets (ATCC 8739) were reconstituted and diluted in PBS to obtain a concentration of 100-150 CFU per plate on controls. We then added five μ l of plasma to 20 μ l of this bacteria solution and 95 μ l of cell culture medium. This solution was incubated 45 minutes at 40°C before plating 50 μ l on LB plate. A negative control without tree swallow plasma was made every hour. All plates were done in duplicate and incubated 24 hours at 40°C. The bactericidal capacity of plasma was recorded as $[1 - (\text{average number of surviving colonies on an individual's plates} / \text{average number of colonies on control plates})] * 100$.

White cell counts: To obtain a leukocyte profile, we smeared and air dried approximately 5 μ l

of fresh blood on a glass slide immediately after collection. Smears were stained with quick-dip stain and counter stain without heat fixation (Toma *et al.*, 2006) before they were scanned for leukocytes using a 10x ocular and 100x oil immersion lens. A total of 100 leukocytes were identified from each slide, noting the frequencies of lymphocytes, heterophils, eosinophils, basophils and monocytes. Leukocytes were identified according to Clark *et al.* (2009). Basophils and monocytes were not considered further due to their rarity. A sub-sample of nine slides were counted four times and repeatability was high ($r = 0.75$).

Proxies of individual performance

We considered three proxies of individual performance: growth between 2 and 16 days after hatching, mass at fledging and parasite burden. Nests were monitored every two days during the breeding season (from early May to mid-July). Nestlings were marked individually by nail clipping until their 12th day of life and thereafter ringed with an official aluminum ring. Nestlings were weighed (± 0.01 g) with a platform scale equipped with a contention device at 2 and 16 days after hatching. Growth was calculated as the mass gained between those two measurements. Mass at day 16 was used as fledging mass. The number of *Protocalliphora* pupae in each nest was counted after the breeding season and used as a measure of parasite burden imposed by a blood-feeding ectoparasite (Daoust *et al.*, 2012). These three measures of performance influence survival and were used since we could not obtain true measures of fitness. Indeed, growth and fledging mass are positively correlated with the probability of recruitment (Monros *et al.*, 2002, McCarty, 2001), whereas a large parasite burden significantly reduces survival (Thomas *et al.*, 2007). All procedures described in this study were approved by the University of Sherbrooke's Animal Care Committee (protocol number FP2009-01) and comply with current Canadian laws regarding animal research.

Statistical analysis

Assessment of the data structure

We only retained nestlings for which all immunity measures were available for analyses ($n = 210$). We checked all immune measures for normality. Bactericidal capacity of plasma was slightly skewed, we therefore normalized it prior to all analyses using the following formula: $-\sqrt{1 - \text{bactericidal capacity}}$ (Legendre and Legendre, 1998). Immune measures were then centered and scaled to unit variance to reduce the impact of measurement units. Given the statistically inherent correlations among the percentages of three types of leukocytes ($-0.67 \leq r \leq -0.30$), only the percentage of lymphocytes was used in multivariate analysis. We conducted a redundancy analysis (RDA) on all immunity measurements using year, agriculture type, and sex as constraining variable to assess the general correlation structure among all five immunity variables as well as the impact of possible grouping variables on their structure. We then used an ANOVA like permutation test for RDA under reduced model with terms added sequentially from first to last using 1000 permutation to have a more quantitative idea of the level of significance (Legendre *et al.*, 2011). The RDA and the permutation test were done using the *vegan* v2.0-4 package in R (Oksanen *et al.*, 2012). Given that both year (2010, 2011) agricultural landscape type (extensive, intensive) and their interaction had highly significant effects (Table 3.1), we considered that our population consisted of four sub-groups, representing different ecological contexts (intensive 2010, intensive 2011, extensive 2010 and extensive 2011). Normalized immune measurements were centered and scaled to unit variance within each sub-group for further analyses. The above analyses were performed in R v2.15. (R Development Core Team, 2012).

Table 3.1. Effects of different grouping variables on the five immune measures based on an ANOVA-like permutation test using 1000 permutations. Sample size is 206 instead of 210 due to 4 individuals of unknown sex which were removed. Measurements were made in 2010 and 2011 on tree swallow nestlings.

Grouping variable	Variance	F-value	P-value
Year	0.75	38.69	<0.001
agriculture type	0.15	7.87	<0.001
Sex	0.02	1.27	0.27
Year : agriculture type	0.13	6.66	<0.001

Correlation among immunity indices

We followed the statistical methods suggested by Buehler et al. (2011) for assessing the relationships among immunity indices and their variability across years and types of agricultural landscape. First, principal component analysis (PCA) and correlation circles were obtained for each of the four sub-groups using the ade4 v1.5-1 package in R (Dray, 2007). We examined correlation circles for principal components (PC) with an eigenvalue higher than one (i.e., for PC 1 and 2 in all cases as well as for PC 3 for extensive-2010) (Electronic supplementary material, Table A2.2). To help interpreting the group specific PCA axes, we used a pair-wise approach to evaluate the correlations among all our immune markers. We thus calculated a Pearson's correlation coefficient and its 95% confidence intervals for r with the fisher Z-score method following Sokal and Rohlf (1994) for every pair of immune measures by agricultural landscape intensity and by year. These correlation analyses were also performed in R.

Correlation between immunity and proxies of individual performance

We used the scores on the first and second principal components provided by the group-specific PCAs as general immunity indices. The relationships between immunity and fledging mass and growth were modelled in R using linear mixed models using the lme4 v0.99 (Bates *et al.*, 2011) in order to take into account the non-independence of nestlings born in the same nest-boxes. None of the models in which the effect of PC1 was allowed to vary by nest-box were significantly better than when only the intercept varied (most significant: $\chi^2 = 3.08$, $P = 0.21$), so a common slope was used. The relationship between immunity and parasite burden was modelled with a linear model since burden was assessed at the nest-box level.

Results

Assessing data structure

We measured the immune responses of 210 tree swallow nestlings over the 2 years of the study (Table 3.2). Preliminary RDAs of the entire dataset suggested a difference between years as well as between habitats of different agricultural intensity (electronic supplementary material, Figure A2.1). RDA1 and RDA2 explained 16.38% and 3.98% of the variance in the 5 immune indices (Table A2.1). The permutation test supported this interpretation: year farmland type and their interaction, but not nestling sex, had significant effects on the nestlings' immune responses (Table 3.1). Hence, further analyses were performed on four sub-groups representing both agriculture types and years.

Table 3.2. Descriptive statistics of seven immune measures taken on tree swallow nestlings in 2010 ($n = 128$) and 2011 ($n = 82$).

Immune marker	Mean	Median	Minimum	Maximum
PHA	1.10	1.11	0.02	2.26
Bacteria killing	0.72	0.85	0.00	1.00
Hemolyse	1.21	1.00	0.00	3.50
Agglutination	8.22	8.250	3.00	12.00
% lymphocytes	44.63	45.00	15.00	79.00
% heterophils	28.89	29.00	6.00	58.00
% eosinophils	25.42	25.00	6.00	51.00

Relationships among immune measures across environments

The first 2 components of the sub-group PCAs explained together from 48% to 60% of the variance (mean = 55.6%). All four sub-groups showed different correlation patterns. Both intensive sub-groups showed a negative correlation (long opposed vectors; 151° and 165° between vectors) between percentage of lymphocytes and hemolysis, which was absent from extensive sub-groups (either 90° or long and small vectors; 99° and 85° between vectors; Figure 3.1). Agglutination was strongly correlated with bacteria-killing capacity in both intensive 2011 (11° between vectors) and extensive 2011 (17° between vectors), but not in extensive 2010 sub-groups (105° between vectors; Figure 3.1). Relationships between hemolysis and agglutination were always low. Bacteria-killing capacity and percentage of lymphocytes showed no correlation except in extensive 2011 where the relationship was

positive (12° between vectors; Figure 3.1).

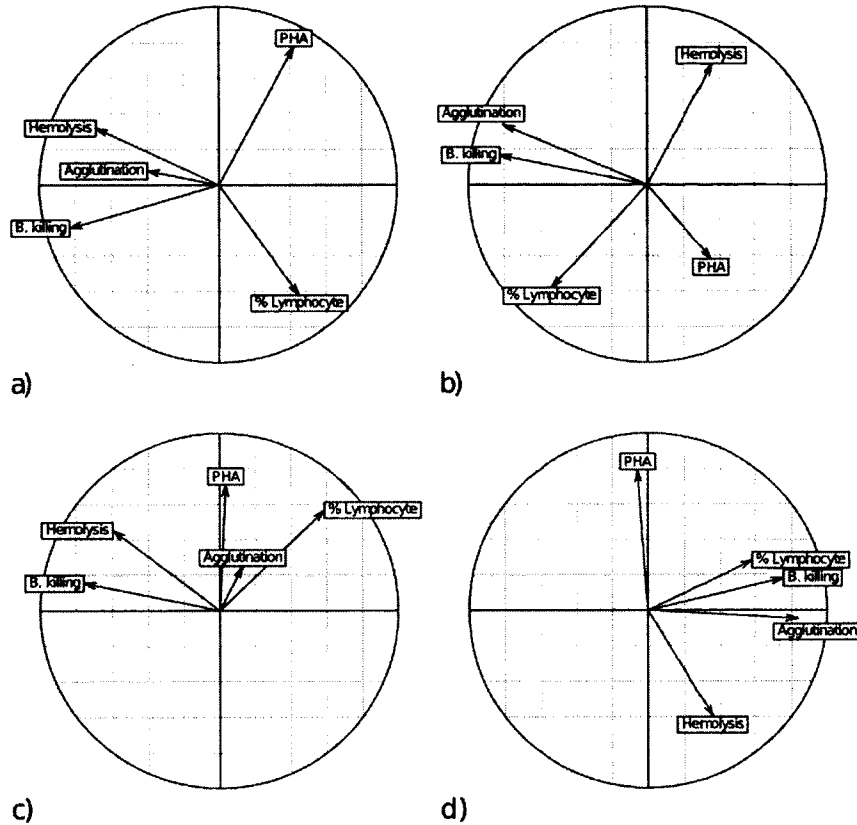


Figure 3.1. Correlation circles representing the association between immune measurements for each sub-group of tree swallow nestlings. The X and Y axes represent PC1 and PC2 of a principal component analysis made per sub-group respectively. The lengths of the vectors indicate the strengths of the relationships. The size of the angle between two vectors indicates the direction of the correlation: a small angle between two vectors represents a positive correlation; a 90° angle indicates no correlation and opposed vectors indicates a negative correlation. A) Intensive 2010: $n = 38$, 57.25 % of variance explained by both PCs; B) Intensive 2011, $n = 27$, 59.77 %; C) Extensive 2010, $n = 90$, 48.37 %; D) Extensive 2011, $n = 55$, 57.01 %.

We used a pair-wise approach to explore correlations among indices which showed that sub-groups differed in the strength and direction of correlations among different immune markers (Figure 3.2). Correlation coefficients ranged from -0.36 to 0.61. There was considerable variation even within sub-groups, resulting in large confidence intervals. Both year and farmland type affected the strength and direction of several pair-wise correlations. For instance, while the positive correlation between bacteria killing capacity and agglutination was significant only in 2011 ($r = 0.07$, $P = 0.45$ and $r = 0.47$, $P < 0.001$ for 2010 and 2011 respectively), the correlation between bacteria killing capacity and lymphocyte percentage was significantly negative in 2010 ($r = -0.18$, $P = 0.04$) and marginally significantly positive in 2011 ($r = 0.21$, $P = 0.06$). Moreover, farmland type significantly affected the correlation between hemolysis and hemagglutination as the correlation was significant only in extensive farmlands ($r = 0.04$, $P = 0.73$) but not in intensive ones ($r = 0.5$, $P < 0.001$). Overall, the relationships between immune indices were inconsistent among sub-groups in accordance with our analyses using principal component analysis.

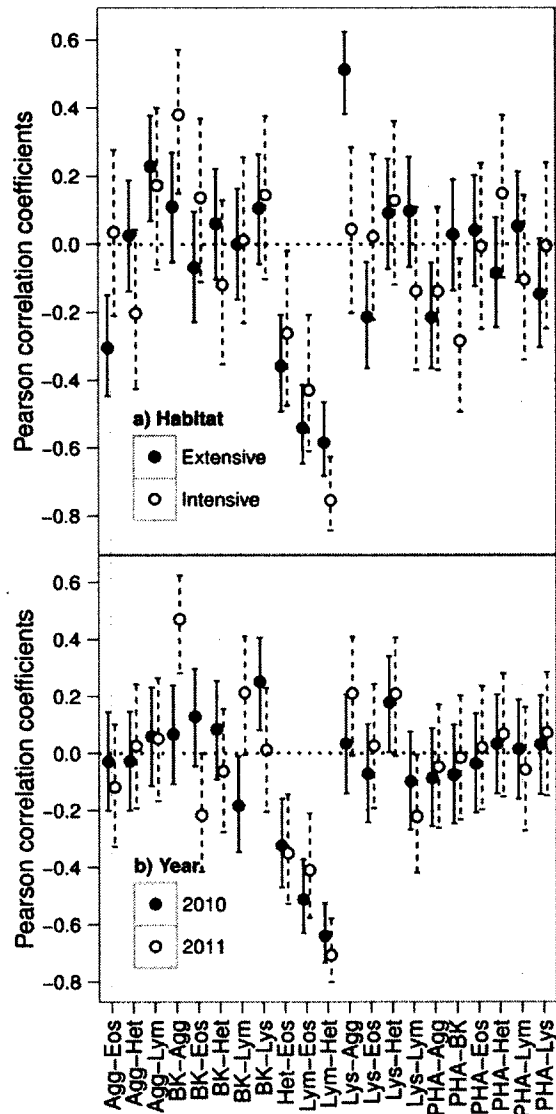


Figure 3.2. Pair-wise Pearson correlation coefficients among immune measures of nestling tree swallows according to A) levels of agriculture intensity (extensive = filled circles; intensive = open circles) and B) year (2010 = filled circles; 2011 = open circles). Error bars represent 95 % confidence intervals calculated by Z-score transformation. All possible pairs between phytohemagglutinin response (PHA), hemagglutination (Agg), hemolysis (Lys), bacteria killing capacity of plasma (BK), percentage of lymphocytes (Lym), percentage of heterophils (Het) and percentage of eosinophils (Eos) were included.

Linking integrated immune score and individual performance

Growth and fledging mass were highly correlated ($r = 0.86$), while parasite burden was moderately correlated with fledging mass and growth ($r = -0.34$ and -0.37 , respectively). Given that PCA was done by sub-group, the integrated immune score differs among sub-group. The relative importance of each immune measure on the PC1 score is represented by the vector length on the horizontal axis of the correlation circles (Figure 3.1). Mass at fledging was negatively affected by PC1 only in the intensive 2010 sub-group (Table 3.3). Similarly, growth was negatively affected by PC1 in intensive 2010; however, the effect was only marginally non-significant (Table 3.3). Individuals with a higher integrated immune response (mostly bacteria killing, hemolysis and agglutination) had higher mass at fledging and growth in intensive 2010 (electronic supplementary material, Figure A2.2 and A2.3). Nest-box identity explained a large portion of the variance in fledging mass (mean = 43.29 %; SD = 11.69 %) and growth (mean = 63.18 %; SD = 6.16 %). None of the sub-groups had a significant relationship between PC2 and fledging mass or growth. The linear regression revealed that the relationship between immunity and parasite burden was significant in two subgroups: the intensive 2010 and the extensive 2011 while not in intensive 2011 and extensive 2010 (Table 3.3). Individuals with a lower immune response had a higher parasite burden (electronic supplementary material, Figure A2.4).

Table 3.3. Effect of integrated immune response (PC1) on A) mass at fledging and B) growth between day 2 and 16 after hatching and C) parasite burden of tree swallow nestlings for each sub-group (intensive 2010, intensive 2011, extensive 2010 and extensive 2011). Estimates (\pm standard error (SE)) were obtained for linear mixed models including nest-box as a random effect for mass at fledging and growth and from a linear model for parasites. % refers to the variance explained by nest-box identity.

Sub-group		Intensive 2010	Intensive 2011	Extensive 2010	Extensive 2011
sample size		38	27	90	55
A) Fledgling mass	Estimate	-0.485	0.235	0.019	0.234
	SE	0.221	0.273	0.173	0.198
	F-value	4.820	0.742	0.012	1.387
	P-value	0.037	0.40	0.91	0.25
	%	39.862	28.527	49.676	55.102
B) Growth	Estimate	-0.034	0.009	0.003	0.000
	SE	0.018	0.028	0.014	0.015
	F-value	3.444	0.101	0.034	0.001
	P-value	0.075	0.76	0.85	0.98
	%	71.248	56.877	60.352	64.236
C) Parasites	Estimate	3.956	-1.342	1.463	-4.94
	SE	1.670	2.617	1.365	1.587
	F-value	5.609	0.263	1.15	6.982
	P-value	0.023	0.61	0.29	0.012

Discussion

Our results highlight the importance of considering environmental heterogeneity when assessing individual immune responses. Indeed, the relationships among immune measures of nestling tree swallows varied across agricultural environments. Both temporal and spatial difference in environments had important effects. Also, while nestlings with greater immune

responses generally performed better, the magnitude of the effect was weak and depended on the environment. Our results also reinforce the claim that the complexity and variability of the immune system may not be captured by one or two immune markers (Matson *et al.*, 2006a, Ardia, 2007). While the use of multiple immune markers is a significant step forward for ecological immunology, future studies will also need to overcome the challenges caused by temporal and spatial environmental differences.

Relationships among immune measures across environments

To face a high diversity of pathogens, the immune system must be adaptable. This might explain why correlations were not consistent between environments. While Matson *et al.* (2006a) showed that relationships among immune indices changed across species and Ardia (2007) showed that they changed across populations, our results now suggest that there is variation even within a single population and that correlations vary in an unpredictable manner. Our results suggest that local environment (both spatial and temporal) can influence both the absolute values of nestlings' immune responses as well as the correlations among immune markers.

We expected that negative correlations, resulting from investment trade-offs among immune functions, would be observable in intensive farmlands, which are composed of lower quality habitats (Arriero, 2009, Ghilain and Bélisle, 2008). The PHA response and the bacteria killing capacity of plasma, our most functionally different measures (PHA response is an induced, mostly cellular immune response while bactericidal capacity of plasma is constitutive and humoral), were significantly negatively correlated ($r = -0.28$) in intensive farmlands but not in extensive ones ($r = 0.03$), lending partial support to our prediction. This result points to a trade-off between different axes of the immune system in low quality habitat, in contrast to the

findings of Palacios *et al.* (2012), who found no evidence of a trade-off between acquired and innate axes of the immune system in tree swallows of Tompkins County, New York. Nevertheless, other pair-wise correlations among immune markers were negatively correlated in high-quality habitat and uncorrelated in low-quality habitat, inconsistent with this hypothesis. Our results suggest that the answer may be more complex than a simple trade-off when resources are limited. Although trade-offs may be present when resources are limiting, negative correlations may also be caused by preferential investment in certain immune components appropriate for the pathogens present in the habitat. Further research is needed in order to determine the relative importance of resources and pathogens in a natural context.

Temporal environmental heterogeneity was also important. For example, the correlation between bacteria killing capacity and lymphocyte percentage was significantly negative in 2010 and marginally, non-significantly positive in 2011. Hence, our results suggest that immune markers might reveal opposing results even in the same population, the same habitat, but for different years. Although we do not have a sufficient number of years to determine how the quality of each year influences the immune responses, our study still shows it produces important differences.

At least two non-mutually exclusive explanations can account for the differences in relationship among immune measures across our farmland-year sub-groups. First, a difference in energetic or nutrient constraints could result from different habitat quality. Nutrition has been shown repeatedly to have an important impact on immune responses (Siva-Jothy and Thompson, 2002, Lochmiller *et al.*, 1993). In our study system, Rioux Paquette *et al.* (in press) showed that insect prey abundance was lower late in the season in intensive farmlands when it is most crucial for nestling development and fledging. Yet this difference in prey abundances was only observed in two out of three years, likely as a result of yearly variations in meteorological conditions. Such differences in resources between years and habitats could

explain the observed differences in the correlations among immune markers. Another possible explanation is that pathogen pressure differs among environments as parasites can have a considerable impact on immune responses (Boughton *et al.*, 2011) and influence the correlation between the humoral and cellular immune responses (Johnsen and Zuk, 1999). Likewise, the presence of bacterial or viral pathogens is likely to influence investment in different axes of the immune system. Although pathogen pressure was not measured in this study, our results are consistent with individuals modulating their immune responses according to local environment in order to optimize their integrated immune response.

Integrated immune score and individual performance

Correlations among immune measurements were inconsistent among sub-groups. Using a single PCA of the pooled data for the whole population would thus be unjustified and could lead to erroneous conclusions (McCoy *et al.*, 2006). Because PCAs were done by sub-group to obtain an integrated immune score, none of the scores used as explanatory variables have the same meaning. Interpretation must then be made individually for each sub-group. While such an approach complicates the interpretation, it is more appropriate given that individuals from different sub-groups have experienced different ecological conditions (environment, pathogen pressure, nutritional status; which causes the differences in immune relationships).

The effect of the integrated immune score on fledging mass and growth varied among sub-groups. More precisely, it was significant only in the intensive-2010 sub-group. It is difficult to conclude on the causes of this difference. Prey abundance is generally lower in the intensive farmlands (Rioux Paquette *et al.*, in press). This was likely reflected in the presence of a trade-off between PHA and bacteria killing capacity. Further, individuals may be able to reallocate some energy from body mass in order to achieve the minimum immune development for

survival. However, this relationship was not present in intensive farmland in 2011. Similarly, parasite burden differed across sub-groups. The intensive-2010 and extensive-2011 sub-groups showed strong negative effects of the integrated immune response on the number of *Protocalliphora* pupae found in nests. Given that parasitism has been linked with reduced survival (Moller *et al.*, 2009, Howe, 1992), our results suggest that the importance of the effect of immune responses on performance could depend on the environment.

Another factor which makes the study of immune responses and individual performance difficult is mortality during early development. Most of the mortality occurs before all immune measures are obtained, and deceased individuals are thus not considered in multivariate analyses. Given that mean nestling mortality by nest is relatively high in our study system and varies across habitats (Ghilain and Bélisle, 2008)(intensive 2010: 28.0 %; intensive 2011: 37.3 %; extensive 2010: 20.4 %; extensive 2011: 25.3 %), measurement of immune responses are partly based on a post-selection sample. Nestlings dying before fledging could have lower immune responses (Christe *et al.*, 1998). The non-random removal of individuals with the lowest integrated immune response could explain the lack of correlation between our proxies of individual performance and immunity in intensive 2011, where mortality was highest. Even though we missed this invisible fraction (Nakagawa and Freckleton, 2008) representing low fitness individuals, we were still able to detect an effect of immunity on fledging mass and parasites in intensive 2010 and on parasites in extensive 2011.

Concluding remark

Our results suggest that studies on a particular species, or even a single population, will not be easily generalised to others. Given that spatiotemporal environmental variation is likely to affect individual immune responses, further studies on multiple immune measures are

critically needed to quantify the importance of environmental variation on immune responses. To understand the underlying mechanisms of organismal immune responses, research must not only use multiple immune measurements, but also investigate how immune responses vary within one individual measure in different ecological contexts using larger-scale, long-term field experiment. Molecular-based immune markers such as β -defensin genes (Hellgren and Sheldon, 2011) or major histocompatibility complex diversity (Piertney and Oliver, 2006) should also be included in studies to assess the complex interactions between genotype and environment as well as to tease apart the relative importance of evolutionary versus ecological variables involved in the immune response.

Acknowledgements

This work was supported by funding from the Natural Sciences and Engineering Research Council (NSERC) of Canada through Discovery grants to M.B., D.G. and F.P., the Canadian foundation for innovation (CFI) grants to M.B. D.G. and F.P., the Fonds Québécois de la Recherche sur la Nature et les Technologies (FQRNT) new researcher grant to F.P. and the Canada Research Chair program (CRC to FP and MB). We are very grateful to H. P.-Gauvin and C. Gayet for help in the field and to several research assistants that have contributed to the long-term tree swallow data collection. We also thank all the farmers who graciously let us conduct our research on their properties.

CHAPITRE 4

CONCLUSION

4.1 Retour sur les résultats

Une des conclusions principales de mon mémoire est que l'environnement a une très grande importance sur le système immunitaire. L'environnement peut causer des variations aussi bien dans la valeur absolue d'une réponse immunitaire que dans la corrélation entre les différentes réponses immunitaires. En effet, l'intensité des pratiques agricoles a un effet aussi bien sur la valeur absolue que sur les corrélations. Un des mécanismes très probable d'action de l'intensification des pratiques agricoles sur le système immunitaire est via l'acquisition de ressources. En effet, l'abondance d'insectes aériens, la source d'alimentation de l'Hirondelle bicolor, est plus faible en milieu intensif qu'en milieu extensif (Evans *et al.*, 2007; Rioux-Paquette *et al.*, 2012). Les individus en milieu de pauvre qualité ont probablement moins de ressources (énergie, nutriment) à investir, menant à une réduction de la réponse immunitaire (Brzek et Konarzewski, 2007). Par exemple, les oisillons Hirondelle rustique (*Hirundo rustica*) avec une diète supplémentée ont une réponse au PHA accrue par rapport aux oisillons contrôles (Saino *et al.*, 1997). Un manque de ressources peut aussi forcer un individu à faire des compromis au sein même des composantes de l'immunité. Ceci se traduirait par une corrélation négative entre deux mesures immunitaires. Nos résultats montrent que chez les oisillons d'Hirondelle bicolor, peu de compromis de cette sorte sont visibles sauf entre le PHA et la capacité bactéricide du plasma. La température affecte également le système immunitaire. La thermorégulation est un processus coûteux et peut donc entrer en compétition avec le système immunitaire pour l'énergie disponible. Notre analyse de 'sliding windows' montre que les oisillons sont particulièrement sensibles durant les 10 premiers jours après leur éclosion. Ceci est probablement dû au fait qu'ils sont alors incapables de réguler leur

température efficacement durant cette période (Marsh, 1980). Finalement, l'année a aussi un effet sur le système immunitaire. Nos résultats montrent effectivement que certaines corrélations entre mesures immunitaires varient d'une année à l'autre.

Un autre point important ressortant de mes travaux de maîtrise est la complexité et la variabilité du système immunitaire. Ce dernier doit faire face à une grande diversité de pathogènes en constante évolution pour trouver de nouvelles façons de lui échapper (Sasaki et Godfray, 1999). Pour faire face à ce défi, le système immunitaire est composé d'une grande variété d'effecteurs. L'expression de ces effecteurs peut être contrôlée de façon complexe au niveau génétique (Kilpimaa *et al.*, 2005), par des facteurs environnementaux (Arriero, 2009) ou encore par d'autres effecteurs immunologiques (Romagnani, 1997). Il se forme donc un réseau complexe entre les différents effecteurs. Le système immunitaire a d'ailleurs plusieurs caractéristiques des réseaux complexes (Segel et Cohen, 2001). Par exemple, plusieurs effecteurs sont redondants, c'est-à-dire qu'ils effectuent des tâches similaires. Ainsi, une infection bactérienne peut être combattue par des macrophages, des anticorps ou les deux. Une autre caractéristique des réseaux très présente dans la réponse immunitaire est l'échafaudage, où l'évolution de la réponse dépend de la présence préalable de certaines autres composantes. Par exemple, lors de la réponse à une infection par des bactéries, les bactéries sont d'abord agglutinées par des anticorps. Ceci permet ensuite de déclencher la cascade de complément qui comprend plus de 30 protéines circulant dans le plasma et menant à la lyse des bactéries et donc leur mort (Janeway *et al.*, 2003). On s'attendrait donc à ce que les mesures fonctionnellement reliées comme les titres d'agglutination, de lyse et la capacité bactéricide soient positivement corrélées. Nos résultats montrent pourtant que ce n'est pas toujours le cas. La structure complexe du système immunitaire lui permet d'être très variable et plastique. C'est ce qui permet d'optimiser (et non maximiser) la réponse immunitaire face aux conditions écologiques locales. Mon projet a une grande importance pour le domaine de l'éco-immunologie en incitant à la prudence. Mes résultats montrent un impact important de l'environnement spatial et temporel sur le système immunitaire. Aussi bien les valeurs des

mesures que les corrélations entre celles-ci changent selon l'environnement. Il est donc nécessaire de faire preuve d'une grande prudence quand on généralise les résultats d'une étude à une autre, même s'il s'agit de la même espèce. Mes résultats incitent même à la prudence lors de l'interprétation des résultats des études déjà publiées. Sachant l'importance de l'environnement, on peut se demander si les conclusions tirées de l'étude d'une seule population dans un seul environnement sont valables (Palacios *et al.*, 2012). La même étude faite l'année suivante aurait pu arriver à des conclusions complètement différentes.

Bien que l'utilisation d'une seule mesure donne une image incomplète du système immunitaire, de telles études ne sont pas inutiles. Mes recherches sur la réponse au PHA ont par exemple permis de montrer l'importance de la température durant les premiers jours après l'éclosion, alors que le développement est rapide (Lamoureux, 2010). L'interaction entre l'effet de l'environnement et de la masse sur l'immunité chez les femelles est aussi très intéressante. Il est possible qu'un indice global d'immunité n'ait pas permis de détecter de telles relations. En effet, certaines mesures immunitaires, telles que la production d'anticorps naturels, sont plutôt constantes durant la vie d'un individu contrairement à la masse (Mendes *et al.*, 2006), ce qui mènerait à une très faible corrélation. L'importance d'utiliser plusieurs mesures immunitaires dépend donc de la question qui est posée. C'est en assemblant chaque petite pièce d'un puzzle que l'on finit par avoir une image complète.

4.2 Perspectives d'avenir

Mes recherches apportent plus de questions que de réponses. En effet, plusieurs pistes existent pour pousser encore plus l'étude de l'éco-immunologie de l'Hirondelle bicolor dans notre système d'étude. Par exemple, un des problèmes de mon deuxième article, comme pour plusieurs études sur des traits intimement liés à la survie, est celui de la fraction invisible

(Nakagawa et Freckleton, 2008). Le problème vient du fait qu'il y a un fort taux de mortalité en bas âge dans notre population (environ 30 %). Il a été montré que chez l'Hirondelle de fenêtre (*Delichon urbicum*), les oisillons mourants en bas âge ont une réponse immunitaire significativement inférieure (Christe *et al.*, 1998). Une fraction non aléatoire de la population, représentant les individus à faible capacité immunitaire, n'est donc pas échantillonnée. Il serait donc fort intéressant de pouvoir poursuivre mon étude en incluant une mesure immunitaire échantillonnée en bas âge, comme par exemple la capacité bactéricide du plasma, qui a été mesurée sur des oisillons n'ayant que 6 jours (Stambaugh *et al.*, 2011) ou une mesure immunitaire génétique comme la diversité du complexe majeur d'histocompatibilité (Piertney et Oliver, 2006) ou les gènes de β -défensine, un peptide antimicrobien à large spectre (Hellgren et Sheldon, 2011). De telles mesures permettraient d'obtenir un aperçu de la capacité immunitaire des individus décédant en bas âge.

Une autre avenue qui serait intéressante à explorer est celle des suivis à long terme. Les suivis à long terme ont une grande importance dans l'avancement de la compréhension de l'écologie et de l'évolution chez les animaux (Clutton-Brock et Sheldon, 2010). Un suivi d'individu marqué, comme le projet hirondelle, permettrait de départager l'effet de l'environnement des effets génétiques et parental sur le système immunitaire. Un tel suivi pourrait aussi permettre d'éclaircir le type de pression de sélection ayant mené à la variabilité et à la plasticité qu'on observe dans le système immunitaire. Finalement, il serait possible de mettre en évidence, de façon solide, des tendances générales dans la structure du système immunitaire.

Une autre faiblesse de mon troisième chapitre à laquelle il serait intéressant de remédier est celle de la causalité. Effectivement, mon deuxième article montre que, dans certains milieux, les individus ayant un système immunitaire plus élevé ont une masse à l'envol plus élevée. Mes analyses n'ont cependant pas permis d'élaborer sur le lien de causalité. Il pourrait effectivement s'agir d'une corrélation fallacieuse. Par exemple, l'environnement local et la

généétique pourraient causer la réponse immunitaire et la masse à l'envol et ainsi donner une apparence de corrélation entre les deux, même s'il n'y a pas de lien réel. Il serait donc très intéressant d'étudier le problème dans le cadre d'une modélisation par équation structurelle ou d'analyse de piste (Shiple, 2002). Le problème pourrait même être élargi pour inclure des interactions avec d'autres systèmes physiologiques régulateurs tels que le système de réponse au stress (Cohen *et al.*, 2012) et la pression parasitaire (Johnsen et Zuk, 1999). Une foule d'études ont montré un lien entre le système endocrinien/nerveux et le système immunitaire (Black, 1994; Wilckens et De Rijk, 1997). Par exemple, un diagramme dirigé de l'éco-immunologie de l'Hirondelle bicolore pourrait ressembler à la Figure 4.1. Aucune étude à ma connaissance n'a utilisé d'analyse de piste pour faire avancer le domaine de l'éco-immunologie même si une telle approche a déjà été proposée (Buehler *et al.*, 2011).

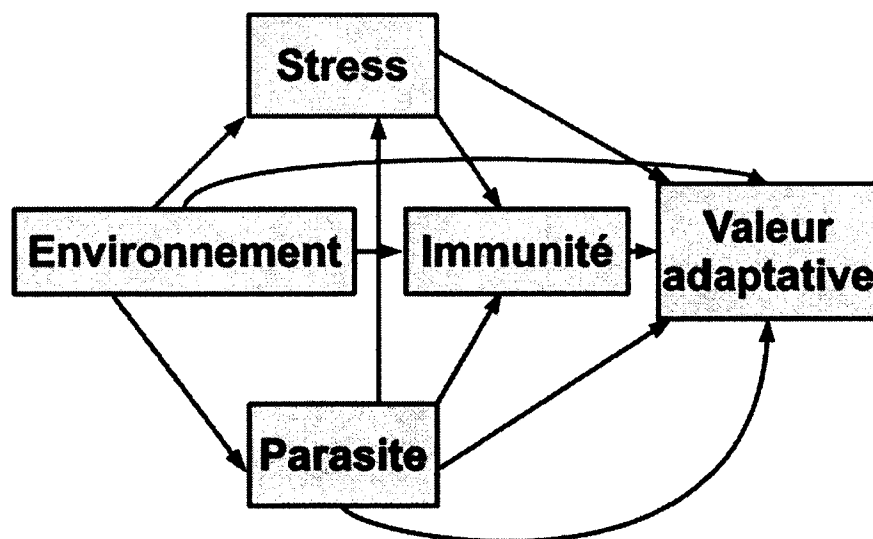


Figure 4.1. Diagramme dirigé représentant les liens de causalités entre l'environnement, l'immunité, la charge parasitaire, le stress et la valeur adaptative.

Une autre avenue à explorer est la non-linéarité des relations immunitaires. Effectivement, énormément de relations en écologie sont non-linéaires (i.e. relation logistique, quadratique, de non-coexistence). Pourtant, très peu d'études en éco-immunologie considèrent cet aspect. Pourtant, des études ont montré, par exemple, la non-linéarité de la relation entre la réponse immunitaire et la probabilité de survie (Raberg et Stjernman, 2003). Malheureusement, les méthodes statistiques non linéaires sont moins communes. Par exemple, les analyses de composantes principales comme utilisées dans mon troisième chapitre assument des relations linéaires (Legendre et Legendre, 1998). Une nouvelle méthode statistique prometteuse est celle du «maximum information coefficient» (Reshef *et al.*, 2011). Cette méthode permet d'obtenir un indice similaire à un r^2 , mais non paramétrique et en ne faisant aucune supposition de linéarité.

Étant donné l'émergence récente du domaine de l'éco-immunologie, il n'est pas étonnant que plusieurs questions restent sans réponse. La complexité du système immunitaire est un grand défi pour les scientifiques, mais un défi important. Une meilleure compréhension du système immunitaire dans un environnement naturel ne fera pas qu'avancer l'écologie, mais pourrait aussi avoir des impacts importants en médecine et en épidémiologie.

ANNEXE 1

Supplementary material for
Effects of agricultural intensification and temperature on immune response to
phytohemagglutinin in tree swallows (*Tachycineta bicolor*)

by

Gabriel Pigeon, Renaud Baeta, Marc Bélisle, Dany Garant, Fanie Pelletier

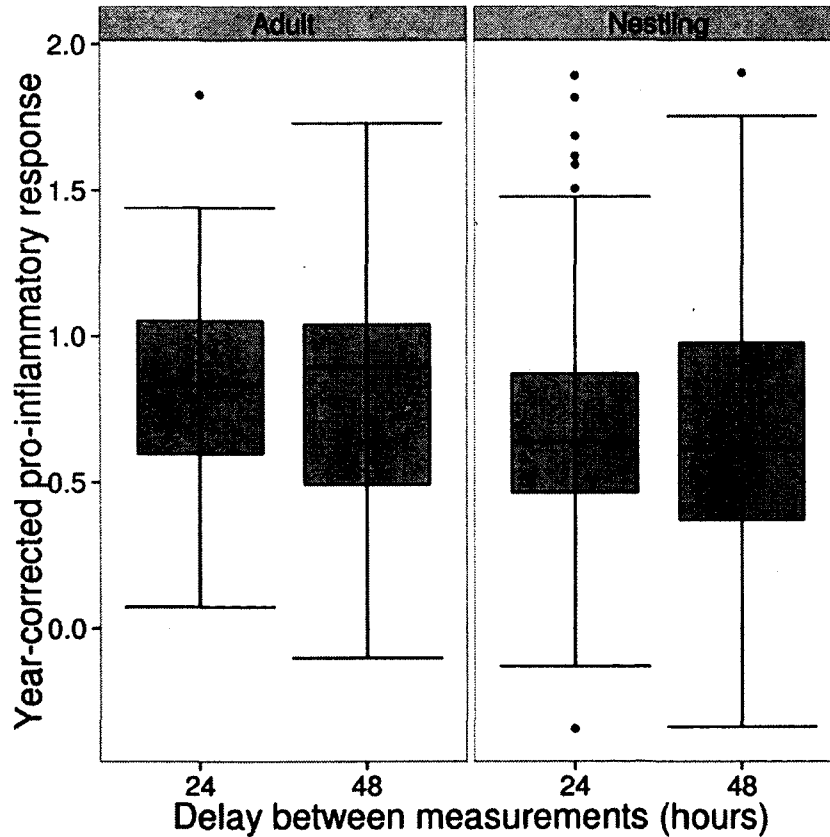


Figure A1.1. Boxplot of the year corrected pro-inflammatory response measured after a 24 and 48 hour delay for both adult female and nestlings tree swallows in Southern Québec, Canada.

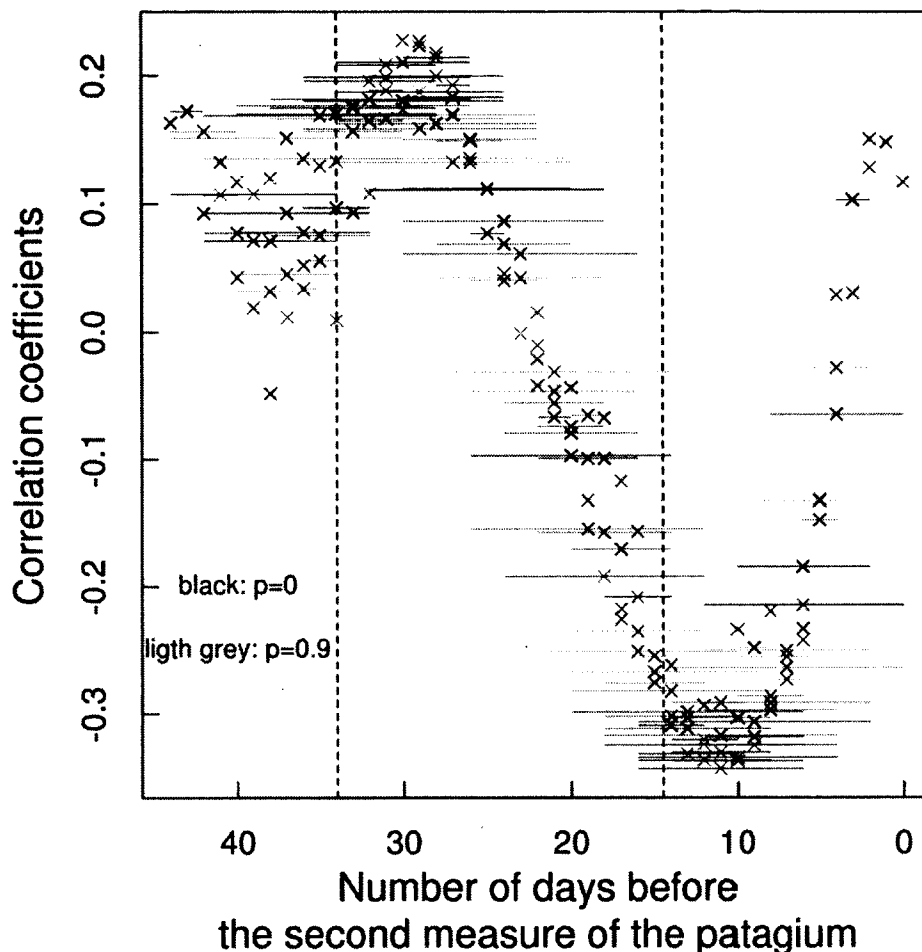


Figure A1.2. Result of the sliding window analysis for the effect of temperature on nestling response to PHA. Each point represents the center of a time interval with its associated Pearson correlation coefficient as the y value and central day as the x value. Horizontal bars represent the period covered by the window. X values are a backward count wherein day 0 is the day of the second patagium measurement after injection of PHA (this usually concurs with day 15 or 16 after hatching depending on the nestling). *P*-values associated to the correlations are represented by the shade of the point. Vertical dashed bars represent the average laying and hatching dates respectively.

Table A1.1. Average and standard deviation of different environmental characteristics in intensive and non-intensive agricultural habitat at fine (500 m radius) and large (5 km radius) scales.

	Variables	Non-intensive (sd)	Intensive (sd)
Environment (5km)	intensive cultures	11.461 (13.032)	72.125 (15.62)
	non-intensive cultures	55.403 (25.345)	15.644 (14.978)
	forest	25.435 (18.223)	1.612 (1.45)
	water	0.878 (1.503)	1.092 (1.486)
Environment (500m)	intensive cultures	6.46 (3.783)	59.234 (13.907)
	non-intensive cultures	59.576 (6.604)	10.853 (6.763)
	forest	0.179 (0.138)	0.924 (2.238)
	water	0.583 (0.308)	0.232 (0.189)

Table A1.2. AIC model selection for the model of response to PHA of adult females of 2009-2010 ($n = 54$) for tree swallows monitored on 10 farms in southern Québec, Canada. Only models with Δ AICc smaller than 2 are shown. Cumulative weight (cum ω) is the sum of AIC weight of models where the variables are present. The final selected model is in bold.

Model	Age	Year	Agriculture type	Mass	Temperature	Agriculture type x mass	Brood size	Agriculture type x brood size	df	AICc	Δ AICc	AIC ω
1	+	+	+	+	+	+	+		10	49.421	0	0.516
2	+	+	+	+	+	+			9	50.633	1.212	0.281
3	+	+	+	+	+	+	+	+	11	51.289	1.867	0.203
cum ω	1	1	1	1	1	1	0.718	0.202				

Table A1.3. AIC model selection for the model of response to PHA of nestlings of 2009-2010 ($n = 408$) for tree swallows monitored on 10 farms in southern Québec, Canada. Only models with $\Delta AICc$ smaller than 2 are shown. Cumulative weight (cum ω) is the sum of AIC weight of models where the variables are present. The final selected model is in bold.

Model	Year	Mean temperature during growth	Lay date	Agriculture type	Mass of mother	Age of mother	Mean temperature during laying	Brood size	Agriculture type x mass of mother	Sex	Delby	df	AICc	$\Delta AICc$	AIC ω
1	+	+										7	-148.071	0.000	0.878
2	+	+	+									8	-144.702	0.370	0.065
3	+	+	+	+								9	-144.523	0.549	0.059
4	+	+	+	+								8	-144.344	0.727	0.054
5	+	+	+	+								7	-144.162	0.910	0.049
6	+	+	+	+	+							10	-144.078	0.993	0.047
7	+	+	+		+							9	-144.073	0.998	0.047
8	+	+					+					8	-144.010	1.061	0.046
9	+	+			+							8	-143.974	1.097	0.045
10	+	+	+	+	+							10	-143.896	1.175	0.043
11	+	+	+	+	+		+					9	-143.786	1.286	0.041
12	+	+			+							9	-143.689	1.382	0.039
13	+	+						+				8	-143.638	1.433	0.038
14	+	+	+		+							9	-143.589	1.483	0.037
15	+	+	+	+				+				9	-143.391	1.680	0.034
16	+	+							+			8	-143.382	1.690	0.033
17	+	+	+	+			+			+		10	-143.332	1.739	0.033
18	+	+	+	+	+			+				11	-143.316	1.756	0.032
19	+	+							+		+	8	-143.281	1.790	0.032
20	+	+										8	-143.218	1.854	0.031
21	+	+	+	+	+							8	-143.192	1.879	0.030
22	+	+	+	+	+			+				10	-143.142	1.929	0.030
23	+	+	+	+	+		+					9	-143.124	1.947	0.029
24	+	+	+	+	+	+	+					11	-143.115	1.957	0.029
cum ω	1	0.92	0.53	0.43	0.25	0.15	0.15	0.07	0.06	0.03	0.03	0.03			

ANNEXE 2

Supplementary material
For
Ecological immunology in a fluctuating environment: an integrative analysis to tree
swallow nestlings immune responses
by
Gabriel Pigeon, Marc Bélisle, Dany Garant, Alan Cohen and Fanie Pelletier

Table A2.1. Eigenvalues of the redundancy analysis for the pooled value.

	Eigenvalue	Proportion Explained
RDA1	0.755	0.151
RDA2	0.155	0.031
RDA3	0.022	0.004
PC1	1.124	0.225
PC2	0.918	0.184
PC3	0.874	0.175
PC4	0.646	0.129
PC5	0.507	0.507

Table A2.2. Eigenvalues of the principal component analysis for the four sub-groups.

Sub-groups	Eigenvalues				
Intensive 2010	1.703	1.159	0.953	0.728	0.457
Intensive 2011	1.871	1.117	0.944	0.698	0.369
Extensive 2010	1.296	1.123	1.046	0.826	0.710
Extensive 2010	1.747	1.104	0.879	0.798	0.472

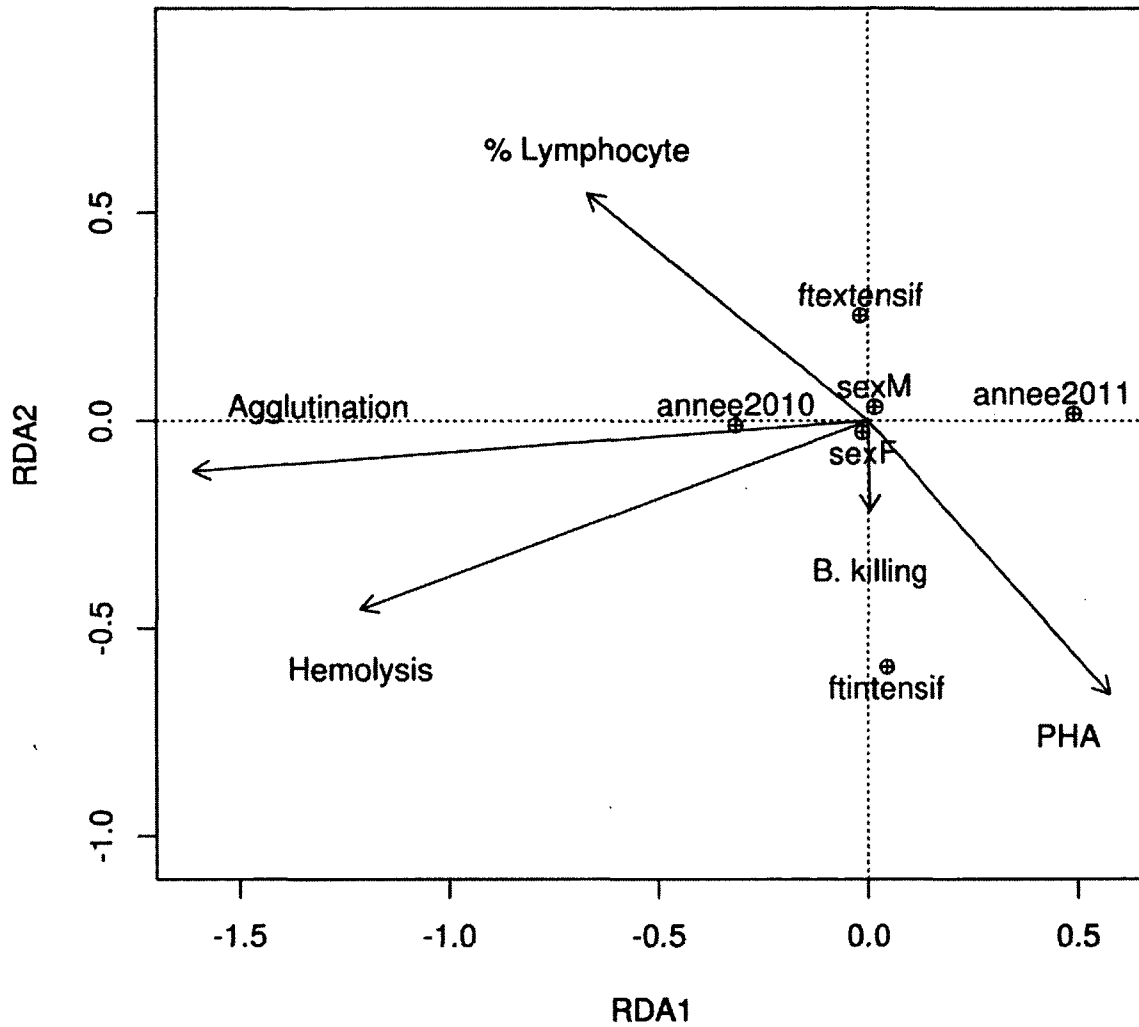


Figure A2.1. Redundancy analysis of pooled nestling's immune measurements with year, farm type and sex as constraining variables. Arrows represent loading values of the different immune measures. Red circles represent centroids of constraining variables. 20.37 % of the variance is explained by RDA 1 and 2. $n = 210$.

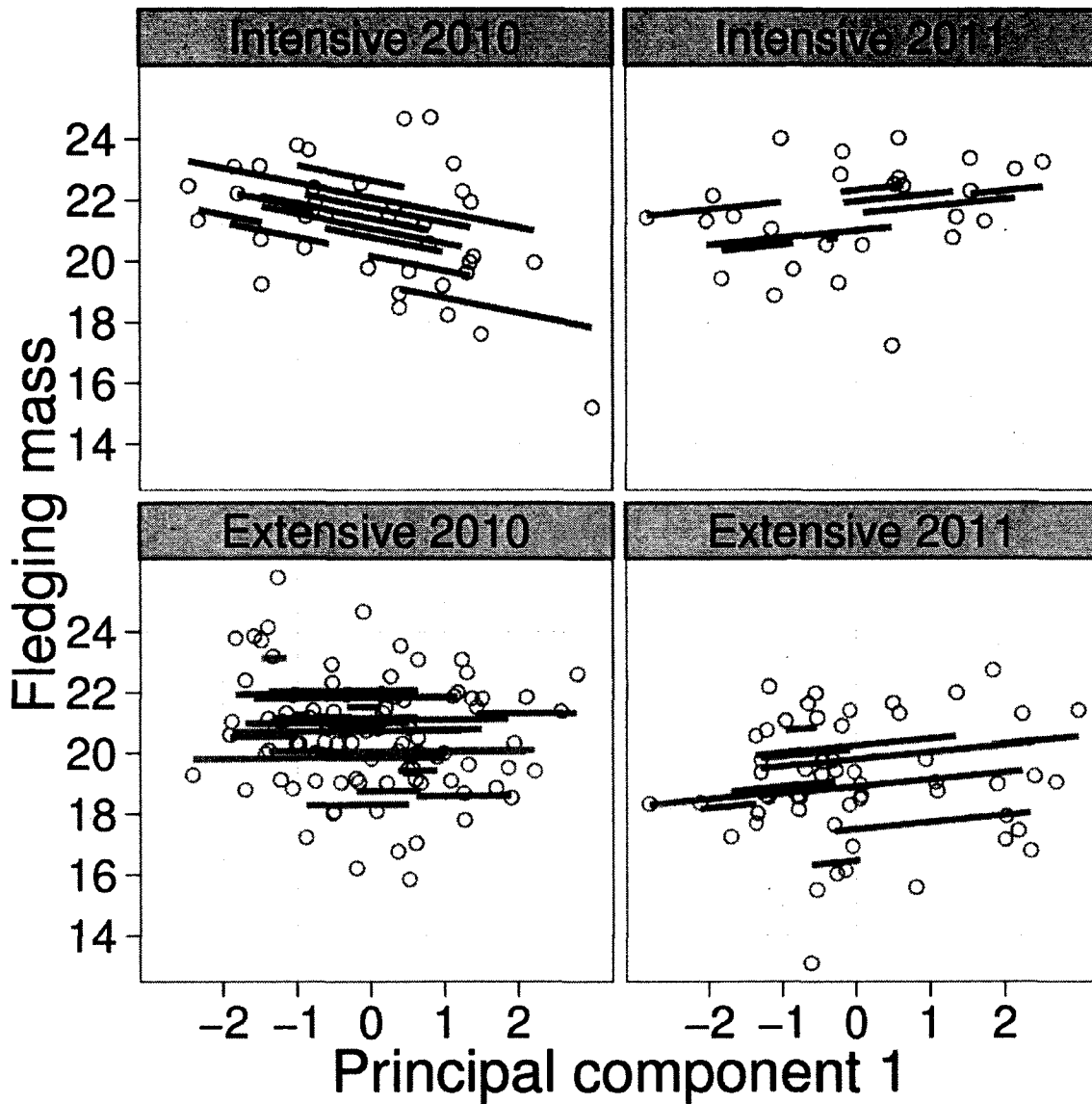


Figure A2.2. Relationship between PC1 and mass at fledging of tree swallow nestlings. Regression lines are obtained from linear mixed model (LME) and represent the regression with a random intercept by nest-boxes. The contribution to PC1 of each immune marker by sub-group is equal to the horizontal loading displayed in Figure 3.1.

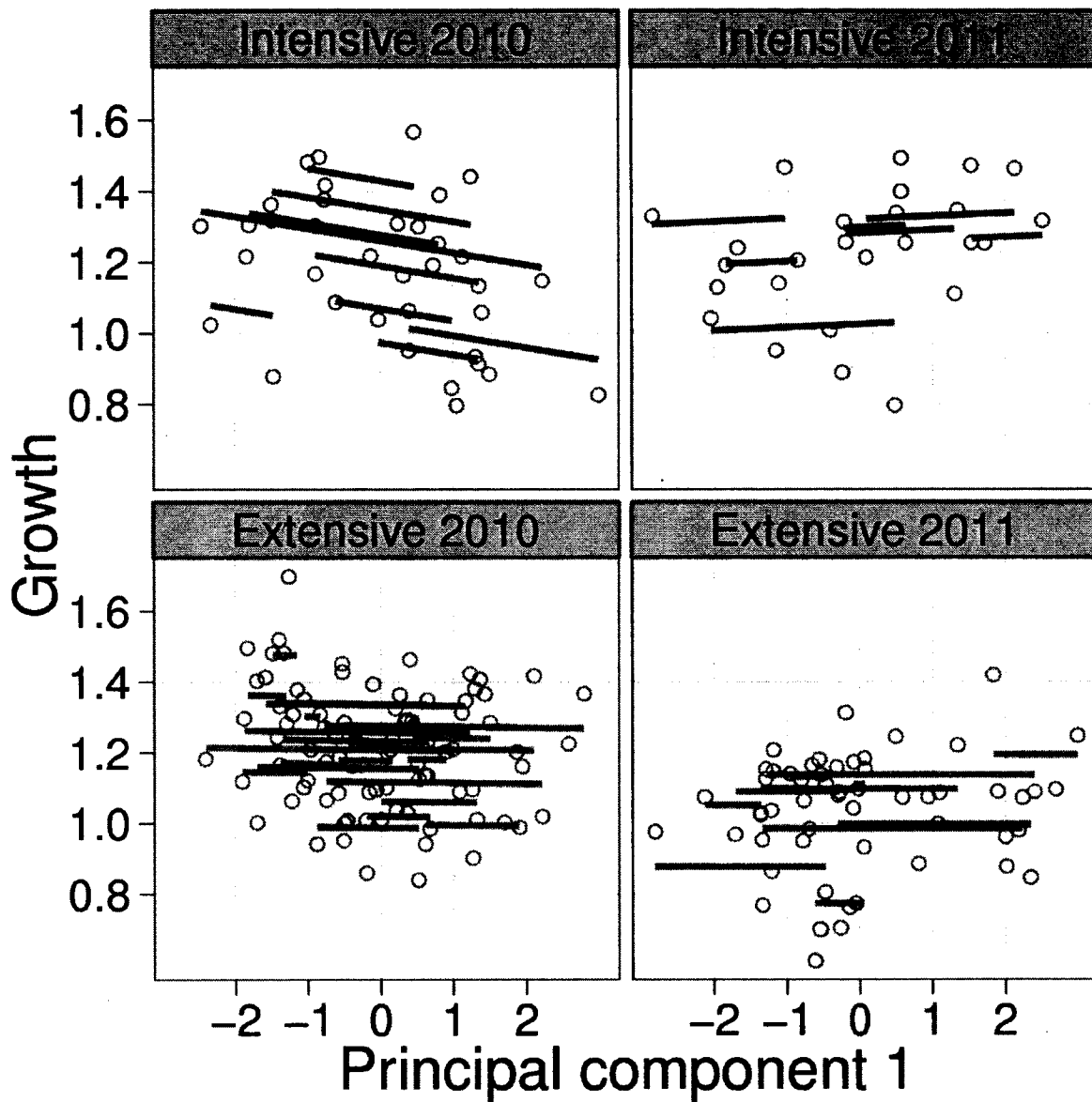


Figure A2.3. Relationship between PC1 and growth of tree swallow nestlings. Regression lines are obtained from linear mixed model (LME) and represent the regression with a random intercept by nest-boxes. The contribution to PC1 of each immune marker by sub-group is equal to the horizontal loading displayed in Figure 3.1.

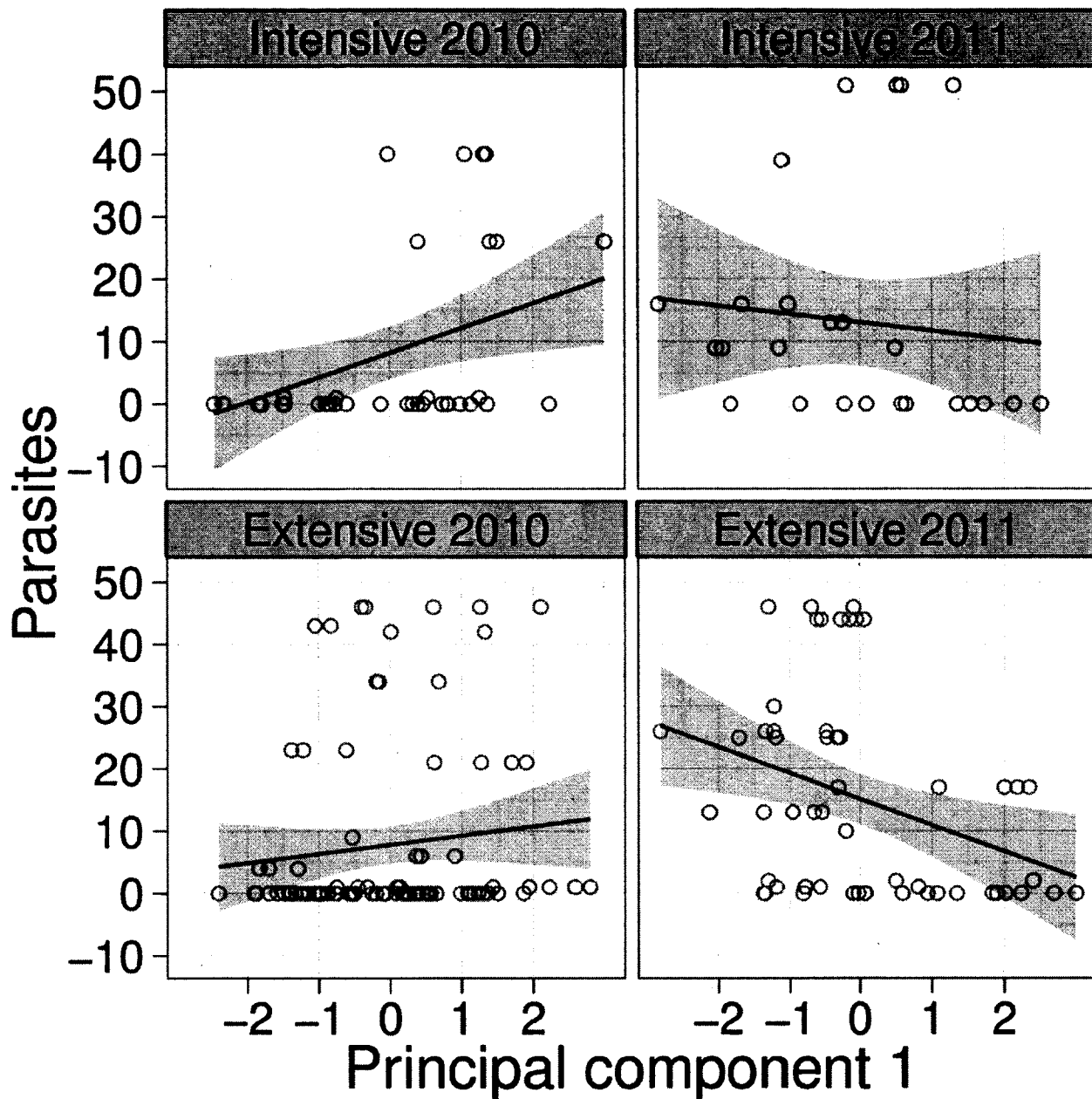


Figure A2.4. Relationship between PC1 and parasite burden of tree swallow nestlings. Regression lines are obtained from linear model. The contribution to PC1 of each immune marker by sub-group is equal to the horizontal loading displayed in Figure 3.1. Shaded region represents the 95 % confidence interval.

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