UNIVERSITE MONTPELLIER II
SCIENCES ET TECHNIQUES DU LANGUEDOC

THESE

Pour obtenir le grade de

DOCTEUR DE L'UNIVERSITE MONTPELLIER II

Discipline : Biologie de l'évolution et écologie

Ecole Doctorale : Systèmes Intégrés en Biologie, Agronomie, Géosciences, Hydrosciences, Environnement

Maladies infectieuses émergentes au sein des zones humides méditerranéennes dans le contexte des changements globaux

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Soutenue publiquement le 23 novembre 2012 devant le jury composé de :

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Résumé

L'émergence de maladies telles que le SRAS et le SIDA au cours des dernières décennies a fait prendre conscience des liens étroits existant entre santé animale, santé humaine et santé des écosystèmes. En effet, les pathogènes émergents ont pour la plupart une origine zoonotique (i.e. ils circulaient à l'origine au sein des populations animales). Les risques sanitaires associés à ces émergences sont en constante évolution sous l'influence des changements globaux qui modifient les écosystèmes et les contacts entre les hôtes. La prévention et le contrôle des maladies infectieuses émergentes nécessitent la compréhension de leur dynamique dans l'ensemble des compartiments dans lesquels elles circulent. Le travail présenté ici avait pour objectif d'améliorer cette compréhension au sein des zones humides méditerranéennes en ce concentrant sur deux pathogènes émergents : les virus Influenza A (VIA) et le virus West Nile. Il a été structuré selon trois axes de recherche : i) Utiliser la surveillance épidémiologique de l'avifaune sauvage pour comprendre la circulation du virus West Nile dans le bassin méditerranéen ii) Comprendre la dynamique des VIA au sein des différents compartiments où ils circulent et à leur interface iii) Comprendre le rôle des conditions environnementales dans la dynamique des VIA notamment au sein des populations humaines. Nos résultats mettent en évidence l'intérêt de mener des études multidisciplinaires sur le long terme pour comprendre l'épidémiologie des maladies émergentes. Ils soulignent également le rôle des activités anthropiques et des conditions environnementales dans la dynamique de ces maladies. Nos études apportent des éléments de réflexion pour allier gestion des risques d'émergence et gestion des écosystèmes et des populations. Elles encouragent à développer ce type d'approche afin de relever le défi de la prévention et du contrôle des pathogènes émergents.

Mots clés : écologie de la santé, maladies infectieuses émergentes, changements globaux, zones humides, Méditerranée, Camargue, virus influenza A, virus West Nile.

Abstract

During the last decades, the emergence of numerous infectious diseases such as SARS and AIDS has raised awareness of the close links that exist between animal health, human health and ecosystem health. Many of the emerging pathogens have a zoonotic origin (i.e. they originally circulated among animal populations). The health risks associated with the emergence of these diseases are progressing under the influence of global changes that affect ecosystems and contacts between hosts. The prevention and control of emerging infectious diseases require an in-depth understanding of their dynamics in all the compartments in which they occur. The aim of the present work is to improve our understanding of these phenomena within the context of Mediterranean wetlands by focusing on two emerging pathogens: Influenza A viruses (IAV) and West Nile virus. The thesis is structured around three research axes i) Using epidemiological surveillance of wild birds to investigate the circulation of West Nile virus in the Mediterranean Basin ii) Exploring IAV dynamics in the different compartments in which they circulate and at their interface iii) Determining the role of environmental conditions in IAV dynamics, especially within human populations. Our results highlight the value of long-term interdisciplinary studies for the understanding of the epidemiology of emerging diseases. They also emphasize the role of human activities and environmental conditions in the dynamics of these diseases. Our studies open up perspectives for combining emerging disease risk management and the management of ecosystems and populations. They also argue in favour of further developing this type of approach in order to meet the challenge of emerging pathogen prevention and control.

Keywords: health ecology, emerging infectious diseases, global changes, wetlands, Mediterranean, Camargue, influenza A virus, West Nile virus.

Remerciements

Au cours de ces trois années j'ai eu la chance de côtoyer des milieux et des personnes très différentes. Des marais de chasse, aux espaces naturels protégés, en passant par les laboratoires de l'Institut Pasteur et les bureaux de la Tour du Valat et de l'IRD, j'ai partagé le quotidien de chasseurs, de vétérinaires, de chercheurs et de virologues. Les personnes qui m'ont apporté leur aide m'apparaissent si nombreuses que j'ai très peur d'en oublier. Je tiens à leur exprimer ici toute ma gratitude.

Bien sur je voudrais d'abord remercier Michel et Fred, directeurs complémentaires et toujours disponibles, qui m'ont guidée et encouragée tout au long de cette thèse. Merci pour votre confiance et votre soutien. Merci pour toutes ces discussions qui m'ont apporté autant sur le plan scientifique que sur le plan humain.

Merci aussi à Camille et Elsa, mes deux « prédécesseurs » qui m'ont apporté leur aide au cours d'échanges toujours fructueux. En espérant que ce travail, bâti sur les bases du leur, soit à la hauteur de ses fondations.

Merci à Sylvie van der Werf sans la volonté de laquelle la collaboration avec l'Institut Pasteur n'aurait pu, ni être mise en place, ni se maintenir depuis 2005, et dont les commentaires sur plusieurs des articles présentés ci-après ont été très constructifs.

J'exprime également une grande reconnaissance à François Renaud qui a toujours gardé un œil sur mon travail, l'a valorisé et a pris le temps de discuter de mes articles et des projets en cours. Merci pour cette confiance que j'espère ne pas décevoir dans le cadre de nos collaborations futures.

Merci à Franck d'Amico, Sarah Randolph, Serge Morand et François Moutou d'avoir accepté, malgré leurs plannings chargés, de se pencher sur mon travail et d'y apporter leur point de vue. En espérant que ce manuscrit aura éveillé leur intérêt.

De même, merci à Franck Prugnolle et Marc Choisy d'avoir participé à mon comité de thèse et ainsi amené du recul et des idées neuves pour améliorer et compléter ce travail.

Merci à la fondation MAVA qui a contribué au financement d'un grand nombre des études présentées ci-après.

Merci à la fondation AXA pour la recherche qui a financé cette thèse, ainsi que ma participation à plusieurs colloques et formations. Merci également à l'ensemble de son personnel qui a suivi mon travail et m'a permis d'améliorer mes compétences de « communicante » tout en me réservant un très bon accueil lors des « Pop days ».

Les études présentées dans cette thèse sont le fruit de la collaboration entre des personnes aux compétences complémentaires à commencer par celles et ceux qui ont réalisé les analyses virologiques :

Viviane Grandhomme, virtuose de la détection et de la caractérisation des virus influenza. Merci pour ta rigueur et ton professionnalisme. Merci aussi d'avoir eu la patience de m'initier aux rudiments de la PCR et du lightcycler.

Sylvie Lecollinet et Steeve Lowenski, qui ont testé en long, en large, et en travers les sérums de pies et ont participé activement à la discussion des résultats de cette étude.

Merci à Gaëlle Simon qui, aidée par Séverine Hervé, a testé les sérums de sangliers et participé à la réflexion sur les résultats de l'étude correspondante.

J'ai également beaucoup appris en matière d'analyse de données et de modélisation au fil des discussions avec Eric Elguero, Benjamin Roche, Hector Rodriguez, Olivier Devineau et Simon Daoust. Merci à tous d'avoir pris le temps de comprendre mes jeux de données complexes et nombreux et de m'avoir donné pistes et conseils.

Les milliers d'échantillons récoltés n'auraient pu l'être sans l'aide de tous ceux qui m'ont accompagnée sur le terrain. Merci à Jocelyn de m'avoir suivie soirs et week-ends, toujours avec bonne humeur, vers les cloaques de milliers d'Anatidés (paix à leurs âmes). Merci à Olivier Pineau, et à toute l'équipe du domaine de la Tour du Valat : dompteurs de sangliers et réparateurs de pièges hors pair. Merci à Sébastien Rambert pour les heures passées devant les pièges, ainsi qu'à Steeve et Anthony qui m'ont assistée dans les prises de sang. Merci aussi à tous les stagiaires qui m'ont un jour aidée sous la pluie, le vent ou les moustiques.

Un immense merci également à tous les propriétaires de chasse, éleveurs, chasseurs, piqueurs et piégeurs qui ont accepté de collaborer à ces études et nous ont toujours bien accueillis, renseignés et aidés. Ce travail n'aurait tout simplement pas existé sans eux. Un mot en particulier à messieurs Herbinger, Coeur et Cordesse, pour leur aide indéfectible depuis 2005.

Merci également à tous les membres et les coordinateurs du réseau des GROG qui ont accepté de partager avec nous leurs précieuses données et nous ont permis d'étudier l'impact des conditions environnementales sur la transmission de la grippe pandémique.

Viennent ensuite ce qui m'ont supportée au bureau, à la pose café, à la cantine…

Merci à tous les membres du Mivegec pour leur accueil et leur soutien. Un mot en particulier à Dorothée, merci pour ton sourire et tes encouragements, ainsi qu'à Anne et Flore pour cette année d'organisation de séminaires qui s'est bien déroulée grâce à elles.

Merci à tous les membres de la Tour du Valat ainsi que de la FIBA, de Wetlands International, de TAKH et de l'ONCFS.

Yves qui m'a aidée sur le terrain et permis de participer à de multiples activités de l'équipe, faisant faire un bond à mes connaissances ornithologiques. Marie, Mireille et Florence toujours disponibles pour un conseil et prêtes à résoudre avec le sourire tous les soucis quotidiens. Jacqueline, à l 'écoute, qui m'a permis d'accéder aux plus récents comme aux plus anciens articles. Cathy et Jean-Claude qui m'ont guidée dans le monde des factures, des frais de missions et de l'administration.

Merci enfin à tous les hôtes de la Tour du Valat, stagiaires, permanents ou gens de passage qui ont partagé soirées, sorties et autres bons moments tout au long de ces trois ans.

Bien sur, un grand merci à Anne-Sophie, qui m'a supportée chaque jour au bureau, m'a hydratée, nourrie et accompagnée jusque dans les endroits les plus improbables tels que cette bergerie une veille de noël…

Merci à Audrey d'avoir partagé travail, chouquettes et crises en tous genres !

Merci à tous les membres de ma famille qui m'ont toujours écoutée et soutenue malgré la distance.

Merci à Thomas, simplement d'avoir toujours été là.

A mes grands-parents,

Table des matières

Introduction **7**

I. Le bassin méditerranéen, un espace en mutation **8**

II. Objectifs de la thèse **10**

Chapitre 1 ..... **17**

I. La surveillance de la faune sauvage **18**

1) Un peu d'Histoire 18

2) Les enjeux de la surveillance 19

3) Programmes de surveillance : des compromis entre coût et efficacité 20

II. Surveillance du virus West Nile et des flavivirus apparentés en Méditerranée **21**

1) Histoire du virus West Nile 21

2) Cycle du virus West Nile 22

3) Circulation récente du virus West Nile et des flavivirus apparentés en Méditerranée 22

4) Etude de la circulation des virus West Nile et Usutu en Camargue 23

5) Etude de la circulation de flavivirus chez le goéland leucophée à l'ouest de la Méditerranée 24

Chapitre 2 ..... **31**

I. Les virus Influenza A : histoire, épidémiologie et enjeux **32**

1) La grippe dans l'Histoire 32

2) Epidémiologie et évolution des virus influenza 34

II. Dynamique des virus influenza A au sein des milieux naturels : l'exemple de la Camargue **35**

1) Dynamique des virus influenza A en Camargue : données existantes 35

2) Dynamique des virus influenza A à l'interface entre Anatidés domestiques et sauvages ... 36

3) Un autre réservoir aviaire naturel : les Laridés 38

4) Un hôte potentiellement important : le sanglier 44

Chapitre 3 ..... **59**

I. Persistance des virus Influenza A dans l'environnement **60**

II. Influence des conditions météorologiques sur la présence du virus H1N1pdm09 en France 63

Discussion **71**

I. Importance de la surveillance de la faune sauvage **72**

1) Des suivis qui doivent être menés à long terme 72

2) Des méthodes innovantes pour minimiser les coûts et maintenir le suivi des pathogènes émergents 74
II. Les réservoirs naturels de pathogènes des compartiments complexes et hétérogènes..... 75
1) Hétérogénéité interspécifique 75
2) Hétérogénéité intraspécifique 76
III. Les compartiments abiotiques : des ensembles à ne pas négliger 77
1) La persistance dans les milieux abiotiques permet la survie lors de l'absence d'hôtes sensibles 77
2) Les conditions environnementales influencent la dynamique des vecteurs et des maladies qu'ils transmettent 78
3) Les conditions environnementales influencent la dynamique des pathogènes au sein des populations humaines 78
IV. Rôle des activités humaines dans la dynamique des maladies émergentes 80
1) Les activités humaines influent sur la démographie des hôtes et des vecteurs 80
2) La gestion cynégétique conditionne la santé du gibier mais aussi celle des espèces sympatriques 81
Conclusion 82
Bibliographie 85
Annexes 103

Liste des publications

Introduction

- **Article 1 :** Vittecoq M., Thomas F., Jourdain E., Moutou F., Renaud F. & Gauthier-Clerc M. Risks of emerging infectious diseases: evolving threats in a changing area, the Mediterranean basin. *Transboundary and Emerging Diseases.* doi: 10.1111/tbed.12012. ..... **15**

Chapitre 1

- **Article 2 :** Vittecoq M., Lecollinet S., Jourdain E., Thomas F., Blanchon T., Arnal A., Lowenski S. & Gauthier-Clerc M. Recent circulation of West Nile and other flaviviruses in Southern France. Accepté dans *Vector-Borne and Zoonotic Diseases* **27**
- **Article 3 :** Arnal A., Gomez Diaz E., Cerdà Cuéllar M., Lecollinet S., Busquets Martí N., Morejón de Girón F.X.A., Garcia Bocanegra I., Pages Martinez N., Pearce-Duvet J., Vittecoq M., Hammouda A., Samraoui B., Garnier R., Selmi S., González-Solís J., Gauthier-Clerc M., Jourdain E. & Boulinier T. Meaban flavivirus in Yellow-Legged Gulls (*Larus michahellis*) and Seabird Ticks (*Ornithodoros maritimus*) in the Mediterranean Basin. Soumis à *Emerging Infectious Diseases*. **29**

Chapitre 2

- **Article 4 :** Vittecoq M., Thomas F., Renaud F. & Gauthier-Clerc M. 2011. Avian influenza viruses: environmental influence. In Encyclopedia of environmental health. Elsevier. pp253-263. ..... **47**
- **Article 5 :** Vittecoq M., Grandhomme V., Champagnon J., Guillemain M., Crescenzo-Chaigne B., Renaud F., Thomas F., Gauthier-Clerc M. & van der Werf S. High Influenza A virus infection rates in Mallards bred for hunting in the Camargue, South of France. *PloS One.* 7(8): e43974. doi:10.1371/journal.pone.0043974. ..... **49**
- **Article 6 :** Arnal A., Vittecoq M., Pearce-Duvet J., Gauthier-Clerc M., Boulinier T. & Jourdain E. Laridae: a neglected reservoir that could play a major role in avian influenza virus dynamics. En préparation. **51**
- **Article 7 :** Arnal A., Pearce-Duvet J., González-Solís J., Vittecoq M., Garnier R., Alioua W., Samraoui B., Selmi S., Gauthier-Clerc M., Boulinier T. & Jourdain E. Spatial patterns of influenza A antibodies in yellow-legged gull (*Larus michahellis*) eggs in the Mediterranean basin. Soumis à *Virology Journal*. **53**
- **Article 8 :** Vittecoq M., Grandhomme V., Renaud F., Blanchon T., Thomas F., van der Werf S. & Gauthier-Clerc M. Is there any seasonality of influenza infections in Charadriiformes? A year-round study in Yellow-legged gulls (*Larus michahellis*) from Southern France. En préparation. **55**
- **Article 9 :** Vittecoq M., Grandhomme V., Blanchon T., Simon G., Herve S., Renaud F., Thomas F., Gauthier-Clerc M. & van der Werf S. 2012. Study of influenza A virus in wild boars living in a major duck wintering site. *Infection, Genetics and Evolution* 12: 483-486. ..... **57**

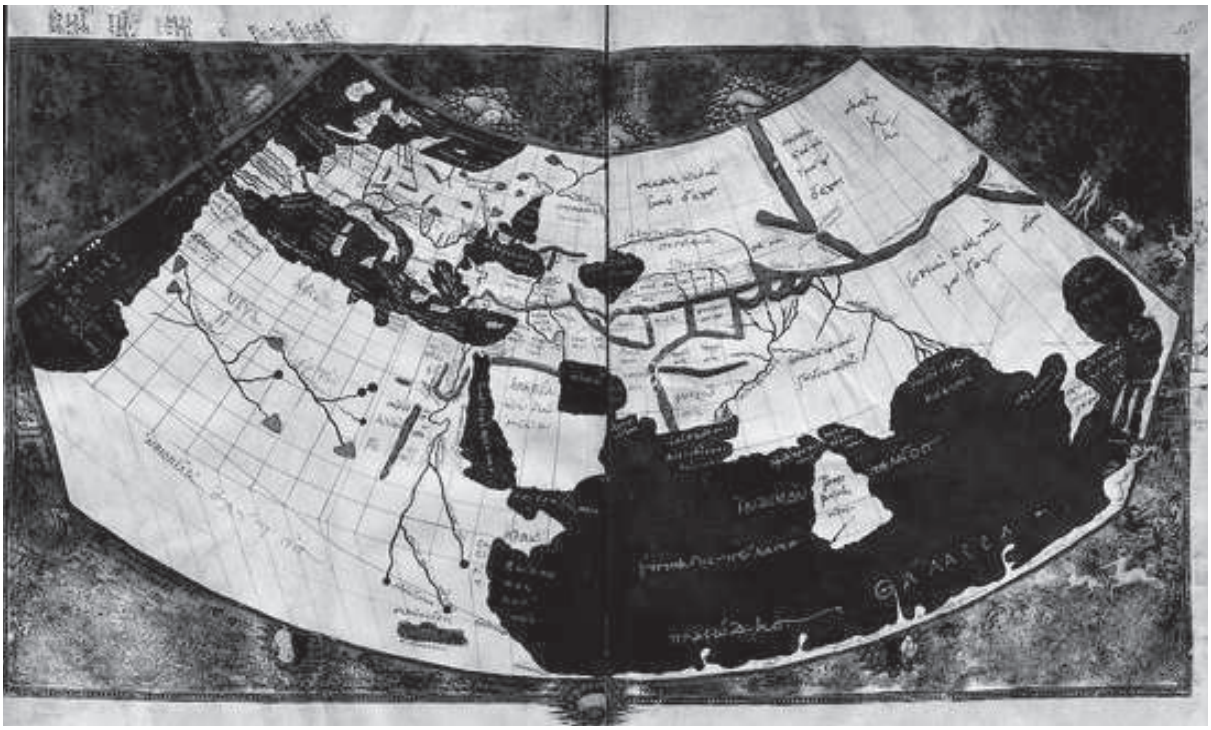
Chapitre 3

- **Article 10 :** Vittecoq M., Ottmann M., Thomas, F., Renaud, F. & Gauthier-Clerc, M. 2011. Persistance des virus influenza A en fonction des paramètres environmentaux. *Virologie* 15: 371-379. ..... **67**
- **Article 11 :** Vittecoq M., Grog I., Roche B., Thomas F. & Gauthier-Clerc M. Does the weather play a role in pandemic influenza spread ? A study of H1N1pdm09 infections in France in 2009-2010. En préparation. **69**

Liste des annexes

- **Annexe 1 :** Vittecoq M., Elguero E., Lafferty K.D., Roche B, Brodeur J., Gauthier-Clerc M., Missé D. & Thomas F. 2012. Brain cancer mortality rates increase with *Toxoplasma gondii* seroprevalence in France. *Infection, Genetics and Evolution* 12: 496-498. ..... **105**
- **Annexe 2 :** Vittecoq M., Lafferty K.D., Elguero E., Brodeur J., Gauthier-Clerc M., Missé D., Roche B. Cat ownership is neither a strong predictor of *Toxoplasma gondii* infection nor a risk factor for brain cancer. *Biology Letters*. doi:10.1098/rsbl.2012.0511. ..... **107**
- **Annexe 4 :** Communications orales & posters **109**
- **Annexe 5 :** Album photos : Prélèvements biologiques **111**

Introduction



Carte du monde connu selon Ptolémée (IIeme siècle ap. JC). Copie exécutée vers 1450 par Giovanni Roso, Biblioteca nazionale Marciana, Venise. Malgré sa longitude exagérée, on observe que le bassin méditerranéen est la zone la mieux connue à l'époque et jusqu'au XVeme siècle en Europe.

I. Le bassin méditerranéen, un espace en mutation

Le bassin méditerranéen est un point chaud de biodiversité dont plus de 30% de la faune et 60% de la flore sont endémiques (Blondel & Aronson 1999; Cuttelod *et al.* 2008). Les écosystèmes abritant cette biodiversité ont été modifiés par l'Homme depuis des millénaires. Dès le Pléistocène supérieur, la colonisation des îles méditerranéennes par *Homo sp.* a conduit à l'extinction de nombreuses espèces de mammifères endémiques (Schule 1993; Alcover, Sans & Palmer 1998). Par ailleurs, l'avènement de l'agriculture et de la domestication de différentes espèces animales à l'est du bassin il y environ 11500 ans puis leur extension progressive dans la région ont profondément transformé les paysages (Zeder 2008, 2011).

Le développement des activités anthropiques a parallèlement été déterminant dans l'histoire des agents pathogènes humains (McMichael 2004). Les liens étroits entre humains et animaux induits par la domestication de ces derniers ont permis à de multiples agents pathogènes de s'adapter à un nouvel hôte : *Homo sapiens sapiens* (Weiss 2001). Parmi ces nouvelles maladies citons la tuberculose, dont la présence en

Egypte est avérée dès 2000 av. JC (Zink *et al.* 2003), ou la brucellose dont on a observé les séquelles sur les ossements d'habitants d'Herculaneum, ville voisine de Pompéi, fuyant l'éruption du Vésuve en 79 ap. JC (Capasso & Capasso 1999).

Activités humaines, modification des écosystèmes et émergence[1] des agents pathogènes apparaissent donc intimement liées depuis des millénaires. A une échelle beaucoup plus récente, depuis le début de l'épidémie du SIDA en 1981, ces dernières décennies ont été marquées par l'émergence ou la réémergence de nombreux agents pathogènes affectant les populations humaines ou les animaux domestiques. Au sein des populations humaines plus de 60% des agents pathogènes émergents ont une origine zoonotique, et parmi eux la majorité est issue de la faune sauvage (Daszak *et al.* 2004; Jones *et al.* 2008). Ces crises sanitaires sont largement associées aux perturbations environnementales telles que les pollutions, les modifications des habitats, et les changements climatiques, regroupés sous le terme de changements globaux, qui se caractérisent par la rapidité de leur évolution et leurs impacts sensibles sur l'ensemble du globe (Gauthier-Clerc & Thomas 2010). La prise de conscience de cette association entre santé humaine, santé vétérinaire et santé des écosystèmes a mis en lumière la nécessité de créer des ponts étroits entre des disciplines jusqu'alors très éloignées donnant naissance à l'écologie de la santé (Daszak *et al.* 2007).

Le bassin méditerranéen occupe une situation clé entre trois continents au carrefour de multiples voies de migrations d'oiseaux, d'échanges de biens et de flux de personnes. La combinaison de sa grande biodiversité avec cette situation géographique et sa grande vulnérabilité aux changements globaux en fait un point chaud potentiel d'émergence de maladies infectieuses (Jourdain *et al.* 2007a). A ce contexte s'ajoutent les événements récents qui ont bouleversé l'échiquier sociopolitique de la région. Au nord, la crise économique met en péril les systèmes de santé, les conséquences sanitaires étant déjà mesurables, en particulier en Grèce (Kentikelenis *et al.* 2011). Au sud les révolutions arabes apportent d'une part des crises sanitaires liées à leur répression violente (WHO 2011a), d'autre part de nouvelles opportunités pour améliorer la prévention et le contrôle des maladies infectieuses au sein des nouvelles démocraties (Jabbour 2012).

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[1] Selon l'organisme mondial pour la santé (OMS) : « une maladie émergente est une maladie qui apparait pour la première fois dans une population ou qui existait précédemment mais connaît une augmentation rapide d'incidence ou de distribution géographique ».

En préambule à mon travail j'ai tenté d'identifier les nouveaux enjeux liés aux maladies infectieuses émergentes au sein du bassin méditerranéen à travers un article de revue, en me concentrant sur l'évolution des ressources, des conditions socio-économiques et des flux de biens et de personnes **[Article 1]**. En s'appuyant sur des exemples récents cet article souligne le caractère primordial de la gestion des risques d'émergence pour assurer la santé de la population méditerranéenne, mais aussi la santé de l'économie de l'espace méditerranéen. Les réponses à ces nouveaux défis passent par la nécessaire adaptation des textes internationaux qui contribuent à la gestion des risques sanitaires afin de prendre en compte les évolutions en cours.

Au sein du bassin méditerranéen, les zones humides[2] qui représentent environ 18,5 millions d'hectares, sont essentielles pour la biodiversité, notamment pour les oiseaux, qui s'y reproduisent et y hivernent en grands nombres, comme pour l'économie ; chasse, pêche, agriculture et culture du sel s'y développant (MWO 2012). Cependant les zones humides sont extrêmement vulnérables aux changements globaux. Environ 50% des zones humides méditerranéennes ont disparu depuis 1900 tandis que celles qui subsistent connaissent des mutations importantes du fait des changements d'occupation des sols, de la pollution et des changements climatiques (MWO 2012). Lieu de contact entre faune sauvage, faune domestique, arthropodes potentiellement vecteurs de maladies et humains, ces zones sensibles aux changements actuels constituent des points privilégiés d'émergence pour les maladies infectieuses.

II. Objectifs de la thèse

L'objectif général de cette thèse était d'étudier les risques d'émergence de maladies infectieuses au sein des zones humides méditerranéennes dans le contexte des changements globaux. La Camargue a été choisie comme zone d'étude principale pour ce travail car elle illustre l'ensemble des enjeux associés à cette problématique. Lieu de rencontre des eaux douces du Rhône et des eaux salées de la Méditerranée, la Camargue est une mosaïque d'habitats d'environ 178000 hectares. Cette zone humide est considérée d'importance internationale pour l'hivernage des canards selon les critères Ramsar. En effet, lors de pics d'occupation elle peut accueillir jusqu'à 150000 individus

[2] *Selon la convention de Ramsar : «* les zones humides sont des étendues de marais, de fagnes, de tourbières ou d'eaux naturelles ou artificielles, permanentes ou temporaires, où l'eau est stagnante ou courante, douce, saumâtre ou salée, y compris des étendues d'eau marine dont la profondeur à marée basse n'excède pas six mètres »

(Deceuninck & Fouque 2010; Gauthier-Clerc & Kayser 2012). Plus généralement 289 espèces d'oiseaux venant aussi bien de Sibérie que d'Afrique subsaharienne fréquentent régulièrement la région qui représente un carrefour migratoire (Kayser *et al.* 2003). Les oiseaux sont les hôtes naturels de nombreux agents pathogènes dont certains peuvent représenter un risque pour la santé humaine et celle des animaux domestiques (Reed *et al.* 2003; Hubálek 2004). La Camargue est ainsi un point chaud en termes de risques d'émergence d'agents pathogènes (Jourdain *et al.* 2007a). Ces risques évoluent avec les changements d'usage des milieux camarguais qui résultent de la mise en balance d'activités agricoles (riziculture, élevage, saliculture) avec des activités écologiques (zones mises en réserve) et des activités cynégétiques, touristiques et récréatives (Picon 1978; Mathevet 2004). Les écosystèmes camarguais sont donc façonnés par les activités humaines en pleine mutation, ce qui entraine des modifications des communautés animales et végétales.

Cette thèse est divisée en trois chapitres correspondant aux trois étapes du travail mené.

La première étape de ce travail, présentée dans le chapitre 1, a été d'explorer des méthodes de surveillance épidémiologique basées sur le suivi sérologique d'espèces sauvages en prenant pour modèle le virus West Nile. En effet pour étudier les risques d'émergence d'une maladie infectieuse dans une région la première étape est de savoir si le pathogène en jeu circule dans cette région. Pour répondre à cette question une surveillance épidémiologique doit être mise en place. Le virus West Nile représente un bon exemple d'agent pathogène ayant récemment réémergé en Camargue et cette réémergence pourrait être en partie liée à l'évolution des pratiques agricoles dans le delta (Ponçon *et al.* 2007). Le virus West Nile se maintient via un cycle impliquant des oiseaux sauvages en tant qu'hôtes naturels et des moustiques du genre *Culex* en tant que vecteurs. Les infections qu'il cause semblent généralement asymptomatiques chez les oiseaux européens. Cependant, l'observation ponctuelle d'épisodes de mortalité (e.g. Malkinson *et al.* 2002) et de symptomes sévères (e.g. Jourdain *et al.* 2007b) indique l'existence possible de formes sévères d'infection passant inapercues au sein de l'avifaune sauvage (Hars *et al.* 2008). Chez les chevaux ou les humains, qui représentent des hôtes accidentels, ces infections peuvent entrainer des syndromes neurologiques graves voire la mort (Dauphin *et al.* 2004). Des cas équins on été observés en 2000 et

2004 en Camargue après 35 ans d'absence de détection (Murgue *et al.* 2001; Zeller *et al.* 2004). Aucun cas équin ou humain n'ayant été signalé en Camargue depuis 2004, le premier objectif était de déterminer si le virus continuait d'être présent dans la région malgré cette absence de détection. Dans ce but, un suivi sérologique a été réalisé chez la pie bavarde (*Pica pica*), précédemment identifiée comme une espèce sentinelle pour le virus West Nile (Jourdain *et al.* 2008) **[Article 2]**. En parallèle de ce travail, une collaboration a été mise en place avec la thèse d'Audrey Arnal pour explorer une nouvelle méthode de surveillance basée sur l'étude des anticorps présents dans les œufs de goéland leucophée (*Larus michahellis*), espèce potentiellement impliquée dans l'introduction et l'amplification du virus West Nile (Jourdain *et al.* 2007c). Cette méthode a été utilisée dans le but de détecter la circulation du virus West Nile dans l'avifaune sauvage à l'échelle de l'ensemble de l'ouest méditerranéen **[Article 3]**. Ces deux études avaient également pour but de détecter la circulation éventuelle d'autres flavivirus apparentés au virus West Nile.

L'étape suivante de cette thèse a été d'étudier la dynamique d'un agent pathogène dans les multiples compartiments où il circule et à leur interface en prenant pour modèle les virus Influenza A (VIA).

Lorsque la surveillance épidémiologique prouve la présence régulière d'un agent pathogène dans une région, l'étape suivante dans la prévention des épizooties ou épidémies liées à ce pathogène est de comprendre sa dynamique au sein des différents compartiments dans lesquels il circule et à leur interface. Cette étape, présentée dans le chapitre 2, a donc consisté à étudier la dynamique des VIA (communément appelés virus grippaux) en Camargue au sein de différents compartiments. Les VIA ont été choisis pour modèle d'une part car il s'agit de pathogènes à enjeu fort en termes de santé publique et vétérinaire (WHO 2009, 2011b; OIE 2012b), d'autre part car leur présence régulière en Camargue chez les canards hivernants a été bien documentée depuis 2005 (Lebarbenchon *et al.* 2007a b, 2009, 2010). En s'appuyant sur les résultats des études antérieures, qui portaient principalement sur la circulation des virus Influenza A chez les Anatidés, nous avons cherché à comprendre le rôle des autres compartiments épidémiologiques et de leurs interactions. Après un travail de revue visant à présenter le modèle **[Article 4],** trois problématiques complémentaires ont été abordées dans ce cadre : i) Les lâchers de canards colverts (*Anas plathyrynchos*) à but cynégétique

établissent une interaction forte entre compartiments aviaire domestique et sauvage. Cette pratique peut-elle avoir un impact sur la dynamique des virus Influenza A chez les oiseaux d'eau hivernant en Camargue ? **[Article 5]** ii) Les Laridés constituent un réservoir méconnu des virus Influenza A. Ont-ils un rôle important à jouer dans la dynamique mondiale des virus Influenza A ? **[Article 6]** Les virus influenza A circulent-ils régulièrement chez les Laridés à l'ouest de la Méditerranée ? **[Article 7]** La saisonnalité des infections dues aux virus Influenza A est-elle semblable en Camargue chez les Laridés à celle observée chez les Anatidés avec qui ils partagent leur site d'hivernage? **[Article 8]** iii) Le sanglier, comme le porc, peut être infecté par des virus Influenza A aviaires, humains et porcins. En Camargue les sangliers partagent l'usage des zones humides avec les canards hivernants et les humains acteurs du territoire. Sachant qu'ils se trouvent à l'interface entre ces différents compartiments, ont-ils un rôle à jouer dans l'épidémiologie des virus Influenza A dans la région ? **[Article 9].**

La dernière étape de ce travail de thèse, présentée dans le chapitre 3, s'inscrit également dans l'étude de la dynamique des virus influenza A au sein des différents compartiments où ils circulent, mais en s'intéressant cette fois aux compartiments abiotiques. Qu'ils soient aviaires, porcins, ou humains, les virus influenza A doivent dans la plupart des cas survivre à l'extérieur de leur hôte pour être transmis. Selon les situations et les groupes d'hôtes cette phase environnementale a lieu soit directement dans l'eau ou l'air soit au sein de différents fluides corporels (fientes, sécrétions nasales, etc.). La persistance en milieu abiotique est donc un élément clé de la dynamique des virus Influenza A. Les connaissances concernant cette persistance restent encore limitées et généralement considérées séparément pour les virus Influenza A circulant chez les humains d'une part et chez les oiseaux d'autre part (Weber & Stilianakis 2008; Stallknecht, Brown & others 2009). C'est pourquoi nous avons souhaité établir un état de l'art concernant cette problématique dans son ensemble. Ce travail avait également pour objectif d'identifier des axes prioritaires de recherche à développer **[Article 10]**. Parmi ces axes figure la compréhension de l'influence des paramètres climatiques sur la transmission des virus Influenza A chez l'Homme et des mécanismes sous-jacents à cette influence. Afin de compléter les connaissances existant quant à cette problématique nous avons étudié le lien entre la présence de la grippe pandémique H1N1pdm09 en France métropolitaine et l'humidité, la température et les rayonnements UV **[Article**

11]. L'objectif de cette étude était de déterminer quelles sont les conditions climatiques favorables à la présence des virus Influenza A dans la population humaine dans un contexte pandémique et à l'échelle d'un pays au climat tempéré.

La dernière partie de cette thèse est constituée par la discussion des résultats obtenus, et des perspectives de recherches qui s'ouvrent à l'issue des études menées. Enfin, une conclusion plus générale souligne la nécessité de mettre en place des études multidisciplinaires sur le long terme pour relever le défi de la prévention et du contrôle des maladies infectieuses émergentes au sein du bassin méditerranéen.

Article 1. Risks of emerging infectious diseases: evolving threats in a changing area, the Mediterranean basin.

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Publié dans *Transboundary and Emerging Diseases.*

Transboundary and Emerging Diseases

REVIEW

Risks of Emerging Infectious Diseases: Evolving Threats in a Changing Area, the Mediterranean Basin

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Keywords:
emerging infectious diseases; Mediterranean region; global changes; risk factors

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Received for publication June 26, 2012

doi:10.1111/tbed.12012

Summary

The Mediterranean basin is a biodiversity hotspot; it has historically had a large human presence that has shaped ecosystems for millennia. As the cradle of many civilizations, the area was one of the main theatres for transitions that punctuated both human and pathogen histories, which are intimately linked. Today we are living through another great historical transition summarized in the expression 'global changes'. In this context, we are witnessing a rise in the emergence of pathogens widely associated with aforementioned global changes. The Mediterranean basin might be especially vulnerable to this phenomenon due to the acute consequences global changes will have in this key intercontinental interface region. In addition, Arab revolutions and European economic crisis are creating both sanitary issues and presenting new opportunities to improve infectious disease control and prevention in the region. The aim of this review is to identify the impacts that ongoing changes might have on the risk of infectious disease emergence in the Mediterranean basin. We focussed on three key domains undergoing transformations: (i) resources, namely safe drinking water and animal products, (ii) socio-economic factors including health inequalities within countries and poor sanitary conditions linked to ongoing conflicts and (iii) movements of people and goods that are reshaped by current changes and are intimately linked to the risk of disease proliferation. Building on recent examples, we try to identify upcoming challenges and discuss ways to meet them in the light of existing international human and veterinary health guidelines and their possible improvements.

Introduction

The twenty-two riparian countries and territories of the Mediterranean Sea share a unique climate coupled with an extremely rich natural and cultural heritage. The economy of the region remains mostly dependent – especially on the southern rim – on natural resources (Plan Bleu, 2009). Zone of exchanges and of conflicts at the same time, the region is still in search of stability. This stability must be built from common approaches on shared issues that include disease risk management.

The Mediterranean basin is unique in its combination of a major biodiversity hotspot and a historically large human presence, which has shaped ecosystems for millennia (Blondel and Aronson, 1999). As an example, during the Middle and Upper Pleistocene, invasions of Mediterranean islands by *Homo* sp. led to the near-complete extinction of the endemic fauna (Schule, 1993; Alcover et al., 1998). In addition, agriculture arose in the eastern Mediterranean and began to modify local environments and biotic communities at least 11 500 cal BP if not earlier (Zeder, 2011). As the cradle of many civilizations, the area was also one of

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the main theatres for transitions that punctuated both human and pathogen histories, which are intimately linked (McMichael, 2004). Contemporary with the beginning of agriculture, the emergence of animal domestication among early human settlements in the eastern Mediterranean enabled enzootic pathogens to enter *Homo sapiens* (Weiss, 2001). New infections spread in human populations: tuberculosis has been identified in Egyptian mummies from as early as 2000 BC (Zink et al., 2003) and bone lesions typical of brucellosis were detected on the skeletons of the fugitives from Herculaneum (a Roman city near Pompeii) as early as 79 AD (Capasso and Capasso, 1999).

Today, the Mediterranean region is inhabited by 460 million people and plays a key interface role between Asia, Africa and Europe as one of the main hubs of the global transport network. The area is thus potentially at high risk for disease emergence and spread (Seimenis et al., 2006; Jourdain et al., 2007). Experts have warned that upheavals, which are currently shaking the region, will modify these risks and may enhance them if not properly dealt with (e.g. Rechel et al., 2011; Jabbour, 2012). The European part of the basin is confronting an important economic crisis, which is already threatening health systems in some countries like Greece (Kentikelenis et al., 2011). The southern part of the region has been experiencing revolutions that bring both sanitary crises (e.g. WHO, 2011) and create new opportunities to improve infectious disease control and prevention in the young democracies (Jabbour, 2012). These phenomena act in synergy with global changes defined as the interactions between natural changes in the Earth's physical and biological structure and the broader effects of human activities (Ecosystems Panel, 2000). The Mediterranean area appears particularly vulnerable to global changes that notably include pollution, climate change, land use change and the exponential increase of people and goods transport. Indeed, the prediction of reduced precipitation in North Africa is of major concern because 22% of the world's water-poor population lives in the Mediterranean basin (Giorgi and Lionello, 2008; Plan Bleu, 2009). Moreover, increasing urbanization and human population density in coastal areas are critical in the Mediterranean area, where land use changes have led to the disappearance of more than 50% of wetlands over a century (MWO, 2012).

In this context, our aim is to identify the impacts that ongoing changes might have on infectious disease (re) emergence risk in the Mediterranean basin. We focussed on three key domains undergoing change: (i) resources, namely safe drinking water, to which a large part of Mediterranean inhabitants have no access and animal products that account for an increasingly large part of the Mediterranean diet, which bring new risks of zoonosis transmission; (ii) socio-economic factors including health inequalities within countries and poor sanitary conditions linked to ongoing conflicts; and (iii) movements of people and goods that are reshaped by current changes and intimately linked to disease spread risks. We discuss ways to mitigate the expected impacts of ongoing changes in the light of existing international human and veterinary health guidelines and their possible improvements (Table 1; WHO, 2005; UNDP, 2010; OIE, 2011; UNODA, 2012).

Resources

Water crisis

Water availability is already a major issue in the southern and eastern parts of the Mediterranean basin, where 180 million people are 'water-poor' (<1000 m^3 per capita per year) and 60 million suffer conditions of water scarcity (<500 m^3 per capita per year; Plan Bleu, 2009). This situation will likely worsen with ongoing changes. The regional effects of climate change on the water cycle may deplete water resources, increase their variability and weaken their exploitability (Giorgi and Lionello, 2008). The most water-poor areas are likely to be the most affected (Giorgi and Lionello, 2008). This trend may be amplified by soil sealing caused by the rapid urbanization of the Mediterranean coast, which prevents infiltration and thus recharge of groundwater (Plan Bleu, 2009). Increasing tourism activities in the region are associated with water use conflicts, especially during summer, when the water needs of both agriculture and tourism facilities are high (Eurostat, 2009; Plan Bleu, 2009). Furthermore, urbanization and conflicts favour the development of slums with poor access to safe drinking water (UNDP, 2010). In 2010, 13% of urban inhabitants of southern Mediterranean countries were living in slums (UNDP, 2010).

These changes may enhance the impact of waterborne diseases such as enteric fevers, which are already problematic in the Mediterranean area. As an example, although very few cholera cases were reported in the Mediterranean basin during the last decade (WHO, 2012a), *Vibrio cholerae* has recently been isolated from freshwater (Eddabra et al., 2011), water of coastal areas (Vezzulli et al., 2010) and fish living in both marine and freshwater habitats (Halpern et al., 2008; Senderovich et al., 2010) in European and North African countries. The increasing numbers of refugees resulting from ongoing conflicts in North Africa and the Middle East might create ideal conditions for new cholera outbreaks, which often occur in overcrowded settlements (Bompangue et al., 2009; Mahamud et al., 2011). Because *V. cholerae* proliferation is dependent on climatic conditions (Lipp et al., 2002; Koelle, 2009), global warming might also increase the risk of cholera re-emergence even in southern European countries (Guégan, 2002).

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Table 1. International organizations and international guidelines related to disease risk management

Organization	International guidelines
WHO The World Health Organization is the directing and coordinating authority for health within the United Nations system. It is notably responsible for providing leadership on global health matters, setting norms and standards, providing technical support to countries and monitoring and assessing health trends	International Health Regulations (IHR) Global rules updated in 2005 to enhance national, regional and global public health security including the assessment of the surveillance and response capacities of the member states and the development and implementation of plans of action to ensure that these core capacities are functioning
OIE The World Organization for Animal Health is the intergovernmental organization responsible for improving animal health worldwide. It aims at promoting veterinary services, food safety, animal welfare and international solidarity	The Terrestrial Code and the Aquatic Code Last updated in 2011, they set out standards for the improvement of animal health and welfare and veterinary public health worldwide, including through standards for safe international trade in terrestrial and aquatic animals and their products
UNDP The United Nations Development Programme is the UN's global development network, advocating for change and connecting countries to knowledge, experience and resources to help people build a better life	The Millennium development goals (MDC) In 2000, 189 nations made a promise to free people from extreme poverty and multiple deprivations. This pledge became the eight Millennium Development Goals to be achieved by 2015
UNODA The United Nations Office for Disarmament Affairs promotes: nuclear disarmament and non-proliferation; strengthening of the disarmament regimes in respect to other weapons of mass destruction and chemical and biological weapons; disarmament efforts in the area of conventional weapons	The Biological Weapons Convention It is one of the disarmament conventions managed by the UNODA (the others are not related to infectious diseases). It became effective in 1975 and prohibits the development, production, acquisition, transfer, stockpiling and use of biological and toxin weapons

The emergence of antimicrobial drug resistance complicates the treatment of common waterborne diseases such as *Escherichia coli* infections worldwide (WHO, 2012b). In the Mediterranean basin, a wide variety of antimicrobial drugs are commonly found in groundwater (Rabiet et al., 2006; Mompelat et al., 2009), and their concentration might increase in the near future by water scarcity (Petrovic et al., 2011). These drug residuals favour the evolution of resistant pathogens that are also commonly isolated in groundwater as well as in aquatic birds in Mediterranean countries (e.g. Bonnedahl et al., 2009; Economides et al., 2012).

Implementing appropriate water treatment and surveillance is crucial to the prevention of disease emergence in the Mediterranean basin. Indeed, even in European countries, most of the existing treatment systems do not eliminate either antimicrobial drugs or all pathogenic bacteria (e.g. Wéry et al., 2008; Mompelat et al., 2009). Water safety is not specifically addressed by international health guidelines, which focus on the risk of diseases spread from one country to another rather than within countries (WHO, 2005). One of the Millennium Development Goals championed by the United Nations (UN) is to 'halve, by 2015, the proportion of the population without sustainable access to safe drinking water and basic sanitation' (UNDP, 2010). This international effort has already significantly improved water access in the Mediterranean region (IME, 2007; UNDP, 2010). Nevertheless, ongoing changes may impair this progress because the countries involved may not be able to sustain their current efforts.

Livestock revolution

On a global scale, economic development tends to be accompanied by an increased demand for animal protein. In the Mediterranean basin, this increase is especially pronounced because the diet traditionally contained little meat. Mediterranean inhabitants still eat less animal products than the European average, but the proportion of animal protein in their diet has increased steadily since the 1960s with strong variability between countries (Gil et al., 1999; Garcia-Closas et al., 2006). This increasing demand for animal products leads to the development of industrial rearing practices that favour the emergence of new pathogens because the animals have genetically poor immune capacities and are raised in stressful and crowded conditions that facilitate rapid disease transmission and evolution (Steinfeld, 2004; Lebarbenchon et al., 2010). In addition, domestic animals, notably poultry, remain a key subsistence source for many poor Mediterranean inhabitants (FAO, 2009). Epizootics are very costly for these inhabitants, whose economic well-being depends on their animals, and they are at high risk for zoonotic disease transmission because animals and humans live in close contact (FAO, 2009). Emerging zoonotic diseases have to

be controlled simultaneously in different kinds of rearing facilities in the Mediterranean region, which entails the implementation of a very complex epidemiological network.

These challenges have been highlighted by the difficulties encountered in the control of the highly pathogenic H5N1 avian influenza viruses (HPAIV). The coexistence of industrial poultry, where conditions are favourable for the evolution of HPAIV, and family poultry, where surveillance and vaccination campaigns are difficult to implement due to the multiplicity of poultry owners, complicates the control of HPAIV. This is notably the case in Egypt, where HPAIV infections have regularly been reported since 2006 in both poultry and humans (Fasina et al., 2010; OIE, 2012a). Epidemiological studies have highlighted the importance of taking into account socio-economic factors to adjust compensation schemes and implement effective collaborations with poultry owners (Meleigy, 2007; Leibler et al., 2009). The current turmoil in the Mediterranean basin raises concerns about the durability of these recently forged collaborations, which represent keystones for the surveillance of zoonotic diseases (Guan et al., 2012). HPAIV epizootics also underscored the importance of wildlife surveillance in the understanding of zoonotic disease epidemiology (e.g. Olsen et al., 2006). Wildlife surveys revealed that wild waterfowl represent a source of new influenza viruses that can evolve into highly pathogenic forms in the context of industrial farming (Lebarbenchon et al., 2010).

For other zoonotic diseases, wild species may be important pathogen reservoirs, adding another compartment to include in the surveillance network. As an example, bovine tuberculosis (caused by *Mycobacterium bovis*) is a zoonotic disease that can affect the health of both cattle and humans (Humblet et al., 2009). Recent studies in France and Italy showed that wild boars (*Sus scrofa*) are spillover hosts of bovine tuberculosis, while in Spain this species is considered a reservoir host that needs to be targeted by vaccination campaigns to prevent outbreaks in cattle (Serraino et al., 1999; Humblet et al., 2009; Gortazar et al., 2011). Interestingly, studies in Spain revealed that the intensification of game management may increase the prevalence of bovine tuberculosis. Indeed, wild boars and red deer living in high densities in fenced hunting estates were shown to be at higher risk of tuberculosis infection than those living in unfenced areas (Vicente Baños et al., 2007; Castillo et al., 2011). In southern and eastern Mediterranean countries, the few available data suggest a high prevalence of *M. bovis* in cattle, representing substantial risks of infection in the population (Ozmen et al., 2005; Hassanain et al., 2009; Bouzid et al., 2010; Ben Kahla et al., 2011). Wild host species are also likely existing in these countries, but their identity and role in tuberculosis transmission dynamics remain to be investigated. In these countries, the priority for now is to implement efficient disease surveillance in domestic animals.

The scarcity of surveillance data in domestic animals in the southern and eastern parts of the Mediterranean basin as well as in other developing countries raises questions about the adequacy of the disease information system implemented by the World Organisation for Animal Health (OIE). This system defines compartments (countries or smaller geographical zones) infected or free of disease for each notifiable disease. As an example, most countries that did not report bovine tuberculosis in 2011 are in fact lacking a bovine tuberculosis surveillance system, which illustrates the ambiguity of national sanitary statuses (OIE, 2012b). This lack of surveillance highlights the need to modify OIE international guidelines so that the world is not represented as closed compartments that contain a disease or not, but as an interconnected network in which different pathogens circulate regardless of national borders.

Socio-economic factors

Growing socio-economic inequalities

Health inequalities, although variable among countries, remain high within most Mediterranean countries and tend to grow due to ongoing changes (e.g. Patel and Burke, 2009; Boutayeb and Helmert, 2011). Urbanization increases the gap between rural and urban areas – as well as between conventional habitats and slums in cities – in terms of sanitary conditions and access to healthcare (Patel and Burke, 2009). Moreover, the economic crisis and the political instability currently experienced in the Mediterranean basin enhance these disparities through the increase in underprivileged populations, including homeless people and undocumented migrants, and the concomitant decrease of health actions aiming at helping them (Busch-Geertsema et al., 2010; Boutayeb and Helmert, 2011; Rechel et al., 2011).

In rural areas, access to health services remains generally more difficult than in urban areas, leading to lower use of medical care and under-reporting of diseases. In rural populations living in close contact with domestic and wild animals, which carry a large diversity of pathogens, this lack of surveillance may favour disease (re)emergence. As an example, the number of West Nile cases reported in humans and horses since 2010 has dramatically increased all over the Mediterranean basin (Papa et al., 2010; Sirbu et al., 2011; OIE, 2012c). Mosquitoes generally transmit this virus from bird to bird, but they occasionally infect mammals, sometimes causing serious clinical signs (Dauphin et al., 2010). The lack of surveillance in both animals and humans in the rural areas where the virus re-emerged did not allow an accurate map to be made of West Nile

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virus circulation foci. This would have allowed the prediction of future outbreaks and an assessment of the risk of disease transmission through blood transfusion, although recent studies showed that blood donors living in risk areas should be systematically tested for West Nile virus infection (Pezzotti et al., 2011; Papa et al., 2012). In addition, the screening of sentinel wild bird species proved to be effective for identifying circulation areas (Jourdain et al., 2008).

The importance of effective surveillance in rural areas was also alarmingly illustrated by local outbreaks of bubonic plague in Algerian villages in 2003 and 2008 (Bertherat et al., 2007; Bitam et al., 2010). These outbreaks presumably originated from endemic rodent foci that were formerly unknown because of a lack of surveillance. The concomitant detection of a rodent plague focus in Libya (Tarantola et al., 2009) raises the fear of plague re-emergence from rural foci to neighbouring cities in the current context of socio-political revolutions and population displacements.

In urban areas, undocumented migrants, homeless people and slum inhabitants, who live in poor sanitary conditions with low access to medical services, have a higher risk of contracting zoonotic, water-borne and vector-borne infections than better-off urban populations (Brouqui, 2011). Indeed, this growing population is often in contact with a wide range of vectors favoured by poor hygiene (e.g. body lice and bed bugs) or the presence of rats or other anthropophilic urban species (e.g. fleas). These vectors can transmit various diseases, some of them lethal. As an illustration, epidemic typhus cases have been increasingly reported from the homeless population of Marseille, France (Brouqui et al., 1996; Badiaga et al., 2011).

The objectives set by the UN through the Millennium Development Goals have led the FAO and WHO to implement action plans that brought considerable improvement in healthcare access in both rural areas and slums in the Mediterranean region (UNDP, 2010). In addition, since the beginning of the H5N1 crisis, OIE and FAO have coordinated international efforts to strengthen surveillance of avian influenza infections in both domestic and wild animals (Dauphin et al., 2010). Unfortunately, the current economical and socio-political crises in the Mediterranean basin have tended to decrease healthcare funding and disrupt the operation of institutions while an increasing number of vulnerable people are at risk of zoonotic infection. Learning from past examples (Suhrcke et al., 2011) and current evidence, medical experts warn Mediterranean governments of the growing epidemiological threat that impacts of the current crises and changes represent for their population because disease emergence could be favoured. Improving healthcare programmes for rural populations and underprivileged urban inhabitants appears crucial to prevent future disease emergence. For zoonotic diseases, efficient surveillance of wild and/or domestic animal reservoirs is also essential.

Ongoing and future conflicts

The Mediterranean basin is characterized by its extensive cultural diversity as well as by major economic contrasts between coexisting developed and developing countries. It has historically been an area of exchanges and conflicts (Bethemont, 2002). Military and commercial contacts between Mediterranean populations have allowed them to swap their germ pools for several millennia. Greek and Roman wars notably favoured the dispersal of bubonic plague throughout the Mediterranean basin, from the Athenian plague that started in 430 BC to the Justinian plague that raged from 541 to 542 AD (Soupios, 2004; Raoult and Drancourt, 2008). As another more recent example, Spanish soldiers imported typhus from America to Europe during the Spanish succession war in the early 18th century (Nguyen-Hieu et al., 2010).

Today, in addition to the long-lasting Israeli–Palestinian tensions, Arab revolutions have faced violent repression, inducing conflicts in the southern and eastern parts of the Mediterranean basin since December 2010. During the same period, Southern European countries have experienced social struggles associated with the implementation of austerity plans by several governments in response to the economic crisis. Alarmingly, these conflicts may worsen in the near future due to climate change, as it may lead to water shortage. Indeed, a recent study showed that climatic events may have had a role in 21% of the civil conflicts in the five last decades (Hsiang et al., 2011).

Conflict areas are particularly good breeding grounds for emerging diseases. They disrupt health institutions, making monitoring programmes ineffective and access to medical care difficult, while people's immunity is critically decreased by stressful conditions (Gayer et al., 2007). For example, the incidence of typhoid fever in Palestinian refugees increased from 4.3 per 100 000 inhabitants in 2006 to 13.0 in 2007, whereas no outbreak has been recorded in Israeli people since 1985 (UNRWA, 2007). The displacement of people induced by conflicts leads refugees to abandon combat areas and gather in areas with poor sanitary conditions. Zoonotic disease outbreaks may be favoured when people return home because of rodent proliferation in places where water and food supplies have been left unprotected. In 2000, for example, Albanian refugees returning home after the Kosovo war faced an outbreak of tularaemia characterized by the sudden emergence of 327 cases although the disease had formerly rarely been reported in the Balkans (Reintjes et al., 2002).

International organizations, such has WHO and different non-governmental organizations (NGOs), have a key role

to play in coordinating healthcare and disease surveillance during conflicts when national institutions are temporarily unable to assume these tasks. As an illustration, WHO warned of disease outbreak risks in Libya in 2011 and coordinated the implementation of medical care measures in collaboration with NGOs and local professionals (WHO, 2011). However, in a context of conflict, the efficient implementation of relevant heath measures may be severely impaired by access limitations, as is currently the case in Syria (ICRC, 2012).

Knowing that active terrorist groups, either religious or separatists, are numerous in the Mediterranean basin (Europol, 2012), another aspect of the risk of infectious disease emergence is linked to the potential use of pathogens as biological weapons. No bioterrorism event has recently been reported, although this has happened in Mediterranean history (Berche, 2009). For example, the bubonic plague outbreak of 1346, which killed a third of the European population, originated from the Genoese fleeing from Sicily after the siege of Kaffa by the Tartars. The besieged had been intentionally contaminated by their attackers, who threw corpses of plague victims into the city (Derbes, 1966). Biological weapons may not be preferred by terrorists because they are difficult to control (Berche, 2009). Nevertheless, the inexpensive nature of biological weapons along with their significant capacity to create panic among affected populations, even from a small number of cases, suggests that the risk of their spread is not negligible. The pathogenic agents most likely to be used are those that are most easily spread, involve severe symptoms and for which treatments are long or nonexistent; these include the agents of smallpox, anthrax, plague, botulism, tularaemia and viral haemorrhagic fevers (e.g. Ebola virus, Lassa virus; Rotz et al., 2002). An essential component on the preparation for bioterrorism is the implementation of surveillance systems that can rapidly detect and monitor the course of an outbreak and efficiently react, with the aim of minimizing the associated morbidity and mortality. However, few surveillance systems have been specifically designed for collecting and analysing data for the early detection of a bioterrorist event (Bravata et al., 2004). A recent debate was raised by research studies aimed at understanding how mutations could increase the capacity of direct mammal-to-mammal transmission of the deadly H5N1 HPAIV (Fidler, 2012). This debate emphasized that neither the international convention on biological weapons (Table 1; UNODA, 2012) nor WHO have the legitimacy to regulate research projects performed for peaceful purposes (Fidler, 2012). Key ethical questions are raised by such research projects because they generate both the knowledge needed to implement efficient health policies and a risk of emergence and spread in the population of dangerous pathogens (Brumfiel, 2012). This recent debate urges the creation of new international guidelines that could be inspired by the existing international framework for handling the smallpox virus (WHO, 2010).

Movements of goods and people

Each component of the current changes experienced in the Mediterranean basin described above generates new flows of good and/or people. Water scarcity, which is predicted to worsen in the near future, forces most Mediterranean countries to import 'virtual water', that is, to increase their importations of various goods whose production requires large amounts of water (MWO, 2012). Industrial rearing practices resulting from the growing demand for animal protein lead to increasing movements of live animals and livestock products (Steinfeld, 2004). Economic inequalities encourage rural inhabitants to move to urban areas and people from the poorest countries to emigrate to wealthier countries, which partly explains why overall human movements linked to permanent immigration have never been so high (OECD, 2011). Conflicts inside and outside the Mediterranean basin increase the numbers of refugees fleeing combat zones and entering safer countries (UNHCR, 2012). In addition, growing tourism is associated with human mobility within the region. In 2007, 30% of worldwide international tourist arrivals (about 275 million people) occurred in Mediterranean countries (Plan Bleu, 2009). This number was expected to rise to 235–300 million people by 2012 (CEPF, 2010). This exponential increase in the movement of people and goods may have major consequences on pathogen dissemination, as illustrated by the extremely rapid and worldwide spread of SARS in 2002–2003 and of the new H1N1 variant in 2009–2010 (Colizza et al., 2007; Khan et al., 2009).

The effect of current changes associated with these new possibilities of spread can favour the emergence of new infectious diseases in the Mediterranean region. As an example, Rift Valley fever, a severe mosquito-borne disease affecting ruminants and occasionally humans, was originally present only in Sub-Saharan Africa. In 1977, it spread in Egypt from Sudan, probably through camel trade (Abd El-Rahim et al., 1999). Rift Valley fever outbreaks have been subsequently regularly observed in Egypt causing human and animal losses (Ahmed Kamal, 2011). The intensification of animal trade and the multiplication of extreme rainfall events linked to climate change will probably increase the risk of Rift Valley fever spread in the Mediterranean basin urging for reinforced surveillance of this disease in the region (Chevalier et al., 2010). In the same way, dengue and chikungunya might spread to regions where one of their mosquito vectors (*Aedes albopictus*) has become established after its introduction by the trade of used tires (Schaffner and Karch, 2000). Although

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A. albopictus has been present in Italy and France since the 1990s (Schaffner and Karch, 2000; Romi et al., 2008), only a single local chikungunya outbreak has been reported in Italy and two autochthonous human cases have been detected in France (Rezza et al., 2007; INVS, 2012). Surveillance in both countries was based on vector surveys and syndromic surveillance of humans leading to reports of suspected cases (Gobbi et al., 2012; INVS, 2012). Encouragingly, this example shows that appropriate surveillance and management measures can efficiently deal with emerging health risks.

Increasing migrant flows across the Mediterranean basin might promote the emergence of diseases in countries where their spread has been limited by effective control measures. Multidrug-resistant tuberculosis is notably of major concern in this context (WHO, 2012c). Countries with a low incidence of human tuberculosis, such as France and Spain, report that the incidence of latent *Mycobacterium tuberculosis* drug-resistant infections is higher in foreign-born residents than in the native population (e.g. Robert et al., 2003; Monge-Maillo et al., 2009). The detection of latent cases is crucial both to treat infected patients and to avoid extensive transmission of such serious diseases. The implementation of syndromic surveillance in migrants proved efficient in some European countries (e.g. Riccardo et al., 2011). However, this surveillance system does not allow the detection of asymptomatic cases, which have to be specifically searched for in migrants arriving from areas with high incidence levels. Such specific control programmes can rapidly decrease the health impact and transmission risk of resistant tuberculosis, as illustrated by the programme implemented in Israel over the last decade (Chemtob et al., 2003). This programme followed WHO guidelines on multidrug-resistant tuberculosis control therefore highlighting the major role of WHO for successfully implementing such programmes.

WHO guidelines provide recommendations for homogeneous implementation of efficient control measures among countries that respect the human rights. Indeed, ethical issues must be considered. For example, the circulation rights of migrants can be denied due to serious infections. In the same way, importation bans linked to sanitary crises can have severe economic outcomes. This crucial point is extensively addressed in the International Health Regulation guidelines (WHO, 2005). Yet no enforceable actions exist to force WHO member states to apply WHO guidelines. This acute issue was recently discussed by the IHR Review Committee following the 2011 *E. coli* European outbreak that harmed the Spanish economy, notably due to a Russian import ban that violated WHO recommendations (Fidler, 2011). Moreover, IHR only deals with the control of international flows but not national disease control programmes. Similarly, the OIE terrestrial code focusses on the flows of animals and their products between countries but not the control of diseases within each member state, even if this issue has been addressed in the control of HPAIV. Each government has to implement its own disease control measures, yet international organizations might play a key role to guide and help set up these programmes.

Conclusion

Intensive current and future changes confronting the Mediterranean basin will have major impacts on the risk of infectious disease emergence. Managing these risks should be a priority for all Mediterranean governments because they may affect both the health of their inhabitants and the country's economy. The ageing of the population is likely to make this challenge even more complex. Indeed, the proportion of older people (>65 years) in southern Mediterranean countries, which is today approximately 5%, will increase over the next decades and reach the level already attained in northern Mediterranean countries, which is over 15% (Christensen et al., 2009; CIA, 2012). Disease risk management has become an important component of good governance that can be a crucial condition to implement fruitful international relations (Fidler, 2004).

Mediterranean countries are intimately related to each other because of their common history, cultural and commercial links, and numerous human flows. These links should favour an increased collaboration in managing the risks of disease emergence. International organizations, notably WHO and OIE, play a key role in guiding such collaboration. Action plans targeting emerging diseases of international concern, such as H5N1 avian influenza or HIV, have led both WHO and OIE to move from actions focussing on human and animals flows, which traditionally benefit developed countries, to actions guiding local control programmes and integrated surveillance network, which benefit both developed and developing countries. Furthermore, as proposed by the One Health Initiative (http://www.onehealthinitiative.com/), collaborations between human, animal and environmental health professionals should be reinforced. The integration of these modifications to the WHO and OIE guidelines are necessary to adapt to an interconnected world, where the identification of separate epidemiological compartments is becoming harder and harder.

Acknowledgements

We sincerely acknowledge Stephen Larcombe for his valuable comments during the preparation of this manuscript. M. Vittecoq is supported by an AXA research fund Ph.D. fellowship. This work was funded by the Agence inter-éta-

blissements de recherche pour le développement, the MAVA foundation and the Région Provence-Alpes-Côte d'Azur.

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Chapitre 1

Surveillance de la circulation du virus West Nile

et des flavivirus apparentés

La mort d'Alexandre. Karl von Piloty, 1886. Plutarque rapportant la mort de corbeaux peu avant le décès d'Alexandre le Grand il a été suggéré que la fièvre précédant sa mort aurait pu être causée par le virus West Nile (Marr & Calisher 2003). Cette hypothèse fait bien entendu débat (Cunha 2004; Oldach, Benitez & Mackowiak 2004).

I. La surveillance de la faune sauvage

1) Un peu d'Histoire

La surveillance épidémiologique de la faune sauvage est une discipline très récente si on la compare à la surveillance des maladies chez l'Homme et les animaux domestiques dont les origines remontent à plusieurs millénaires. En Europe les premiers programmes de surveillance ciblant la faune sauvage ont été mis en place vers 1930 au Danemark et une dizaine d'années plus tard en Suède (Christiansen 1935; Borg 1975). Ces études ont notamment permis de mettre en évidence des cas d'empoisonnement au mercure grâce à l'examen de cadavres collectés en Suède (Borg *et al.* 1966). La première coordination européenne entre plusieurs programmes de surveillance nationaux a fait suite à l'expansion de la rage vulpine sur le vieux continent à la fin des années 1960. L'échange d'informations épidémiologiques entre les pays concernés, coordonné par le centre de collaboration de l'organisme mondial pour la

santé (OMS), a permis de mener des actions de contrôle efficaces, principalement via la vaccination orale des renards (Aubert 2003; WHO 2012a). Depuis 1993 il existe à l'organisation mondiale de la santé animale (OIE) un groupe de travail spécialement chargé de traiter les données concernant la faune sauvage. La récolte de ces données empreinte le même circuit que celle des données concernant les animaux domestiques au sein des 172 états membres via la base de données centrale de l'OIE (WAHID ; http://web.oie.int/wahis/public.php), la surveillance chez les uns et les autres étant considérée d'une égale importance (OIE 2012a). En France depuis 1986 le réseau SAGIR coordonne l'essentiel de la collecte d'informations concernant la santé de la faune sauvage sur le territoire national grâce un réseau de vétérinaires, de laboratoires d'analyses, mais aussi de chasseurs (ONCFS 2011). Des réseaux semblables existent dans plusieurs pays européens contribuant à l'examen annuel d'environ 18000 et 50000 animaux respectivement dans le cadre de programmes de surveillance généraux ou ciblant une maladie (Kuiken *et al.* 2011).

2) Les enjeux de la surveillance

La surveillance des maladies de la faune sauvage revêt des enjeux multiples et complémentaires (Mörner *et al.* 2002) :

- Un enjeu de conservation. En effet les effectifs d'espèces rendues vulnérables par les divers impacts des changements globaux peuvent être diminués de façon critique par des épidémies mettant en péril la survie même de l'espèce. On peut citer l'exemple des populations de bouquetins ibériques (*Capra pyrenaïca*) décimées par la gale sarcoptique (*Sarcoptes scabei* ; Pérez *et al.* 2002).

-Un enjeu cynégétique, car toute pathologie peut influencer la qualité et la quantité de gibier. Ainsi les effets conjugués de l'introduction de la myxomatose et de l'arrivée récente du virus de la maladie hémorragique ont conduit à une diminution très forte des populations de lapin européen (*Oryctolagus cuniculus* ; Delibes-Mateos, Ferreras & Villafuerte 2009).

-Un enjeu économique, dès que la maladie est transmissible aux animaux domestiques. La problématique actuelle de la tuberculose bovine en Europe illustre cet enjeu. Le sanglier a en effet été identifié comme un hôte favorisant l'expansion des foyers épizootiques en France et en Italie, et comme un réservoir en Espagne, où les

pratiques cynégétiques augmentent conjointement les densités d'individus et la prévalence de tuberculose dans les populations (Serraino *et al.* 1999; Humblet, Boschiroli & Saegerman 2009; Gortazar *et al.* 2011; Castillo *et al.* 2011).

-Un enjeu de santé publique dès que la maladie est transmissible à l'Homme. Citons l'exemple de l'echinococcose alvéolaire (causée par *Echinococcus multilocularis)* dont le renard est un hôte et peut contaminer par ses déjections des baies ou l'eau ingérée par les humains. Plus de 800 cas humains sont recensés chaque année en Europe causant des troubles hépatiques graves (ECDC 2012).

-Un enjeu scientifique puisque la connaissance de la répartition géographique des pathogènes circulant dans la faune sauvage est la première étape nécessaire à la compréhension de leur épidémiologie (Mörner *et al.* 2002).

3) Programmes de surveillance : des compromis entre coût et efficacité

Tout programme de surveillance de la faune sauvage est limité par son coût et doit être optimisé au vu d'un budget donné. Ainsi le programme de contrôle de la rage en France comprenait jusqu'en 1989 à la fois des campagnes de vaccination orale des renards et une incitation financière à les tuer. Cette seconde mesure ayant été jugée inefficace, le budget y étant alloué à été en partie réinvesti dès 1990 pour étendre le rayon d'action des campagnes de vaccination, ce qui a contribué à augmenter leur efficacité (Aubert 2003). L'optimisation de la surveillance et la mise en place des programmes efficaces, qui peuvent être financés sur le long terme, nécessitent d'évaluer le besoin de surveillance pour un pathogène donné, notamment en fonction de la probabilité et des conséquences de sa présence, et de tester différentes méthodes de suivi pour déterminer laquelle doit être privilégiée.

Dans ce contexte les deux études présentées dans ce chapitre avaient un triple objectif : i) Déterminer si le virus West Nile circule dans les zones étudiées sans que des cas humains ou équins ne soient forcément détectés ii) Déterminer si d'autres flavivirus apparentés circulent dans ces zones. Ces deux points permettant d'évaluer la pertinence de la mise en place d'un suivi régulier sur le long terme de la présence de ces virus. iii) Evaluer l'efficacité des deux méthodes utilisées (l'une étant éprouvée l'autre innovante) dans la surveillance des virus ciblés.

II. Surveillance du virus West Nile et des flavivirus apparentés en Méditerranée

1) Histoire du virus West Nile

Le virus West Nile est détecté pour la première fois en 1937 en Ouganda dans le sang d'une femme venant du West Nile district qui lui donnera son nom (Smithburn *et al.* 1940). Les nombreuses études menées à la suite de cette découverte prouvent que le virus est largement répandu dans l'Ancien Monde, des hauts plateaux de l'Afrique du Sud (McIntosh *et al.* 1967), à l'Inde (Smithburn, Kerr & Gatne 1954) en passant par Madagascar (Fontenille *et al.* 1989). Le virus est initialement considéré comme peu pathogène car les infections humaines semblent rarement associées à un syndrome clinique (Hamilton & Taylor 1954). Cependant, dès 1994, la multiplication d'épidémies importantes d'encéphalites causées chez l'Homme par le virus West Nile dans le bassin Méditerranéen et jusqu'en Russie remettent en cause son statut d'agent peu pathogène (Murgue *et al.* 2001; Dauphin *et al.* 2004). En parallèle, de nombreuses épizooties sont observées chez les chevaux dans l'ensemble de la région méditerranéenne (Tber 1996; Murgue *et al.* 2001; Autorino *et al.* 2002; Steinman *et al.* 2002). De plus, en août 1999, des cas humains d'encéphalites virales sont rapportés à New York (Garmendia, Van Kruiningen & French 2001). Ces cas sont accompagnés d'encéphalites équines et de la mort de plusieurs centaines d'oiseaux sauvages (Garmendia et al. 2001). La souche du virus West Nile impliquée est très similaire à la souche israélienne isolée en 1998 et a très certainement été introduite aux États-Unis à partir du Proche-Orient (Lanciotti et al. 1999). Les modalités de cette introduction demeurent inconnues mais l'hypothèse d'une origine anthropique, par exemple par importation d'oiseaux exotiques infectés, semble la plus plausible (Rappole & Hubalek 2003). Le virus s'est par la suite rapidement répandu dans le nouveau monde jusqu'à en occuper une grande partie aujourd'hui (Petersen & Hayes 2008). L'intérêt suscité par le virus a été considérablement augmenté par les conséquences dramatiques de cette extension, ainsi, 812 cas humains ont été répertoriés en 2011 en Amérique du Nord et 340 au sein de l'Union européenne. A ces cas s'ajoutent un réel problème de conservation lié à la forte mortalité constatée chez certaines espèces d'oiseaux américaines telles que le tétras des armoises (*Centrocercus urophasianus;* Walker et al. 2007) et au bouleversement des communautés aviaires lié à cette mortalité (LaDeau, Kilpatrick & Marra 2007).

2) Cycle du virus West Nile

Le cycle du virus West Nile implique des oiseaux en tant que réservoir naturel et des moustiques (principalement du genre *Culex*) en tant que vecteurs (Dauphin *et al.* 2004). L'homme et les chevaux ne peuvent transmettre le virus et représentent donc des hôtes accidentels cul-de-sac pour le virus West Nile. Cependant les infections à virus West Nile provoquent parfois chez l'Homme et le cheval un syndrome fébrile pouvant se compliquer de troubles neurologiques graves et entrainer la mort (Dauphin *et al.* 2004). Chez les oiseaux les épisodes de mortalité importante sont rarement observés dans l'Ancien Monde tandis qu'ils sont fréquents en Amérique (Zeller & Schuffenecker 2004).

3) Circulation récente du virus West Nile et des flavivirus apparentés en Méditerranée

En 2010 et 2011 les cas équins et humains se sont multipliés dans l'ensemble du bassin Méditerranéen y compris dans les pays frontaliers de la France que sont l'Espagne et l'Italie (OIE 2012c). Toutefois aucun cas équin ou humain n'a été détecté en France depuis 2006 (OIE 2012c).

Par ailleurs, le virus Usutu, phylogénétiquement proche du virus West Nile a émergé en Autriche en 2001 (Weissenböck *et al.* 2002). Il a ensuite été détecté en Hongrie (Bakonyi *et al.* 2007), en Espagne (Busquets *et al.* 2008), en Italie (Lelli *et al.* 2008; Manarolla *et al.* 2010), en Suisse (Steinmetz *et al.* 2010) au Royaume-Uni (Buckley, Dawson & Gould 2006) et enfin en Allemagne (Becker *et al.* 2012). Le cycle du virus Usutu implique, comme celui du virus West Nile, des moustiques principalement du genre *Culex* en tant que vecteurs et des oiseaux en tant qu'hôtes naturels (Weissenbock *et al.* 2010). L'introduction du virus dans plusieurs pays européens a causé des épisodes de mortalités importants chez des oiseaux captifs et sauvages notamment des merles noirs (*Turdus merula*; Becker *et al.* 2012). Toutefois ces épisodes semblent se raréfier en Autriche malgré la circulation continue du virus sans doute grâce à une immunisation de groupe qui se met peu à peu en place contre ce virus dont les souches circulantes semblent très peu varier d'une année à l'autre (Chvala *et al.* 2007). Peu de données existent quant à la pathogénicité du virus Usutu pour l'Homme et les chevaux. Toutefois l'existence de syndromes neuro-invasifs chez l'homme liés à

l'infection par le virus Usutu a été rapporté en Italie (Cavrini *et al.* 2009; Pecorari *et al.* 2009).

De plus de récents travaux ont mis en évidence la circulation d'autres flavivirus en Espagne tels que le virus Bagaza chez des perdrix rouges (*Alectoris rufa*) et des faisans de Colchide (*Phasianus colchicus*; Agüero et al. 2011) ou différents flavivirus, jusqu'alors non décrits, chez de multiples espèces de moustiques (Sánchez-Seco et al. 2010).

4) Etude de la circulation des virus West Nile et Usutu en Camargue

Dans ce contexte nous souhaitions dans un premier temps déterminer si le virus West Nile circulait dans l'avifaune sauvage en Camargue en l'absence de détection de cas humain et équin depuis 2004 dans la région. Nous souhaitions également savoir si le virus Usutu, détecté dans plusieurs pays voisins, était présent en France bien qu'aucun épisode exceptionnel de mortalité aviaire qui puisse être un indice de sa présence n'ait été signalé. Enfin, un autre objectif était de confirmer l'efficacité d'une méthode précédemment utilisée en Camargue : le suivi sérologique de la pie bavarde en tant que sentinelle (Jourdain *et al.* 2008). Dans ce but nous avons mis en place un suivi sérologique des pies bavardes en Camargue via trois campagnes de piégeage réalisées entre novembre 2009 et décembre 2010. Cette étude est décrite dans l'**article 2**. Ces travaux ont permis de mettre en évidence la circulation récente du virus West Nile en Camargue et ainsi de confirmer l'efficacité de la méthode utilisée pour la surveillance active de ce virus. Par ailleurs des anticorps spécifiques au virus Usutu ont été détectés chez plusieurs individus dont l'un était juvénile. Toutefois l'existence de réactions croisées entre les anticorps spécifiques au virus Usutu et au virus West Nile lorsque les titres observés sont faibles ne permet pas de conclure avec certitude à la présence récente du virus Usutu au sein de l'avifaune camarguaise. Néanmoins, nous pouvons conclure que le virus West Nile, ainsi que potentiellement un autre flavivirus qui lui est étroitement apparenté, ont circulé récemment en Camargue sans détection chez les animaux domestiques ou les populations humaines. Ces résultats incitent à poursuivre l'étude des flavivirus circulants dans l'avifaune camarguaise en pérénisant le suivi décrit ainsi qu'en l'étendant à l'ensemble du littoral méditerranéen français. Par ailleurs, la mise en place de tels suivis à l'échelle du bassin méditerranéen permettrait d'avoir une

vision plus nette de la circulation des flavivirus dans la région et ainsi de mieux prévenir les épizooties et les épidémies potentielles qui peuvent y être liées.

5) Etude de la circulation de flavivirus chez le goéland leucophée à l'ouest de la Méditerranée

Malheureusement le coût de la mise en place et du maintien d'un tel réseau de surveillance à large échelle apparaît important. C'est pourquoi, dans le cadre de la thèse d'Audrey Arnal, l'efficacité pour la surveillance de la circulation du virus West Nile et des flavivirus apparentés d'une méthode innovante, le suivi des anticorps dans les œufs, a été testée au cours d'une seconde étude. Il a été montré que les taux d'anticorps présents dans les œufs de différentes espèces d'oiseaux, dont les goélands leucophées, sont corrélés à ceux présents chez la mère lors de la ponte (Gasparini *et al.* 2002; Hammouda *et al.* 2011). Le suivi des anticorps dans les œufs d'oiseaux coloniaux, pouvant être récoltés rapidement et de façon massive, permet donc de disposer facilement d'une illustration de l'exposition de la population concernée à l'agent pathogène recherché (Pearce-Duvet *et al.* 2009). Le goéland leucophée a été choisi ici car il fait partie de la famille des Laridés au sein de laquelle le virus West Nile a déjà été détecté en milieu naturel tout comme des anticorps spécifiques au virus Usutu (Malkinson *et al.* 2002; Hubálek 2008). Des infections expérimentales ont par ailleurs prouvé la sensibilité du goéland à bec cerclé (*Larus delawarensis)* à l'infection par le virus West Nile (Komar *et al.* 2003). De plus, le goéland leucophée a été identifié comme potentiellement impliqué dans l'introduction et la propagation du virus West Nile en Méditerranée (Jourdain *et al.* 2007c). En outre, les individus de cette espèce sont fidèles à leur site de reproduction et seuls les jeunes dispersent sur de longues distances (Sadoul & Pin 2009). Cette caractéristique permet d'inférer approximativement le lieu de circulation du pathogène recherché à partir de la situation de la colonie dans laquelle les anticorps qui lui sont spécifiques sont détectés. Enfin, il s'agit d'une espèce aux effectifs très importants dans l'ensemble du bassin Méditerranéen, ce qui permet de prélever des œufs sans risquer de porter atteinte aux populations étudiées (Birdlife International 2012c). L'étude réalisée entre 2009 et 2011 sur 1098 œufs récoltés dans des colonies françaises, espagnoles et algériennes est présentée dans l'**article 3**. Aucun anticorps spécifique au virus West Nile n'a été détecté au cours de cette étude. En revanche des anticorps spécifiques au virus Meaban ont été observés au sein d'une

colonie au nord-est de l'Espagne. La séroprévalence observée dans les œufs de cette colonie augmentait de 2009 à 2011. De plus, des tiques prélevées dans la colonie étaient également porteuses du virus dont elles semblent représenter un vecteur. Ce flavivirus méconnu avait précédemment été décrit chez des tiques prélevées dans des colonies de goélands argentés (*Larus argentatus*) en Bretagne et des anticorps spécifiques à ce virus avaient été détectés chez les oiseaux de ces colonies (Chastel *et al.* 1985). La capacité de ce virus à infecter l'Homme et son éventuelle pathogénicité sont pour lors inconnues. Ces résultats suggèrent que l'espèce choisie n'est pas idéale pour suivre la circulation du virus West Nile puisqu'aucun anticorps spécifique n'a été détecté y compris dans les zones où des cas équins ou humains ont été signalés pendant l'étude (OIE 2012c). Cependant, la mise en évidence de la circulation du virus Meaban incite à étudier plus avant son écologie et sa distribution. La méthode utilisée pourrait être mise à profit pour remplir ces objectifs en l'étendant à d'autres colonies Méditerranéennes.

Article 2. Recent circulation of West Nile and other flaviviruses in Southern France

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Accepté dans *Vector-Borne and Zoonotic Diseases.*

Running title

Circulation of flaviviruses in Southern France

Keywords

West Nile virus, Flavivirus, Serology, Birds

Title

Recent circulation of West Nile and other flaviviruses in Southern France

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Abstract (272 words)

In recent years the number of West Nile cases reported in horses and humans dramatically increased throughout the Mediterranean basin. Besides, the emergence of Usutu virus in Austria in 2001 as well as its subsequent expansion to Hungary, Spain, Italy, Switzerland, UK and Germany raised further concern about the impact of flavivirus spread on human and animal health in Western Europe. Despite frequent West Nile and Usutu cases detection in neighbouring countries, no West Nile case has been detected in France since 2006 and Usutu virus has never been reported. Yet, recent investigations aiming at detecting the circulation of flaviviruses in France were missing.

We investigated the circulation of West Nile and Usutu viruses in wild birds in southern France through a serological survey conducted on a sentinel species, the magpie (*Pica pica*), in the Camargue area. We detected West Nile virus neutralizing antibodies at a high titre (160) in a two-year-old bird showing recent exposure to WNV although no West Nile case has been detected in human or in horse since 2004 in the Camargue. Additionally, we observed low titres (10 or 20) of Usutu virus specific antibodies in 5 magpies among which 2 were also seropositive for West Nile virus. Because cross-reactions at low titres may occur between closely antigenically related flaviviruses, such low titres do not allow concluding that these birds had been exposed to Usutu virus. Yet, these results urge for further investigations about flavivirus circulation in Southern France and emphasize the need of implementing epidemiological studies on the long term, rather than during short periods following sanitary crises, to gain insight into viral dynamics within natural reservoirs.

Text (1000 words)

Introduction

In recent years veterinary and medical scientists recognized numerous (re)emergences of flaviviral zoonoses worldwide (Weissenböck et al. 2010). In Western Europe there were numerous reports on the multiplication of West Nile cases in horses and humans (ECDC 2012) and the emergence of Usutu virus (USUV) in Austria in 2001 as well as its subsequent expansion to Hungary, Spain, Italy, Switzerland, UK and Germany (Becker et al. 2012). USUV and West Nile virus (WNV) are maintained through enzootic cycles implying wild birds as reservoir hosts and ornithophilic mosquitoes as vectors. WNV principally infects wild birds but spillover infections occasionally occur in humans and horses, sometimes leading to severe neurological problems (Weissenböck et al. 2010). Besides, a large diversity of flaviviruses has recently been identified in Spain in birds and mosquitoes (Vasquez et al. 2012).

In Southern France, WNV infections were reported in horses, humans and birds in the 1960s and between 2000 and 2006 (Jourdain et al. 2007 and 2008; Balança et al. 2009). Since 2006, no WN case has been detected, although multiple outbreaks have occurred throughout Southern Europe (ECDC 2012). Despite frequent detection of WNV and USUV in neighbouring countries, the French flavivirus surveillance network has been limited since 2007 to reporting important mortality events in wild birds and clinical cases in horses and humans. Therefore, two major issues remain unresolved: a) Does WNV regularly circulate in Southern France in the absence of human and equine cases? b) Do other flaviviruses circulate in Southern France without being detected? We aimed at investigating these questions through a serological study conducted on wild birds in the Camargue area.

We chose to focus on magpies (*Pica pica*) because this species is a sensitive sentinel to detect WNV enzootic activity (Jourdain et al. 2008) and because USUV has already been isolated in this species (Savini et al. 2011). Additionally, the sedentarity of the magpie ensures that positive individuals have been infected in the study area or a few kilometers away from it. We chose the

Camargue as a study area because WNV circulation has repeatedly been reported there, the last local WN case detection dating from 2004 (Jourdain et al 2008; Balança et al. 2009). Moreover, this region is a hotspot for the potential introduction of bird pathogens due to its situation at the crossroads of several bird migration routes.

Materials and Methods

The study was conducted from November 2009 to December 2010 in two sites, i.e. A1, which includes dry and wet habitats, and A2, which is a wetland area. WNV had been reported in both sites in 2004 (Jourdain et al. 2007) in horses (A1) or wild birds (A2), and seropositive magpies had been detected in 2005 (A1 and A2) (Jourdain et al. 2008). Circular multi-catch magpie traps were set every working day during three capture sessions: November–December 2009, May-June 2010 and November–December 2010. Using plumage criteria magpies were classified as juveniles (i.e. born in the preceding spring), 2nd year (i.e. born in spring of the previous year) or adults (Svensson 1992). They were ringed and sampled for blood before being released. Blood samples were centrifuged and resulting serum samples were stored at -20C°. Neutralizing antibody (NAb) titres for WNV (IS-98-ST1 and France00 strains) and USUV (SAAR-1776 strain) were determined using a 96-well plate neutralization test as described (Figuerola et al. 2007). All strains were provided by the National Reference Centre for arboviruses of the Institut Pasteur of Paris. Serum samples with a neutralizing activity (absence of cytopathic effect) at dilution ≥1:10 were considered positive. To assess the specificity of the methods, we tested serum samples collected on magpies in the Camargue area during and after the 2004 WN epizootic in horses (Jourdain et al. 2008), for which we strongly expected seropositivity for WNV. These samples were tested for antibodies against WNV, USUV and Tick-borne

encephalitis virus (TBEV, Hypr strain), a flavivirus belonging to the tick-borne flavivirus group which is not closely related to WNV.

Results

Among the 63 magpies sampled, a 2nd year bird trapped in spring 2010 on site A1 was seropositive for WNV with NAb at titre 160 (Table 1). Additionally, three adult magpies, trapped in June 2010 and a 1st year bird trapped in December 2009, both in site A2, were seropositive for USUV with NAb at low titre (10 to 20; Table 1). Finally, two adult birds caught in site A2 presented NAb for both WNV and USUV. It remained unclear whether USUV seropositive birds had been exposed to USUV, WNV, both, or a closely related virus. Indeed, cross-reaction between WNV and USUV antibodies can be visualized at low titres in virus neutralization tests since WNV and USUV are closely antigenically related.

The tests implemented on samples collected in 2004-2005 aimed at investigating this cross-reactivity. Out of 51 sera, 12 were seropositive for WNV only and 24 were seropositive for both WNV and USUV (Supplementary material). In the latter, WNV NAb titres were superior or equal to USUV Nab titres, while in 5 of our 2009-2010 samples USUV NAb titres were higher than WNV Nab titres. No sample was positive for TBEV showing that, although cross-reactivity can occur between WNV and USUV, our seroneutralization tests were specific for flaviviruses antigenically close to WNV.

Discussion/Conclusions

This serosurvey shows that WNV and potentially other closely related flaviviruses have recently circulated in the Camargue area, although no human or equine cases have been reported

since 2004. These results urge for further investigations of flavivirus circulation in Southern France and highlight the fundamental importance of implementing epidemiological studies on the long term, rather than during short periods following sanitary crises, to gain insight into viral dynamics within natural reservoirs. They also confirm that the magpie is a sensitive indicator species for the detection of WNV circulation. We suggest that a long-term multi-focus serosurvey implemented on magpies throughout the Mediterranean basin would be an efficient tool to provide an overview of WNV dynamics in the region and would greatly help predicting and facing future outbreaks linked to flaviviruses.

Acknowledgments

M. Vittecoq was supported by an AXA Ph.D. research fellowship. We are grateful to trappers and students who helped us to collect samples. We also thank the MAVA foundation for its financial support and the Centre Scamandre for authorizing bird captures on its land.

Disclosure Statement

No competing financial interests exist.

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Table 1. Neutralizing antibody titres against West Nile virus (WNV) and Usutu virus (USUV) observed in serum samples from 7 out of 63 magpies tested in the Camargue, France.

Sampling date	Sampling site	Age	Neutralizing antibody titres		
			WNV (IS-98)*	WNV (Fr00)†	USUV
09/12/2009	A2	Juvenile	<10	<10	10
31/05/2010	A1	2nd year	160	160	<10
03/06/2010	A2	Adult	<10	<10	20
09/06/2010	A2	Adult	<10	<10	20
23/06/2010	A2	Adult	20	10	20
23/06/2010	A2	Adult	<10	<10	10-20
25/06/2010	A2	Adult	10	10	20

*IS-98, IS-98-ST1 WNV strain. †Fr00, France00 WNV strain

Supplementary material. Neutralizing antibody titres against West Nile virus (WNV), Usutu virus (USUV) and Tick-borne encephalitis virus (TBEV) in serum samples from magpies collected in 2004-2005, Camargue, France.

Sampling date	Neutralizing antibody titres		
	WNV (Fr00)†	USUV	TBEV
21/10/04	160	10	ND
28/07/05	160	20/40[a]	<20
02/09/04	80/160[a]	20	ND
29/09/05	80/160[a]	10	<20
12/08/05	80	20/40[a]	<20
22/09/05	80	10	<20
27/09/05	80	10	<20
29/09/05	80	20	<20
18/08/05	80 (40 with IS-98)	10	<20
11/08/05	40	10	<20
02/09/05	40	10/20[a]	<20
02/09/05	40	<10	<20
22/09/05	40	10/20[a]	<20
29/09/05	40	20	<20
29/09/05	40	40/80[a]	<20
09/08/05	20/40[a]	<10	<20
09/08/05	20/40[a]	<10	ND
15/07/05	20	20	<20
22/07/05	20	10	<20
28/07/05	20	<10	<20
04/08/05	20	<10	<20
11/08/05	20	10	<20
23/08/05	20	<10	<20
01/09/05	20	<10	<20
01/09/05	20	<10	<20
15/09/05	20	10	<20
22/09/05	20	<10	<20
29/09/05	20	20	<20
07/07/05	20	10	<20
20/09/05	20(40 with IS-98)	40	<20
28/07/05	10 (20 with IS-98)	20	<20
11/08/05	10 (20 with IS-98)	<10	<20
29/09/05	10 (20 with IS-98)	<10	<20
22/07/05	<10 (10 with IS-98)	<10	ND

†Fr00, France00 WNV strain; titres against IS-98-ST1 WNV strain (IS-98) are indicated when avalaible and different from titers against France 00 WNV strain. [a]Two values are indicated when the duplicates of a test gave a different result. For 15 samples, which are not included in the table, all titres were <10.

Article 3. Meaban flavivirus in Yellow-Legged Gulls (*Larus michahellis*) and Seabird Ticks (*Ornithodoros maritimus*) in the Mediterranean Basin.

Arnal A., Gomez Diaz E., Cerdà Cuéllar M., Lecollinet S., Busquets Martí N., Morejón de Girón F.X.A., Garcia Bocanegra I., Pages Martinez N., Pearce-Duvet J., **Vittecoq M.**, Hammouda A., Samraoui B., Garnier R., Selmi S., González-Solís J., Gauthier-Clerc M., Jourdain E. & Boulinier T.

Soumis à *Emerging Infectious Diseases*.

Title

Meaban flavivirus in Yellow-Legged Gulls (*Larus michahellis*) and Seabird Ticks (*Ornithodoros maritimus*) in the Mediterranean Basin

Running title (Max 50 characters)

Meaban virus in gulls and seabird ticks in Spain

Keywords

Arboviruses, Epidemiology, Spain, Charadriiformes, Maternally-Acquired Immunity

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One sentence summary

We provide evidence for Meaban virus presence in a yellow-legged gull colony localized on Medes Islands, Spain, whereas 18 other colonies sampled in the western Mediterranean basin do not appear to be significantly infected by flaviviruses.

Abstract - 148

In recent years, flaviviral zoonoses, whose reservoirs include wild birds, have emerged worldwide. We characterized flavivirus presence in the Mediterranean basin, using yellow-legged gull eggs obtained over 3 years from 19 breeding colonies; chick sera and soft ticks were also collected from the Medes Islands colony (Spain). At Medes and the nearby village of L'Escala, significant numbers of eggs and chicks had antibodies against the flavivirus E-protein. However, eggs were negative for West Nile, Usutu, and tick-borne encephalitis neutralizing antibodies. Other colonies had, at most, one egg containing antibodies against the flavivirus E-protein. Soft ticks screened for flaviviral RNA carried a virus that was 95% similar to a fragment of Meaban virus' NS5 gene. All antibody-positive eggs sampled from Medes Islands neutralized Meaban virus. This study is the first to detect Meaban virus in the Mediterranean basin and highlights the utility of maternal antibody detection in flavivirus surveillance.

Text - 3,467

Introduction

Over the last decades, climate change and human activities including international air traffic expansion, increase in world population, commercial transportation, urbanization and deforestation have led to the emergence of numerous human and animal arboviruses (*1*). Arboviruses are transmitted from one vertebrate host to another via hematophagous arthropods (*2*). They are classified into 5 families (*Bunyaviridae, Flaviviridae, Reoviridae, Rhabdoviridae,* and *Togaviridae*) according to antigenic relationships, morphology and replicative mechanisms (*3*). In humans, most arboviral infections may be asymptomatic. In particular, several flaviviruses are human pathogens that, while generally asymptomatic or resulting in mild illness, can also cause central nervous system diseases, coma, or death (*3*). The genus *Flavivirus* consists of about 70 antigenically related viruses that are geographically widespread (*3*). A full understanding of the role of the natural reservoirs of these viruses is critical to assess and predict the risk of disease transmission to humans and domestic animals. Wild birds represent one of the most specific animal reservoirs (*3*) for flaviviruses that present the greatest risk to public health, some of which are present in Europe (*4,5*).

West Nile virus (WNV) is one such re-emerging flavivirus that is most often transmitted by mosquitoes of the genus *Culex* (*6*). Wild birds are the natural reservoirs for WNV; humans, horses, and other mammals are considered dead-end hosts in whom infection may nonetheless lead to febrile illness or fatal neurological disorders (*6*). In Europe, WNV transmission to humans and horses has been sporadically reported since the 1960s (*6*). However, in 2010, multiple outbreaks occurred in North Africa, and some European countries, which triggered renewed concern about the potential economic and public health impact of this virus (*7*). Usutu virus (USUV) is a mosquito-borne flavivirus that is closely phylogenetically-related to WNV and whose natural reservoir is also wild birds (*8*). USUV has been reported in different European countries (*8,9*). Unlike for WNV, the medical relevance of USUV for humans is not fully understood. In 2009, USUV-related illnesses were reported in two immunocompromised patients in the Emilia-Romagna region of Italy (*10*).

Tick-transmitted flaviviruses are also known to circulate in Europe in wild bird populations. Tyuleniy virus was isolated in 1969 from *Ixodes uriae* collected in a seabird colony on the Tyuleniy Island, which is located in the Okhotsk Sea in of eastern Russia (*11*). Tyuleniy virus was later detected in the same tick species on the French Atlantic coast (*12,13*), the Lofoten Islands in Norway, and the western coast of USA (*11*). Based on serological data, it was been suggested that Tyuleniy viruses might be mildly pathogenic in

human (*13*). Tick-borne encephalitis virus (TBEV) is a highly pathogenic flavivirus known to circulate in Europe (*14*). TBEV can affect humans and cause fatal neurological infection (*14*). The principal vector of TBEV is the hard tick *Ixodes ricinus*, which is widely distributed in Europe (*14*). Finally, Meaban virus was isolated in 1985 from soft ticks *Ornithodoros maritimus* sampled from herring gulls (*Larus argentatus*) nests on the west coast of France (*4*). Antibodies against this virus were also found in colonies of this seabird host species (*12*). This flavivirus has never been reported in any other parts of the world (*11*). Its potential occurrence and pathogenicity in humans and other domestic and wildlife species are unknown.

The yellow-legged gull (*Larus michahellis*) is a relevant biological model when investigating the detection of flaviviruses in wild bird populations for several reasons. First, they also have colonized some urban areas and can exploit resources resulting from human activities (*15*), thus increasing the risk of pathogen transmission to human. They can breed in various habitats, which may be more or less shared with other seabirds. Because they are colonial and nest in large and dense colonies (*16*), the sharing of infectious agents between individuals may be facilitated. Although non-breeder birds may traverse large areas (*17*), breeding adults show relatively restricted movements and high breeding site fidelity, which means this long lived species is potentially useful in detecting the local circulation of infectious agents.

Secondly, this species belongs to the Laridae family, a taxonomic group that host several flaviviruses. For instance, white-eyed gulls (*Ichthyaetus leucophthalmus*) were found to be exposed to WNV in nature (*18*). Yellow-legged, Mediterranean (*Larus melanocephalus*) and black-headed (*Larus ridibundus*) gulls were identified as species potentially involved in the introduction and spread of WNV in the Mediterranean basin (*19*). USUV-specific antibodies have been detected in black-headed gulls (*5*) and Meaban virus has been isolated from soft ticks (*Ornithodoros maritimus*) sampled from herring gull nests (*4*).

Lastly, research on yellow-legged gulls is facilitated by local regulations. Population control efforts, including egg sterilization campaigns, are implemented because this species is perceived as a nuisance and threat to other species on which it can prey (*16*). The sampling of eggs for epidemiological purposes (*20,21*) is therefore straightforward.

Different methods may be used to assess whether a bird population has been exposed to a particular infectious agent. Serological methods can detect pathogen-specific antibodies synthesized by the host immune system following infection (*22*). In wild bird species for which adults are difficult to catch, detecting maternal antibodies deposited in eggs may prove a useful tool. Indeed, the amount of antibody detected in the egg yolk correlates positively

with the amount detected in the plasma of breeding females at the time of laying (*23*) and therefore reflects their prior exposure to infectious agents (*20,21*).

A drawback of these indirect methods is that they are only useful if the host immune response is detectable for a relatively long period of time (*22*). A study of the duration of flavivirus-specific antibodies in wild birds suggests that antibodies remain detectable for at least one year (*24*). Because cross-reactivity with antibodies directed against phylogenetically related pathogens may occur (*25*), direct detection of a virus or its genome are necessary to precisely identify the flaviviruses responsible for the serological reactions observed.

Objectives

This study aimed to characterize the exposure of wild birds to flaviviruses in the western Mediterranean basin. We screened egg yolks of yellow-legged gulls from 19 colonies in four countries for flavivirus-specific antibodies. In tandem, nestlings, soft ticks found on hosts and mosquitoes were sampled from a colony in which antibodies against flaviviruses were detected, so as to elucidate potential transmission pathways in this system.

Materials and methods

Sampling

From 2009 to 2011, early during the incubation period (March/April), one egg per clutch was collected from a sub-sample of nests within each of 19 breeding colonies located in France (n = 9), Spain (n = 5), Tunisia (n = 2), and Algeria (n = 3) (Figure). In total, 1098 eggs (8-50 eggs per colony) were sampled. In the laboratory, the egg yolk was separated from the albumen, homogenized and frozen at -20°C until analysis. Antibodies were extracted from egg yolks using chloroform as previously described (*26*), and the product was stored at -20°C.

In addition to the egg survey, additional sampling of chick blood, ticks and mosquitoes was performed in Medes Islands where significant flavivirus antibody prevalence had been detected. Yellow-legged gull chick plasma was collected over three consecutive years (2009 to 2011). For each chick, up to 2 ml of blood (for chicks more than 15 days old) was drawn from the tarsal or brachial vein using a sterile syringe. Each sample was immediately transferred into heparinized tubes and maintained in a cooler while in the field. In the lab, the blood was centrifuged at 2500 rpm for 15 min and the resulting plasma was stored at -80 °C until analysis. Because maternal antibody concentration decreases with age (*27*), each chick was weighed and its bill measured so that its approximate age could be estimated. In parallel, soft tick (*O. maritimus*) nymphs were sampled directly from yellow-legged gull chicks

following visual inspection, and maintained alive until they could be returned to the laboratory. Ticks were then placed in 36 groups (mean size of 5-6), with each group corresponding to one individual chick. In addition, 4 CDC light traps baited with CO_2 were set out over 24h in August 2011 in order to capture *Culicidae* mosquitoes. The captured mosquitoes were morphologically identified and pooled by species into 10 groups containing up to 13 mosquitoes. Both ticks and mosquitoes were kept frozen at -20°C until analysis.

Serological analyses

Egg extracts and chick plasma were screened for antibodies directed against the WNV envelope protein (E-protein), which contains epitopes shared with other viruses of the Japanese Encephalitis serocomplex (*28,29*). We used a commercially available ELISA kit (ID Screen® West Nile Competition, ID VET, Montpellier, France) in accordance with the manufacturer's instructions. Results were expressed as a percentage of competition (PC) calculated using the optical density (OD) of the sample and the mean OD of the negative control (NC) of the kit as follows: $PC=(OD_{sample}/OD_{NC}) \times 100$. According to kit instructions, samples with $PC \leq 40\%$ were considered positive, those with $PC > 50\%$ were considered negative, and those with PC between 40% and 50% were considered doubtful. For statistical analyses, doubtful samples were grouped together with negative samples.

A sample of randomly selected ELISA positive eggs (n = 74) and all the ELISA positive plasma (n = 13) were further screened for neutralizing antibodies against WNV IS-98-ST1 strain for egg samples (analyses performed in France) and Eg101 strain for plasma samples (analyses performed in Spain) using 96-well plate neutralization tests as previously described (*30*). The presence of neutralizing antibodies against USUV (SAAR-1776 strain) was also assessed by a similar 96-well plate neutralization test for 15 eggs negative for WNV neutralizing antibodies. A plaque reduction neutralization test ($PRNT_{90}$) was performed on the same 15 eggs to detect antibodies against TBEV, as well as on a sample of 71 ELISA-positive eggs to detect antibodies against Meaban virus.

Briefly, 6-well plates were seeded with $8x10^5$ Vero (TBEV) or SW13 cells (Meaban virus) per well 1 day before the neutralization assay. Serially diluted egg extracts (1/20 to 1/320) were incubated with TBEV (Hypr strain) or Meaban virus (Brest ART707 strain) as a suspension containing 400 plaque-forming unit per mL of Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) for 1h30 in a CO_2 incubator at 37°C. Then, the culture medium was removed from the wells and 0.5 mL of the virus-serum mixture was added to the wells and left for 1h30 at 37°C. One mL gelosa obtained by mixing equal volumes of

carboxymethylcellulose (VWR) (TBEV) or Agarose Seaplaque (Lonza) (Meaban) and DMEM with 2% fetal calf serum was added. After 5 days of incubation at 37°C, the cells were rinsed twice, fixed with paraformaldehyde 4%, and stained with crystal violet for easy plaque counting. The extract was considered positive if it prevented the formation of viral plaques, i.e. if the number of viral plaques was less than 10% the number counted in the control well (without plasma or egg extract).

Genetic analyses

The 36 tick pools and 10 mosquito pools were analyzed using a generic nested reverse-transcription polymerase chain reaction (RT-nested PCR) previously designed to detect flavivirus ribonucleic acid (RNA) genome (*31*). Total RNA was extracted using a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Reverse transcription of RNA to cDNA and subsequent amplification were carried out using the Access RT-PCR System (Promega, Madison, WI, USA). A fragment of the viral NS5 gene (143bp) was amplified using degenerate primers and conditions as previously described (*31*). PCR products were electrophorezed on 2% agarose gels and visualized with ethidium bromide. Amplification products were sent for direct sequencing (Macrogen, Inc.).

Sequence chromatograms were checked manually and assembled using Geneious v. 5.3.6 (Biomatters Ltd.). We then performed a similarity analysis against published sequences in GenBank (http://www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Search Tool (BLAST) to find the best match (E-value $\leq 10^{-8}$).

Statistical analyses

We used generalized linear models (GLM) to assess whether flavivirus antibody prevalence in the Medes colony differed among years. The most parsimonious model was selected by Akaike information criterion (AIC). The analyses were done using R software version 2.12.0 (R development Core Team, 2010).

Results

Antibodies against flaviviruses in eggs from western Mediterranean yellow-legged gull colonies

We found a high number of ELISA-positive eggs in the Medes Islands, Spain. The proportion of antibody-positive nests increased over time, with 37%, 49% and 67% respectively in 2009, 2010 and 2011 (Generalized linear model: slope=0.6, Std. error = 0.2, Z

value = 2.8, p = 0.004). In addition, in 2011, 6 nests were sampled, from gulls breeding on terraces and roofs of the L'Escala village (11 km from Medes); of these 6 eggs, 4 were also ELISA-positive. The observed prevalence of antibodies against flavivirus E-protein was low to null in the other study sites (Table 1). Only 3 eggs were ELISA-positive: 1 from the Jijel colony in Algeria in 2010 and the other 2 from the Corrège colony in France (1 in 2009 and 1 in 2010).

Only 1 of the 74 eggs tested by neutralization tests (Table 2) presented a low neutralization effect against WNV at titer 10. No egg presented neutralizing effects against USUV or TBEV. Conversely, neutralizing antibodies against Meaban virus were detected in the 71 ELISA positive-eggs tested (Table 2).

Antibodies against flaviviruses in the plasma of Medes Islands chicks

Antibodies against flaviviruses were found in 13 out of 256 plasma samples tested by ELISA (5.1%; binomial $CI_{95\%}$: 2.4-7.8). All of them were negative for WNV neutralizing antibodies.

Detection of a Meaban virus RNA in soft ticks sampled from Medes Islands

Flavivirus RNA was detected in one of the 36 tick pools tested. BLAST sequence analyses in GenBank indicated a 95% similarity to the NS5 gene fragment of a flavivirus first described as Meaban virus and isolated from *O. maritimus* ticks (*32*). No flaviviral RNA was detected in the mosquito pools.

Discussion

Flavivirus exposure in several yellow-legged gull colonies across the western Mediterranean basin

Our study is unique in that we used the same protocol to simultaneously survey flavivirus exposure in several breeding colonies of a common wild bird species across the western Mediterranean basin. Using ELISA, we found that there are strong differences in flavivirus exposure among yellow-legged gull colonies.

With the exception of one colony in Spain, the prevalence of antibodies against flavivirus E-protein was low to null. The presence of a few positive eggs in the colonies of Jijel (Algeria) and Corrège (France) might reflect past on-site flavivirus exposure or be explained by the rare dispersal of gulls among colonies (*6,7,19*).

Our ELISA screening procedure detects antibodies against the flavivirus E-protein, an antigen commonly used in WNV serodiagnosis (*29*). This E-protein mediates both receptor-binding and fusion activities after virus uptake by receptor-mediated endocytosis and, because

of these functions, is the major target for virus-neutralizing antibodies (*29*). However, a significant drawback of current immune assays utilizing the E-antigen is the high degree of cross-reactivity observed with other antigenically-related flaviviruses (*28*). These flavivirus cross-reactive responses can confound the interpretation of serological tests, and it is often impossible to truly determine the infecting virus without performing neutralization tests (*28*).

In this study, neutralization tests were conducted on egg samples to identify the flavivirus(es) against which exposure had been revealed by ELISA. Neutralization tests showed that the flavivirus-specific antibodies detected by ELISA did not neutralize USUV or TBEV, which suggests that these two flaviviruses are not present on the Medes Islands. One egg sample from Medes Islands was positive for WNV neutralizing antibodies at a very low titer, suggesting either past WNV exposure of the breeding female or cross-reaction with another flavivirus. Because WNV has recently been detected in the Mediterranean basin, notably in Spain (*7,30*), north Africa and southern France (*6*), higher flavivirus antibody prevalence might have been expected, particularly in the Ebro Delta in Spain (*33*) or in the Camargue in France (*19*). However, our results suggest that adult yellow-legged gulls from the western Mediterranean basin have not recently been exposed to WNV, USUV, or TBEV.

First detection of a Meaban virus in the Mediterranean basin: the Medes Islands

Out of the 19 breeding colonies sampled, the Medes Islands and the nearby village of L'Escala (Spain) were the only ones to show a high prevalence of flavivirus-specific antibodies as detected by ELISA. The increasing proportion of nests with antibody-positive eggs from 2009 through 2011 suggests a local flavivirus circulation. Indeed, if the pattern had been due to a past epidemic event, demographic effects (natural mortality and recruitment of naive individuals) would likely have led to a decrease of local antibody prevalence.

Antibodies against flavivirus were also detected in the plasma of yellow-legged gull chicks sampled from the Medes Islands. As most chicks were > 15 days old, the detected antibodies were unlikely to be of maternal origin if one assumes a similar decay rate for maternal antibodies among gull species (*27*). This result suggests that the chicks were likely exposed to flaviviruses after birth.

In order to identify the flavivirus(es) present in the Medes colony, potential arthropod vectors were collected and tested for flaviviral RNA. All mosquitoes tested were negative, suggesting that mosquitoes did not play a major role in flavivirus transmission to gulls at the time of sampling. Conversely, sequence analyses of RNA extracted from seabird ticks (*Ornithodoros maritimus*) on yellow-legged gull chicks were 95% similarity to a fragment of

the NS5 gene of a seabird tick-borne virus first described as Meaban virus (*4*). The *O. maritimus* tick has been reported to infest 90% of gull nests in the Medes Islands (*34*) and it is from this same seabird host species that Meaban virus was previously isolated in France (*4*). Given these results, Meaban virus neutralization tests were conducted on a subset of ELISA-positive egg extracts and all were able to neutralize Meaban virus (Table 2). This result confirms the flavivirus' identity and represents the first detection of a Meaban virus in the Mediterranean basin. Neutralization of Meaban virus was also detected for one sample from the Corrège colony (France) and two samples from L'Escala (Spain). This result might reflect dispersion of this virus by gulls respectively between colonies and in urban area.

O. maritimus ticks are known to be present on several of the yellow-legged gull colonies where antibodies against flaviviruses were not detected (e.g., Riou, Frioul, Porquerolles and Sidrière colonies, southern France; K.D. McCoy and R. Garnier, *pers. obs.*). High antibody prevalence may be explained by the fact that infected vectors and viremic adult gulls show restricted movements. Yellow-legged gulls are relatively faithful to their breeding sites (*35*), and adults on the Atlantic coast and Mediterranean basin do not move much during the winter (*36*), which may restrict Meaban virus dispersal. Furthermore, *O. maritimus* dispersal is likely restricted because of its biology and because prospecting movements of yellow-legged gulls among colonies are limited (*37*). Recent work, however, reports that *Ornithodoros* ticks may be able to disperse long distances via the movements of the seabird hosts (*38*).

The possibility that the virus may be slowly dispersed to novel locations should not be overlooked, notably because yellow-legged gulls are known to colonize urban areas (*15*). In this study, the antibody-positive eggs from L'Escala might reflect urban colonization by gulls originating from the Medes Islands, which is only 11 kilometers away. It is crucial to understand the implications of this dispersal to urban habitats because there is little knowledge about the zoonotic potential of Meaban viruses. Chastel *et al.* (*13*) did not detect any neutralizing antibody against Meaban virus in 562 sera collected from human beings living in Brittany. However, other tick-borne flaviviruses associated with wild birds are either demonstrated human pathogens (e.g., WNV, TBEV) (*5*) or suspected human pathogens (Saumarez reef and Tyuleniy viruses) (*13*). Data on the diversity, distribution, and pathogenicity of the virus for seabirds and its potential risk for humans are still lacking.

Conclusion and perspective

Our study reports the first detection of Meaban virus in the Mediterranean basin. This flavivirus was only detected in samples from the Medes Islands (Spain) despite a widespread sampling effort that included 19 breeding colonies in 4 countries. Our results suggest that Meaban virus, whose zoonotic potential is unknown, is locally present (*4*). The seabird tick *O. maritimus* has also been reported on other avian species such as European shags (*Phalacrocorax aristotelis*) (*39*) which breed on the coasts of the Iberian Peninsula. Whether this tick in the Medes Islands or in other colonies shows differential feeding preferences for certain host species is not known (*38*) but would be important to determine because tick-vector specialization may lead to seabird-specific virus transmission and disease risk (*40*). Further investigations targeted on other bird species nesting on the Medes Islands, a more comprehensive analysis on the ticks, vector competence, infection rates and population genetic structure, should help clarify the risks of Meaban virus spread within the Mediterranean basin. This study also highlights the utility of eggs, which contain maternal antibodies, in broad-scale surveillance procedures aimed at detecting potential and known pathogens.

Acknowledgments

We thank the many people who helped with various aspects of field work. J. Pearce-Duvet was supported by a Chateaubriand postdoctoral fellowship and a National Science Foundation postdoctoral fellowship. E.G.-D. was supported by a Juan de la Cierva contract from the Ministerio de Educación y Ciencia, Spain, and a Marie Curie reintegration grant from the European Commission (PERG08-GA-ERG-2010- 276838). Boudjèma Samraoui was supported by the Algerian Ministère de l'Enseignement Supérieur et de la Recherche Scientifique (DGRSDT/M.E.S.R.S.) and by the DSFP program, King Saud University, Saudi Arabia. We thank S. Moutailler (ANSES), P. Desprès and M. Grandadam (National Reference Center for Arboviruses, Institut Pasteur) for the virus strains for the neutralization tests, X. de Lamballerie for providing SW13 cells as well as information about Meaban virus cultivation and S. Lowenski for performing virus neutralization tests. ELISA analyses on eggs were conducted at the Plateforme d'Analyses Chimiques en Ecologie (CEFE). Support from OSU OREME, ANR (11 BSV7 003 EVEMATA grant), INIA-MICINN (FAU2008-00012-C02-01), AIRD and CNRS is acknowledged. Authorizations for working in the study sites were obtained from: Direction régionale de l'Environnement, de l'Aménagement et du Logement (DREAL); Syndicat Mixte des Etangs Littoraux (SIEL); Conservatoire Etudes des Ecosystèmes de Provence-Alpes du sud (CEEP); Parc national de Port-Cros; Service

Environnement de la Mairie de Gruissan (Aude); Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural de la Generalitat de Catalunya; Parc Natural de El Montgrí, les Illes Medes i el Baix Ter; Dirección General de Patrimonio Natural y Biodiversidad de Murcia; Conselleria de Medi Ambient i Mobilitat del Govern de les Illes Balears; Tomás Montalvo (Agència de Salut Pública de Barcelona); Parc Natural del Delta de l'Ebre; Cabildo de Gran Canaria; Conselleria de Medi Ambient, Aigua, Urbanisme i Habitatge de la Generalitat Valenciana; the forest service in the Tunisian Ministry of Agriculture; the Algerian DGRSTD/MESRS and DSFP, King Saud University (Saudi Arabia). We thank C. Chastel for his articles, S. Barry, F.X. Hallé, N. Rode; and F.X. Abad for their help on the manuscript and the many students of the Barcelona University and the staff of CReSA for help in the fieldwork. We would like to dedicate this article to the memory of Àlex Lorente, director of the Parc Natural de El Montgrí, les Illes Medes i el Baix Ter who passed away unexpectedly on 6 of August 2012 from an apnea accident.

Biographical sketch

Audrey Arnal studies the transmission dynamics of zoonotic parasites. Her research combines immunological analyses with field sampling to gain insight into the circulation of infectious agents such as West Nile or Avian Influenza viruses in wild populations of colonial seabirds.

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Table.1. Prevalence ` f antib` dies directed against the flavivirus envel` p pr` tein f` und in yell` w-legged gulls f` r all c` l` nies sampled

C` untry	C` l` ny	Year	Sample size	Number ` f p` sitive samples	Number ` f d` ubtful samples
France	Plane (43°11'14"N-5°23'13"E)	2010	20	0	0
	Ri` u (43°10'39"N-5°23'04"E)	2009	32	0	0
		2010	8	0	0

	Fri` ul (43°16'27"N-5°18'15"E)	2009	32	0	0
		2010	32	0	0
	Carteau (43°22'40"N-4°51'27"E)	2009	32	0	0
		2010	32	0	0
		2011	30	0	0
	Bess` n (43°29'15"N-4°27'47"E)	2009	32	0	0
		2010	32	0	0
	Grau-du- r` i (43°32'15"N-4°08'17"E)	2009	32	0	0
	Villeneuve (42°02'50"N-3°13'21"E)	2009	32	0	0
		2010	32	0	0
	Gruissan (43°06'36"N-3°06'25"E)	2009	32	0	0
		2010	32	0	0
	C` rrège (42°51'15"N-3°01'21"E)	2009	32	1	1
		2010	32	1	0
Spain	L'Escala (42º07'32"N-3º07'58"E)	2010	6	4	0
	Medes (42°02'50"N-3°13'21"E)	2009	38	14	6
		2010	49	24	1
		2011	49	33	3
	Ebr` delta (40°34'17"N-0°39'37"E)	2009	32	0	0
		2010	29	0	0
		2011	30	0	0
	C` lumbretes (39º53'52"N-0º41'06"E)	2010	50	0	0
	Drag` nera (39º35'00"N-2º19'00"E)	2010	39	0	0

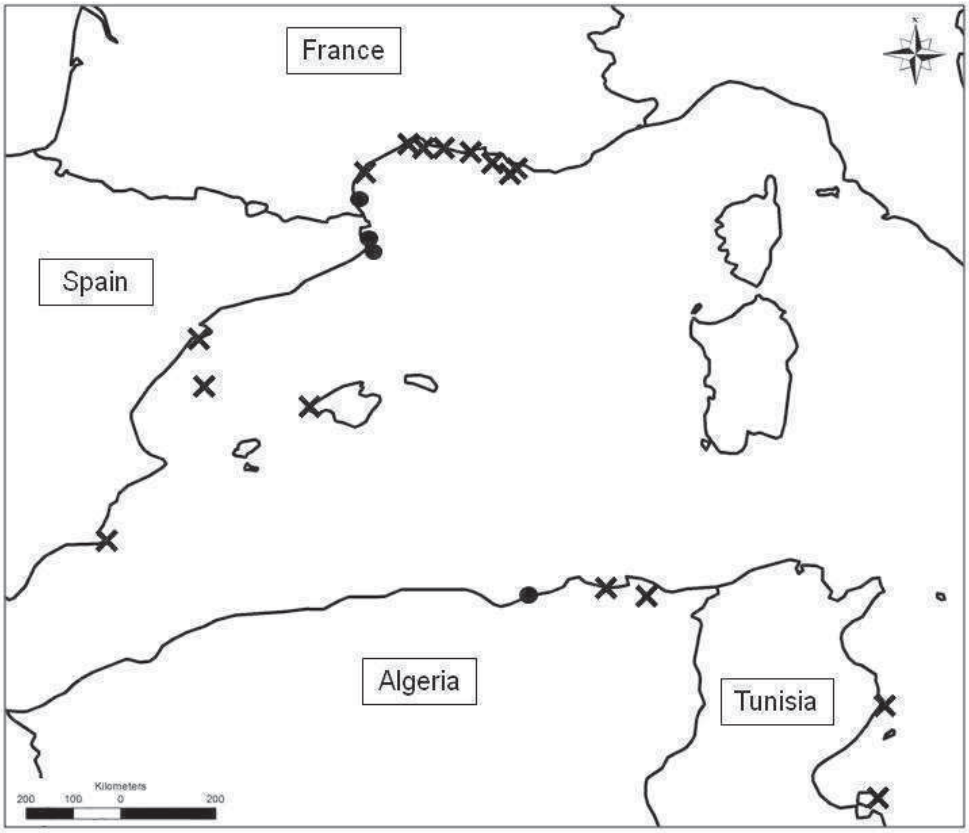
	Isla Gr` sa (37°43'40"N-0°42'27"O)	2009	32	0	0
		2010	32	0	0
		2011	30	0	0
Algeria	Chetaïbi (37°05'79"N, 7°17'53"E)	2009	32	0	0
		2010	32	0	0
	Jijel (36°47'21"N, 5°36'19"E)	2010	31	1	0
	Skikda (36°56'15"N, 6°53'09"E)	2010	17	0	0
Tunisia	Djerba (33°39'10"N-10°58'59"E)	2009	32	0	0
	Sfax (34°42'28"N-10°45'02"E)	2009	32	0	1

Table.2. Results ` f virus neutralizati` n tests t` identify if the antib` dies were specific t` West Nile, Usutu, Tick-b` rne encephalitis, and Meaban viruses

C` untry	C` l` ny	Year	West Nile virus sample size(p` sitive)	Usutu virus sample size(p` sitive)	Tick-b` rne encephalitis sample size(p` sitive)	Meaban virus sample size(p` sitive)
France	C` rrège	2009	1(0)	1(0)	1(0)	1(1)
		2010	1(0)	1(0)	1(0)	
Spain	L'Escala	2010	2(0)	2(0)	2(0)	2(2)
	Medes	2009	14(0)	2(0)	2(0)	13(13)
		2010	22(1)	3(0)	3(0)	22 (22)
		2011	33(0)	3(0)	3(0)	26 (26)

Figure. Location of colonies sampled in the western Mediterranean

Circles indicate colonies that had at least one egg positive for antibodies against the flavivirus E-protein while crosses indicate antibody-negative colonies.



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Chapitre 2

Comprendre le rôle des compartiments épidémiologiques et leurs interactions : la dynamique des virus Influenza A en Camargue

Autoportrait à la grippe espagnole. Edvard Munch. 1919. Munch peint cet autoportrait alors qu'il est atteint par la grippe espagnole[3] et se croit proche de la mort. Il survivra finalement à sa maladie contrairement à plusieurs artistes de l'époque tels que Guillaume Apollinaire et Edmond Rostand.

I. Les virus Influenza A : histoire, épidémiologie et enjeux

1) La grippe dans l'Histoire

Le mot influenza, terme scientifique pour désigner les virus grippaux, signifie influence en italien et se rapporte au rôle qu'étaient supposés avoir les astres sur la survenue de cette maladie. Tout aussi imagé, le mot grippe, utilisé plus couramment en français, vient de l'allemand « greifen » qui signifie saisir brusquement et se réfère au caractère soudain de la fièvre généralement associée aux infections grippales. Ces deux termes illustrent le caractère mystérieux et brusque que revêtaient les infections dues aux virus Influenza dans le passé.

[3] La grippe espagnole doit son nom à la croyance que l'épidémie débuta en Espagne (Tognotti 2003). Le virus tua en effet nombre de Madrilènes et infecta le roi d'Espagne lui-même. Pourtant on sait aujourd'hui que les premiers cas furent détectés aux Etats-Unis et en France (Potter 2001). Cependant la censure empêcha cette information de circuler dans les pays en guerre tandis que les cas furent rapidement reportés en Espagne, pays neutre où la censure n'avait pas cours.

Bien que l'agent infectieux responsable de la grippe n'ait été isolé qu'en 1912, la description de symptômes caractéristiques de la grippe est présente dans de nombreux textes historiques, ce qui suggère que les épidémies de grippe ont touché les populations humaines depuis plus de 2000 ans. La première description de symptômes grippaux est attribuée à Hippocrate qui dépeint une épidémie de toux à Périnthe en 412 av. JC dans le livre 6 des Epidémies (Martin & Martin-Granel 2006). Une autre épidémie qui pourrait avoir été due à un virus influenza est rapportée par Tite-Live au sein de l'armée romaine assiégeant Syracuse en 212 av. JC. La première pandémie grippale sur laquelle tous les auteurs s'accordent est quant à elle décrite en 1580 et commença en Asie avant de s'étendre à l'Europe et à l'Afrique puis d'atteindre finalement l'Amérique (Potter 2001). A la suite de cette pandémie plus de 8000 morts furent comptabilisés à Rome et certaines villes espagnoles furent décimées (Beveridge 1991). Depuis le XVIIIème siècle, les rapports médicaux sont plus précis et plus nombreux ce qui permet d'identifier avec plus de certitude la présence de la grippe. De 1800 à nos jours 9 pandémies grippales ont été reconnues (Potter 2001; Taubenberger & Morens 2010). La pandémie ayant débuté en 1918 dite de « grippe espagnole » fut la plus meurtrière puisqu'on considère aujourd'hui qu'elle tua entre 40 et 50 millions de personnes (Potter 2001).

En parallèle, la grippe dite saisonnière, c'est-à-dire celle causée par des virus influenza qui diffèrent peu de ceux ayant circulé précédemment dans la population, est présente presque chaque année dans presque tous les pays du monde. Les données historiques manquent quant à ces épidémies plus discrètes. Toutefois, dans tous les pays où une surveillance des infections grippales a été mise en place depuis 50 ans les virus influenza ont été régulièrement détectés dans la population visée (Potter 2001). Il est encore aujourd'hui difficile d'évaluer l'impact annuel mondial des épidémies saisonnières de grippe, notamment du fait du manque de suivi dans les pays tropicaux au sein desquels l'enjeu représenté par les infections grippales a été longtemps sous-estimé (Simonsen 1999). Au vu des données actuelles on évalue le nombre de décès causés par les infections grippales de 250000 à 500000 par an dans le monde (WHO 2009).

2) Epidémiologie et évolution des virus influenza

L'évolution et l'épidémiologie des virus influenza ainsi que les enjeux économiques et de santé publique qui y sont associés sont décrits dans l'**article 4**. C'est pourquoi nous nous limiterons ici à une brève description des éléments d'épidémiologie et de classification nécessaires à la compréhension de la suite de ce manuscrit.

Les virus influenza sont des virus à ARN simple brin appartenant à la famille des Orthomixoviridae. On distingue parmi eux des virus de type A, B et C. Les virus de type C infectent principalement l'Homme, sont peu variables d'un point de vue antigénique et ont un impact limité en termes de santé publique. Les virus de type B infectent presque exclusivement l'Homme et sont impliqués dans les épidémies de grippe saisonnière. Enfin, les virus de type A touchent un grand nombre d'oiseaux et de mammifères dont l'Homme. Nous nous concentrerons ici sur ces derniers car ils sont les plus variables au niveau antigénique et sont de ce fait impliqués à la fois dans les épidémies saisonnières et les pandémies grippales.

Les virus influenza A (VIA) sont classés en sous–types HxNy en fonction de la combinaison de deux protéines de surface, l'hémagglutinine (HA) et la neuraminidase (NA) qui constituent les cibles principales des anticorps. A ce jour, 17 types moléculaires de HA et 9 types de NA ont été répertoriés (Webster *et al.* 1992; Fouchier *et al.* 2005; Tong *et al.* 2012). Les VIA se caractérisent par l'évolution rapide de leurs sites antigéniques, qui peut avoir lieu selon deux mécanismes : i) L'accumulation de mutations ponctuelles sélectionnées principalement par l'immunité des hôtes ii) Le réassortiment entre les segments de souches différentes infectant une même cellule (Scholtissek 1995). Le premier mécanisme est à l'origine de l'évolution progressive des virus de la grippe saisonnière tandis que le second peut conduire à l'émergence de variants très différents des virus ayant précédemment circulé dans la population humaine et causer des pandémies.

Les virus influenza A peuvent infecter une large gamme d'hôtes, dont de nombreux oiseaux, les humains, les chevaux, les porcs ainsi que certains carnivores et cétacés (Wallensten 2007). Les oiseaux sauvages, en particulier les Charadriiformes (limicoles, mouettes et goélands) et les Ansériformes (Oies, canards et cygnes) constituent le réservoir naturel des virus influenza A et la source initiale de tous les virus circulant chez les mammifères (Webster *et al.* 1992). On distingue, en fonction de leur virulence,

deux types de souches de VIA. Les souches faiblement pathogènes (FP) circulent naturellement chez les oiseaux chez lesquels elles causent généralement des infections asymptomatiques ou bénignes (Webster *et al.* 1992; Latorre-Margalef *et al.* 2009). Les souches hautement pathogènes (HP) qui peuvent évoluer lorsque des virus FP sont placés dans des conditions particulières telles que celles rencontrées dans les populations de volailles élevées de façon intensive (Lebarbenchon 2010; Ito et al. 2001). Seuls les sous-types H5 et H7 sont aujourd'hui connus pour leur capacité à évoluer vers des formes hautement pathogènes. Les virus H5N1 HP qui circulent actuellement en Asie et au Nord de l'Afrique, bien qu'ils n'aient pas la capacité de se transmettre d'Homme à Homme, ont causé au 2 aout 2012 la mort de 358 personnes dans le monde (WHO 2012b). De plus, ces virus ont un impact économique très important au niveau mondial du fait du coût des mesures de prévention et de contrôle mises en œuvre, comme des restrictions commerciales liées à ces mesures (FAO 2009).

II. Dynamique des virus influenza A au sein des milieux naturels : l'exemple de la Camargue

1) Dynamique des virus influenza A en Camargue : données existantes

L'étude des VIA au sein de l'avifaune sauvage a débuté à l'automne 2005 en Camargue (pour une synthèse des travaux menés avant la présente thèse voir Lebarbenchon 2008). L'échantillonnage réalisé de 2005 à 2008 sur 121 espèces d'oiseaux appartenant à 14 ordres a montré que seuls les Ansériformes et les Charadriiformes étaient porteurs de VIA dans la région (Lebarbenchon *et al.* 2007a; Lebarbenchon 2008).

Parmi eux la sarcelle d'hiver (*Anas crecca*) et le canard colvert présentent les plus fortes prévalences (respectivement 5,3% et 4,4% en moyenne entre l'hiver 2005/2006 et l'hiver 2007/2008 ; Lebarbenchon 2008). Une prévalence importante (10,3%) a également été relevée chez la sarcelle d'été (*Anas querquedula*) entre septembre 2005 et juillet 2008 sans que le faible échantillonnage (n=29) ne permette de conclure au rôle de cet hôte qui n'est présent en Camargue qu'au printemps, à la fin de l'été et au début de l'automne (Lebarbenchon 2008). Chez le canard colvert et la sarcelle d'hiver un pic d'infection survenant chaque année en septembre a pu être mis en évidence

(Lebarbenchon *et al.* 2010). Ce pic pourrait être expliqué par l'arrivée massive de canards juvéniles au système immunitaire naïf à cette période en Camargue.

Chez les Charadriiformes, des VIA ont été détectés de 2005 à 2008 chez les deux espèces de Laridés qui ont fait l'objet d'un échantillonnage important : la mouette mélanocéphale (*Larus melanocephalus*) et le goéland leucophée (prévalences moyennes respectives : 2,8% et 0,5% ; Lebarbenchon 2008). Parmi les virus isolés chez ces espèces la découverte d'un virus H9N2 issu d'un réassortiment entre une souche américaine et une souche eurasienne chez une mouette mélanocéphale a souligné le rôle que peuvent jouer les Charadriiformes dans les échanges intercontinentaux de VIA (Lebarbenchon *et al.* 2009).

Enfin, l'ensemble des 150 VIA détectés chez les oiseaux d'eau en Camargue entre 2005 et 2008 était de type FP (Lebarbenchon *et al.* 2007b). Les travaux menés entre 2005 et 2008 ont établi une base de connaissances sur la dynamique des VIA en Camargue qui a permis au cours de la présente thèse d'aborder des problématiques nouvelles présentées ci-après: i) Impact des lâchers de canards colverts sur la dynamique des VIA ii) Rôle des Laridés (goélands, mouettes et sternes) et dynamique temporelle des infections dues aux VIA dans ce réservoir iii) Rôle potentiel du sanglier dans la dynamique des VIA.

2) Dynamique des virus influenza A à l'interface entre Anatidés domestiques et sauvages

Le canard colvert est l'un des hôtes naturels principaux des virus influenza A (Stallknecht & Brown 2007). Il a de plus été prouvé qu'il peut être un porteur sain de virus H5N1 HP et donc potentiellement participer à leur propagation (Keawcharoen *et al.* 2008). Il s'agit par ailleurs de l'espèce de canard la plus abondante au Monde avec plus de 20 millions d'individus sauvages recensés sur tous les continents excepté l'Antarctique (Birdlife International 2012b). De plus, la domestication du canard colvert, qui a eu lieu en Asie il y a plus de 3000 ans, a conduit à la sélection de différentes races de canards domestiques, dont la population globale maintenue en captivité s'élève aujourd'hui à environ 681 millions d'individus (Tanabe 1995). Parallèlement à cette population domestique élevée pour sa viande, depuis le début du XXème siècle les élevages de canards colverts destinés au renforcement des populations à des fins

cynégétiques se sont multipliés. La pratique des lâchers de canards colverts pour le tir, d'abord mise en place aux Etats-Unis (Leopold 1933), est adoptée en Europe dès 1950 au Danemark, en Tchécoslovaquie et au Royaume-Uni. En France ces lâchers se pratiquent depuis les années 1970. L'estimation actuelle du nombre de canards lâchés en France annuellement dans ce cadre est de l'ordre de 1,4 millions d'individus (Champagnon 2011).

En Camargue l'apparition de chasses commerciales dans les années 1970 a entrainé le développement local de cette pratique. On estime aujourd'hui à au moins 30 000 le nombre d'individus lâchés dans la région chaque année. Les individus lâchés en Camargue sont élevés en plein air en contact avec les canards présents dans le milieu naturel via le partage de l'usage de l'eau et le dépôt possible de fientes par les oiseaux sauvages dans les enclos dont le toit est constitué de filets. Du fait de ces conditions d'élevage et du lâché en milieu naturel des individus élevés, il est probable que des échanges de VIA existent entre populations captives et populations sauvages de canards colverts en Camargue. Dans ce contexte, nous avons mis en place une étude, présentée dans l'**article 5,** afin de tester les hypothèses suivantes : i) Les conditions d'élevage (forte densité, faible diversité génétique) favorisent les infections par les VIA chez les canards colverts captifs ii) Ces conditions permettent les échanges de VIA entre canards vivant en milieu naturel et en captivité iii) Les souches potentiellement isolées dans les élevages présentent des caractéristiques particulières (forte virulence, résistance aux antiviraux, capacité de passage vers l'Homme) associées à leur circulation dans les populations d'élevage.

Pour répondre à cet objectif nous avons effectué des prélèvements (écouvillons cloacaux) au sein d'un élevage de canards en 2009 et de quatre élevages en 2010. En parallèle nous avons échantillonné des individus tués à la chasse durant les hivers 2008/2009 et 2009/2010. Les résultats de cette étude ont montré que les canards d'élevage sont effectivement particulièrement sensibles aux infections par les VIA puisque les prévalences observées allaient jusqu'à 99% tandis qu'en milieu naturel les prévalences observées excèdent très rarement les 20%. La souche impliquée dans l'épizootie détectée en 2009 était de type H10N7, comme toutes les autres souches détectées au cours de l'étude elle était faiblement pathogène. Cette souche ne présentait pas de facteur de risque lié à sa circulation dans une population d'élevage. De plus, elle

n'a pas été identifiée chez les canards tués à la chasse ni avant, ni après le lâché des individus captifs chez lesquels ce virus avait été isolé. Enfin, de nombreux virus de type H5 LP ayant potentiellement la capacité d'évoluer vers une virulence forte (forme HP) ont été détecté au cours des deux années d'échantillonnage chez les canards tués à la chasse. Les échanges de VIA entre canards domestiques et sauvages n'ont donc pas été mis en évidence au cours de cette étude. Cependant, la probabilité qu'ils existent étant données les conditions d'élevage des canards de lâché est très importante et incite vivement à renforcer les contrôles sanitaires des colverts captifs avant leur lâcher au vu des résultats de cette étude.

3) Un autre réservoir aviaire naturel : les Laridés

(a) Les Laridés un réservoir peu étudié à ne pas négliger

Les Charadriiformes constituent avec les Ansériformes le réservoir naturel des VIA (Webster *et al.* 1992). Parmi les Charadriiformes les VIA ont été détectés chez quelques espèces de limicoles telles que le tournepierre à collier (*Arenaria interpres*) ou le bécasseau maubèche (*Calidris canutus*) principalement en Amérique du Nord (Olsen *et al.* 2006; Hanson *et al.* 2008; Munster & Fouchier 2009). Cependant, c'est, au sein de cet ordre, parmi les Laridés que les VIA sont le plus fréquemment détectés (e.g. Munster *et al.* 2007; Winker, Spackman & Swayne 2008). Les Laridés regroupent les mouettes, les goélands et les sternes, soit un total de 102 espèces aux écologies très variées (IOC 2012). La plupart de ces espèces sont inféodées aux milieux marins et se reproduisent en colonies (del Hoyo 1996). Certaines d'entre elles, comme la sterne arctique (*Sterna paradisaea*), sont très abondantes (e.g. > 2millions d'individus chez la sterne arctique ; Birdlife International 2012a) et sont présentes de l'Arctique à l'Antarctique (del Hoyo 1996). Pour différentes raisons incluant la facilité d'échantillonner des espèces chassées et les plus fortes prévalences généralement observées chez les Anatidés, les VIA ont été beaucoup moins étudiés chez les Laridés que chez les Anatidés. Pourtant, comme l'illustre l'**article 6**, les données existant sur la circulation des VIA dans cette famille, associées à l'écologie des espèces qui la constituent, suggèrent que ce groupe représente un réservoir naturel de VIA à ne pas négliger.

En premier lieu, l'existence des sous-types H13 et H16 détectés principalement chez les Laridés (Fouchier *et al.* 2005; Olsen *et al.* 2006) suggère qu'ils ont évolué

indépendamment des sous-types circulant chez les Anatidés, peut-être via la sélection de voies de transmission privilégiées différentes. La capacité de ces virus à se transmettre par la voie aérienne pourrait faciliter leur transmission vers les oiseaux domestiques ou l'Homme, d'autant qu'il a été montré que des virus H16 peuvent se lier aux cellules des voies respiratoire supérieures humaines (Lindskog *et al.* In Prep.). Par ailleurs, les VIA circulant en Amérique et en Eurasie sont phylogénétiquement distincts (Ito *et al.* 1991; Suarez & Perdue 1998; Widjaja *et al.* 2004). Cependant, des réassortiments entre les clades de ces deux ensembles géographiques existent (Wallensten *et al.* 2005; Dugan *et al.* 2008; Lebarbenchon *et al.* 2009). Ils ont été pour la plupart isolés chez des Laridés, qui apparaissent de ce fait jouer un rôle important dans le brassage génétique des VIA (Pereda *et al.* 2008; Lebarbenchon *et al.* 2009; Ramey *et al.* 2010; Wille *et al.* 2011). Ce rôle est sans doute lié à la capacité de certaines espèces du groupe à effectuer des migrations intercontinentales, et parfois même de l'Arctique à l'Antarctique. Enfin, des VIA de type HP ont été détectés chez plusieurs espèces de Laridés, y compris chez des individus ne présentant pas de symptômes cliniques (Muzinic *et al.* 2010; Savić *et al.* 2010). Des infections expérimentales ont confirmé la capacité de certaines espèces de Laridés à être porteurs sains de VIA de type HP (Perkins & Swayne 2001; Brown *et al.* 2006; Brown, Stallknecht & Swayne 2008). Ces données associées aux importantes migrations entreprises par certaines espèces du groupe soulignent leur rôle potentiel dans la dispersion de ces pathogènes.

(b) Circulation des VIA chez les Laridés : étude sérologique dans le bassin méditerranéen

Les prévalence des infections par les VIA observées sont généralement plus faibles chez les Laridés que chez les Anatidés (Olsen *et al.* 2006). Les études sérologiques représentent donc le moyen le plus efficace pour obtenir des informations sur la circulation de ces virus au sein de ce groupe dans une zone étendue sans avoir à effectuer un nombre trop important d'échantillons. L'intérêt de ce type d'approche a été illustré par de multiples études impliquant la capture d'adultes suivie de prélèvements sanguins. Ces études ont mis en évidence l'exposition de plusieurs espèces de Laridés aux VIA en Amérique du Sud, en Tasmanie (Haynes *et al.* 2009) ou encore en Mongolie (Ishtiaq *et al.* 2012). La majorité des espèces de Laridés étant coloniales, l'étude des anticorps maternels dans les œufs, présentée dans le chapitre 1, apparaît comme une

alternative idéale à ce type de méthode pour optimiser l'échantillonnage au sein de ce groupe chez lequel la capture d'adultes est difficile (Pearce-Duvet *et al.* 2009).

Pour étudier la circulation des VIA chez les Laridés à l'ouest du bassin méditerranéen le goéland leucophée a été choisi comme espèce modèle dans le cadre de la thèse d'Audrey Arnal (**article 7**). Nous évoquions dans le chapitre 1 le caractère localement abondant de cette espèce et sa fidélité au site de reproduction, ainsi que la faible dispersion des adultes. Ces caractéristiques favorisent respectivement l'échantillonnage sans mettre en péril les populations, et l'interprétation des résultats obtenus. De plus, le goéland leucophée fait partie des espèces de Laridés opportunistes qui ont colonisé les habitats urbains au cours des dernières décennies, se nourrissant de déchets et de restes de pêche (Duhem *et al.* 2003). L'espèce est donc fréquemment en contact avec les humains et leur environnement. Par ailleurs, généralement inféodée aux milieux côtiers, les goélands leucophées fréquentent également les étendues d'eau douce proches où se regroupent les Anatidés dont ils se nourissent régulièrement (Tamisier & Dehorter 1999). En outre, des VIA ont déjà été isolés dans les fientes de cette espèce (Lebarbenchon *et al.* 2010) et des anticorps spécifiques aux VIA ont été détectés dans ses œufs en France en 2008 (Pearce-Duvet *et al.* 2009) et en Tunisie en 2009 (Hammouda *et al.* 2011). Ainsi, le goéland leucophée représente un lien potentiel entre plusieurs compartiments dans lesquels les VIA circulent, (Laridés, canards sauvages et domestiques, et populations humaines).

L'étude, à laquelle j'ai collaboré (participation à l'échantillonnage et aux analyses), a été menée sur deux ans (2010-2011) dans trois pays (Algérie, Espagne, France). Elle avait pour objectif de répondre à trois questions concernant la circulation des VIA chez les Laridés à l'ouest de la Méditerranée : i) La circulation des VIA est-elle régulière chez le goéland leucophée dans l'ensemble de la région? ii) Les infections ont-elles lieu au sein des colonies ou à l'extérieur de celles-ci ? iii) Quelles souches de VIA circulent au sein des populations étudiées ? Pour répondre à ces questions 1384 œufs ont été récoltés au sein de 11 colonies réparties dans les 3 pays étudiés. La présence d'anticorps spécifiques aux VIA a été testée dans ces œufs et parmi les échantillons positifs les types H5, H7, H13 et H16 ont été recherchés.

Dans l'ensemble des colonies une partie importante des œufs prélevés contenait des anticorps spécifiques aux VIA (≥ 21% des œufs dans chaque colonie). De plus, la

proportion de ces œufs positifs augmentait de 2009 à 2010 dans 9 des 11 colonies étudiées. Ces résultats, confirment la circulation des VIA chez les goélands leucophées dans l'ensemble de la zone d'étude. Cette circulation semble régulière puisque si les mères n'avaient pas été infectées entre les 2 saisons de reproduction étudiées la proportion d'œufs positifs aurait du diminuer ou rester stable, or elle augmente. Toutefois, le maintien de l'étude à long terme serait nécessaire pour confirmer cette régularité de circulation.

Aucune corrélation spatiale n'a été détectée entre la position des nids et la présence d'anticorps spécifiques aux VIA dans les œufs des 3 colonies où cette corrélation a pu être testée. Cette absence de corrélation suggère que les mères ne s'infectent pas à l'intérieur des colonies mais à l'extérieur de celles-ci, soit sur les sites de nourrissage pendant la période de reproduction, soit pendant l'hiver, par exemple au sein des reposoirs où les goélands se rassemblent pendant la nuit.

Parmi les anticorps détectés aucun n'était spécifique aux virus H5 ou H7, ce qui confirme que ces sous-types sont rares chez les Laridés (Krauss *et al.* 2007; Munster *et al.* 2007). Enfin, contrairement à ce qui a été observé chez d'autres espèces de Laridés (Velarde *et al.* 2010; Toennessen *et al.* 2011), les anticorps spécifiques aux virus H13 et H16 ne représentaient qu'une faible proportion des anticorps spécifiques aux VIA détectés (<25% au total). Ce résultat suggère que, soit les tests utilisés ne sont pas assez sensibles pour détecter une positivité en H13 ou H16 lorsque les taux d'anticorps sont faibles, soit d'autres sous-types, circulant aussi chez les Anatidés, représentent la majeure partie des VIA qui infectent les goélands leucophées au sein de la zone d'étude. Ce point pourrait être tranché en déterminant la spécificité des anticorps trouvés au sein des œufs qui sont négatifs aux sous-types H5, H7, H13 et H16.

(c) Saisonnalité de la circulation des VIA chez les Laridés : étude chez le goéland leucophée en Camargue

Au-delà des types de VIA circulant chez les Laridés et de leur répartition spatiale se pose la question de la dynamique temporelle des infections. Comme exposé précédemment (Chap. 2 II.1), chez les Anatidés, une saisonnalité marquée des infections à VIA est observée en Europe comme en Amérique du Nord (Olsen *et al.* 2006; Wallensten 2007). Les pics d'infection sont généralement observés entre août et septembre lorsque de nombreux individus nés durant l'été et immunitairement naïfs se

rassemblent durant la migration vers les sites d'hivernages et à l'arrivée dans ces zones (Olsen *et al.* 2006). Chez les Laridés, aucune saisonnalité claire n'a été mise en évidence. La période de détection des pics d'infection varie en fonction des sites d'étude. Par exemple, dans la Baie du Delaware (Etats-Unis), ces pics sont observés en mai (Hanson *et al.* 2008), alors qu'en Europe du Nord, les taux d'infections les plus forts sont détectés de juin à août, tandis que dans certaines colonies aucune circulation de VIA n'est détectée pendant la saison de reproduction (Munster *et al.* 2007).

La dynamique temporelle des infections à VIA reste donc méconnue chez les Laridés, d'autant que les études suivant ces infections dans une population tout au long de l'année font défaut chez ce groupe, alors qu'elles commencent à se développer chez les Anatidés (Pearce *et al.* 2010; Hill *et al.* 2011; Hoye *et al.* 2011). Pour contribuer à améliorer nos connaissances en ce domaine, nous avons mis en place une étude suivant les oiseaux tout au long de leur cycle annuel en Camargue **[Article 8]**. La temporalité de la circulation des VIA ne peut être étudiée à l'échelle d'une année grâce à une approche sérologique dans l'état actuel de nos connaissances. En effet, la présence d'anticorps chez un individu ne permet pas de dater son exposition aux VIA car la persistance des anticorps chez les Laridés est méconnue. Seule l'observation de séroconversion permettrait de détecter la circulation de VIA entre deux mois d'échantillonnage et nécessiterait de recapturer les même individus d'un mois sur l'autre toute l'année, ce qui est très difficile chez ce type d'espèce.

Nous avons donc choisi d'étudier l'excrétion des VIA en récoltant des fientes fraiches au sol sur un îlot servant à la fois de zone de reproduction au printemps et de reposoir le reste de l'année et exclusivement fréquenté par l'espèce modèle. L'efficacité de cette méthode a été démontrée au cours de recherches précédentes portant sur l'étude des VIA chez les Anatidés (Hoye *et al.* 2011) comme chez les Laridés (Lebarbenchon *et al.* 2010; Wille *et al.* 2011). Grâce à cette approche nous souhaitions tester deux hypothèses : i) Il existe une saisonnalité marquée des infections à VIA chez les Laridés ii) Cette saisonnalité est similaire à celle observée chez les canards. En effet, en Camargue les goélands leucophées étudiés partagent l'usage des plans d'eau avec une population très importante des canards hivernants, dont nous avons évoqué les forts taux d'infection à VIA en septembre. De plus, ces canards sont régulièrement consommés par les goélands leucophées. Du fait de ces possibilités d'échange

importantes nous avons supposé que les dynamiques temporelles d'infection à VIA étaient similaires chez les populations sympatriques d'Anatidés et de Laridés en Camargue.

Pour tester ces hypothèses 1082 échantillons de fiente ont été effectués de février à décembre 2010. Les données issues de l'analyse de ces prélèvements ont été comparées à celle obtenues à partir des 1123, 652 et 1085 écouvillons cloaquaux prélevés sur les canards tués à la chasse respectivement de septembre à janvier 2007/2008, 2008/2009 et 2009/2010. Chez les canards un pic d'infection a été observé en septembre au cours des trois années consécutives. Un pic semblable a également été observé en 2010/2011 chez les canards selon les données de l'ONCFS (Hars *et al.* 2011). A contrario, les taux d'infection (0 à 4%) ne variaient pas significativement en fonction du mois d'échantillonnage chez les goélands leucophées.

Contrairement à ce qui était attendu aucune saisonnalité n'a donc été mise en évidence dans la circulation des VIA chez les goélands leucophée en Camargue. Par ailleurs, les prévalences faibles observées contrastent avec les forts taux de séropositivités aux VIA observés dans les œufs de cette espèce sur des colonies distantes de moins de 100 km (cf. **article 7**). Différentes explications peuvent être données à ces résultats : i) Les VIA circulent tout au long de l'année en faible quantité dans la population étudiée et cette faible circulation suffit à expliquer la présence des anticorps spécifiques aux VIA dans les œufs du fait de la persistance longue des anticorps chez les Laridés, observée pour d'autres pathogènes (Staszewski *et al.* 2007a b) ii) Des pics importants d'infection ont lieu ponctuellement mais pas nécessairement chaque année, des taux d'infection très élevés ayant été ponctuellement observés chez des poussins de Laridés (Fouchier *et al.* 2005; Velarde *et al.* 2010) iii) Les goélands sont infectés au cours de leurs premières années, hors de Camargue, durant l'hiver au cours duquel une partie d'entre eux rejoint la côte atlantique ou remonte le Rhône.

Pour trancher entre ces différentes explications des études supplémentaires sont nécessaires. Un premier élément de réponse quant à la régularité du schéma observé pourra être amené par l'analyse des 965 prélèvements de fientes effectués de février 2011 à janvier 2012 qui n'a pu avoir lieu pour le moment. La mise en place de prélèvements sur les lieux où les jeunes individus hivernent permettrait également

d'apporter des indices pour mieux comprendre la dynamique temporelle des VIA chez les goélands leucophées en France.

4) Un hôte potentiellement important : le sanglier

Les premiers cas cliniques de grippe chez le porc ont été rapportés en 1918 (Koen 1919), tandis que le premier isolement de VIA chez cette espèce date de 1930 (Shope 1931). Depuis, des souches adaptées à cet hôte ont évolué en parallèle des souches aviaires et humaines (Brown 2000). Les VIA sont actuellement des pathogènes courants dans les élevages porcins sur tous les continents où ces derniers existent (Brown 2000; Olsen *et al.* 2000; Van Reeth *et al.* 2008). Ils causent une faible mortalité, en revanche ils sont associés à une morbidité pouvant atteindre les 100% (OIE 2010). Ces infections entraînent notamment des retards dans l'engraissement et causent du fait de ces retards, ainsi que du coût des soins et des vaccinations, des pertes économiques importantes (OIE 2010). Les sous-types H1N1, H3N2 et H1N2 circulent au sein des populations porcines (Brown 2000).

Le porc constitue un lien entre le réservoir aviaire et le compartiment humain. En effet, les porcs possèdent dans leur système respiratoire supérieur les récepteurs reconnus par les VIA aviaires, porcins et humains et peuvent donc être infectés par ces trois types de virus (Ito *et al.* 1998). Ainsi, des réassortiments entre souches aviaires, humaines et porcines peuvent avoir lieu au sein des cellules du porc à la faveur de co-infections. Ces réassortiments contribuent à l'évolution des VIA circulant chez le porc (Vincent *et al.* 2008), mais peuvent aussi donner naissance à des variants transmissibles à l'Homme et pour lesquels aucune immunité acquise n'existe au sein de la population humaine (OIE 2010). Il semble que le virus H1N1 à l'origine de l'épidémie dite de « grippe espagnole » qui éclata en 1918 soit issu d'un réassortiment entre une souche aviaire et une souche humaine qui pourrait avoir eu lieu chez le porc (Dos Reis, Hay & Goldstein 2009). De même, le variant H1N1 qui causa une pandémie en 2009 (H1N1pdm09) était issu d'un réassortiment entre une souche aviaire, une souche humaine et deux souches porcines de VIA. Ce réssortiment a probablement eu lieu chez un porc (Neumann, Noda & Kawaoka 2009).

Le sanglier (*Sus scrofa scrofa)* appartient à la même espèce que le porc (*Sus scrofa domesticus*). Les données concernant la circulation des VIA chez cet hôte sont cependant

limitées à quelques études sérologiques ponctuelles qui illustrent la grande variabilité géographique de l'exposition des sangliers aux VIA en Europe (Vicente *et al.* 2002; Markowska-Daniel 2003; Vengust, Valencak & Bidovec 2006; Kaden *et al.* 2008) comme en Amérique du Nord (où l'on parle de cochons sauvages ; Saliki, Rodgers & Eskew 1998; Gipson *et al.* 1999; Hall *et al.* 2008; Corn *et al.* 2009). De façon intéressante la majorité des anticorps détectés sont spécifiques aux virus de type H1N1(e.g. Kaden et al. 2008; Vicente et al. 2002) qui sont les plus fréquents chez les porcs d'élevage en Europe (Van Reeth *et al.* 2008; Kyriakis *et al.* 2011) comme en Amérique du Nord (Brown 2000). De plus, de plus fortes prévalences ont été observées chez les cochons sauvages dans les zones où les élevages de porcs industriels sont les plus nombreux en Amérique du Nord (Corn *et al.* 2009).

En Camargue il n'existe aucune porcherie industrielle et les porcs domestiques se limitent à quelques individus élevés par certaines familles. Cependant une importante population de sangliers (2000 à 3000 individus) occupe le territoire et partage l'usage des zones humides avec les nombreux canards hivernant dans la région et les différents acteurs du territoire (riziculteurs, chasseurs, gestionnaires d'espaces naturels etc.). Les canards, comme les humains, sont régulièrement infectés par des VIA en Camargue (Lebarbenchon *et al.* 2010; GROG 2012). De plus ,le virus H1N1pdm09, dont le passage de l'Homme vers le porc a été avéré dans plusieurs pays (Hofshagen *et al.* 2009; Forgie *et al.* 2011), a circulé dans la population humaine de la région de septembre à février 2009, puis en novembre et décembre 2010. Or, les sangliers locaux sont en contact avec ces deux groupes d'hôtes et devraient donc être exposés aux VIA qui circulent au sein de ces derniers. Ils pourraient dans ce cas jouer un rôle important dans la dynamique des VIA en Camargue, notamment en créant des possibilités de réassortiment entre les différents VIA circulant dans des groupes d'hôtes distincts.

Dans ce contexte, nous avons souhaité étudier la circulation des VIA chez les sangliers camarguais afin de déterminer s'ils jouent effectivement un rôle important dans la dynamique locale des VIA **[Article 9]**. Nous avons privilégié l'étude des virus excrétés car celle-ci permet d'isoler les souches impliquées et ainsi de déterminer si elles sont d'origine aviaire, humaine ou porcine. Des échantillons sanguins (n=20) ont pu être prélevés en complément afin de rechercher des anticorps spécifiques aux VIA.

Entre septembre 2009 et novembre 2010, 315 écouvillons nasaux ont été récoltés. Au cours de cette période le virus H1N1pdm09 a circulé au sein de la population humaine de la région (INVS 2010; GROG 2012). Par ailleurs, les taux d'infection à VIA variaient en fonction des mois entre 4% et 15% chez les canards tués à la chasse entre septembre 2009 et Janvier 2010 en Camargue (**article 4**). Pourtant aucun VIA n'a été détecté au sein des écouvillons collectés chez les sangliers de la région. De même, aucun anticorps spécifique aux VIA n'a été observé au sein des échantillons de sérum collectés. Ces résultats n'excluent pas l'existence d'une circulation faible ou ponctuelle des VIA chez les sangliers en Camargue. Cependant, ils montrent que le sanglier n'a pas un rôle épidémiologique majeur dans la dynamique des VIA au sein de la zone d'étude en comparaison des Anatidés. Ces conclusions sont en accord avec celles d'une étude sérologique menée simultanément (septembre 2009 à août 2010) dans 5 autres départements français (Aveyron, Ille-et-Vilaine, Nord, Pyrénées-Atlantiques et Finistère) chez les sangliers (Payne *et al.* 2011). Celle-ci montre en effet que dans le département étudié le plus proche de la Camargue (l'Aveyron) le taux de présence des anticorps spécifiques aux AIV était très faible (0,9% n=110 ; Payne *et al.* 2011). En outre, les résultats de cette étude mettent en évidence une association géographique entre les sous-types retrouvés chez les porcs et chez les sangliers soutenant l'hypothèse d'une transmission des VIA des élevages de porcs vers la faune sauvage (Payne *et al.* 2011).

Au vu de ces résultats, il serait intéressant de mener en parallèle des études couplant sérologie et isolement des souches excrétées au sein des élevages porcins et des populations de sangliers en France afin de savoir si les souches circulant dans ces deux compartiments sont bien identiques ou très proches et de comprendre quels sont les échanges viraux entre ces deux groupes d'hôtes.

Article 4. Avian influenza viruses: environmental influence.

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Publié en tant que chapitre dans *Encyclopedia of environmental health*. Elsevier.

Avian Influenza Viruses: Environmental Influence

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Abbreviations

AIV	avian influenza virus
HA	hemagglutinin
HPAI	highly pathogenic avian influenza
LPAI	low pathogenic avian influenza
NA	neuraminidase

The epidemiology of avian influenza viruses (AIVs) has undergone a revolution in the past 10 years. Previously, the outbreaks were confined in time and space; today AIVs have become an increased international concern. This unprecedented situation resulted in the failure to control and eradicate infections in poultry due to highly pathogenic avian influenza (HPAI) H5N1 and low pathogenic avian influenza (LPAI) H9N2 viruses. Moreover new reassortments implying AIVs, like the novel H1N1 human virus that has recently become pandemic, represent a serious threat to public health.

Structure and Nomenclature

Influenza viruses are RNA viruses belonging to the Orthomyxoviridae family. They have a very small genome with only eight RNA segments. Based on variants of the M1 and NP proteins, they are classified into types A, B, and C. Type A influenza viruses are divided into subtypes defined on the antigenicity of the glycoproteins, hemagglutinin (HA), and neuraminidase (NA), which interact with receptor sites of host cell membranes. The HA allows the virus to attach to the surface of a cell, whereas the NA allows the virus to be released. Sixteen forms of HA (H1 to H16) and nine forms of NA (N1 to N9) have been described and their combinations form the classification of AIVs by subtypes. Strains are described by the influenza type, the host where isolated, the strain number, the year of isolation, and finally the subtype.

Evolution

Surface antigenic proteins undergo two types of evolution: drift and shift. The genetic variability within each subtype in RNA sequences explains the antigenic drift.

Antigenic shift induces major changes creating new viral subtypes through genetic reassortment by replacing gene segments when the host is infected simultaneously by different subtypes. Coinfections have been shown to be frequent in wild ducks in North America. This phenomenon increases the possibility of genetic reassortment and, consequently, the emergence of new viral subtypes. This has already been described in waders in North America, wild ducks in Japan, and common guillemots (*Uria aalge*) in Sweden, involving exchanges of genes between American and European viral lineages. These exchanges between continental lineages seem limited but may occasionally occur where the migratory paths of American and European birds cross.

Host Range

AIVs are present in a wide range of hosts. They are widespread in the animal kingdom, occurring mainly in birds, humans, horses, pigs, and sometimes in cetaceans and mustelids. Wild birds are the central element of the viral ecology of AIVs. AIVs have been isolated in wild birds worldwide for the 16 HAs and 9 NAs, except in Antarctica where only serological data exist. AIVs have been found in at least 12 orders of birds, including ducks, passerines, waders, gulls, terns, pheasants, and falcons. Genotypes of AIVs vary geographically and between years, as well as between species and populations of birds, but different lineages diverge on a geographic rather than temporal or host basis. The majority of species concerned by AIVs are waterbirds, and in particular the Anseriforms (ducks, geese, and swans) showing a wide variety of subtypes and having a major role in dissemination and perpetuation of AIVs in the wild. The mallard *Anas platyrhynchos* is particularly important because virtually all the subtypes have been isolated from it. AIVs are also widespread in the Charadriiformes (gulls, terns, and shorebirds). H13 and H16 subtypes have been isolated only in gulls. Genetic analysis of viruses from ducks and shorebirds also suggests that their viral gene pools are not separated. The role of Charadriiformes in AIVs epidemiology is believed to be important, but yet not clear.

Ecology

Little is known about the ecology of AIVs in natural populations, as most studies concerned either domestic

animal or human health. However, until recently, the epidemiology of AIVs consisted of the perpetuation of LPAI viruses in wild birds where they caused no outbreaks and of their irregular spread to poultry. The role of waterbirds in the dispersal of AIVs has been well established for many years. The postbreeding and premigratory periods seem to be crucial periods for disease transmission in staging areas. During these periods, a high AIVs prevalence of infection, which can exceed 30%, occurs in juvenile ducks in Delaware Bay in Canada. Hinshaw and colleagues in 1985 showed that the perpetuation of AIVs in waterfowl is transmitted from adult to juvenile birds on lakes where they congregated before migration. The prevalence decreases in the course of southward migration, indicating a loss of viral infection during migration. The ducks migrating northwards in spring have much lower prevalence of AIVs, but high enough viral titers to reintroduce the virus in their breeding areas. In America, shorebirds have a higher prevalence of carrying the viruses north during the spring migration than in ducks. The pattern in gulls is less known, but they have a higher prevalence in late summer and early fall. Much information is lacking with regard to the competition between virus subtypes; it is not yet clear if temperate regions in Palearctic areas have their own endemic subtypes or are colonized every spring by African subtypes, or every autumn by Siberian subtypes.

Susceptibility and Symptoms

The structural changes of the HA and NA proteins reduce the ability of the host's immune system to recognize variant subtypes, requiring a continual replacement of vaccine strains. Two phenotypes of virulence, a high virulence and a low virulence, have been described. LPAI subtypes are asymptomatic or lead to benign respiratory symptoms. HPAI is responsible for clinical signs ranging from unapparent to high levels of mortality, the so-called 'fowl plague.' There is marked variation in the susceptibility of different bird species to the same AIVs. The shift from LPAI to HPAI subtypes is achieved by the insertion of basic amino acids at the cleavage site of the polypeptide precursor of the HA. The factors of this evolution from LPAI to HPAI genotypes are not known. Wild birds have a long evolutionary history with AIVs and have repeated exposure to an important diversity of virus subtypes. LPAI are the common forms circulating in wild reservoirs. Virulent strains of AIVs have never been collected from apparently healthy waterfowl, other than waterfowl located near a poultry outbreak. This low virulence of subtypes in wild birds is doubtless the consequence of a long coevolution, allowing the viruses to remain in their hosts without destroying them. Yet Latorre-Margalef in 2009 showed that LPAI infection can be costly for wild mallards. Indeed, they found that the body mass of infected individuals was significantly lower than that of the healthy ducks although no effect on subsequent migration could be highlighted. When bird densities are low and bird diversity high, such as in wild birds, a very virulent subtype leading to high host mortality may disappear because of the impossibility of transmitting quickly to healthy birds before the death of sick ones. Until recently HPAI has been mainly isolated in poultry and there was a general consensus that it had in contrast little or no impact on wild birds. Current theories are that HPAI viruses arise by mutation after LPAI viruses have been introduced to poultry by wild birds, which constitute a permanent source of LPAI subtype gene fragments. It is not possible to predict when this mutation occurs; at times it is quite rapid after the introduction. The LPAI forms can circulate for several years in poultry before manifesting as HPAI, as seen in poultry in the United States in 1983. At the moment, only subtypes H5 and H7 have been shown to be responsible for highly pathogenic phenotypes. Whereas AIVs maintain low virulence in wild birds, highly pathogenicity phenotypes can be selected by practices prevailing in some systems of poultry production. Immunologically naïve poultry with very low genetic diversity can be housed in high density and mass-produced conditions, providing ideal ecological conditions for evolution, preservation, and fast transmission of very virulent strains. This scenario seems to apply, for example, to the epizootic of the H5N2 subtype in 1994 in Mexico. An LPAI H5N2 strain had been isolated in a wader, the Turnstone (*Arenaria interpres*), in 1991 in the United States. In October 1993, a H5N2 strain presenting similarities with the wild strain was discovered in poultry in Mexico. The strain was still an LPAI. But in 1994, a succession of mutations in domestic poultries made it more and more pathogenic. At the end of 1994, the strain had become highly pathogenic and caused a high mortality in the poultry. LPAI H5N2 strains circulate at present in Europe, simultaneously in both wild birds and poultry. They do not raise economic problems as long as they do not evolve to more virulent forms. In theory, it is likely that HPAI subtypes appear regularly in wild individuals. The fact that epizooties are not detected in wild populations results probably from ecology unfavorable to the HPAI subtypes of the viruses, in particular lower densities of hosts and stronger competition with other LPAI viruses.

Transmission, Introduction to Poultry, and Spreading

The AIVs are particularly abundant in the intestinal part of the digestive tract of birds, which explains why high concentrations of infectious virus are found mainly in the

feces. They are transmitted very efficiently via bird-to-bird contact transmission and fecal deposits into the water supply. AIVs have been isolated from surface water. They can remain infective in water, outside a live avian host for long periods , experimentally from a few days in water at temperatures between 22–35 °C and up to a month or more in water between 0–4 °C. AIVs RNA was claimed to be recovered from Siberian lake ice, giving the theorical possibility of the reemergence of lineages after long period of absence but this result was shown to be due to laboratory contamination. Waterbirds' habitats provide an ideal mode of viral dispersal. The persistence also depends on environmental factors such as pH, temperature, UV radiation, and salinity. Now, waterborne transmission being the main determinant in AIVs dynamics, this persistence appears as a key factor in AIVs epidemiology. Yet the presence of infectious particles in water also depends on viral excretion duration and inactivation rate.

The conventional situation is the infection of the gastrointestinal tract in avian host species, but this changed with the HPAI H5N1 subtype. Experimental inoculation of HPAI H5N1 of domestic mallards showed that the digestive tract was not the main site of replication for this subtype, but that viruses replicated rapidly in the trachea, suggesting an oral transmission path. The classic scenario is a primary introduction of LPAI viruses to poultry by wild birds. AIVs then spread from farm to farm mainly by human activities, such as movements between farms by veterinarians, farmers' staff, or vehicles. Long-distance spread is possible by national or international trade of poultry, eggs, or feathers.

HPAI Outbreaks

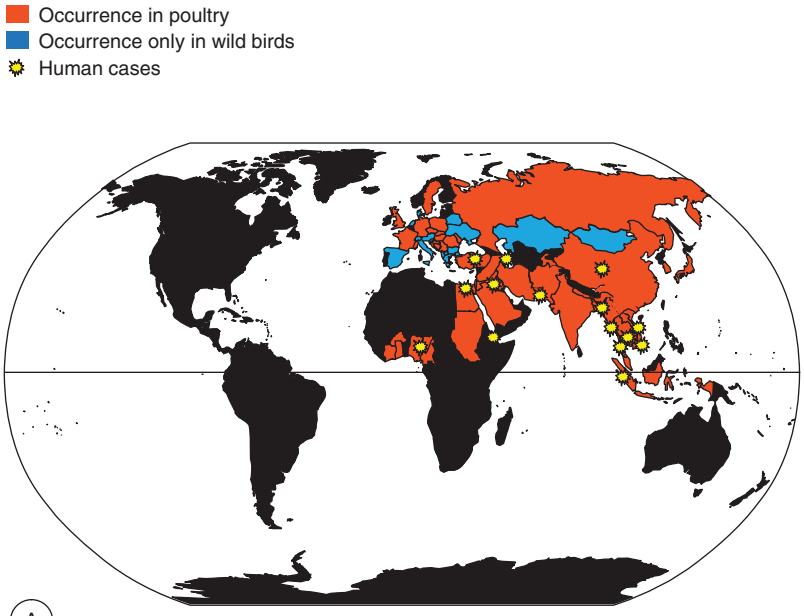
HPAI viruses in general can be considered as emerging infectious diseases with increased frequency of outbreaks in recent years in addition to the particular emergence of HPAI H5N1 virus. During the past 50 years, 25 disease outbreaks due to highly pathogenic subtypes have been identified in birds, primarily from poultry. One outbreak in wild birds has been described, due to HPAI H5N3 in common tern (*Sterna hirundo*) in 1961 along the coast of South Africa. With the HPAI H5N2 outbreak in chickens in Texas, the United States experienced their first outbreak among poultry in 20 years. Before the Asian HPAI H5N1 outbreaks, HPAI had rarely been isolated from free-living wild birds. When isolated, it was usually in cases concerning wild birds living in the vicinity of HPAI outbreaks in poultry, through the transmission from domestic birds to wild birds. These outbreaks have generally remained localized in limited geographic areas, except recently in the United States in 1983, in Mexico in 1994, in Pakistan in 1995, and now on a larger scale in Asia since 2003 (**Box 1A**). HPAI H5N1 is now endemic in this region, cases being regularly reported in poultry in a dozen South Asian countries. African countries are also from time to time affected. Ecological niche modeling has made it possible to link H5N1 outbreaks with climatic variables, population density, and farming data at regional scale. Thanks to this approach, risk areas can be identified and understanding of AIVs epidemiology can be improved.

Species Barrier Crossing

Some AIVs have the peculiarity of crossing barriers between species and, when highly pathogenic, can lead to mortality in mammals, notably humans. Harbor seals *Phoca vitulina* died from a H7N7 subtype in the United States in 1980 and farmed mink from H10N4 subtype in Sweden in 1984. AIVs do not usually infect humans but cases have been reported for H5N1, H7N7, H7N2, H7N3, and H9N2. Most human infections have occurred following direct contact with infected, domestic birds. Human clinical illness ranged from conjunctivitis to pneumonia and death. In 2003 in Holland, the H7N7 subtype caused the death of a veterinarian. Eighty-nine other human cases were confirmed to be infected, mostly among poultry workers showing mild cases of conjunctivitis. Following the large expansion of the HPAI H5N1 among poultry, hundreds of human infections with HPAI H5N1 have been reported in Asia, Europe, and Africa since 2003, causing the death of more than 282 people (**Box 1B**). Nevertheless the number of reported HPAI H5N1 human cases seems quite low with regard to the numerous outbreaks that occurred in birds since 2003. One can wonder why H5N1 avian influenza is not more efficient in infecting humans. Chandrasekaran and colleagues in 2008 partially answered this question by highlighting a difference in cellular receptors that could explain the rarity of the transmission of the H5N1 avian influenza from birds to humans (**Box 2A**).

The HPAI H5N1 has been transmitted to a diversity of mammals. Some domestic carnivores have died after eating contaminated birds or through experimental inoculation; cases were reported in cats as early as 2004 in Thailand and also detected in Germany and Austria at the beginning of year 2006. A study conducted in Thailand in an infected zone showed that 25% of dogs and 7% of cats carried antibodies. Wild carnivores can also be infected as seen with the Owston's civet (*Chrotogale owstoni*), a globally threatened viverrid, in Vietnam, a marten (*Martes foina*) in Germany on the island of Rügen and a mink *Mustela* sp. in Sweden. In October 2004, there was an outbreak in tigers in the Srinacha zoo in the east of Thailand; the infection was apparently caused by the consumption of contaminated chicken meat (tigers were fed whole carcases of infected chickens), leading to the euthanasia of 147 tigers. Taubenberger and colleagues in

Box 1



A

Country	Total since 2003	
	Cases	Deaths
Azerbaijan	8	5
Bangladesh	1	0
Cambodia	9	7
China	38	25
Djibouti	1	0
Egypt	90	27
Indonesia	161	134
Iraq	3	2
Lao	2	2
Myanmar	1	0
Nigeria	1	1
Pakistan	3	1
Thailand	25	17
Turkey	12	4
Viet Nam	112	57
Total	467	282

B

(A) Countries reporting confirmed occurrence of H5N1 avian influenza since 2003 (status as of 4 January 2010). Modified from WHO: http://gamapserver.who.int/mapLibrary/app/searchResults.aspx

– It is noted that North America, South America, and Australia are not currently concerned by H5N1 avian influenza. If the wild bird migration is the main parameter in charge of the distribution of this virus, as it is still considered, why is Africa not completely affected and why are only the poultry of certain countries affected by this pathogen? Are wild birds culprits or victims of the evolution of the H5N1 avian influenza caused by intensive agriculture?

(B) Cumulative number of confirmed human cases of H5N1 avian influenza since 2003 (status as of 30 December 2009). Modified from WHO (2009) Cumulative Number of Confirmed Human Cases of Avian Influenza A/(H5N1). Reported to WHO: http://www.who.int/csr/disease/avian_influenza/country/cases_table_2009_12_30/en/index.html (accessed March 2010)

– The Ministry of Health in Bangladesh announced on 26 May 2008 the discovery of the first human case of H5N1 avian influenza in the country. The number of countries affected by the H5N1 increases in time, but how many human cases have not been declared? The current situation of the disease in human populations leads to various questions:

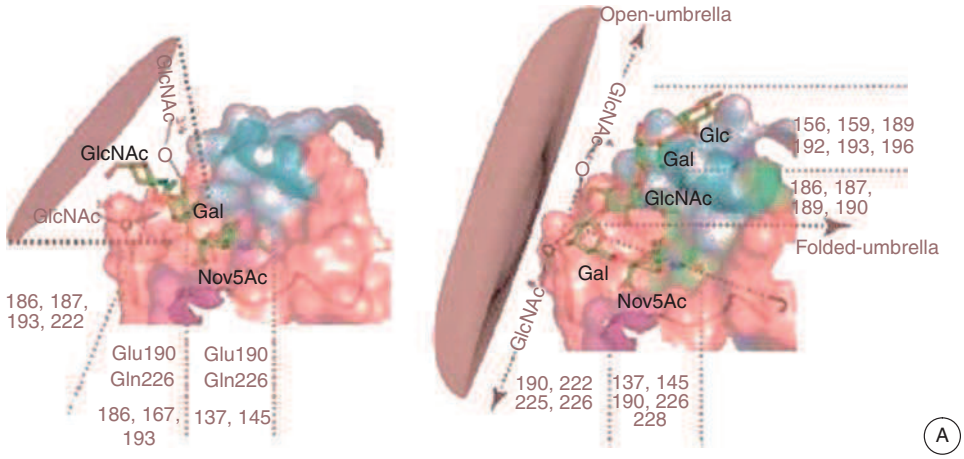
(a) Why are certain countries more concerned than the others?

(b) Do these differences reflect particular societal organizations or particular genetic and cellular configurations favorable for the development of the virus in human populations?

(c) Why is the H5N1 avian influenza not more efficient in infecting humans?

2005 showed that the 1918 H1N1 human pandemic AIVs originated from avian viruses shortly before the pandemic and crossed the avian–human species barrier. Likewise, pandemic influenza H1N1 virus, which has been reported on 15 December 2009, in more than 208 countries, including at least 9596 human deaths, includes avian segments. Indeed it resulted from the reassortment of recent North American H3N2 and H1N2 swine viruses (i.e., avian/human/swine 'triple' reassortant viruses) with Eurasian avian-like swine viruses. LP AIVs are also regularly transmitted to humans who are frequently in contact with birds. In Europe, millions of wild birds are shot, cooked, and consumed by hunters and their dogs every year. A recent study in Iowa, United States, showed the transmission of H11N9 virus from wild birds to hunters and wildlife professionals. After outbreaks in poultry in 1999, two children in Hong Kong were infected by the LPAI H9N2 subtype and developed respiratory disorders, but later recovered. In the United Kingdom in 2007, four people were also infected by the

Box 2



(A) Graph representing the virus receptors of type H5 (left) in the form of cone, and of type H1, H2, and H3 (right) in the form of umbrella. Adapted from Chandrasekaran A, Srinivasan A, Raman R, et al. (2008) Glycan topology determines human adaptation of avian H5N1 virus hemagglutinin. *Nature Biotechnology* 26: 107–113.

- A difference in cellular receptors explains the rarity of the transmission of the H5N1 avian influenza from birds to humans. These results answer, at least partially, the questions asked at the end of Box1. But this discovery offers new perspectives for the detection of possible pathogenic sources to humans.
- The first step of a viral infection is its fixation on the host cell. This happens through the connection of a viral protein to a receptor on the cell surface, a glycoprotein for avian influenza viruses. The viruses have their preferences and not all of them collide with the same receptors. It is the reason why transmission from birds to humans is so rare. The results of the study show that avian influenza viruses of type H5 and H7 settle only on a glycoprotein of conical shape, which is only present in the lower respiratory system of humans. They are not found in the nasal mucus membranes or the throat where the receptors are in the form of an umbrella; these are more adequate for human influenza viruses of the type H1, H2, and H3. The preference of viruses infecting humans through upper respiratory tracts has already been demonstrated, but it remained unexplained. The confrontation here is with a biophysical specificity of the viral attack.
- This characteristic puts the humans shielded from the transmission of the avian influenza virus via droplets rich in virus particles resulting from coughing and sneezes of infected individuals. This is why the disease is passed on with difficulty between human beings. However, birds can easily transmit the virus to other birds through the ingestion of food contaminated by droppings or excrement.
- This specificity of the avian and human influenza viruses could allow to identify more quickly strains susceptible to infect humans. It could also allow the detection of a possible mutation that, giving the avian virus the capacity to settle in other receptors, would facilitate its transmission in human populations. A new strain of virus presenting this particular characteristic could then thwart immune systems and existing vaccines, making such distribution at the same time more effective and mortal for humans. This mutation must be watched closely.

LPAI H7N2 and hospitalized with influenza-like illness after a poultry outbreak.

The HPAI H5N1: A Revolution in AIVs Ecology

The emergence of the HPAI H5N1, in particular since 2003, dramatically changed the perception and the epidemiology of AIVs. The infections occurred at an unprecedented geographic scale, including several continents, involved an unprecedented number of birds and the destruction of millions of them, and an increase of the host range. The mode of transmission changed from the primarily fecal and oral route to respiratory routes.

Origin

The origin of this unprecedented situation resulted in the failure to control and eradicate HPAI H5N1 infections in poultry caused by the industrialization of the poultry production systems worldwide, and not changes in ecology of wild birds. HPAI H5N1 was not detected for several years but probably circulated in a low-level but endemic situation. From December 2003 to February

2004, major epizootic outbreaks in chickens were reported almost simultaneously in eight countries of South-East Asia. This outbreak was not due to a unique genotype, but to multiple genotypes, which had gradually diverged genetically. The geographic extension and the genetic evolution of the virus since 1996 probably have taken place without any link to wild birds.

The lack of precise and long-term studies in domestic and wild birds does not allow exactly the reconstitution of phylogenetic origin of this subtype. Several hypotheses based on genetic analyses may explain the origin of the HPAI H5N1 subtype. The progenitor HPAI H5N1 was discovered in 1996 in domestic geese in the province of Guangdong, South China. It shows segments of genes similar to those found in Hong Kong in a LPAI H9N2 subtype of the Japanese quail (*Coturnix coturnix japonica*), and in a LPAI H6N1 subtype of a green-winged teal *Anas carolinenis*. This last subtype itself shows similarities to a LPAI H6N5 subtype isolated in a shearwater in Australia in 1973. The domestic goose viruses have been dispersed from the region of Guangdong to Hong Kong through the commercial movements of poultry. Domestic chickens became a new host in 1997. From 2000, it seems that domestic ducks frequently became hosts of the virus without expressing the disease and silently amplified the virus in poultry populations.

Maintenance and Spreading of HPAI by Poultry Trade and Wild Birds

Trade

During the previous epizooties of HP subtypes of H5 and H7, it was shown that the expansion of these viruses was due to human activities, in particular, movements of poultry or their products. The same mechanism seems to explain the dispersal of HPAI H5N1. This commercial scenario explains the expansion and the maintenance of the virus in South-East Asia until 2004, via the legal and illegal poultry trade. In their study conducted in the east of China, Chen and colleagues in 2006 show that southern China was the epicenter of the disease. The virus was repeatedly introduced into neighboring countries such as Vietnam, by way of the poultry trade, thus creating new genetic sublineages of the virus. The regional virus sublineages suggest that HPAI H5N1 virus is perpetuated in poultry primarily through their movements and their products rather than by continued reintroduction by migrating birds.

Long-distance dispersion of the HPAI H5N1 by live and dead poultry has been highlighted. It was introduced from Lanzhou into Lhasa in Tibet, 1500 km away, by live chickens. Duck meat infected by the virus has been discovered in Korea and Japan imported from China. In January 2006, the virus was detected in the impoverished Kurdish regions of Anatolia in eastern Turkey, but subsequent epidemiological inquiries showed that the virus was already very widespread in Turkey, in particular along the main highway connecting Ankara to Anatolia. Trade in low-value poultry, collected by trucks and sold throughout Turkey to low-income farmers, allowed the virus to spread quickly. A scenario of virus expansion identical to that in South-East Asia in 2004 is now present in Africa. In February 2006, the first outbreak in Africa originated on a 46 000 laying hen industrial farm in Jaji in the State of Kaduna, one of the most extensive poultry industrial zones in Africa. The commercial trade probably brought the virus because, in spite of bans on such movements, the importation of poultry from contaminated zones continued throughout 2005. Owing to the poultry trade, the virus quickly spread throughout Nigeria and reached adjacent countries, creating a new large endemic area.

Millions of wild birds, mostly passerines for aviculture, are imported legally and illegally into Europe from Asia. The first virus entered the European Union in October 2004 with two Thai eagles *Spizaetus nipalensis* smuggled from Bangkok and seized in customs at the Brussels International Airport. The cause of their infection is not known but they had probably been fed infected chicken carcasses. On 16 September 2005, a Pionus parrot *Pionus* sp., native to Surinam, kept in quarantine with Mesias *Leiothrix argentauris* sp. officially stated to be from Taiwan, was found dead and declared infected by the virus. It appeared later that the Mesias, and not the parrot, were infected by the virus, but the samples had been mixed by the UK authorities. The transport of droppings or waste to fertilize fish farms in Asia, Eastern Europe, and Africa could also be a major route of dispersal.

Wild birds

Migratory birds had first been accused as the primary cause of the spread of this virus in Asia in 2004. However, until 2005, the number of cases of wild birds reported as having contracted the HPAI H5N1 was still sporadic and few, usually scavengers or bird predators, compared with the geographic distribution of the virus. In spite of searches for the virus in wild birds in Asia in 2003 and 2004, very few individuals were found to be positive. In 2004, in Hong Kong, the sampling of 2200 wild birds showed only negative results. A study based on samples taken from 13 100 'migratory ducks' and 'migratory geese' between December 2002 and March 2005 in the zone infected by the virus in eastern China showed that, until 2004, none of these wild birds were infected by HPAI H5N1. The wild bird infections remained localized and limited to resident wild birds, victims of the virus circulating in domestic birds, and played no major role in the dispersal of the virus by migratory birds. Evidence

emerged in Asia that the contamination was primarily spread by human activities.

From mid-2005, international institutions, non-governmental organizations, and media again believed that migratory wild birds were the main cause of the dispersal of HPAI H5N1 outside Asia. This assumption led to debate between epidemiologists and ecologists. The claim was based on the discovery in May 2005 that hundreds of wild birds, mainly bar-headed goose (*Anser indicus*), brown-headed gulls (*Larus brunnicephalus*), and Pallas's gulls (*Larus ichthyaetus*), had died on Lake Quinghaihu, in the province of Qinghai, on the high Asian plateau. In July 2005, the virus crossed the Russian border. From February 2004, the virus was present in North-West China, not far from the Russian Altai and border of Kazakhstan. The virus started its progress westward again in summer 2005 by crossing the Russian border. The summer 2005 marked a new epidemiological phase with a rapid progression of the virus westwards to the Balkans. In early 2006, the virus continued its extension in poultry, along several axes; one from Turkey and the south of Astrakhan to Azerbaijan, the Middle-East, and Egypt, another from southern Russia and China to Kazakhstan, Afghanistan, Iran, and northwestern India, and a third from Nigeria to Niger, Cameroon, and Burkina Faso.

The role of wild birds has been largely debated. It has been widely asserted in the press, international agencies, and even in scientific literature that HPAI H5N1 virus genotypes were spread to domestic poultry through contact with wild birds. Even if wild birds are associated with some outbreaks, they are unlikely to be the major spreading factors. The wild bird mortality at Quinghaihu was an important basis for claims of the role of migratory birds in spreading H5N1 to the west and the south because the lake was considered as remote. It seems much more likely that these migratory birds were the victims of the H5N1 by arriving in spring 2005 on breeding areas, which were already infected by the virus. Moreover, one year later, in May 2006, it was revealed that the main species affected, the bar-headed goose, was artificially reared near the lake, raising the possibility that farmed birds were the source of the outbreak.

From February 2006, the virus began to spread further west in Europe, affecting numerous European Union countries. This time, the great majority of reported cases concerned dead wild birds. This expansion of the disease seems to be caused by harsh-weather movements of wild birds during a cold spell around the Black Sea. These cases showed that virus can spread through infected wild birds traveling short distances, but no evidence for long-distance transmission during seasonal migration has yet been found.

Although research has detected the existence of healthy wild birds carrying the HPAI H5N1 only few times, their regular existence is highly likely, particularly in dabbling ducks. The number of domestic breeds infected by the virus as well as their wide geographic distribution increases the probability of contact between wild birds and the virus. However, it is intriguing that the number of wild birds contaminated by the virus seems so small, and that the virus apparently passes difficultly from domestic birds to wild birds. In 2004, it was demonstrated that domestic mallard ducks, which belong to the genus *Anas*, could be healthy carriers of the virus and play a central role in the production and the preservation of HPAI H5N1. There is no reason why wild mallards should not possess the same capacities. Recently, experimentally infecting wild ducks with HPAI H5N1, showed that dabbling ducks belonging to the genus *Anas* were clinically unaffected and that mallards show abundant virus excretion without clinical or pathological evidence of debilitating disease.

Research Needed

Ecological research is urgently needed to control and anticipate HPAI outbreaks. The global poultry production system with its extensive trade and poultry products are the most likely source for the repeated evolution of HP from LPAI. Research is needed to understand the evolution from LPAI to HPAI AIVs from an ecological point of view, to understand the host–parasite interactions and why the environment of domestic poultry production is more favorable to the emergence of HPAI AIVs than wild environment. This knowledge would allow for the anticipation of emergence and adaptation in the poultry production systems to avoid these emergences. Moreover, ecological modeling appears as a useful tool to understand the factors involved in outbreak occurrence and to target surveillance projects on high-risk areas. The traditional approach to controlling disease is to eradicate the pathogenic parasite. The elimination of LPAI subtypes may, however, result in an increase in the prevalence of more virulent forms. The fear also remains that suboptimal vaccination may result in the reduction of disease but without affecting transmission, resulting in an endemic situation. In addition there is little information on immunity to AIVs in wild birds. In this context, the immunoecological approach may aid in understanding how to improve control strategies by increasing the penalty of virulence for the parasite. The interest of maternal antibody transfer study has for example recently been pointed out. Indeed, antibodies in eggs and hatchlings can reflect the mother's past exposure to pathogens and both life stages are more easily sampled than the adult stage, thus quantifying antibodies found in avian young could help clarify AIVs epidemiology.

Conclusions

The emergence of the HPAI during the past decades and the recent global spread of the HPAI H5N1 since the end of 2003 have dramatically changed the perception of risks, causing the death of a considerable number of domestic birds, important economic losses, and preparation of plans to fight potential pandemics. LPAI H9N2 is now circulating in domestic chicken and ducks worldwide and has already acquired receptor specificity found in humans, being consequently a better candidate than HPAI H5N1 to become pandemic. Wild birds have a long history of coevolution with AIVs. The origin of this unprecedented situation did not result from natural changes in the environment but is a human-made problem from anthropic modifications in poultry production systems for human food provisioning, where the AIVs can evolve in more virulent forms, and the globalization of exchanges without controlling and eradicating infected poultry. Despite the fact that migrating wild birds were frequently presented as the main source of the problem, and notably of the global dispersal, wild birds pose less risk of HPAI evolution, introduction, and spread than the poultry production and trade systems.

Overwhelming evidence supports the hypothesis that human movements of domestic poultry have been the main agent of global dispersal of the virus to date. The natural globalization of the exchanges of migratory birds seemed to hide the globalization of poultry products without strict health control, as the mechanism for disease spread. The inappropriate emphasis on wild birds contributed to reduce the probability that alternative mechanisms were fully considered. When HPAI viruses are widespread in poultry, spillover into wild populations is inevitable, potentially causing significant population losses of endangered species, such as the bar-headed goose in Asia. To date, the HPAI H5N1 was apparently not able to become endemic in wild birds and gradually died out despite repeated reintroduction from poultry.

Very high densities of animals and increased stress factors are particularly favorable for the maintenance and transmission of virulent agents, in particular HPAI. Paradoxically, the HPAI virus coupled with a fear of transmission by wild birds could lead to a reversion to battery farming, which increases risk of emergence of more virulent pathogens, rather than maintaining the current trend to better animal welfare resulting from free-range agriculture.

Global trade of poultry and domestic animals, legal or illegal, need improved regulation to decrease the capacity for infected poultry to spread the disease. The solution lays in controlling the poultry trade and production systems, by training farmers and developing veterinary infrastructures.

Considerable financial supports by richer countries and international cooperation are needed to help some affected countries. Given that the current controls for animal production and transport are poor, there is strong probability that the HPAI H5N1 subtype will remain endemic in many parts of the world. Comparisons between countries show that it is possible to control the epizootic disease. In Taiwan, Japan, and South Korea, the virus has disappeared. Control of trade and strong veterinarian surveillance were the keys to this success. Conversely in China, Vietnam, and Cambodia, where means were not implemented to control the epizootic disease, the virus is now endemic.

Acknowledgments

The authors sincerely acknowledge Lisa Ernoul for her editorial help during the preparation of this article. This work was funded by the French 'Agence Nationale de la Recherche' (ANR) 'Santé-Environnement/Santé au travail.'

See also: SARS.

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Article 5. High Influenza A virus infection rates in Mallards bred for hunting in the Camargue, South of France.

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Publié dans *Plos One*

OPEN ACCESS Freely available online

PLOS ONE

High Influenza A Virus Infection Rates in Mallards Bred for Hunting in the Camargue, South of France

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Abstract

During the last decade, the role of wildlife in emerging pathogen transmission to domestic animals has often been pointed out. Conversely, far less attention has been paid to pathogen transmission from domestic animals to wildlife. Here, we focus on the case of game restocking, which implies the release of millions of animals worldwide each year. We conducted a 2-year study in the Camargue (Southern France) to investigate the influence of hand-reared Mallard releases on avian influenza virus dynamics in surrounding wildlife. We sampled Mallards (cloacal swabs) from several game duck facilities in 2009 and 2010 before their release. A very high (99%) infection rate caused by an H10N7 strain was detected in the game bird facility we sampled in 2009. We did not detect this strain in shot ducks we sampled, neither during the 2008/2009 nor the 2009/2010 hunting seasons. In 2010 infection rates ranged from 0 to 24% in hand-reared ducks. The 2009 H10N7 strain was fully sequenced. It results from multiple reassortment events between Eurasian low pathogenic strains. Interestingly, H10N7 strains had previously caused human infections in Egypt and Australia. The H10 and N7 segments we sequenced were clearly distinct from the Australian ones but they belonged to the same large cluster as the Egyptian ones. We did not observe any mutation linked to increased virulence, transmission to mammals, or antiviral resistance in the H10N7 strain we identified. Our results indicate that the potential role of hand-reared Mallards in influenza virus epizootics must be taken into account given the likely risk of viral exchange between game bird facilities and wild habitats, owing to duck rearing conditions. Measures implemented to limit transmission from wildlife to domestic animals as well as measures to control transmission from domestic animals to wild ones need to be equally reinforced.

Citation: Vittecoq M, Grandhomme V, Champagnon J, Guillemain M, Crescenzo-Chaigne B, et al. (2012) High Influenza A Virus Infection Rates in Mallards Bred for Hunting in the Camargue, South of France. PLoS ONE 7(8): e43974. doi:10.1371/journal.pone.0043974

Editor: Patricia V. Aguilar, University of Texas Medical Branch, United States of America

Received March 27, 2012; **Accepted** July 27, 2012; **Published** August 27, 2012

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Funding: M. Vittecoq was supported by an AXA Ph.D. research fellowship. The authors thank the French "Agence Inter-e¨tablissement pour la Recherche et le De¨veloppement", the Centre National de la Recherche Scientifique, and the MAVA foundation for their financial support. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

During the last decade, awareness concerning the intimate links between human and animal health has rapidly increased in the context of disease emergence [1]. Indeed, approximately 80% of the infectious diseases that recently emerged were zoonotic [2]. The role of wildlife in emerging pathogen transmission to humans and domestic animals has in many cases been pointed out [3–5]. Conversely, pathogen transmission from domestic animals to wildlife has received far less attention, although the importance of this issue was often mentioned [6,7]. Indeed, contacts between wildlife and livestock or their environment sometimes result in wildlife diseases with conservation issues [8,9]. Besides, hand-reared animal releases into the wild for either conservation or exploitation purposes represent a particular case in which hand-reared individuals eventually share natural habitats with their wild congeners. In both cases such releases can dramatically influence disease dynamics in the surrounding wild animal populations [10–14].

In the present study we focused on the case of game restocking, which implies the release of millions of individuals worldwide each year [15]. Birds are the most frequently involved, with millions of individuals being released annually in Europe only. For example more than 3 millions red-legged partridges are released annually in Spain [15], and ca. 1.4 million Mallards are being so in France [16]. The Camargue region, a complex network of wetlands situated in the Rhone delta, is a major duck winter quarter [17] and a central place for wildfowl hunting in France [18]. Hunting is also among the most important economic activities in the area, which is one of the reasons for the massive Mallard releases in the Camargue [19]. At least 30 000 hand-reared individuals are released annually in the region [20]. Maximum Mallard numbers

in the wild are reached in September after the beginning of the hunting season, with 56 500 individuals on average over the last seven years (Gauthier-Clerc, unpubl. data), these numbers certainly include a mixing of wild and released Mallards. Given its central position on the flyway of many European migratory species, the Camargue is also a potential hotspot for the introduction and transmission of bird-borne pathogens [21].

For this reason, avian influenza viruses (AIV) have been studied since 2004 in the area. These negative-sense single stranded RNA viruses belonging to the *Orthomyxoviridae* family are commonly characterized by the combination of their surface proteins: hemagglutinin (HA) and neuraminidase (NA) [22,23]. AIVs are highly variable and undergo continuous genetic evolution via two mechanisms: i) accumulation of point mutations at each replication cycle, ii) reassortment involving gene segment exchanges that occur when a cell is co-infected by different viruses [24]. These mechanisms contribute to the emergence of new variants with the ability to transmit to new hosts and/or with epidemic or even pandemic potential. Aquatic birds, particularly Anseriforms (ducks, geese and swans) and Charadriiforms (gulls, terns and shorebirds) constitute their major natural reservoir [23,25]. The AIV circulating in wild birds are usually low pathogenic ones (LPAIV). LPAIV generally have little impact on their host [26,27], although some studies have reported a possible influence on migration capacities [28]. Besides, when LPAIV of H5 or H7 subtypes are transmitted from wild birds to domestic ones reared in artificial environments, their virulence can evolve to high pathogenicity [29,30]. Highly pathogenic avian influenza viruses (HPAIV), such as HP H5N1 strains currently circulating in Asia and Africa, are still of great economic concern, notably due to the cost of preventive actions including vaccination and massive birds culling [31]. Moreover, HPAIV infections represent a threat for human health since 603 HPAIV H5N1 human infections including 356 fatal cases have been reported worldwide since 2003 [32].

Relatively high prevalence of AIV was regularly detected in the wintering Mallard population of the Camargue (e.g. 5.4% prevalence during the 2006–2007 hunting season) [33]. Moreover a seasonal infection pattern was identified in Mallards during autumn and winter, with higher infection rates in early fall [33]. Mallards hence represent a focal study species in AIV research. Indeed wild Mallards are one of the main low pathogenic AIV natural reservoir host [34], and have proven to be healthy carriers of some of the H5N1 HPAIV strains [35]. However, to our knowledge no study ever aimed at investigating the potential role of hand-reared Mallards released for hunting in the epidemiology of AIV, despite the very large number of ducks being released in the wild annually.

To clear this gap we conducted a 2-year study in the Camargue to investigate the potential influence of hand-reared Mallard releases on AIV dynamics in surrounding wildlife. We first hypothesized that, owing to high density rearing conditions and to their genetic uniformity, hand-reared Mallards should be highly susceptible to AIV infections and could play an amplification role in AIV dynamics. This phenomenon has already been pointed out in red-legged partridge (*Alectoris rufa*) reared for hunting in Spain, where *Escherichia coli* prevalence was much higher in hand-reared populations before their release than in the wild ones [36]. To test this assumption we collected cloacal swabs from Mallards reared for hunting in several game bird facilities (GBF) in 2009 and 2010, and analysed these samples to measure AIV prevalence. Second, we hypothesized that AIV exchange occurs between wild and hand-reared Mallards, potentially leading either to the circulation of new strains in wild populations or to the amplification and dispersal of wild strains. Indeed, no barrier prevents AIV exchange between wild birds and hand-reared ducks in the GBF since water flows exist between pens and ponds used by wild birds. Moreover, as the GBF roofs are made of nets wild birds can deposit feces in the pens. Finally, hand-reared ducks are in direct contact with wild ones after their release. To investigate these issues, we tested shot waterfowl before and after hand-reared Mallards were sampled. Noteworthy, genetic analyses suggest that 76% of hunted Mallards in the Camargue have a captive origin [37]. Considering the low annual survival of released Mallards (0.8–15.9% depending on the release site) [37], the individuals we tested from the Camargue hunting bags certainly included a large proportion of ducks released some months before being shot. These released ducks cannot be differentiated morphologically from the wild ones [38]. Here we hence analyzed shot ducks as a whole since they are a representative sample of the Mallard population wintering in the Camargue, which is composed of individuals of both wild and captive origin that share habitats and can thus be considered as a single epidemiological unit.

Our third hypothesis was that any AIV strain potentially found in captive reared Mallards might present genetic characteristics linked to its circulation in domestic populations. We therefore performed a molecular study of the identified strains in order to look for such characteristics, and in particular to test for the presence of mutations known to be associated with increased virulence, since it has been highlighted that artificial environments are favorable to the appearance of such mutations in birds [30]. We also searched for mutations linked with transmission to other species including humans, since some studies proved that they can be acquired during their transmission among birds [39]. Thirdly, we tested for the presence of mutations conferring resistance to common antiviral drugs, since such resistance has recently been recorded in wild birds, notably in Sweden [40]. Lastly, full sequence analysis of some strains was performed, so as to get insight into their geographic origin through a phylogenetic study, and to determine their relatedness with strains, which have caused human infections in the past.

Results

Infection Rates and AIV Strains Involved

The infection rates in the different GBF were determined by real-time RT-PCR targeting the conserved M gene of AIV (Table 1). A very high infection rate (99%) was observed in the single GBF sampled in 2009. In 2010 the infection rates ranged between 0 and 24%, being of 8% in the farm infected in 2009. Initial subtyping, searching for H5, H7, H9, N1, and N7 by real-time RT-PCR performed on positive samples led to the identification of an H10N7 strain in the GBF in 2009 and further testing for H10 by real-time RT-PCR showed that the outbreak involved a single H10N7 virus (named H10N7 Camargue below). The viral strains involved in the infections observed in 2010 were LPAIV. H5, H7, H9, H10, and N7 subtypes were searched among them but none was detected.

In shot ducks, mean AIV infection rates were 15% and 5% during the 2008/2009 and the 2009/2010 hunting seasons, respectively (Table 2). During these two periods the maximum monthly infection rates (respectively 20% and 16%) were observed in September (Table 2). All the involved strains were LPAIV but an important proportion of these were of the H5 subtype (2008/2009:17%; 2009/2010:30%). No H7 or H10N7 subtype was detected in AIV found in shot ducks.

Table 1. AIV infection rates in hand-reared ducks.

Year	GBF	Number of samples	Duckling age (weeks)	Days before release	AIV prevalence (%)
2009	GBF1	222	6	21	99
2010	GBF1	99	6	2	8
	GBF2	100	7	0	0
	GBF3	100	12.5	0	24
	GBF4	100	8	0	0

doi:10.1371/journal.pone.0043974.t001

H10N7 Camargue Strain

Phylogeny. The H10N7 Camargue strain was isolated in embryonated chicken eggs and amplified. Determination of the sequence of the whole genome was performed in order to gain insights into its phylogeny and molecular characteristics. The phylogenetic analysis of four H10N7 Camargue isolates confirmed that they were very closely related to each other and formed a cluster (Figures 1 and S1). Analysis of each of the sequences of the 8 viral segments highlighted some shared characteristics (Figures 1 and S1). First, the sequences of all segments were clearly distinct from those of LPAIV strains from the North-American lineage. Second, none were closely related to highly pathogenic viruses (H5N1 or H7N7) that circulated in Europe and Asia. However, the PB2 segment was more closely related to the Asian HP H5N1 cluster than to most of the European LPAIV strains we included in our phylogenetic analysis. This finding suggests an ancient Asian origin of this segment. Third, the sequences of all segments were closely related to Eurasian strains. Interestingly, the most closely-related Eurasian strains differed between segments. As an example the LPAIV strain A/mute swan/Hungary/5973/2007 (H7N7) was very closely related to the H10N7 Camargue virus for the N7 segment, but less so for the M segment and belonged to a distant phylogenetic group for the PB2 segment. In the same way, the LPAIV strain A/mallard/Netherlands/9/2005 (H7N7) was closely related to the H10N7 Camargue virus for the M segment, whereas its PB2 segment belonged to a distinct phylogenetic group. African strains that we included in our analysis were part of Eurasian clusters and clearly distinct from American strains. For one of them, A/Pekin duck/South Africa/AI1642/2009 (H10N7) the NS, N7 and H10 sequences were closely related to those of the H10N7 Camargue virus but available sequences of two other segments (NP and M) were clearly distinct. Finally, it is important to note that based on the analysis of partial sequences of the H10 (data not shown) the H10N7 strains that caused human infections in Australia (Genebank accession numbers ADG58106 and ADG58107; Ratnamohan,V.M. and Dwyer, D.E. unpublished) are clearly distinct from the H10N7 Camargue strain. On the contrary Egyptian H10N7 strains that circulated in birds in 2004, i.e. at the time of the detection of two human infections [41], are relatively closely related to the H10N7 Camargue strain. However, this relatedness is difficult to interpret since the involved nodes are not strongly supported statistically (Figure 1).

Study of the extremities of the viral segments. We also determined the sequences of the extremities including the full non-coding sequences of all segments of the H10N7 Camargue strain, which to our knowledge was not performed before for any H10N7 strain. The extremities of the N7 segment revealed two characteristics that confirmed the phylogenetic distinction between the H10N7 Camargue strain and those of the American lineage. First, in the non-coding region of the 5′ extremity, an A was found at position 1440, as commonly seen in Eurasian strains, while strains of the American lineage possess a T at this position. Second, at position 145–147 of the 3′ extremity that belongs to the coding region the Camargue strain, like other Eurasian ones, has a supplementary amino acid compared to strains of the American lineage.

Molecular characterization. The molecular analysis of the H10N7 Camargue strain is detailed in Table 3. The focus strain is a low pathogenic one since the HA segment possesses a monobasic cleavage site. HA, NA and PB2 sequences all presented some typical avian characteristics (see Table 3). No known determinants of adaptation to mammals (humans or mice) were detected. In addition, we did not identify any mutation known to be linked with increased virulence in mammals. Yet, we detected the V149A mutation in PB2, which is associated with high virulence in chickens. However, 149A was also observed in low pathogenic H7 strains sampled from wild and domestic ducks in China. This suggests that this mutation alone does not increase the virulence in ducks [42]. Furthermore, no mutation was detected in the H10N7 Camargue strain at positions known to be linked with reduced susceptibility or resistance to neuraminidase inhibitors or M2-blockers.

Table 2. AIV infection rates in shot ducks.

	2008/2009		2009/2010	
Month	Number of samples	AIV prevalence (%)	Number of samples	AIV prevalence (%)
Sep.	170	20	97	16
Oct.	71	4	166	2
Nov.	22	18	139	2
Dec.	1	0	88	8
Jan.	1	0	63	0

doi:10.1371/journal.pone.0043974.t002

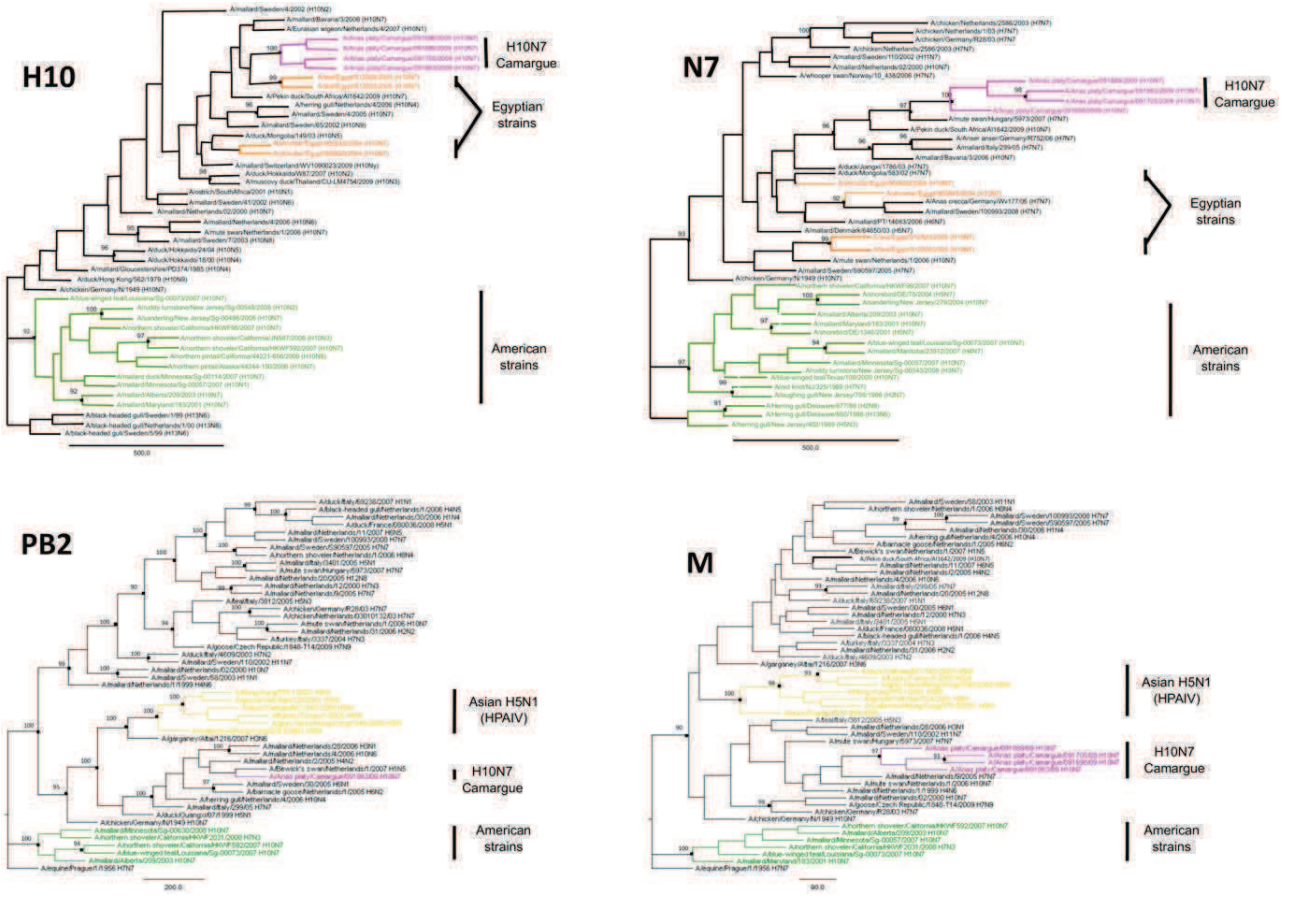

Figure 1. Phylogenetic trees of H10N7 Camargue viruses. ML phylogenies reconstructed from sequences of the H10, N7, PB2 and M segments. Topological supports summarized from 100 ML bootstrap replications are shown when ≥90. H10N7 Camargue viruses are in purple. Egyptian strains are shown in orange. American viruses are in green. Asian H5N1 highly pathogenic avian influenza strains are in yellow.
doi:10.1371/journal.pone.0043974.g001

Table 3. Molecular characteristics of the H10N7 Camargue strain. Amino acid substitutions related to known phenotypic traits identified for the H10N7 Camargue strain are in bold.

Characteristics/Phenotype	Segment	Sequence/Substitution	Reference	H10N7 Camargue
High pathogenicity	HA	RERRRKKR	[60]	Absent
Typical avian RBS	**HA**	**138A, 190E, 194L, 225G, 226Q, 228G (H3 numbering)**	**[61]**	**Present**
Adaptation to human α2,6 receptor	HA	H183N	[62]	Absent
High hemadsorption activity (typical of avian viruses)	**NA**	**367S, 370S, 372S, 400N, 403W, 432K**	**[63,64]**	**Present except for the 432 position (432E)**
Binding specificity for avian sialic acids	**NA**	**275I, 431P**	**[65]**	**Present**
Mammalian Host range determinant	PB2	D701N	[66]	Absent
Mammalian Host range determinant	PB2	E627K	[67,68]	Absent
High virulence in pigs	PB2	D92E	[69]	Absent
High virulence in chickens	**PB2**	**V149A**	**[70]**	**Present**
Typical avian motif	**PB2**	**Motif ESEV on the C-terminal position**	**[71]**	**Present**
Amantadine resistance	M2	A30T	[72]	Absent
Amantadine resistance	M2	S31N	[73]	Absent
Amantadine resistance	M2	G34A	[74]	Absent

doi:10.1371/journal.pone.0043974.t003

Discussion

AIV Infections in Hand-reared Mallards

This study confirms our first hypothesis that hand-reared Mallards are highly sensitive to AIV infections. Indeed, we detected high infection rates in the GBF we sampled in 2009 (99%) and in 2010 (up to 24%), whereas the prevalence usually observed in duck populations very rarely exceeds 20% in the wild [33,43,44]. This shows that massive outbreaks can occasionally affect GBF, potentially impacting on AIV dynamics in the wild populations with which hand-reared Mallards are in contact before and after their release into the wild. This pattern has already been highlighted in Spain where rabbit (*Oryctolagus cuniculus*) restocking caused sarcoptic mange dispersion in the wild rabbit population, leading to massive decrease in numbers [11]. The possible outcomes of GBF infection onto wild duck populations should here depend on the origin of the strain involved. Indeed, low pathogenic AIV strains that naturally circulate in wild ducks have little impact on their health [26]. Our data did not permit to determine the origin of the strain we identified in 2009. Indeed, we did not detect the H10N7 Camargue strain among the samples collected on shot Mallards, neither during the hunting season that preceded the release of the infected individuals, nor during the following one.

AIV Exchanges between Hand-reared and Wild Ducks

Our sample size for hunted Mallards was reasonably large (n = 299 in 2008/2009 and 555 in 2009/2010). Moreover, the sites where we sampled hand-reared and shot Mallards were close to each other (see Figure 2; for instance one of the hunting estates we sampled was within 4 km of the GBF where we detected an AIV outbreak). Nevertheless, our sampling effort was limited in time to the hunting seasons: we do not have any data from February to August 2009. We therefore cannot exclude that the H10N7 strain originated from wild birds. Population density during the breeding season is low in wild Mallard [45], and thus, even if the H10N7 Camargue virus originated from wild birds, hand-reared Mallards would then have played an amplifying role. In addition, commercial trade of Mallard chicks is common and most of the juveniles sampled in this study traveled 600 km from the producer when one-day old. It is also possible that the H10N7 Camargue strain originated from the birthplace of the chicks. If so, domestic ducks would have both played an amplifying role and potentially a dispersal one, since they could have spread a new strain in wild duck populations.

The lack of samples during the weeks that directly followed the release of the positive Mallards (because the hunting season had not yet started) could explain why we did not detect the H10N7 Camargue virus in wild individuals. Furthermore, the lack of large-scale dispersal of that H10N7 strain could be due to different parameters. First, we sampled the hand-reared ducks of the GBF1 21 days before their release. It is possible that only few birds were still excreting viruses when released into the wild, since excretion time in birds can be as short as 2 days (although it can also last up to 30 days in some cases) [46–48]. Second, a parallel capture-recapture demographic study ran in the Camargue has shown that hand-reared Mallards exhibit low monthly survival before the hunting season (only 44% survive from release until the onset of the hunting period on average) [49]. Moreover, their dispersal capacities appear to be very low [49]. The same pattern has been observed for red-legged partridges released in Spain [13]. Although intestinal parasites were much more numerous in the individuals living in the hunting estates where domestic birds were released, this was interestingly not observed in the neighboring estates [13]. It is possible that the poor survival and low dispersal of the hand-reared Mallards after release limited the potential spread of the H10N7 Camargue virus within the Mallard population in the wild.

Besides, we did not detect any H5 subtype circulating in the GBF whereas 23% of all the AIV we isolated from samples collected on shot Mallards in the Camargue belonged to this subtype. Again we could not highlight any AIV exchange between ducks living in captivity and in the wild. Yet, hand-reared ducks are exposed to wild bird feces and GBF are connected to wild bird habitats through common use of water. Thus, AIV exchanges are very likely to exist. Our results highlighted that: i) GBF represent an epidemiological compartment into which important AIV outbreaks can occur; ii) a significant proportion of Mallards wintering in the Camargue are infected by LPAIV, including H5 strains that are known to be able to evolve to HPAIV in domestic birds [30]. Knowing that rearing conditions in the GBF may favor AIV exchanges between wild and hand-reared Mallards, these findings outline the need for influenza surveillance in GBF to prevent HPAIV outbreaks in both wild and domestic birds in the region.

Geographical Origin of the H10N7 Camargue Strain

We used the phylogenetical analysis of whole-genome sequence of the H10N7 Camargue strain as a supplementary tool to get insights into its evolutionary and geographical origins. The four isolates studied clearly belonged to a cluster that did not include other strains. The infections we detected were therefore certainly due to a single introduction event. All the viral segments of the H10N7 Camargue strain belong to the Eurasiatic lineage also including African strains. The sequences of the extremities of the N7 segment, that exhibit features characteristic of the Eurasian lineage viruses, confirmed this finding. The Eurasian lineage is clearly distinct from the American lineage and our observations further support the fact that inter-hemispheric AIV exchanges are rare. The Camargue strain was also distinct from the H10N7 viruses that previously caused human infections in Australia, but belonged to the same large cluster as the Egyptian H10N7 strains that circulated in birds when H10N7 human infections occurred in this country. These Egyptian strains did not cause severe disease. Nevertheless, this information raises concern about a possible transmission to humans of strains such as the Camargue one, in particular to the GBF owners and hunters who live in close contact with ducks. Interestingly, the strains that are genetically closest relatives differ for each segment of the H10N7 Camargue strain. The Camargue strain thus seems to result from multiple reassortments between different Eurasiatic strains, a common phenomenon which has been illustrated by many studies [50,51]. Regrettably, we could not determine if the H10N7 Camargue strain was more closely related to strains observed in the wild or in GBF. Indeed, the data associated with viral sequences from AIV infected Mallards in GenBank are often too scarce to determine if the sample was taken on a wild or a domestic individual. This underlines the need for more detailed information on AIV sequences included in common databases.

Molecular Characteristics of the H10N7 Camargue Strain

The molecular analysis of the H10N7 Camargue strain allowed us to question whether it may exhibit some characteristics related to its circulation in an artificial environment, namely some mutations linked to increased virulence, resistance to antiviral drugs or transmission to humans. Our results do not support this assumption. The sequences of the studied segments presented typical avian characteristics. No mutation known to contribute to

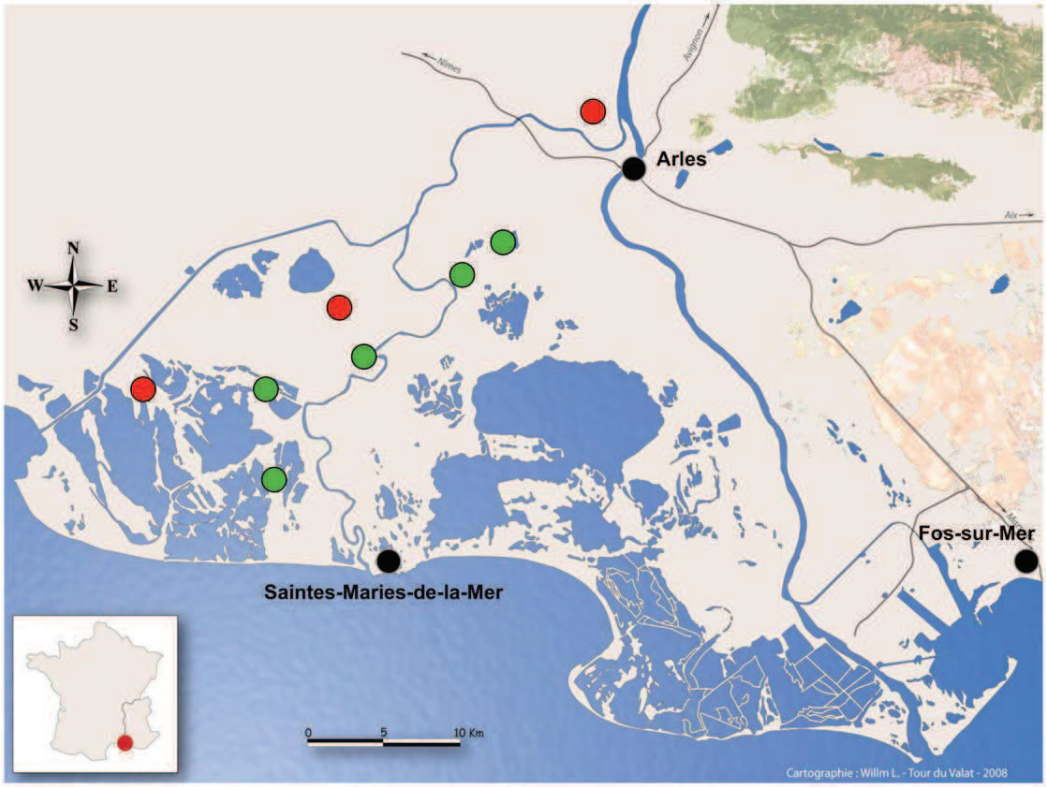

Figure 2. Situation of the sampling sites. Green circles represent the hunting estates where dead Mallards were sampled. Red circles represent the game bird facilities. One of the rearing estate (further named GBF3) is not represented since it is situated 50 km east from Arles, but the Mallards were sampled just before their release in several ponds of Fos–sur–Mer.
doi:10.1371/journal.pone.0043974.g002

transmission to humans was detected. Moreover, the Camargue strain is a low pathogenic one like all viruses isolated during our research program in the Camargue since 2005 [33,52]. No mutation conferring antiviral resistance was detected. This analysis therefore failed to highlight any risk factor linked to this particular strain. Yet, one must keep in mind that we only looked for mutations previously described in the literature, while many others may remain undiscovered so far. It is also important to stress that most experimental studies focusing on mutations modifying host specificity and virulence are carried out on chicken, although it has been demonstrated that phenotypic outcomes of a given mutation can greatly differ from one host species to another [42].

In conclusion, our results point out the important role that could be played by hand-reared ducks released for hunting in AIV dynamics. As we did not detect similar AIV strains in shot and hand-reared ducks we could not prove that AIV exchanges exist between these two epidemiological compartments. Yet, due to rearing conditions in GBF, AIV exchange risks seem to be high enough to urge for sanitary control of hand-reared animals prior to their release into the wild, which appears to be highly insufficient so far. Such surveillance would also prevent HPAIV circulation that may arise from the evolution in GBF of H5 LPAIV that proved to be commonly infecting free-living ducks in the Camargue. The world organization for animal health (OIE) stresses that surveillance of AIV infection should be applied to all domesticated birds including those used "for restocking supplies of game" [53], but control measures generally appear to be poor in game bird rearing estates. This surveillance gap has recently been highlighted in the USA, where game bird holders reported very variable sanitary practices [54]. The problem appears similar in Europe, including France. Additionally, according to French law, game birds should be ringed to allow for the differentiation of wild and released individuals [55]. Most of GBF owners ignore this obligation. If released birds were clearly identified the specific role they may have in AIV dynamics could be addressed both before and after their release. Our study illustrates the reality of the epidemiological consequences that can result from surveillance gaps and the knowledge that could be gained if released individuals were recognizable. Sharing knowledge and strictly controlling viral exchanges between wild birds and all kinds of domestic ones represent major steps to anticipate and face HPAIV epizootics.

Methods

Ethics Statement

This study has been submitted for approval and the results reviewed by the Scientific Council of the Tour du Valat Foundation. Birds were handled, ringed and sampled under the supervision of a veterinarian (Michel Gauthier-Clerc) and a registered duck ringer of the « Museum National d'Histoire Naturelle » of Paris (Matthieu Guillemain). All procedures were carried out in accordance with the permit delivered by the prefect of Paris to the « Office National de la Chasse et de la Faune Sauvage » (permit n 2009–014).

Bird Sampling

Cloacal swabs were taken in the Camargue between August 2008 and July 2010 to collect faecal samples from hand-reared Mallards and wild ducks (see sampling sites in Figure 2). Using a network of hunters, 299 and 552 samples were taken on locally shot Mallards during the 2008/2009 and 2009/2010 hunting seasons (September to January), respectively. Samples were collected on hand-reared Mallards during the pre-release ringing sessions of these birds in one GBF in 2009 and 4 GBF (including the one sampled in 2009) in 2010. Ducks were juveniles (5 to 10 weeks old), reared in open-air and released immediately or within a few days or weeks after sampling (Table 1). Samples were stored in 3 ml of universal transport medium (Biolys kit) and frozen at −80°C until molecular analysis was performed.

RNA Isolation and Virus Detection

RNA isolation from 140 µl of each sample was carried out using a Macherey-Nagel NucleoSpin 96 virus system with RNA elution into a final volume of 60 µl. Influenza A virus was detected by real-time RT-PCR targeting the conserved matrix as describe previously [56]. All real-time RT-PCR assays were performed on a LightCycler 480 (Roche Diagnostic) in a final volume of 10 µl with 2.5 µl RNA, 0.5 µM of each primer, 0.2 µM probe and 0.4 µl enzyme mix using a Superscript III Platinum One-Step quantitative RT-PCR system (Invitrogen). The reaction was carried out with the following temperature profile: 15 min at 45°C, 3 min at 95 C, 50 cycles of 10 s at 95°C, 10 s at 55°C, 20 s at 72°C, and finally 30 s at 40°C. Positive samples were further tested for H5, H7, H9, N1 and N7 subtypes using the same real-time RT-PCR technique (primers available upon request).

Virus Isolation and Characterization

For 10 RT-PCR positive samples, 200 µl of specimen was inoculated in the allantoic cavity of 10-days-old embryonated hen's eggs. The allantoic fluid was harvested after 72 h at 37°C and influenza A virus was detected by hemagglutination assay with hen erythrocytes. Viral RNA was purified from allantoic fluid using a QIAamp viral RNA mini kit (Qiagen) following manufacturer's instructions. HA subtype of virus isolates was determined by RT-PCR and sequencing of the HA_0 cleavage site region using a universal set of primers as described previously [57]. HA sequence was analyzed with the basic local alignment search tool available from NCBI (data not shown) and confirmed by sequencing of the whole HA gene using a set of H10-specific primers (primer sequences available upon request). The NA subtype was deduced from this analysis and confirmed by RT-PCR and sequencing of 172 nt of the NA using a set of H10-specific primers (primer sequences available upon request).

Gene Sequencing and Phylogenetic Analysis

Amplification of viral RNA extracted from 4 virus isolates was carried out using a Superscript Platinum One-step RT-PCR system (Invitrogen) and primers specific of each segment. Sequencing was done using a Big Dye Terminator V1.1 kit and a sequencer ABI DNA Analyzer 3730XL (Applied Biosystems). Sequences of all primers are available upon request. Sequences of the whole viral genome were analyzed with CLC Main Workbench 5.6.1. We performed alignments for the 8 segments using sequences available from the Influenza Sequence Database with CLC Main Workbench 5.6.1. Phylogenetic trees were constructed using maximum parsimony (MP) methods with the dnapars program of the PHYLIP 3.68 package and the maximum likelihood (ML) with the software PhyML 2.4.4. Evolutionary model was selected using Model Generator 0.85 [58]. Nodal supports were assessed with 100 bootstrap replicates generated for each method.

Determination of 3′ and 5′ End Non Coding Sequences of the 8 Segments of the H10N7 Virus

Viral genomic RNA was extracted using the QIAamp Viral RNA Mini kit (Qiagen) from 140 µl of allantoic fluid according to the manufacturer's recommendations. The RNA was eluted in 60 µl of RNase-free water and the 3′ and 5′ NC regions were amplified as previously described [59] using an anchored $(dT)_{14}$ oligonucleotide and primers specific for the coding sequences of both segments for reverse transcription and amplification. After purification, the PCR products were sequenced with internal oligonucleotides using a Big Dye terminator sequencing kit and an automated sequencer (Perkin Elmer). All sequences of the primers are available from the authors upon request.

Rapid Molecular Diagnostic

One-step real-time RT-PCR assays were developed to be specific of the virus identified. All influenza A positive samples detected between August 2008 and July 2010 on wild and domestic Mallards were tested for H10 and N7 subtypes respectively. Detection was performed in the same conditions as described above except annealing temperature was decreased from 55°C to 50°C using the following primers and probes: H10-780Fw (5′-TCT-GAT-AGC-ACC-GAG-CC-3′) and H10-888Rv (5′-ACC-CCC-TCT-CCA-AAA-AC-3′) primers and H10-832 +MGB 5′ [6Fam]-CAA-TCA-GAC-GCA-CCA-AT-[MGBNFQ]3′) probe specific of H10 subtype; N7-1260Fw (5′-CAA-TCC-ATG-CTT-TTA-CGT-AG-3′) and N7-1334Rv (5′-CTG-TTA-CTT-GTC-CAC-CAT-3′) primers and N7-1306Probe(-) (5′ [Fam]-CCT-CTT-CGG-GTC-TTC-CTC-T-[BHQ-1]3′) probe specific of N7 subtype.

Supporting Information

Figure S1 Phylogenetic trees of H10N7 Camargue viruses. ML phylogenies reconstructed from sequences of the NP, PA, NS and PB1 segments. Topological supports summarized from 100 ML bootstrap replications are shown when ≥90. H10N7 Camargue viruses are in purple. American viruses are in green. Asian H5N1 highly pathogenic avian influenza strains are in yellow.
(TIF)

Acknowledgments

We are grateful to game bird facility owners, hunting managers and hunters who authorized us to collect samples from Mallards. We also thank two anonymous referees for their valuable comments.

Author Contributions

Conceived and designed the experiments: MGC FT MG. Performed the experiments: MV VG JC BCC MGC. Analyzed the data: MV VG SW MGC FT MG JC FR. Wrote the paper: MV VG SW MGC FT MG JC FR BCC.

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Article 6. Laridae: a neglected reservoir that could play a major role in avian influenza virus dynamics.

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En préparation.

Title

Laridae: a neglected reservoir that could play a major role in avian influenza virus dynamics.

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Abstract

Highly pathogenic avian influenza viruses remain of great concern worldwide due to their economic impacts and the threat they represent to human health. Anatidae and Laridae are two taxa that contain the main natural reservoir hosts of avian influenza viruses (AIVs). Species belonging to these families may act as important epidemiological links between wildlife, domestic birds, and humans. They represent key interfaces between their epidemiological compartments: wildlife, domestic birds and humans. In order to prevent and control emerging infectious diseases, it is very important to address all potential reservoirs. However, the potential relevance of Laridae to AIV dynamics has remained unexplored. Here, we aim to shed light on current knowledge about AIVs in Laridae using an evolutionary approach, and explain why this group may represent a reservoir that should not be neglected. We focus on the three main steps of all parasitic cycles: transmission, multiplication and propagation. We point out the ecological differences that exist between Laridae and Anatidae and that could explain the preferential transmission route differences detected and the evolution of specific AIV subtypes in Laridae. Second, we stress the variability in temporal patterns of AIV exposure in Laridae and discuss the role of immunity in shaping these patterns. Third, we highlight the important role Laridae may play in intercontinental

exchanges of AIVs. Fourth, we emphasize that Laridae occupy an important epidemiological position at the interface between wildlife, domestic birds, and humans. We conclude by encouraging transdisciplinary research and propounding the need for further progress in understanding AIV dynamics.

Keywords: Gull, terns, evolutionary ecology, life history traits, dispersal.

Contents

I. Introduction 1
II Preferential transmission routes 2
III Temporal variability in AIV infection patterns 4
IV Spatial patterns of AIV dynamics 7
V. AIV exchanges between Laridae and other host groups 8
VI. Conclusion 10

I. Introduction

Over the last decades, human activities including animal rearing practices, land use changes, and commercial transport have given pathogens more opportunities to infect new hosts (Harvell *et al.* 1999; Patz *et al.* 2004; Lebarbenchon *et al.* 2008; Lebarbenchon *et al.* 2010). Such opportunities have led to the emergence of numerous infectious diseases in domestic animals and humans, most of which were originally circulating in wildlife (Daszak *et al.* 2000, Gortazar *et al.* 2007). As an example, at the beginning of the century, SARS (Heymann and Rodier 2004, Peiris *et al.* 2004, Wang & Eaton 2007) and Ebola (Leroy *et al.* 2005; Pourrut *et al.* 2005) were pathogens of fruit bats yet caused outbreaks in humans in Asia and Africa. These emergences were eventually linked to anthropogenic activities, in particular the consumption and trade of bushmeat. Thus, understanding pathogen dynamics in natural reservoirs is a critical part of protecting the health of humans and domestic animals.

Highly pathogenic avian influenza viruses (HPAIVs) provide a good example of pathogens emerging from wildlife that are of great concern worldwide due to their economic impacts and the threat they represent for human and animal health (Chen *et al.* 2005, De Wit & Fouchier 2008, Ferguson *et al.* 2005, Li *et al.* 2004). Avian influenza viruses (AIVs) are classified into different subtypes of the form HxNy based on their combination of two surface proteins, Hemagglutinin (H1-17) and Neuraminidase (N1-9), which are important targets for the immune system (Webster *et al.* 1992; Earn, Dushoff & Levin 2002; Olsen *et al.* 2006; Tong *et al.* 2012). Low pathogenic avian influenza viruses (LPAIVs) that circulate in poultry can evolve into more virulent HPAIVs; one example includes H5N1 HPAIV strains, which cause high mortality rates in poultry (Toshihiro Ito et al. 2001; Camille Lebarbenchon 2010). To date, only H5 and H7 subtypes are known to be able to evolve from low to high pathogenicity in this system (Alexander 2000, Banks *et al.* 2001, Fouchier *et al.* 2007).

Wild waterbirds, in particular Anseriformes (mainly Anatidae: ducks, geese, and swans) and Charadriiformes (Charadriidae and Scolopacidae: waders; Laridae: gulls and terns), are the natural reservoirs of LPAIVs, which generally cause no or few symptoms in their hosts (Alexander 2000, Webster *et al.* 1992). Nevertheless, their impact may be exacerbated by other infections or environmental conditions, potentially resulting in delayed migration or weight loss (Latorre-Margalef *et al.* 2009). Most combinations of the two surface proteins have been found in Anseriformes and Charadriiformes (Webster *et al.* 1992; Earn, Dushoff & Levin 2002; Olsen *et al.* 2006), except for H17, which was recently discovered in bats (Tong

et al. 2012). Phylogenetic analyses suggest that influenza viruses have evolved from an initial aquatic bird reservoir into host-specific lineages (Horimoto & Kawaoka 2001). Because they host a high diversity of virus subtypes, wild waterbirds have been and are still a source for viruses that can evolve specificity for domestic species and humans.

For various reasons, including the ease with which hunted species can be sampled as well as high contact rates between wild ducks and poultry, most epidemiological studies have focused on AIV circulation in Anatidae, whereas much less attention has been given to Charadriiformes (Fig. 1). Nevertheless, in order to anticipate and respond to the emergence of new viruses, it is essential to be better informed about virus circulation within wild reservoirs, without neglecting any key species (Haydon *et al.* 2002). Among Charadriiformes, waders (Charadriidae and Scolopacidae) can be distinguished from gulls and terns (Laridae) (IOC 2012). Surveillance activities performed at other geographical locations, such as in Europe, Alaska, and Australia, found AIV circulation in wader populations to be limited (Hurt *et al.* 2006; Munster *et al.* 2007; Winker, Spackman & Swayne 2008; Hanson *et al.* 2008; Munster & Fouchier 2009). Knowing that AIV circulation in waders appears to be restricted (Stallknecht & Brown 2007), we focused in this review on the Lariae group.

The Laridae family comprises 102 species, some of which are present on every continent and ocean, e.g., the Arctic tern (del Hoyo 1996)(IOC 2012). In the Laridae family, there is variability in species ecology (Table 1). Some Laridae species that may carry AIVs are long distance migrants, abundant in freshwater habitats which are favourable to AIV persistence (Brown *et al.* 2009), and in contact with humans and domestic animals, which highlights the potential value of this group in veterinary and public health. The aim of this review is to shed light on the current knowledge about influenza viruses in Laridae using an evolutionary approach and explain why they represent an AIV reservoir that should not be neglected. To achieve this goal, we focused on the three main steps of all parasitic cycles: transmission, multiplication and propagation (Choisy 2010).

II Preferential transmission routes

Transmission of LPAIVs in wild waterfowl is mainly fecal/oral; individuals are infected when they ingest water contaminated by feces (Webster *et al.* 1992). Aerial transmission may also occur (Costa *et al.* 2011). To enter host cells and then replicate, LPAIVs need to attach to receptors displayed at the surface of target cells. Most receptors are glycans terminating in sialic acids (SAs) (Suzuki 2005; Nicholls *et al.* 2008). Two main types of SA receptors are

distinguished depending on the linkage between the terminal SA and the glycan chain: the α2,3-linked SA, which is known to be present in the mallard trachea and colon, and the α2,6-linked SA, which is present in the human upper respiratory tract. Virus histochemistry studies suggest that α2,3-SA-linked receptors are present in both the intestinal and respiratory tracts of some Laridae species. Furthermore, mallard H6N1 virus has been shown to attach to both Herring (*Larus argentatus*) and Franklin's gull (*Leucophaeus pipixcan*) intestinal and respiratory tract cells (Jourdain *et al.* 2011).

However, LPAIV replication appears to take place in different sites in the two groups and shedding patterns are different, which may subsequently impact the likelihood of transmission between them. In mallards, LPAIV replication predominantly occurs in the intestinal tract, and high concentrations of infectious virus are shed in feces (Ellstrom *et al.* 2008, Webster *et al.* 1978), even if oropharyngeal excretion is also observed (Costa *et al.* 2011, Ellstrom *et al.* 2008, Fereidouni *et al.* 2010, Jourdain *et al.* 2010, Kleijn *et al.* 2010). In contrast, in laughing gulls *(Leucophaeus atricilla*), LPAIVs are primarily shed via the oropharynx, suggesting that viral replication in the upper respiratory tract should not be neglected in Laridae (Costa *et al.* 2011). In Franklin's gulls (*Larus pipixcan*) and laughing gulls (*Leucophaeus atricilla*), experimental infection revealed that virus was shed via the oropharynx for up to 24 days (Bahl & Pommeroy 1977; Costa *et al.* 2011).

There are also important ecological differences between Laridae and Anatidae. Because the two groups often share wetlands, they theoretically could become infected through direct contact or contaminated fresh water (del Hoyo 1996). However, Laridae alimentation is distinct from that of Anatidae, which are herbivorous or granivorous and forage in freshwater to feed on plants. Laridae feed on invertebrates and fish, and some opportunistic species can even eat sick or dead birds, thus favoring the direct transmission of AIVs (Brown *et al.* 2008). Also, for Laridae species that feed exclusively in saltwater, the transmission through water may be impaired since high salinity is unfavorable to LPAIV persistence (Brown *et al.* 2009, Stallknecht *et al.* 1990).

As a consequence of these ecological differences, an alternative AIV transmission route for AIV may be evolutionarily maintained in this group. Because of the high density of individuals in breeding colonies, contact rates may also be higher, facilitating direct aerial transmission of viruses. In contrast, because of the adverse effects of salinity on LPAIV persistence, orofecal transmission could be infrequent in coastal habitats in which gulls and terns forage.

The maintenance of differential transmission routes could explain the evolution of different subtypes in the two taxonomic groups. Indeed, specific AIV-subtypes H13 and H16 are almost exclusively maintained in gull populations (Hinshaw *et al.* 1983; Kawaoka *et al.* 1988; Yamnikova *et al.* 2003; Fouchier *et al.* 2005; Olsen *et al.* 2006), while accounting for a small proportion of the AIVs found in other avian taxa, including Anatidae (Sivanandan *et al.* 1991; Munster *et al.* 2007). Gull viruses H13 and H16 have gene segments that are genetically distinct from those of other AIV viruses that circulate in different wild bird hosts, which suggests that these subtypes diverged from other LPAIVs relatively recently (Webster *et al.* 1992), although long enough ago to allow genetic differentiation (Munster & Fouchier 2009).

Although available data are consistent with this scenario, further investigations are clearly needed to confirm these hypotheses. Whenever possible, both cloacal and oropharyngeal swabs should be collected, which will provide information about the respective importance of these transmission routes in Laridae and Anatidae. Further experimental studies are also needed to directly examine AIV transmission in wild birds. In particular, future work on birds should be guided by already published work on influenza virus transmission between mammals in controlled conditions (e.g., [22-24]). Studies should investigate aerial, water-borne, and contact transmission dynamics of the strains associated with both Anatidae (e.g., H4 or H7) and Laridae (H13 or H16).

III Temporal variability in AIV infection patterns

Phylogenetic analyses suggest that influenza viruses have evolved from the aquatic bird reservoir into host-specific lineages (Horimoto & Kawaoka 2001). Within this bird reservoir gull specific lineages (H13 and H16) have evolved into a distinct group.

In Anatidae, AIV dynamics follow a clear seasonal pattern. Infection peaks are observed in the late summer or at the beginning of fall in both North America and Europe (Webster *et al.* 1992; Wallensten *et al.* 2007; Lebarbenchon *et al.* 2010). These peaks are thought to be primarily linked to the presence of large numbers of juveniles, which gather during and after their migration to wintering grounds and are immunologically naïve (Olsen *et al.* 2006). These infection peaks detected in Anatidae could also be predicted to occur in Laridae. However, current data do not support the existence of a parallel temporal infection pattern. For example, in the Delaware Bay (North America), where a large AIV surveillance program

is in place (877 Laridae sampled), most positive samples have been collected during the breeding period in May (Hanson *et al.* 2008). In contrast, in Northern Europe, infection rates in the 2,602 Laridae sampled were highest from June to August, and AIVs were not detected at all in many colonies during the breeding season (Munster *et al.* 2007). Furthermore, punctual infection peaks are seen in gull chicks but not in duck chicks, for which no data exist concerning natural LPAIV infection (Fouchier *et al.* 2005; Velarde *et al.* 2010). Infection rates reported in Laridae are generally lower than those detected in Anatidae. Olsen et al. (2006) reported a mean AIV prevalence level of 1.4% in gulls (n = 14505) and 9.5% in ducks (n = 34503). Considering these low prevalence levels in Laridae, sample sizes need to be very large to detect seasonal patterns, which could explain why no similar infection peak has yet been detected in this group. The infection peak detection may also depend on the species, the virus subtype, or the environment studied.

The average life span of birds in the two families, and thus the chance to acquire immunity, may explain the differences in temporal infection patterns. Laridae are generally long-lived birds, while the lifespan of Anatidae is usually short. This difference is even greater for duck and goose species that are hunted, such as the mallard (*Anas Platyrhynchos*), which is the main natural host species for AIVs (Stallknecht & Brown 2007). As an example, mean annual survival rates in North American and European adult Mallards are about 50% (Schekkerman and Slaterus 2008) while they usually reach 90% in adult gulls (Altwegg *et al.* 2007; Breton, Fox & Chardine 2008).

This difference in life spans might influence the immunity developed in Anatidae and Laridae populations and consequently modify AIV dynamics. On one hand, a long lifespan favors the development of acquired immune responses, and the protection afforded by these responses is supposed to last longer than in short-lived birds (Lee 2006). On the other hand, the establishment of a strong acquired immune response in adults may have an impact on the offspring by inducing a strong transfer of maternal antibodies in chicks through egg yolks (Gasparini *et al.* 2001; Boulinier & Staszewski 2008). Thus, the low prevalence levels of AIV observed in Laridae could be due to stronger/longer immune responses, linked to longer lifespan. This pattern could be reinforced by the fact that only two AIV-subtypes (H13/H16) are predominant in Laridae, potentially reducing the diversity of antibodies needed, while subtypes are much more diverse in Anatidae (e.g.Munster *et al.* 2007). Indeed, AIV antibodies seem to be subtype specific in birds even if cross-immunity exists between related subtypes (based on the molecular sequencing of the hemagglutinin and neuraminidase genes;

Latorre-Margalef 2012; Fereidouni et al., 2010) or non-related subtypes (Jourdain *et al.* 2010).

Although few data on immunity in Laridae are available, serological studies thus far support that the idea that immunity may be play a more important role in gulls. AIV seroprevalence levels are high and infection rates are low in Laridae populations (De Marco *et al.* 2005; Velarde *et al.* 2010). The few studies that have sought to determine the duration of antibodies persistence tend to confirm that they last longer in Laridae than in Anatidae. A study of pink-footed geese (Anatidae: *Anser brachyrhynchus*) showed, using ELISA tests, that LPAIV-specific antibodies persisted 343 days on average (Hoye *et al.* 2011). Experimental infection of mallards with different LPAIV strains demonstrated, using ELISA tests, that a strong immune response is detectable after viral inoculation, but that this response lasted less than a year in 88% of the ducks studied (Fereidouni *et al.* 2010). The duration of any anti-influenza antibodies in Laridae has yet to be assessed. However, a study focusing on another pathogen naturally circulating in Laridae colonies, *Borrelia burgdorferi,* showed an interannual persistence of detectable antibodies levels in a wild population of black-legged kittiwakes (*Rissa tridactyla*) (Staszewski *et al.* 2007b). Indeed, this species are primarily inhabit marine environments, which may limit their re-exposure to AIVs. Additionally, one year after vaccination against Newcastle disease (a pathogen not naturally encountered by the study population), 13 black-legged kittiwakes still had high levels of NDV-specific antibodies (Staszewski *et al.* 2007a). Moreover, a strong correlation between antibody titers in egg yolks and maternal plasma was observed (Staszewski *et al.* 2007a). Furthermore, the detection of AIV specific antibodies in 14% of the eggs sampled in French yellow-legged gull colonies (*Larus michahellis*; n = 466) tend to confirm that such transfers could influence AIV infection dynamics in Laridae populations (Pearce-Duvet *et al.* 2009).

Knowledge about AIV immune responses in wild birds remains scarce, and we are still a long way from fully understanding the mechanisms underlying AIV dynamics. Experimental infection studies are needed to clarify the course of AIV-specific immune responses at the individual scale. An adapted study design, that includes the rearing of Laridae chicks in the lab, should allow reveal the duration of protection afforded to chicks by maternal antibodies, which appears to play an essential role in temporal dynamics of AIVs. Long-term experimental infections would allow us to assess the persistence of antibodies against AIVs and their variability across Laridae and Anatidae species after single or successive LPAIV infections. AIV studies designed to follow host populations throughout their annual cycle

should be implemented and include both virological and serological sampling to shed light on the temporal dynamics of both infection and immunity.

IV Spatial patterns of AIV dynamics

It is possible that some Laridae species maintain a circulation of AIVs due to their ecology, which limits contact with other species. Yet, data are too scarce to confirm the existence of such an independent cycle. Indeed, AIV studies generally target a few species. For the 102 Laridae species known, epidemiologic data on AIV circulation is only available for 20 of them (Tab. 2).

Most existing studies tend to show that LPAIV dynamics emerge at the community rather than the population level. Indeed, previous studies investigating the effect of host species, geographic location, and sampling time on AIV prevalence levels across broad geographic areas and time scales found weak support for a species effect but evidence for phylogenetic clustering by space and time (Chen & Holmes 2009; Pearce *et al.* 2009; Ramey *et al.* 2010). As a result, transmission and reassortment of AIVs between species may be frequent (Chen & Holmes 2009; Pearce *et al.* 2009; Ramey *et al.* 2010; Reeves *et al.* 2011).

In North America, Anatidae migration flyways seem to represent important barriers to geneflow among LPAIVs (Lam *et al.* 2012). Similarly, molecular analyses of LPAIVs isolated from waterbirds (mostly Anatidae) revealed substantial levels of sequence divergence between Eurasian and North American strains due to the geographic separation (Ito *et al.* 1991; Kawaoka *et al.* 1998; Suarez & Perdue 1998; Widjaja *et al.* 2004; Olsen *et al.* 2006). A few Anatidae species, like Northern pintails (*Anas acuta*) and Steller's eiders, (*Polysticta stelleri*) have been identified as potential agents of exchange for Eurasian and American AIV strains, due to their migration routes and the isolation of intercontinental AIV reassortants in some individuals (e.g Pearce *et al.* 2009; Ramey *et al.* 2010). In contrast, numerous Laridae species undergo intercontinental migration, not only between Eurasia and North America, but also from North America to South America and from Eurasia to Oceania (del Hoyo 1996; Elphick 2007; Winker & Gibson 2010). Such movements appear to result in intercontinental AIV exchange. Most of the intercontinental reassortants that have been identified to date have been found in Laridae (e.g Pereda *et al.* 2008; Lebarbenchon *et al.* 2009; Wille *et al.* 2011), which seem to be the main carriers of AIVs, followed by Anatidae and shorebirds (Krauss *et al.* 2007; Ramey *et al.* 2010).

These studies suggest Laridae migratory patterns have a major role in mediating AIV intercontinental exchanges (Winker & Gibson 2010; Wille *et al.* 2011). Genetic exchanges are concentrated at key sites, where migratory flyways overlap and birds wintering in different continents gather in high densities. Alaska (Ramey *et al.* 2010), Delaware Bay (Wille *et al.* 2011), and the Camargue (Lebarbenchon *et al.* 2009) have all been shown to serve as intercontinental exchange hubs. In Alaska, where birds from up to six continents come to breed (Winker *et al.* 2007), reassortant Eurasian/North American LPAIV strains represent up to 85% of those isolated from both Anatidae and Laridae (Ramey *et al.* 2010).

To date, few studies have been conducted on other potential AIV exchanges. Efforts should concentrate more on the Southern hemisphere as data on AIV circulation in wild birds from this region, apart from Southeast Asia, are lacking. Researchers could take advantage of readily available ornithological knowledge and focus on species known to undergo intercontinental migrations, as well as on habitats situated at the crossroads of several waterbird migratory routes. For example, the Kamchatka region is the Eurasian counterpart of Alaska (Wille *et al.* 2011). The few studies that detected AIVs in both Laridae and Anatidae in Africa, Oceania, and South America were mostly conducted(??) in IBA, such as the Djoudj delta in Senegal (Gaidet et al. 2007), the coast of Tasmania (Haynes et al. 2009), and the Parana River basin in Argentina (Pereda *et al.* 2008). These studies will allow us to assess the relative role of Laridae and Anatidae in AIV intercontinental exchanges.

Such studies are the first steps toward a better understanding of AIV spatial dynamics, which are a crucial part of modelling AIV dispersion risks. In particular, an understanding of the timing and occurrence of infection within the annual cycle of migratory populations is required (Hoye *et al.* 2011). Studies focusing on the role of Northern pintails have shown that this migratory species can bring new strains from Eurasia to North America, which can then be spread into populations of sympatric species (Koehler *et al.* 2008; Pearce *et al.* 2009, 2010). Given such findings, it also appears essential to expand studies to include species that are sympatric with long-distance migratory birds during part of their life cycle.

V. AIV exchanges between Laridae and other host groups

At least one LPAIV epizootic in poultry was linked to a LPAIV subtype found in wild gulls (Sivanandan *et al.* 1991), which highlights their potential capacity to exchange AIVs with domestic birds. Few other data exist that attest to such exchanges. However, virus

histochemistry studies shows that a mallard H6N1 LPAIV stain was capable of attaching to both herring gull and domestic chicken (*Gallus gallus*) receptors (Jourdain et al., 2011). Moreover, Laridae species sometimes share wetlands with domestic species, which can favor AIV exchange (del Hoyo 1996).

Laridae species and humans can and do share viruses. Laridae species can be infected by LPAIV subtypes that are known to cause mild infections (asymptomatic to mild conjunctivitis) in humans, such as H9 and H7 (Sandrock, Kelly & others 2007). Moreover, Ellström et al. (2009) showed the presence of α2,6 SA receptors using lectine studies (Jourdain *et al.* 2011). Accordingly, H3N2 human influenza virus can attach to the trachea of the herring Gull (*Larus argentatus*) and the Franklin's gull (*Leucophaeus pipixcan*; (Lindskog *et al.* In Prep.)). Additionally, H16N3, which is most commonly found in gulls, can attach to human upper respiratory tract (Lindskog et al., in prep.).

What is more worrisome is that Laridae can host highly pathenogenic subtypes. At least 6 species of Laridae can be infected by H5N1 HPAIVs under natural conditions (Tab. 3). H5N1 HPAIVs have occasionally been isolated from dead or severely sick birds (Ellis *et al.* 2004; Liu *et al.* 2005), but also from apparently healthy birds (Muzinic *et al.* 2010; Savić *et al.* 2010). Experimental infection studies have shown that laughing gulls (*Leucophaeus artricilla*) and herring gulls (*Larus argentatus*) can serve as healthy carriers of H5N1 HPAIVs, although sometimes the infections were fatal (Perkins & Swayne 2001; Brown *et al.* 2006; Brown, Stallknecht & Swayne 2008). Additionally, it has been demonstrated that laughing gulls can get infected by H5N1 HPAIVs through the consumption of contaminated meat, which they are likely to encounter in nature due to their scavenging habits (Brown, Stallknecht & Swayne 2008).

Contact between humans and Laridae as a result of hunting is limited. Nevertheless, it may occur. For example, terns are regularly trapped on West African beaches (Boere & Dodman 2011). Contact between humans and Laridae is more commonly due to habitat sharing. Indeed, during the past decades, several large gull species have dramatically increased in abundance, especially in Europe and North America (Blokpoel & Spaans 1991). These species have colonized urban areas worldwide, taking advantage of anthropogenic resources such as garbage and trawling discards (Raven & Coulson 1997; Duhem *et al.* 2008; Lisnizer, Garcia-Borboroglu & Yorio 2011). They exist at the interface between pathogens circulating in humans and wildlife, which is emphasized by antibiotic-resistant bacteria originating from human populations being found in gulls (Dolejska *et al.* 2007; Gionechetti *et al.* 2008; Bonnedahl *et al.* 2009). Laridae might hypothetically mediate AIV transmission from wild

birds to humans and, occasionally, to domestic birds. This factor seems crucial to consider, especially knowing that Laridae can carry HPAIVs (Krauss et al. 2007; Suarez et al. 2004; Winker & Gibson 2010). Finally, as antiviral-resistant influenza strains have already been isolated from mallards (Järhult *et al.* 2011; Orozovic *et al.* 2011), Laridae might also favor the dispersal of this antiviral-resistant that start to spread in some human populations. Thus, future studies should focus on opportunisitically urban Laridae species that live in close contact with humans to gain insight into the AIV exchanges that may take place at this interface.

VI. Conclusion

This review highlighted the potentially crucial role of Laridae species in all steps of AIV cycle. The study of epidemiological systems must not neglect any reservoir and should not focus on the most frequent host group only. Further research is clearly needed to progress toward the understanding of AIV dynamics and should be optimized by taking advantage on evolutionary ecology approaches and ornithological knowledge.

Acknowledgements

Support from OSU OREME, ANR (11 BSV7 003 EVEMATA grant), AIRD and CNRS is acknowledged. J. Pearce-Duvet was supported by a Chateaubriand postdoctoral fellowship and a National Science Foundation International Research Fellowship. M. Vittecoq was supported by an AXA research fund PhD fellowship.

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Table 1. Variability in Laridae species ecology.

Life history traits				Implications
Demography	**Abundant** (e.g., American herring gull; *Larus smithsonianus*)		**Rare** (e.g., lava gull; *Leucophaeus fuliginosus*)	Parasite transmission more or less important
Habitat	**Live in marine environments** (e.g., black-legged kittiwake; *Rissa tridactyla*),	**Live in both marine and freshwater habitats** (e.g., yellow-legged gull; *Larus michahellis*)	**Prefer freshwater habitats** (e.g., ring-billed gull; *Larus delawarensis*)	Parasite transmission through abiotic reservoir
Migration and Movements	**Intercontinental migration** (e.g., Arctic tern; *Sterna paradisaea*)	**Slightly dispersive migration** (e.g., black-tailed gull; *Larus crassirostris*)	**Sedentary** (e.g., black-bellied tern; *Sterna acuticauda*)	Parasite transmission over more or less long distances
Contacts	**Direct contacts** (e.g., kelp gull; *Larus dominicanus*).	**Share habitats** (e.g., European herring gull; *Larus argentatus*)	**Very limited contact** (e.g., common tern; *Sterna hirundo*).	Interface between pathogens circulating in humans, domestic animals, and wildlife

Table 2. Laridae low pathogenic avian influenza (LPAI) subtypes.

This table presents the different subtypes of LPAIV and the different Laridae species in which they were detected.

Species	Haemagglutinin Influenza Virus	Neuraminidase Influenza Virus	References
Arctic tern *Sterna paradisaea*	H5	N3	(Obenauer *et al.* 2006)
Black-backed Gull *Larus fuscus*	H7,H13	N2	(Zakstel'skaja *et al.* 1972; Hinshaw *et al.* 1982; GenBank 2012; NIAID 2012)
Black-headed Gull *Chroicocephalus ridibundus*	H2,H4,H5,H6,H7,H13 ,H16	N1,N3,N5,N6,N4, N8	(Gresikova *et al.* 1979; Janout *et al.* 1979; Fouchier *et al.* 2003; Munster *et al.* 2007; Spackman *et al.* 2009; Germundsson *et al.* 2010)
Black-legged Kittiwake *Rissa tridactyla*	H4,H13,H16	N2,N3,N6	(Toennessen *et al.* 2011)
Black-tailed Gull *Larus crassirostris*	H1,H2,H4,H6,H13	N1,N2,N3,N6	(Slepuskin *et al.* 1972; Tsubokura *et al.* 1981; Otsuki *et al.* 1987)
Common Gull *Larus canus*	H5,H6,H13,H16	N1,N4,N6,N8	(Munster *et al.* 2007; Germundsson *et al.* 2010; Kohls *et al.* 2011)
Common Tern *Sterna hirundo*	H1,H2,H4,H7	N1,N7	(Becker 1966; Röhm *et al.* 1995)
Franklin's Gull *Leucophaeus pipixcan*	H6,H13	N2,N6,N9	(Bahl & Pomeroy 1977; Hinshaw *et al.* 1982, 1983)
Glaucous Gull *Larus hyperboreus*	H3,H5,H6,H13,H16	N1,N3,N8,N9	(Ramey *et al.* 2010; USDA 2012)
Great Black-backed Gull *Larus marinus*	H4,H6,H13,H16	N2,N6,N8	(Munster *et al.* 2007; Germundsson *et al.* 2010; Wille *et al.* 2011)

Great Black-headed Gull *Ichthyaetus ichthyaetus*	H5,H9,H13	N2,N3,N6	(L'vov *et al.* 2001)
American Herring Gull *Larus smithsonianus*	HA 1 to 13	NA 1 to 9	(Widjaja *et al.* 2004; Obenauer *et al.* 2006; NIAID 2012)
European Herring Gull *Larus argentatus*	H1,H2,H5,H6,H7,H10 ,H13,H14,H16	N1,N2,N3,N4,N5, N6,N8	(Zakstel'skaja *et al.* 1972; Hinshaw *et al.* 1982; Kawaoka *et al.* 1988; L'vov *et al.* 2001; Munster *et al.* 2007; Germundsson *et al.* 2010; Marchenko *et al.* 2010; Kohls *et al.* 2011; NIAID 2012)
Kelp gull *Larus dominicanus*	H13	N9	(Pereda *et al.* 2008)
Laughing Gull *Leucophaeus atricilla*	HA 1 to 13	NA 1 to 9	(Lee *et al.* 2001; Widjaja *et al.* 2004; Obenauer *et al.* 2006; NIAID 2012)
Mediterranean Gull *Ichthyaetus melanocephalus*	H9	N2	(Lebarbenchon *et al.* 2007)
Relict Gull *Ichthyaetus relictus*	H2,H9,H10	N2,N7	(Chen *et al.* 2006)
Ring-billed Gull *Larus delawarensis*	H6,H11,H13,H16	N6	(Graves 1992; Gaidet *et al.* 2010; Velarde *et al.* 2010; USDA 2012)
Sabine's Gull *Xema sabini*	H5	N3	(Obenauer *et al.* 2006)
Silver Gull Chroicocephalus novaehollandiae	H13	N6	(NIAID 2012)
Slaty-backed Gull *Larus schistisagus*	H4	N8	(NIAID 2012)
Slender-billed Gull *Chroicocephalus genei*	H4,H16	N2,N3	(L'vov *et al.* 1978)
Sooty Tern *Sterna funcata*	H7,H15	N2,N6,N9	(Mackenzie *et al.* 1984; Obenauer *et al.* 2006)

Vega gull *Larus vegae*	H13	N6	(Spackman *et al.* 2009)
Yellow-Legged Gull *Larus michahellis*	H9,H13	N2	(Lebarbenchon *et al.* 2007; Lin *et al.* 2009)
Whiskered Tern *Chlidonias hybrida*	H6	N2	(NIAID 2012)

Tab. 3. Laridae highly pathogenic avian influenza (HPAI) subtypes.

This table presents the different HPAIV subtypes detected naturally or experimentally in different Laridae species.

Species	Influenza virus subtypes	Infection type	Infectious status	References
Black-headed Gull *Chroicocephalus ridibundus*	H5N1	Natural	Healthy carrier and fatal disease	(Ellis *et al.* 2004; Savić *et al.* 2010)
Brown-headed Gull *Chroicocephalus brunnicephalus*	H5N1	Natural	Pathology	(Liu *et al.* 2005)05/10/12 14:28
Common Gull *Larus canus*	H5N1	Natural	Healthy carrier	(Sharshov 2010)
Common Tern *Sterna hirundo*	H5N1 H5N3	Natural	Healthy carrier, pathology and fatal disease	(Becker 1966; L'vov *et al.* 2006)
Great Black-headed Gull *Ichthyaetus ichthyaetus*	H5N1	Natural	Pathology	(Liu *et al.* 2005)
European Herring Gull *Larus argentatus*	H5N1	Experimental	Pathology and fatal disease	(Brown, Stallknecht & Swayne 2008)
Laughing Gull *Leucophaeus atricilla*	H5N3, H5N1	Experimental	Healthy carrier, pathology and fatal disease	(Perkins & Swayne 2001; Brown *et al.* 2006)
Slaty-backed Gull *Larus schistisagus*	H5N1	Natural	Healthy carrier	(Kou *et al.* 2009)
Slender-billed Gull *Chroicocephalus genei*	H5N2	Natural	Healthy carrier	(L'vov *et al.* 1978)

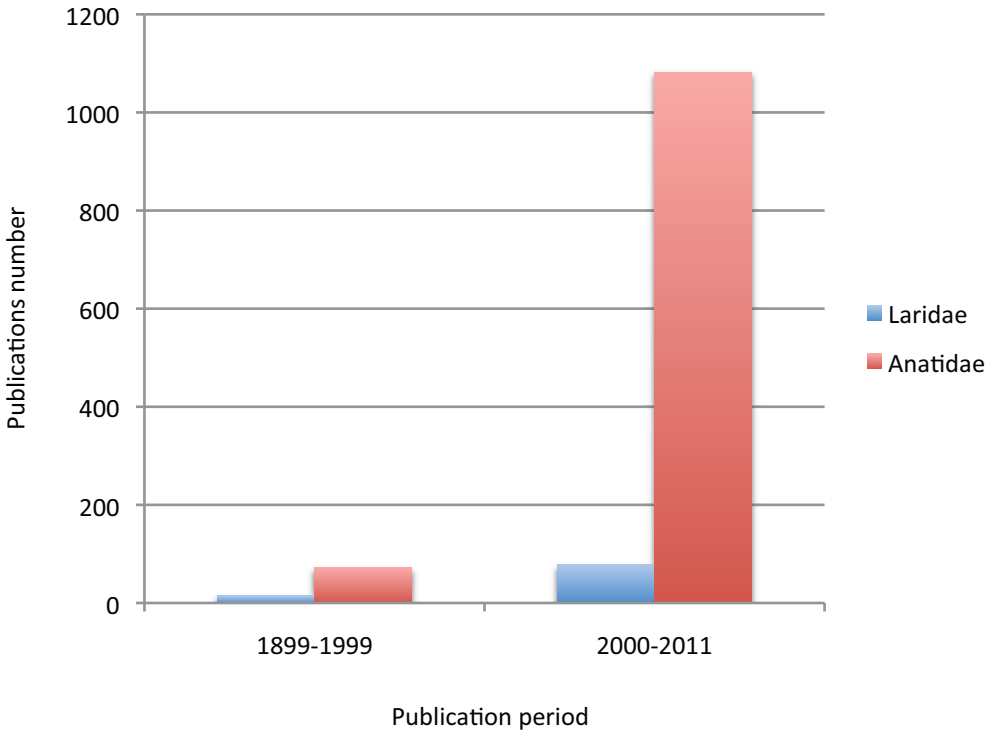

Figure. Number of studies that have focused on AIV circulation in Anatidae versus Laridae from 1899 to 2011.

Article 7. Spatial patterns of influenza A antibodies in yellow-legged gull (*Larus michahellis*) eggs in the Mediterranean basin.

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Soumis à *Virology Journal.*

Title

Spatial patterns of influenza A antibodies in yellow-legged gull (*Larus michahellis*) eggs in the Mediterranean basin.

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Abstract

Background

Highly pathogenic avian influenza viruses (AIVs) remain of great concern worldwide due to their economical impacts. To date, most epidemiological studies have focused on AIV circulation in Anatidae, while less attention has been given to Laridae. In order to better understand the circulation of AIVs in Laridae, we studied the prevalence and subtype specificity of antibodies against AIVs in eggs sampled from an abundant and anthropophilic seabird species, the yellow-legged gull (*Larus michahellis*). Sampling took place over 2 years in 11 breeding colonies across 3 western Mediterranean countries.

Results

We found that yellow-legged gulls are exposed to AIVs and that antibody prevalence was high across all colonies. No spatial autocorrelation within the colonies was detected, suggesting that individuals do not necessarily become infected within the colony. Prevalence levels were heterogeneous across colonies and positively correlated along a south/north gradient. We detected the presence of antibodies specifically against H13 and H16 AIV subtypes, which commonly occur in Laridae species. In contrast, we did not find any evidence of gull exposure to subtypes H5 and H7, which can evolve from low to high pathogenicity in poultry.

Conclusion

This study reports the prevalence of specific AIV antibodies in yellow-legged gull eggs across the western Mediterranean. Our results show that the yellow-legged gull, a species that is in close contact with humans and inhabits urban areas, is exposed to AIVs and may potentially play a role in the circulation of some AIV subtypes. A multisite survey of gull colonies may help further clarify the involvement of this species in the epidemiology of AIVs.

Keywords

Avian influenza, Laridae, disease ecology, colonial waterbird, maternal antibody transfer, serology.

Résumé

Introduction

Les virus de l'Influenza Aviaire (IA) hautements pathogènes représentent une préoccupation mondiale en raison de leurs impacts économiques et en termes de santé publique. À ce jour, la plupart des études épidémiologiques ont mis l'accent sur la circulation des virus IA chez les Anatidés et peu d'attention a été accordée aux Laridés. Afin de comprendre au mieux la circulation des virus IA chez les Laridés, nous avons étudié la prévalence et la spécificité des anticorps dirigés contre les sous-types IA dans les œufs d'une espèce d'oiseaux de mer anthropophile, le goéland leucophée (*Larus michahellis*). L'échantillonnage a été effectué en 2009 et 2010 sur 11 colonies situées en France (n = 7), en Espagne (n = 3) et en Algérie (n = 1).

Résultats

Nous avons constaté que les goélands leucophées sont exposés aux virus IA et que les prévalences en anticorps sont élevées dans l'ensemble des colonies. Ces niveaux de prévalences sont hétérogènes entre colonies et positivement corrélées à un gradient sud/nord. Aucune auto-corrélation spatiale des échantillons porteurs d'anticorps maternels n'est détectée au sein des colonies, ce qui suggère que les individus ne s'infectent pas sur le site de reproduction. Enfin, nous avons mis en évidence la présence d'anticorps spécifiques contre les sous-types H13 et H16, sous-types fréquemment détectés chez les espèces de Laridés. En revanche, les goélands leucophées ne semblent pas exposés aux sous-types H5 et H7, seul sous-types capables d'évoluer en hautement pathogène chez les volailles.

Conclusion

Cette étude rapporte la prévalence des anticorps spécifiques aux virus IA dans les œufs de goéland leucophée de l'ouest Méditerranéen. Nos résultats montrent que le goéland leucophée, espèce en contact étroit avec l'Homme, est exposé aux virus IA et qu'ils pourraient jouer un rôle dans la circulation de certains sous-types. Une étude multi-sites des colonies de goéland leucophée pourrait donc aider à préciser d'avantage l'implication de cette espèce dans l'épidémiologie des virus IA.

Mots clefs

Influenza aviaire, Laridés, écologie de la santé, oiseaux coloniaux, transfert d'anticorps maternel, sérologie.

Introduction

Over the last two centuries, advances in hygiene, the development of many vaccines, and the discovery of penicillin in 1929 resulted in the impression that the battle against infectious agents had been won [1]. However, many anthropogenic environmental disturbances have contributed to pathogen emergence [2]. Indeed, rearing practices and the expansion of international air traffic are just some factors that have increased opportunities for infectious agents to transfer to new hosts [2, 3]. These new infection opportunities have led to the emergence of numerous infectious diseases in domestic animals and humans, most of which originally circulated in wildlife [4-6]. Studying infectious agent circulation in wild reservoirs is essential to describe, understand, and predict potential outbreaks in humans and domestic animals.

Avian influenza viruses (AIVs) well illustrate that knowledge about wild animal reservoirs of human and livestock pathogens is essential to understanding transmission dynamics. AIVs remain of great concern worldwide due to their economic impacts and the threat they represent to human health [7, 8]. These viruses have complex epidemiological dynamics [9], characterized by the ability to jump from wild birds to domestic hosts as well as to wild and domestic mammals, including humans [10, 11].

Wild waterbirds, in particular Anseriformes (mainly Anatidae: ducks, geese and swans) and Charadriiformes (Waders and Laridae: gulls and terns), are considered to be the natural reservoirs for AIVs [9]. Two forms of AIVs can be distinguished according to their pathogenicity in poultry. Low pathogenic avian influenza viruses (LPAIVs) naturally circulate in wild waterbirds, in which they seem to cause no or mild clinical symptoms [9, 12]. While highly pathogenic avian influenza viruses (HPAIVs) do not seem to have wild reservoirs, the introduction of LPAIVs strains from wild animals into poultry can result in the evolution of more virulent forms (HPAIVs) [13, 14]. To date, H5 and H7 are the only subtypes that have been able to evolve from low to high pathogenicity [12, 15]. However, a wide variety of subtypes circulate in wild birds [9, 12]. This diversity, as well as the close contact between wild birds, domestic animals, and humans can induce reassortment between viruses that allows the emergence of new subtypes [16].

For various reasons, including the fact that Anatidae species represent the main natural host reservoir for AIVs [17], given the global distribution of the taxon [18], most studies have focused on AIV circulation in Anatidae and given far less attention to Laridae [19]. However,

studies that have investigated AIV dynamics in Laridae have shown that several species are regularly infected by LPAIVs [20, 21] and may be healthy carriers of HPAIVs [11, 22, 23]. Moreover, specific subtypes such as H13 and H16 are essentially maintained in gull populations [11, 24, 25], although they are also occasionally detected in other avian taxa, including the mallard (*Anas platyrhynchos)* [26], the domestic turkey (*Meleagris gallopavo)* [27], or the American white pelican (*Pelecanus erythrorhynchos*) [28].

Some Laridae species, such as the yellow-legged gull (*Larus michahellis*), are colonial seabirds that nest in large and dense colonies [29], creating good opportunities for virus spread through aerial and feacal transmission [9, 30]. Yellow-legged gulls breed in various habitats and may share the use of wetlands with Anatidae, which can promote AIV circulation through direct bird-to-bird transmission or through contact with contaminated fresh water [31, 32]. Yellow-legged gulls are also predators for ducks [33], and it has been shown that Laridae species can be infected with HPAI H5N1 through the consumption of contaminated meat [34]. Finally, they have colonized some urban areas and can exploit resources resulting from human activities [35, 36], thus promoting the risk of transmitting and spreading zoonotic diseases.

Different methods may be used to assess whether a wild bird population is exposed to AIVs. Most of the studies are based on virus isolation or direct detection of the viral genome, which provides information about immediate infection status and allows the phylogeographic tracking of strains [26, 37]. This approach is useful when the proportion of individuals shedding pathogen at any point in time is high, which does not seem to be the case for AIVs in yellow-legged gulls. Indeed, AIVs were detected in only 0.9% of more than 200 yellow-legged gulls sampled in the Camargue wetlands (France) from September 2006 to July 2007 [13].

When prevalence is too low to conduct surveys based on direct methods, serological surveys may be used to detect the host's specific immunological response following exposure to the infectious agent [38]. A limitation of serological methods is that they are only useful if the host's immune response is detectable for a relatively long period of time [38]. The persistence of antibodies against the influenza nucleoprotein has never been assessed in gulls. However, field studies performed on a natural population of black-legged kittiwakes (*Rissa tridactyla*) showed that antibodies against both a naturally circulating infectious agent and a vaccine antigen were detectable for at least one year [39]. Moreover, the long lifespan of Laridae [18] could favor the development of persistent acquired immune responses [40].

In wild bird species for which adults are difficult to catch, detecting maternal antibodies in eggs may prove a useful tool. Indeed, the amount of antibodies detected in the egg yolk correlates with the amount detected in the plasma of breeding females at the time of laying [41-43] and therefore reflects a female's prior exposure to infectious agents [43-45].

In this study, we aimed to improve the understanding of AIV dynamics in Laridae in the western Mediterranean basin. Using the yellow-legged gull as a model species, we investigated spatial variation in the presence of AIV-specific antibodies in eggs sampled over a 2-year period. Two previous studies, conducted on pairs of yellow-legged gull colonies in France and Tunisia, detected a high prevalence of AIV maternal antibodies in eggs [44, 45], but found different results with regards to spatial variation between colonies. We conducted a study at the scale of the western Mediterranean basin in order to better estimate prevalence levels and clarify spatial variation among colonies.

Current knowledge on the ecology of AIVs suggests that the highest rates of infection in waterbirds are observed mainly during gathering periods, for example during pre-migration gathering [46-49]. Knowing that yellow-legged gulls breed in dense colonies [50], we hypothesized that the high contact rate between breeding females within a colony could increase viral transmission. We therefore investigated the spatial aggregation of AIV antibodies present among nests in a subsample of colonies.

We characterized the AIV subtypes targeted by the antibodies we detected. Knowing that previous studies had found high proportions of H13 and H16 AIV subtypes in Laridae populations [11, 15, 24], we expected a similar result. We also tested for antibodies against H5 and H7 subtypes, in order to assess the role played by yellow-legged gulls in the dispersal of these viruses known to evolve from low to high pathogenicity in poultry. However, we did not expect high levels of H5 or H7-specific antibodies because these subtypes are not frequently reported in gulls [22, 23].

Material and methods

Sampling

In 2009 and 2010, during the breeding season (March/April), we sampled 11 breeding colonies located in France (n=7), Spain (n=3), and Algeria (n=1) (Figure 1). In total, 1,384 eggs (8-127 eggs per colony) were collected (Table 1). One egg per clutch was collected from a sub-sample of nests within each colony, given that nest status (antibody positive or negative) can be reliably estimated from a single egg [44].

Serological analyses

In the laboratory, the egg yolk was separated from the albumen, homogenized, and frozen at -20°C until analysis. Antibodies were obtained using chloroform extraction, as previously described [51], and stored at -20°C.

Egg extracts were screened for antibodies directed against the influenza A virus nucleoprotein using a commercial ELISA kit (ID Screen® Influenza A Antibody Competition Multi-Species, ID VET, Montpellier, France) in accordance with the manufacturer's instructions. Results were expressed as a percentage of competition (PC), using the optical density (OD) of the sample and the mean OD of the negative controls (NC) of the kit as follows: $PC = (OD_{sample}/OD_{NC}) \times 100$. According to kit instructions, samples with PC ≤45% were considered positive, those with PC >50% were considered negative, and those with PC between 45% and 50% were considered doubtful.

Characterization of specific influenza subtypes

With the exception of a few samples, all of the ELISA-positive eggs were screened for antibodies against H5 and H7 subtypes using two commercial ELISA kits (ID Screen® Influenza H5 and H7 Antibody Competition, ID VET, Montpellier, France) in accordance with the manufacturer's instructions. As described above, results were expressed as PC. According to kit instructions, samples with PC ≤ 50% were considered positive, those with PC > 60% were considered negative, and those with PC between 50% and 60% were considered doubtful.

These same extracts were tested for the presence of hemagglutinating antibodies specific for H13 and H16 subtypes using Hemagglutination-Inhibition Tests (HIT) following standard procedures [52, 53]. A HIT titer of ≥ 1:20 was considered positive.

Statistical analyses

All analyses were performed using R.2.15.0 (R development Core Team 2012). We used generalized linear models (GLM) to assess whether influenza antibody prevalence differed among colonies. The significance of factors in our models was determined using model comparison following backwards elimination of terms. The most parsimonious model was selected by AIC criterion [54]. We used a generalized linear mixed model (GLMMPQL; binomial; with colony as a random effect, library MASS) to evaluate if the prevalence levels differed over time. Mantel tests (library ade4) were used to assess if AIV-positive samples

were homogeneously distributed [55] within three randomly chosen colonies: Ebro Delta (Spain), Corrège (France), and Frioul (France). We controlled for multiple comparisons using the false discovery rate correction, which employs $q^* = 0.05$ [56]. This approach controls the proportion of erroneously rejected hypotheses as opposed to the possibility of a single false rejection, thus resulting in more power than in traditional multiple comparison methods [57].

Results

ELISA detection of influenza A virus circulation

Over the two years of study, high prevalence levels were found in each colony (Table 1), with averages (±Standard Error, SE) of 33.2 % ±11.5 in 2009 and 47.2 % ±9.4 in 2010. Prevalence levels varied significantly between the two years of the study (Generalized linear mixed model: β±SE = 0.6±0.2, *t*-value = 3.3, I = 0.009). Differences in prevalence levels were detected among colonies and were positively correlated along a south/north gradient (Generalized linear mixed model: β±SE = 0.1±0.05, *t*-value = 2.8, *P* = 0.021). No spatial autocorrelation was detected within the colonies of Ebro Delta (*P* = 0.2), Corrège (*P* = 0.2), or Frioul (*P* = 0.3).

Detection of antibodies specific for H5, H7 (competitive ELISA) and H13, H16 (HIT) influenza subtypes

No H5 and H7 specific antibodies were detected using ELISAs. The prevalence of antibodies specific for H13 or H16 influenza subtypes is presented in Table 2. Average prevalence levels (±SE) over the two years were 14.5 % ±7.9 and 6.7 % ±3.4 for H13 and H16, respectively. Nineteen samples, i.e. 1% of all samples tested by HIT, contained antibodies with an hemagglutination inhibition effect for both H13 and H16 viruses; the titer observed for the H16 AIV-subtype was generally lower than that for the H13 (Figure 2, Table 3).

Discussion

Our rigorous study used the same protocol to simultaneously survey AIV exposure in several breeding colonies of a common wild bird species across the western Mediterranean basin.

We found that yellow-legged gulls are exposed to AIVs, and that all the breeding colonies showed a high prevalence of antibodies against the AIV nucleoprotein (Table 1).

These high levels of AIV antibody prevalence confirm previous results obtained using yellow-legged gull eggs sampled locally in the western Mediterranean basin [43-45]. They are also consistent with serological results observed in adults of black-legged kittiwakes (*Rissa tridactyla*) living in Norway [58]. Our large-scale study allows us to more broadly assess yellow-legged gull exposure to AIVs in a spatial context, both within and between breeding colonies.

No spatial autocorrelation within the colonies of Ebro Delta, Corrège or Frioul was detected. The absence of ELISA-positive patches of nests [59] suggests that breeding females did not become infected within the colony.

Because antibodies in the plasma are believed to persist at least a year in Laridae [39], seropositive yellow-legged gull females may have been exposed to AIVs at any time of year. During the breeding season, yellow-legged gulls may become infected on feeding sites, where aggregation is likely to lead to AIV transmission between individuals [60, 61]. During the winter, when temperature conditions are favourable to the persistence of AIVs in the environment [31], gulls also nocturnally gather in resting areas; such aggregation can also favor AIV transmission.

This study does not allow us to assess if there is potential transmission within the colony after laying. It would be interesting to analyze the presence of antibodies against AIVs in the plasma of yellow-legged gull chicks older than 15 days. Such chicks no longer have antibodies of maternal origin and could thus reveal transmission within the colony [62].

Prevalence levels are not homogeneous among colonies. This result confirms the variation in prevalence previously detected in two colonies in Tunisia [44]. However, our study shows an increase in prevalence along a south/north gradient (Figure 1). This trend could be due to differences in environmental factors following the south/north gradient. For instance, mallards (*Anas platyrhynchos*), which represent the main natural reservoir host for AIVs [17], are only marginally present in Algeria [18, 63, 64], as opposed to certain areas of France (Camargue; [13]) or Spain (Ebro Delta, [65]). The differences in the proximity between gulls and mallards could potentially explain the differences in prevalence among our colonies.

We detected an increase in the proportion of nests with antibody positive eggs between 2009 and 2010. This result suggests a local, continued circulation of AIVs. Indeed, if the pattern had been due to past transmission events, we would expect demographic factors (natural mortality and recruitment of susceptible individuals) to have led to stable or reduced local antibody prevalence. However, this result must be confirmed by a longer term study.

Antibodies against H5 and H7 subtypes were not detected. This finding is consistent with other studies suggesting that AIV subtypes containing these hemagglutinins are rarely detected in Laridae species [22, 23].

Knowing that H13 and H16 AIV subtypes are common in gull populations [11, 15, 24], we expected to find antibodies against H13 and H16 hemagglutinins. H13 and H16 specific antibodies were detected, but their prevalence levels varied from low to null depending on the study site and year, a result that contrasts with the high prevalence detected by studies conducted in Norway on black-legged kittiwakes [58, 66]. These differences in prevalence may be linked to the fact that yellow-legged gulls and black-legged kittiwakes are two species with different ecologies that live in different environments. The H13 AIV-subtype prevalence levels detected in our study also differed from those reported for ring-billed gull (*Larus delawarensis*) colonies in Canada [67], where 92% and 80% of the adult had antibodies against H13 AIVs in 2000 and 2004 [67].

Serological analyses are classically performed on adult or chick serum, using H13 and H16 antigens and the same HI positivity threshold (titre $\geq$ 1:20) as in our study [58]. These methods can detect both Immunoglobulin type M (IgM) and Immunoglobulin type Y (IgY, which is a functional equivalent of the mammalian IgG [68]). In birds, the acquired immune response is mainly mediated by IgY, which is the only immunoglobulin that can be transferred into the egg yolk [68]. Therefore, HIT analyses carried out on eggs can only detect IgY antibodies, which may partly explain the difference between our results and those from studies that analyzed Laridae serum [58]. In order to verify that the lower prevalence levels observed in our study were not due to a problem of maternal antibody detectability, we tested 12 kittiwake eggs sampled in 2010 from the same Norwegian colony (Hornøya) as in the Toennessen *et al.* study [58]. Antibodies against H13 and H16 subtypes were detected in 7 of 12 and 2 of 12 samples, respectively. One sample contained antibodies able to inhibit the hemagglutination of both H13 (titer = 1:20) and H16 (titer = 1:40) AIVs. Although we tested only a few eggs, this result suggests that the lower H13 and H16 prevalence levels observed on yellow-legged gull eggs are unlikely to be linked to detectability issues.

Finally, only 1% of all the samples tested by HIT contained antibodies able to inhibit the hemagglutination of both H13 and H16 AIVs. This percentage is relatively low as compared to the 37.5% (18/48) of kittiwake samples found positive for both H13 and H16 antigens in Norway [58]. Seropositivity for both H13 and H16 antigens may be explained by a possible co-exposure to different AIV subtypes [69] or, at low virus titer, HIT cross-reactions.

Indeed, a cross-reaction was observed between the H13 and H16 reference serum at the lowest titer levels 1:20 and 1:40 (Table 3).

Conclusion

This study reports a high prevalence of antibodies against AIVs in yellow-legged gull eggs across the western Mediterranean basin. Our results indicate that yellow-legged gulls are exposed to AIVs and that H13 and H16 subtypes are present in the Mediterranean basin. This is a notable result because the circulation of diverse AIV subtypes in wild animals that occur in close proximity to urban and farm animals may potentially be responsible for the emergence of new virulent subtypes [70, 71]. It would be useful to extend this study to other host-pathogen systems with different patterns of transmission and circulation, including pathogens of humans or domestic animals for which wild birds represent the main reservoir.

Acknowledgements

We thank the many people who helped with various aspects of field work, especially Telailia Saleh and Patrick Mayet. J. Pearce-Duvet was supported by a Chateaubriand postdoctoral fellowship and a National Science Foundation International Research Fellowship. The ELISA analyses were conducted at the Plateforme d'Analyses Chimiques en Ecologie (CEFE). Support from OSU OREME, ANR (11 BSV7 003 EVEMATA grant), AIRD and CNRS is acknowledged. Boudjéma Samraoui was supported by the Algerian Ministère de l'Enseignement Supérieur et de la Recherche Scientifique (DGRSDT/ M.E.S.R.S.) and by the DSFP program, King Saud University, Saudi Arabia. Authorizations for working in the study sites were obtained from: Direction régionale de l'Environnement, de l'Aménagement et du Logement (DREAL); Syndicat Mixte des Etangs Littoraux (SIEL); Conservatoire Etudes des Ecosystèmes de Provence-Alpes du sud (CEEP); Parc national de Port-Cros; Service Environnement de la Mairie de Gruissan (Aude); Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural de la Generalitat de Catalunya; Parc Natural de El Montgrí, les Illes Medes i el Baix Ter; Dirección General de Patrimonio Natural y Biodiversidad de Murcia; Conselleria de Medi Ambient i Mobilitat del Govern de les Illes Balears; Ajuntament de Barcelona; Parc Natural del Delta de l'Ebre; Cabildo de Gran Canaria; Conselleria de Medi Ambient, Aigua, Urbanisme i Habitatge de la Generalitat Valenciana; the forest service in the Tunisian Ministry of Agriculture; the Algerian DGRSTD/MESRS and DSFP, King Saud University (Saudi Arabia). We thank Frederick A. Leighton for helpful hints about performing HIT in egg samples. We would like to dedicate this article to the

memory of Àlex Lorente, director of the Parc Natural de El Montgrí, les Illes Medes i el Baix Ter who passed away unexpectedly on 6 of August 2012 from an apnea accident.

Competing interests

The authors declare that they have no competing interest.

Authors' contributions

EJ, TB, and AA were responsible for the conception and design of the study. AA and JP organized and conducted the field work in France and Spain, assisted by JG, MV, RG, and WA. WA organized and conducted the field work in Algeria in 2009 and BS in 2010. AA and JP conducted the cELISAs tests to detect anti-influenza A antibodies and AA the cELISAs tests specific for H5 and H7 subtypes. AA performed the HIT, assisted by EJ. All authors read, revised, and approved the final manuscript.

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Figure legends

Figure 1: **Prevalence patterns of antibodies against AIVs estimated from eggs in a) France; b) Spain; c) Algeria.**

The locations of the study colonies are indicated by black dots. The pie charts represent the number of eggs found to be positive (in black) and negative (in gray) for anti-AIV antibodies; details are provided in Table 1.

Figure 2: HI titer observed for AIV-subtypes a) H13 and b) H16.

Tables

Table 1. Prevalence of antibodies directed against the nucleoprotein of AIVs in yellow-legged gull eggs

Country	Colony	Year	Sample size	Number of positive samples	Number of doubtful samples
France	Riou (43°10'39"N-5°23'04"E)	2009	127	42 (33%)	10
		2010	8	4 (50%)	0
	Frioul (43°16'27"N-5°18'15"E)	2009	79	38 (48%)	9
		2010	51	17 (33%)	3
	Carteau (43°22'40"N-4°51'27"E)	2009	102	57 (56%)	6
		2010	56	25 (45%)	5
	Besson (43°29'15"N-4°27'47"E)	2009	79	34 (43%)	2
		2010	59	35 (59%)	5
	Villeneuve (42°02'50"N-3°13'21"E)	2009	70	20 (29%)	6
		2010	64	34 (53%)	3
	Gruissan (43°06'36"N-3°06'25"E)	2009	99	33 (33%)	12
		2010	91	49 (54%)	3
	Corrège	2009	70	15 (21%)	5

	(42°51'15"N-3°01'21"E)	2010	54	27 (50%)	1
Spain	Medes (42°02'50"N-3°13'21"E)	2009	38	11 (29%)	2
		2010	49	27 (55%)	0
	Ebro delta (40°34'17"N-0°39'37"E)	2009	55	12 (22%)	0
		2010	29	15 (52%)	1
	Isla Grosa (37°43'40"N-0°42'27"O)	2009	66	13 (20%)	6
		2010	50	16 (32%)	1
Algeria	Chetaïbi (37°05'79"N, 7°17'53"E)	2009	55	17 (31%)	4
		2010	33	11 (33%)	1

Table 2. Prevalence of antibodies against H13 and H16 subtypes by inhibition of hemagglutination test

Country	Colony	Year	AIV positive (N;%)	H13 (%)	H16 (%)
France	Riou (43°10'39"N-5°23'04"E)	2009	40 (127 ; 31%)	9 (22.5%)	0
		2010	4 (8 ; 50%)	2 (50%)	1 (25%)
	Frioul (43°16'27"N-5°18'15"E)	2009	37 (79 ; 47%)	11 (30%)	0
		2010	17 (51 ; 33%)	2 (12%)	1 (6%)
	Carteau (43°22'40"N-4°51'27"E)	2009	55 (102 ; 54%)	8 (15%)	0
		2010	25 (56 ; 45%)	8 (32%)	2 (8%)
	Besson (43°29'15"N-4°27'47"E)	2009	34 (79 ; 43%)	12 (35%)	0
		2010	35 (59 ; 59%)	12 (34%)	2 (6%)
	Villeneuve (42°02'50"N-3°13'21"E)	2009	20 (70 ; 29%)	10 (50%)	0
		2010	34 (64 ; 53%)	22 (65%)	3 (9%)
	Gruissan (43°06'36"N-3°06'25"E)	2009	32 (99 ; 32%)	5 (16%)	1 (3%)
		2010	49 (91 ; 54%)	21 (43%)	4 (8%)
	Corrège (42°51'15"N-3°01'21"E)	2009	14 (70 ; 20%)	12 (86%)	1 (7%)
		2010	27 (54 ; 50%)	7 (26%)	0
Spain	Medes (42°02'50"N-3°13'21"E)	2009	10 (38 ; 26%)	5 (50%)	1 (10%)
		2010	27 (49 ; 55%)	6 (22%)	2 (7%)
	Ebro delta (40°34'17"N-0°39'37"E)	2009	12 (55 ; 22%)	3 (25%)	0
		2010	15 (29 ; 52%)	9 (60%)	2 (13%)
	Isla Grosa (37°43'40"N-0°42'27"O)	2009	13 (66 ; 20%)	7(54%)	4 (31%)
		2010	16 (50 ; 32%)	4 (25%)	4 (25%)
Algeria	Chetaïbi (37°05'79"N, 7°17'53"E)	2009	17 (55 ; 31%)	6 (35%)	1 (6%)
		2010	11 (33 ; 36%)	5 (45%)	0

Table 3. Neutralizing antibody titers for eggs containing antibodies that inhibit the hemagglutination of H13 and H16 subtypes

Country	Colony	Year	Titer H13	Titer H16
France	Riou	2010	1:80	1:40
	Carteau	2010	1:320	1:80
			1:40	1:40
	Besson	2010	1:40	1:20
			1:20	1:20

	Villeneuve	2010	1:80	1:20
			1:320	1:20
			1:320	1:160
	Gruissan	2010	1:80	1:20
			1:40	1:20
			1:80	1:20
	Corrège	2009	1:40	1:20
Spain	Medes	2009	1:20	1:80
		2010	1:160	1:40
			1:20	1:20
	Ebro Delta	2010	1:40	1:80
	Isla Grossa	2009	1:20	1:20
			1:80	1:80
Algeria	Chetaïbi	2009	1:160	1:20

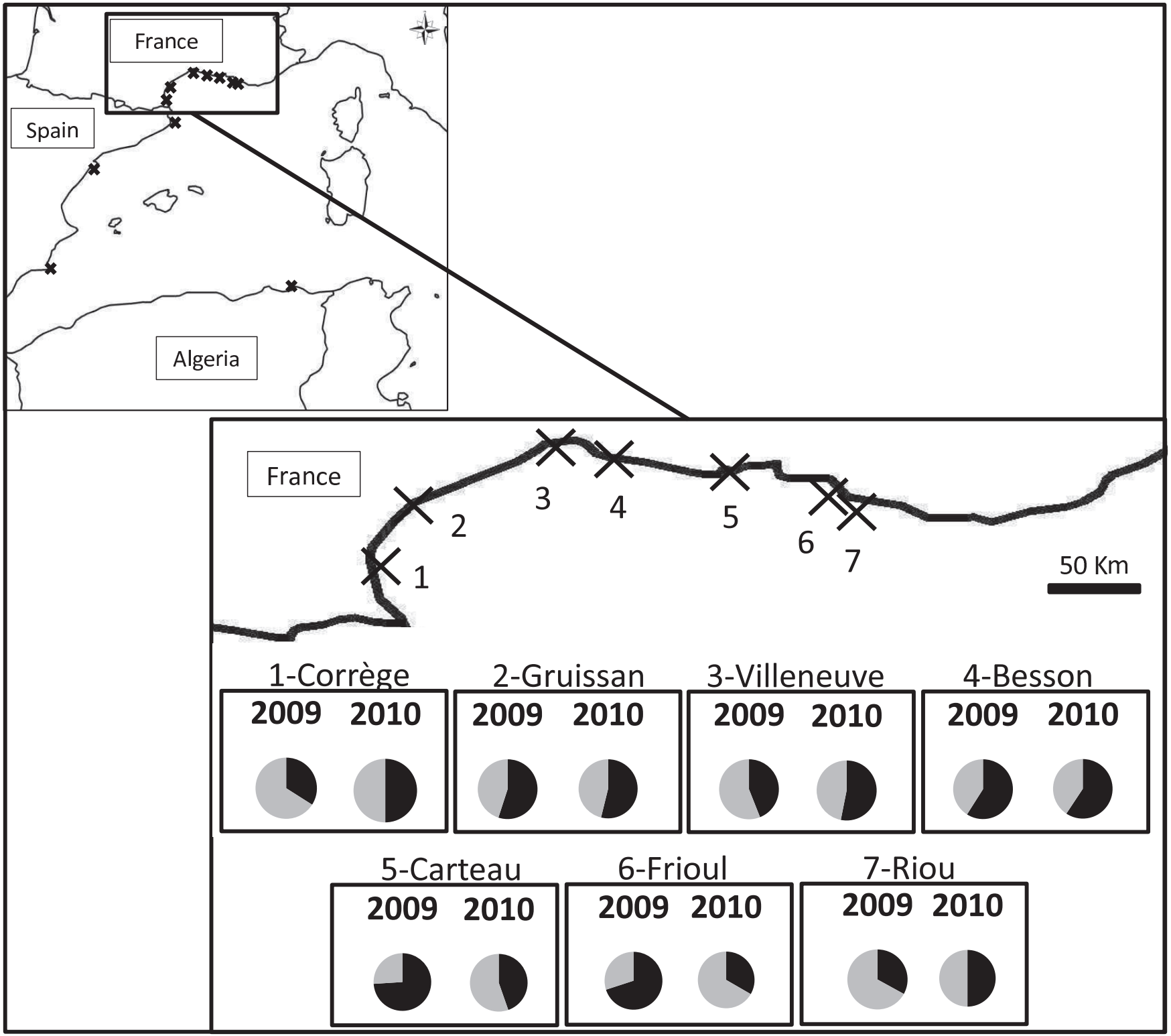
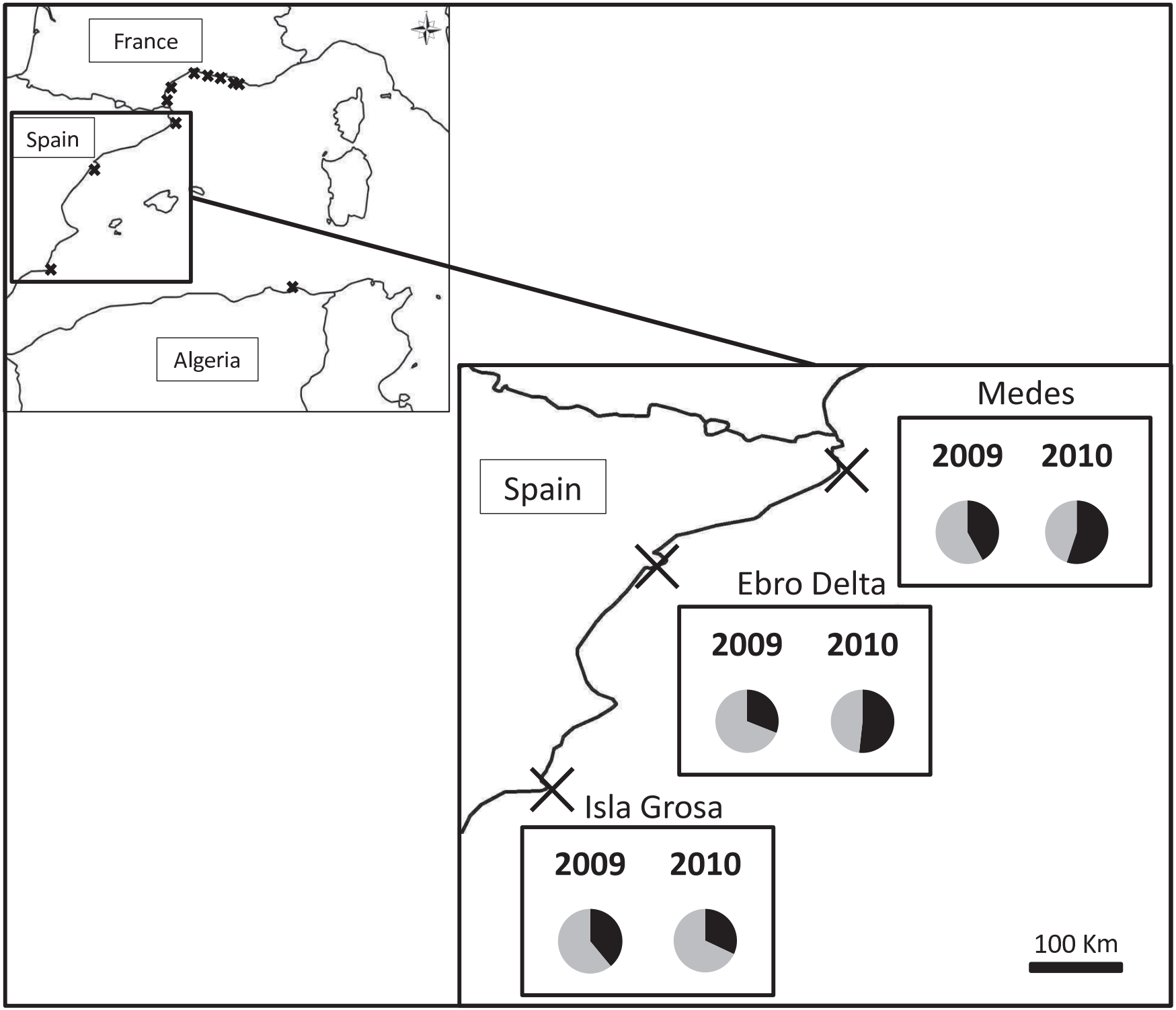
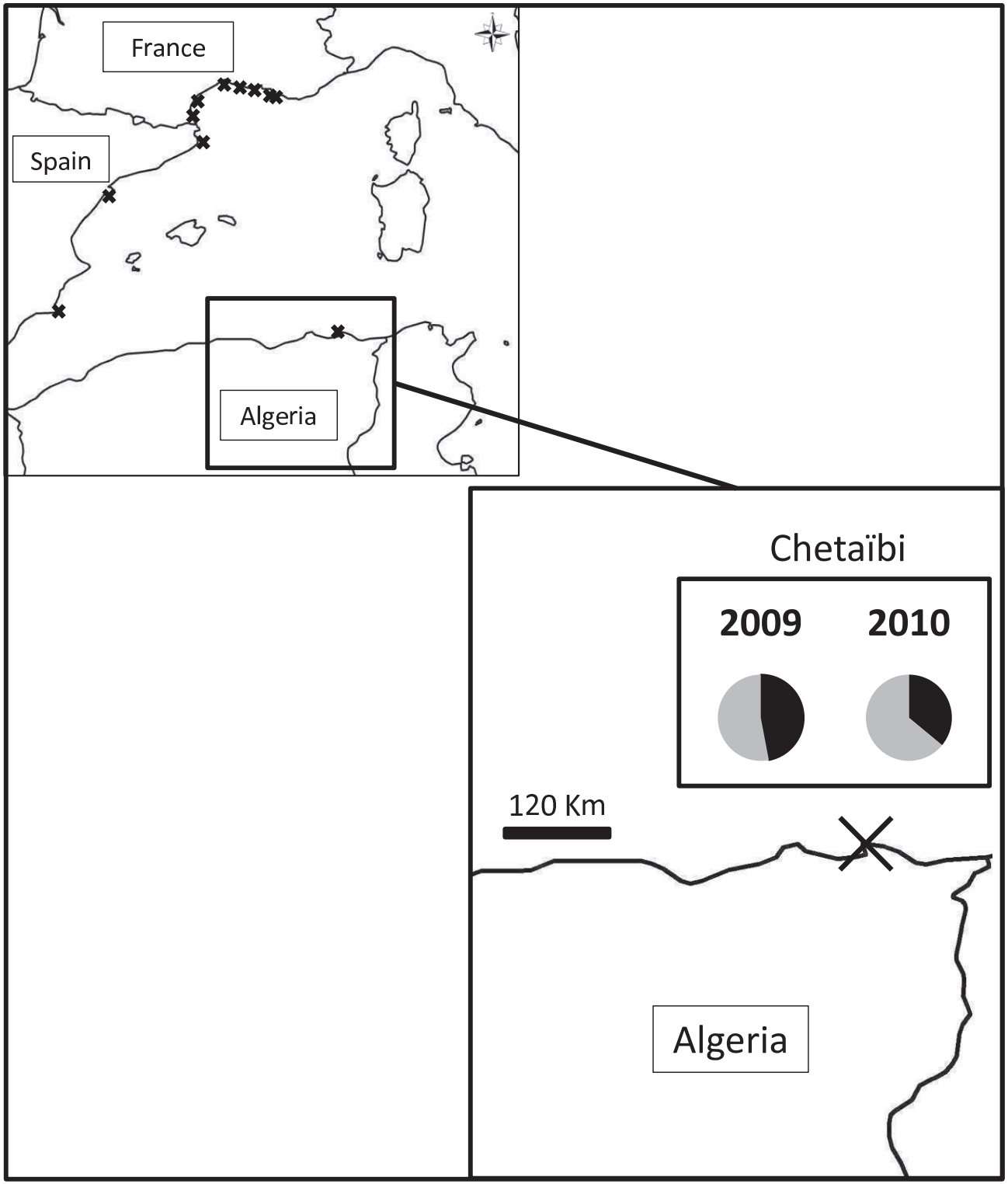
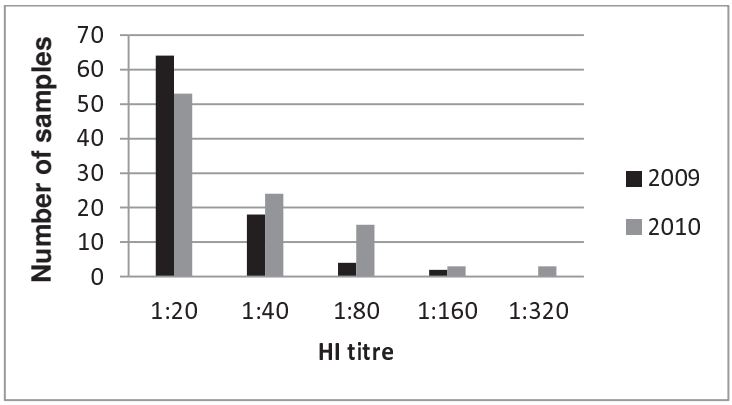

France
Spain
Algeria
France
1
2
3
4
5
6
7
50 Km
1-Corrège
2009 2010
2-Gruissan
2009 2010
3-Villeneuve
2009 2010
4-Besson
2009 2010
5-Carteau
2009 2010
6-Frioul
2009 2010
7-Riou
2009 2010

Figure 1


France
Spain
Algeria
Spain
Medes
2009
2010
Ebro Delta
2009
2010
Isla Grosa
2009
2010
100 Km


France
Spain
Algeria
Chetaïbi
2009
2010
120 Km
Algeria


Number of samples
70
60
50
40
30
20
10
0
1:20
1:40
1:80
1:160
1:320
HI titre
2009
2010

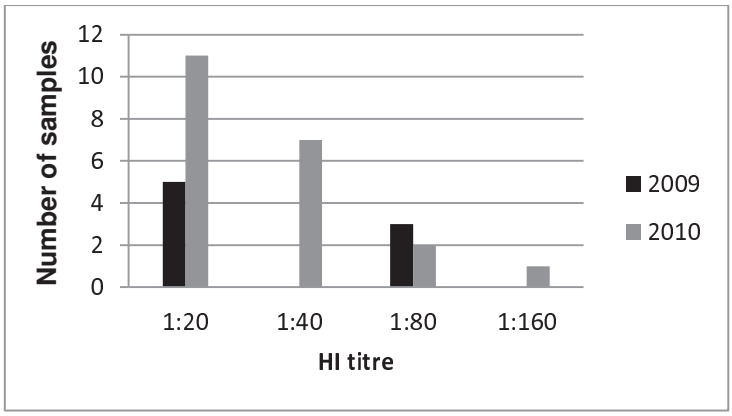

Number of samples
12
10
8
6
4
2
0
1:20
1:40
1:80
1:160
HI titre
2009
2010

Figure □

Article 8. Is there any seasonality of influenza infections in Charadriiformes? A year-round study in Yellow-legged gulls (*Larus michahellis*) from Southern France.

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En préparation.

SHORT TITLE

Seasonal pattern of avian influenza infections in Yellow-legged gulls

TITLE

Is there any seasonality of influenza infections in Charadriiformes? A year-round study in Yellow-legged gulls (*Larus michahellis*) from Southern France

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WORDS COUNT : 2412

ABSTRACT (296 words)

Wild birds, particularly Anseriformes and Charadriiformes, are natural reservoirs of influenza viruses that can be transmitted to domestic animals as well as humans. Understanding avian influenza dynamics in wild birds, especially infection seasonality, is therefore of great importance to prevent potential epizootic and/or pandemic threat. Avian influenza virus infection peaks are observed in Anseriformes populations, especially in ducks, just before and during the fall migration period. Less attention has been paid to Charadriiformes so far. To investigate this issue, we conducted a year-round study in the Camargue, a potential hub of bird pathogen introduction and exchange situated in Southern France. We focused on the Yellow-legged gull (*Larus michahellis*), which expanded on the French Mediterranean coast during last decades, and shares wetlands use with numerous duck species. We sampled fresh droppings of Yellow-legged gulls between February and December 2010 and collected cloacal swabs on wild shot ducks during hunting seasons from September 2007 to January 2010. We observed very low avian influenza virus infection rates with no infection seasonal pattern in the focus Yellow-legged gull population. In contrast, we highlighted a clear seasonal infection peak in sympatric duck populations. Different infection temporal patterns can be caused by several mechanisms including the host specificity of avian influenza virus strains circulating in gulls, the divergent feeding behaviour of the two taxa and the differences between their immune responses against avian influenza viruses. Interestingly, a previous study showed that a large proportion of the eggs of the focus Yellow-legged gulls contained avian influenza virus specific antibodies. This paradox can be explained either by regular low circulation combined with long lasting antibodies, punctual short infection peaks in adults or chicks, or juvenile infection during their dispersal. Long-term data are needed to determine which of these mechanisms underlies the infection pattern observed.

KEYWORDS

Anseriformes, Avian influenza virus, Charadriiformes, *Larus michahellis,* Infection seasonality

TEXT

INTRODUCTION

Avian Influenza viruses (AIV) are negative-sense single stranded ARN viruses belonging to the Orthomyxoviridae family. They are commonly characterized by the combination of their surface proteins the hemagglutinin (HA) and the Neuraminidase (NA) of which respectively 17 and 9 types have been documented so far (Webster et al. 1992, Fouchier et al. 2005, Tong et al. 2012). Aquatic birds, particularly Anseriforms (ducks, geese and swans) and Charadriiforms (gulls, terns and shorebirds) constitute the major natural reservoir of AIV (Webster et al. 1992, Alexander 2000). The AIV circulating in wild birds are usually low pathogenic ones (LPAIV). LPAIV generally have little impact on their host (Latorre-Margalef et al. 2009, Velarde et al. 2010), although some studies have reported a possible influence on migration capacities (Van Gils et al. 2007). Besides, when LPAIV of H5 or H7 subtype are transmitted from wild birds to domestic ones reared in artificial environments, their virulence can evolve to high pathogenicity (Ito et al. 2001, Lebarbenchon 2010). Highly pathogenic avian Influenza virus (HPAIV), such as HP H5N1 strains currently circulating in Asia and Africa, are still of great economic concern, notably due to trade bans and to the cost of preventive actions including vaccination and massive bird cullings (Nicita 2008). Moreover, HPAIV infections represent a threat for human health since 603 HPAIV H5N1 human infections including 356 fatal cases have been reported worldwide since 2003 (WHO 2012). In this context, understanding the ecology and evolution of AIV in their natural habitat through the study of AIV infection dynamics in wild birds appears essential.

Infection seasonality is a crucial part of virus dynamics, which offers important insights into how pathogen-host systems operate (Altizer et al. 2006). Understanding infection seasonality can also help to accurately design research and surveillance programs. Interestingly, such phenomenon has been observed in AIV infections among Anseriformes. Indeed, AIV infection peaks occur in duck populations just before and during the fall migration period, probably due to the high number of immune-naive juveniles present after the breeding season (Olsen et al. 2006). Less attention has been paid to Charadriiforms in which no clear infection seasonal pattern has been highlighted so far. In gulls, for example, the observed temporal pattern of AIV infection is highly variable across regions. As an example, in the Delaware Bay (North America) where a large AIV surveillance program is led, most of the positive samples have been collected in May during the breeding period (Hanson et al. 2008) whereas in Northern Europe infection rates are higher from June to August and AIV are not detected at all in many colonies during the breeding season (Munster et al. 2007). Despite an increasing interest for year-round data collection in wild birds (e.g.

Hoye et al. 2011), this approach remains limited at the moment, especially in Charadriiformes.

Here, we conducted a year-round study on AIV in a biodiversity hotspot situated in Southern France crossroads between several bird migration routes: the Camargue (Berthold 2001, Kayser et al. 2008). This region is a potential hub of pathogen introduction and exchange (Jourdain et al. 2007). Since 2005 a long-term study is led on AIV in wild birds in this area. The first results highlighted a clear and repeatable seasonal pattern in wild ducks, with a marked infection peak occurring in September, when migrating juveniles begin to gather in wintering sites (Lebarbenchon et al. 2010). Samples were also punctually collected on gulls, but sample sizes were too small to lead to any conclusion on AIV infection temporal pattern (Lebarbenchon et al. 2010). In this study we focused on the the Yellow-legged gull (*Larus michahellis*), which is a relevant species to consider in a health ecology perspective as it lives in close contact with humans and their activities, notably due to its feeding habits (Yellow-legged gulls often feed on dumps; Duhem et al. 2003, 2008). In addition, it also appears as an ideal model species to study AIV in gulls in the focus region. Indeed, this species is abundant year-round on the French Mediterranean coast, where it recently expanded, and local populations are well known (Sadoul and Pin 2009). Yellow-legged gulls share wetlands use with numerous duck species including Common Teal (*Anas crecca*) and Mallard (*Anas platyrhynchos*) which represent both the most abundant duck species and the two main AIV hosts in the region (Lebarbenchon et al. 2010). Interestingly, AIV specific maternal antibodies were recently studied in the eggs of Yellow-legged gulls in different places along the Mediterranean coast including Southern France. Important AIV specific antibodies presence in the eggs collected in breeding colonies situated 80km east from the Camargue proved AIV circulation in the local population (Pearce-Duvet et al. 2009). As Yellow-legged gulls and ducks use the same wetlands and breed at the same period, AIV exchanges between ducks and gulls are likely to be frequent. Yellow-legged gulls also sometimes prey on ducks, which could further facilitate such viral transmission. Besides, AIV exchanges between Anseriforms and Charadriiforms have often been highlighted (e.g Widjaja et al. 2004, Wille et al. 2011). Considering these data we developed two hypotheses: i) There is an AIV infection seasonality in the focus gull population. ii) This seasonality is similar to the one observed in the sympatric duck population. To test these assumptions we collected fecal samples on a site occupied by Yellow-legged gulls during eleven months in 2010, and compared the AIV infection rates detected with those observed in local ducks during the previous three years.

MATERIAL AND METHODS

Bird sampling

Fresh dropping swabs were collected each month between February and December 2010 on an islet exclusively used by Yellow-legged gulls both as a resting and a breeding place (43°21'22.9"N; 4°40'00.79"E). This islet was situated in a pound, which was part of a salt exploitation in the Salin-de-Giraud village (Fig. 1). Samples were not collected in January 2010 because we could not access the islet during that month. Besides, using a network of hunters, cloacal swabs were taken on wild shot ducks during the 2007/2008, 2008/2009 and 2009/2010 hunting seasons (September to January). All swabs were stored in 3ml of universal transport medium (Biolys kit) and frozen at -80°C until molecular analysis was performed.

RNA isolation and virus detection

RNA isolation from 140µl of each sample was carried out using a Macherey-Nagel NucleoSpin 96 virus system with RNA elution into a final volume of 60µl. Influenza A was detected by real-time RT-PCR targeting the conserved matrix as describe previously (Duval et al. 2010). All real-time RT-PCR assays were performed on a LightCycler 480 (Roche Diagnostic) in a final volume of 10µl with 2.5µl RNA, 0.5µM of each primer, 0.2µM probe and 0.4µl enzyme mix using a Superscript III Platinum One-Step quantitative RT-PCR system (Invitrogen). The reaction was carried out with the following temperature profile: 15 min at 45°C, 3 min at 95°C, 50 cycles of 10 s at 95°C, 10 s at 55°C, 20 s at 72°C, and finally 30 s at 40°C.

Statistical analysis

Logistic regressions by Generalized Linear Models (GLM; binomial; R software, version 2.11.1) were fitted to the data to investigate the effect of the sampling month on the infection status.

RESULTS

We collected a total of 1082 gull fecal swabs between February and December 2010 (see Fig. 1). Among these samples 10 were AIV positive, which represent a 0.92% mean observed infection rate (95% confidence interval [CI]: 0.35, 1.49; Fig. 2). Observed infection rates varied between 0 and 4% (95% CI: 0, 7.82). The infectious status of Yellow-legged gulls was

not correlated to the month of sampling (GLM; for all months $P > 0.05$). Besides, we collected 1123, 652 and 1085 cloacal swabs on wild shot ducks during the 2007/2008, 2008/2009 and 2009/2010 hunting season respectively. Among these samples 224 were AIV positive, which accounted for a 7.8% mean infection rate (95% CI: 6.85, 8.82). Each year an infection peak was observed in ducks in September, the probability to find a positive sample in September being significantly higher than during the over months (GLM; odds ratio: 2.97; 95% IC: 1.92, 4.76; $P < 0.001$).

DISCUSSION

Our results do not support the hypothesis that AIV infection follow a seasonal pattern in the focal Yellow legged-gulls population, nor the existence of significant exchanges between ducks and gulls. Indeed, under the reverse scenarios, we expected larger AIV infection rates in Yellow-legged gulls, as well as an infection peak in early fall, when migrating ducks massively arrive in the Camargue, an important proportion of them being AIV infected (Lebarbenchon et al. 2010). During early fall, high numbers of young immune naive gulls are present and share wetland use with ducks. A early fall AIV infection peak in gulls could either originated from remaining AIV local strains amplified by newly arriving migrators or from new strains spread by them from their northern breeding grounds. This expected peak was not observed in Yellow-legged gulls, whereas a clear infection peak occurred in the sympatric duck population during each of the previous three years and during the study period as confirmed by the results of an active AIV surveillance program led in the Camargue during the 2010-2011 hunting season by another research team (Hars et al. 2011). Several factors may explain the decoupling of infection in ducks and gulls.

First, gulls are known to be mainly infected by AIV strains which are specific to the reservoir constituted by Charadriiformes, namely H13 and H16 subtypes (e.g. Fouchier et al. 2005, Toennessen et al. 2011). Thus, AIV strains circulating in ducks may not efficiently infect gulls, which could explain that the infection peak in ducks is not linked to an infection peak in gulls. Nevertheless, AIV strains circulating in ducks have also often been isolated in gulls, showing that this specificity is not strict (Widjaja et al. 2004, Wille et al. 2011).

Second, different alimentation habits between gulls and ducks, implying different transmission probabilities, may partly explain the contrasting infection patterns. Indeed, Yellow-legged gulls sometimes forage in freshwater like ducks, but, in the study area, they mainly feed on landfills and sea fishes (Duhem et al. 2008). Nevertheless, they often rest and sometimes forage in freshwater pounds used by ducks and frequently prey on them. Thus,

despite different eating habits, viral transmission should be facilitate between ducks and Yellow-legged gulls.

Third, Yellow-legged gulls have a long lifespan, which favors the development of acquired immune responses, the protection afforded by these responses being also supposed to last longer than in short-lived birds (Lee 2006). Indeed, studies proved that Laridae (gulls family) generally exhibit long lasting antibodies. For instance, a serological study led on the Black-legged kittiwake (*Rissa tridactyla*) proved that AIV specific antibodies could be observed more than a year after natural AIV infection (Toennessen et al. 2011). In contrast, AIV antibodies have been shown to be short-lived in Anatidae such as Mallards and Pink-footed geese (*Anser brachyrhynchus*) in which they generally last less than a year (Fereidouni et al. 2010, Hoye et al. 2011) .

These contrasts between gulls and ducks can help understanding why AIV seasonality is not similar in these two groups of birds. Still, a paradox subsists between the very low mean AIV infection rate (0.92%), and the high proportion of Yellow-legged gull eggs containing AIV antibodies in the study area (Pearce-Duvet et al. 2009). Our sampling method has already proved to be appropriate in several AIV study focusing on wild birds (e.g.Hoye et al. 2011, Wille et al. 2011). Aditionnally, our sampling effort was important, our monthly sample size permitting us to detect at least one infected individual if population prevalence was 3% or higher with 95% confidence. The contrast observed between antibodies presence and very low infection rates may be explained in 3 ways: i) A very low AIV circulation is maintained in the adult population year-round and is sufficient to explain the high proportion of seropositive individuals due to the long lasting immunity observed in gulls. ii) Short intense AIV infection peaks occur in Yellow-legged gulls either in immune naïve chicks or punctually (and not necessarily each year) in adults. iii) AIV infections occur in juveniles when they disperse during their first years out of the focus area.

As discussed before, antibodies are supposed to be long lasting in gulls which would corroborate the first assumption. Moreover such paradox between low AIV prevalence and high seropositive rates has already been highlighed in another gull species (*Rissa Tridactyla*, (Toennessen et al. 2011). Besides, other studies observed high AIV prevalence in gull chicks (e.g. Fouchier et al. 2005, Velarde et al. 2010) which supports the second assumption. However, although we did not specifically sampled chicks, when chicks were present on the sampling site (in May and June) it is unlikely that we only sampled adults. Indeed, since we collected fresh droppings all over the focus islet, notably near some nests, we certainly sampled both chicks and adults feces. So, considering that experimental infections determined

that LPAIV are excreted for approximately a week (and up to 16 days) in gulls (Costa et al. 2011), we should have detected an infection peak, even if it occurred in chicks, unless it was very short. Thus, if infection peaks occur, whether in chicks or adults, in the focus gull population, they are likely to be punctual and may not take place every year. Finally, juveniles of the focus population often spend a large part of their first 3 years on the atlantic coast (mainly in France and Spain) or in terrestrial areas, mainly the Loire Valley (Pin & Sadoul unpubl. data). They may commonly get AIV infected during that dispersal time, since they are likely to still be immune naïve and they reach some areas, like the Grand-Lieu region in the south of Brittany, where AIV circulation is regularly observed (Hars et al. 2011).

As a conclusion, we observed very low AIV infection rates with no infection seasonal pattern in the focus Yellow-legged gull population. This scheme significantly differs from the one observed in sympatric wild ducks. Long-term data are needed to determine which mechanisms underly the infection pattern we highlighted. Additionaly, collecting samples from other parts of the Yellow-legged gull distribution area, including juvenile dispersal sites, should be of great interest. More generally, the study of the AIV natural reservoir constituted by Charadriiformes should not be neglected, otherwise our understanding of the AIV dynamics in their natural habitat will always be partial. Such important gaps in our epidemiological knowledge could simply make us unable to efficiently face the challenge of the management of AIV circulation in poultry and humans.

ACKNOWLEDGMENTS

M. Vittecoq was supported by an AXA Ph.D. research fellowship. We thank the "Compagnie des salins du midi" for permission to conduct sampling on their land. We are grateful to hunting managers and hunters who authorized us to collect samples from shot ducks. We also thank the Centre National de la Recherche Scientifique, the Agence Inter-établissements de Recherche pour le Développement and the MAVA foundation for their financial support.

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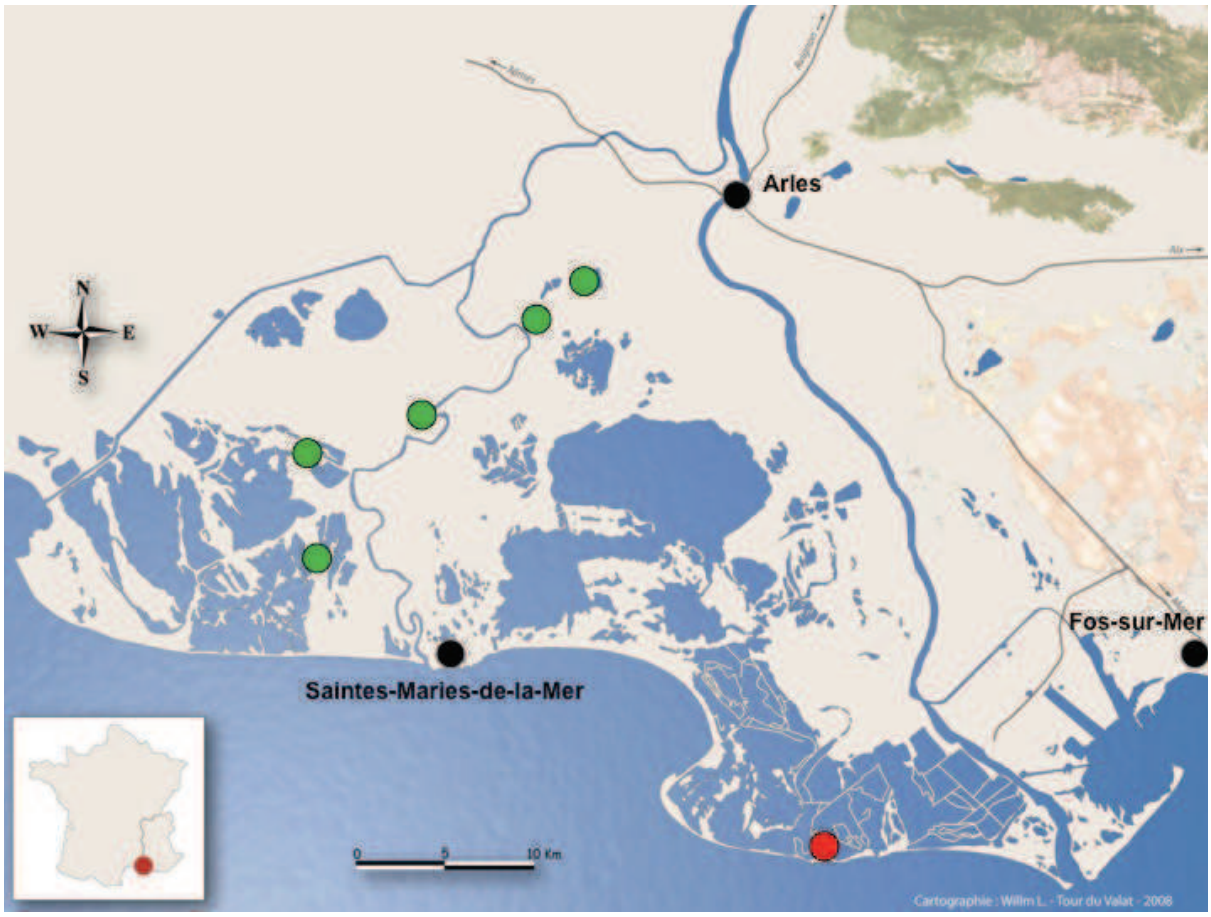

FIGURE 1. Localization of the sampling sites. The red circle represents the islet where gull droppings were sampled. Green circles represent the places where the shot ducks were swabed.

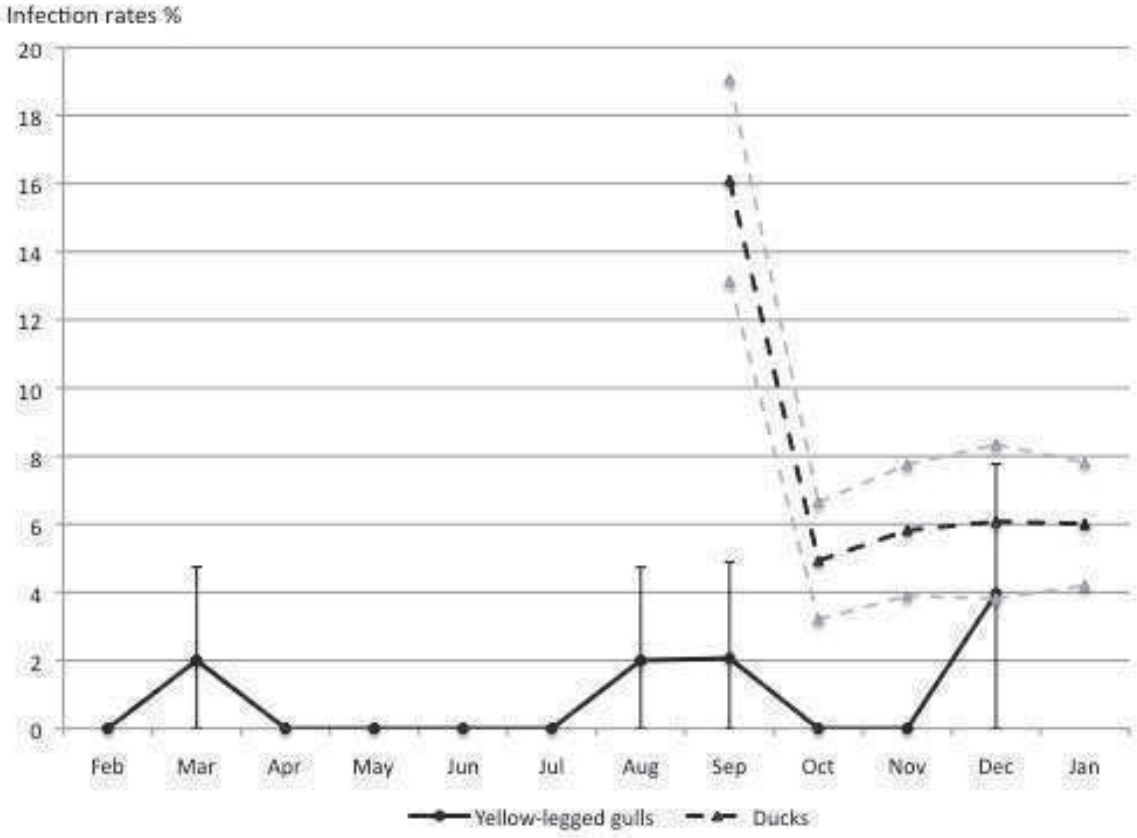

FIGURE 2. Temporal pattern of avian influenza virus (AIV) infections in Yellow-legged gulls and wild ducks in the Camargue. The solid line represents AIV prevalences in Yellow-legged gulls between February and December 2010. The dotted line shows AIV prevalences in Mallards and Common teal from September to January, these values correspond for each month to the mean AIV prevalence in the two species (data pooled) over the three hunting seasons studied (2007/2008 to 2009/2010). Confidence intervals (95%) are indicated by vertical bars for gulls and by grey dotted lines for ducks.

Article 9. Study of influenza A virus in wild boars living in a major duck wintering site.

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Publié dans *Infection, Genetics and Evolution*.

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Infection, Genetics and Evolution 12 (2012) 483–486

Contents lists available at SciVerse ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Short communication

Study of influenza A virus in wild boars living in a major duck wintering site

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ARTICLE INFO

Article history:
Received 18 July 2011
Received in revised form 6 December 2011
Accepted 8 December 2011
Available online 17 December 2011

Keywords:
Wild boar
Influenza virus
Inter-species transmission
France
Health ecology

ABSTRACT

Wild birds, which are reservoirs of influenza viruses, are believed to be the original source of new influenza viruses—including highly pathogenic ones—that can be transmitted to domestic animals as well as humans and represent a potential epizootic and/or pandemic threat. Despite increasing knowledge on influenza A virus dynamics in wild birds, the viral circulation in wild boars remains largely unknown. This is of particular interest since pigs can be infected with both human and avian viruses; upon co-infection, they can act as a mixing vessel through reassortment, a mechanism that resulted in the emergence of the pandemic H1N1 virus in 2009. The Camargue (Southern France) appears as an ideal study area to investigate inter-species transmission of influenza A viruses from wild birds and possibly humans to wild boars. Indeed, the important local wild boar population shares wetland use with humans and the largest concentration of wintering ducks in France, that are both susceptible to infection by influenza A viruses. Additionally, wild boars occasionally prey on ducks. We conducted a virological and serological survey on wild boars in the Camargue (Southern France) between September 2009 and November 2010. No influenza A virus was detected in the collected nasal swabs ($n = 315$) and no influenza specific antibodies were observed in the serological samples ($n = 20$). As the study was mainly focused on viral excretion, which is limited in time, we cannot exclude that low or occasional influenza A virus circulation took place during the study period. Although, wild boars did not seem to be a key element in the dynamics of influenza A virus circulation in the Camargue, wild boar influenza A virus infections should be more widely studied to determine if the pattern observed here represents the normal situation or an exceptional one.

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1. Introduction

Influenza A viruses (IAV) are segmented RNA viruses belonging to the *Orthomyxoviridae* family. They are widespread in the animal kingdom, occurring mainly in birds, humans, pigs, horses, dogs, and sometimes in cetaceans, mustelids, cats, and minks. Wild waterfowl, especially Anseriformes (ducks, geese, and swans) and Charadriiformes (gulls, terns, and waders), play a central role in their ecology (Alexander, 2000). Waterfowl are the reservoir of all known IAV subtypes from which IAVs can be transmitted to other avian or mammalian species and eventually establish new lineages in a new host.

IAVs are highly variable and undergo continuous genetic evolution via two mechanisms related to their RNA and the segmented nature of their genome. These mechanisms consist in the accumulation of point mutations at each replication cycle and reassortment events involving gene segment exchange that occur when a cell is co-infected by different viruses (Webster, 1998). These mechanisms contribute to the emergence of new variants with the ability to transmit to new hosts and/or with epidemic or even pandemic potential. Analysis of the viruses from past pandemics indicates a key role of pigs in the emergence of IAVs with the potential to initiate human-to-human transmission. Indeed, although the virus responsible for the Spanish influenza pandemic in 1918–1919 was not isolated at the time, its genomic sequence has been determined and revealed an avian-like H1N1 virus that contains human-like signature amino acids in several proteins (Taubenberger et al., 1997) that may, according to recent phylogenetic analyses, have been acquired through replication and reassortment events in pigs (e.g., Dos Reis et al., 2009). Similarly, the human H1N1 virus that caused

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doi:10.1016/j.meegid.2011.12.003

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the 2009 pandemic (H1N1pdm09) resulted from a reassortment between a triple (avian/human/swine) reassortant swine influenza virus originating from North America and an Eurasian avian-like swine virus (Neumann et al., 2009). The fact that pigs can be naturally and experimentally infected with human and avian viruses (see Brown, 2000 for a review) further supports the concept of their role as a mixing vessel allowing human and avian strains to reassort.

A better understanding of the epidemiology of IAV in avian, human, and swine compartments and their interplay appears essential. In particular, there is much to learn about the dynamics of IAV in wildlife populations. Since the beginning of the ongoing H5N1 epizootic, wild bird surveillance programmes have been launched worldwide, leading to a rapid increase in our understanding of the epidemiology of avian IAVs (Germundsson et al., 2010; Lebarbenchon et al., 2010a; Siembieda et al., 2010). Despite this increasing knowledge of IAV dynamics in wild birds, very little is known about their circulation in feral pigs and wild boars (both belonging to the *Sus scrofa* species like domestic pigs; Kuntz-Simon and Madec, 2009). Influenza A virus infections in wild boars have rarely been reported. Serological studies performed in several European countries such as in Poland (Markowska-Daniel, 2003, $n = 746$), Spain (Vicente et al., 2002, $n = 78$), Slovenia (Vengust et al., 2006, $n = 178$), and Germany (Kaden et al., 2008, $n = 1221$) emphasize the strong variability of swine influenza A virus (SIV) seroprevalence, which varies from 0% (e.g. Slovenia) to 26% (e.g. Southern Germany) depending on the region. Antibodies directed against the three European subtypes of swine influenza virus (SIV), H1N1, H3N2, and H1N2, have been detected in the different wild boar populations studied, but H1N1 seems to be the most prevalent subtype (e.g., Kaden et al., 2008; Vicente et al., 2002). Interestingly, H1N1 is also the most prevalent subtype in domestic pig populations in Europe (Kyriakis et al., 2011; van Reeth, 2007). In the USA, the few studies that have focused on SIV antibodies in feral swine populations also reported highly variable seroprevalence (0–91%) depending on the region and year (Corn et al., 2009, $n = 118$; Gipson et al., 1999, $n = 20$; Hall et al., 2008, $n = 215$; Saliki et al., 1998, $n = 120$). Moreover, in a recent study performed in South and North Carolina (USA) high seroprevalence was found in feral pigs living near high-density commercial swine production sites while the seroprevalence was null in areas with low-density domestic pig farms (Corn et al., 2009). Nevertheless, disease transmission between domestic and feral pigs or wild boars is poorly understood (Corn et al., 2009), and transmission between birds and wild boars appears to be undocumented.

In this context, studying IAV in populations of feral pigs and wild boars is necessary to understand all components of influenza virus epidemiology. The Camargue situated in the Rhône delta (Southern France) appears as an ideal study area to investigate inter-species transmission of influenza A viruses from wild birds and possibly humans to wild boars. Indeed, although there has been no recent large population study, the total number of wild boars in the Camargue is estimated to range from 2000 to 3000 individuals (Olivier pers. com.). This important local population shares wetlands use with the largest concentration of wintering ducks in France (e.g., Deceuninck and Fouque, 2010), which are commonly infected by IAV (Lebarbenchon et al., 2007a,b). Interestingly, wild boars occasionally prey on dead ducks. During the winter of 2010, up to 160 150 ducks were observed in the region (Gauthier-Clerc unpubl. data). In addition, studies indicate that IAV levels are high in these ducks during early fall and winter (mean prevalence in winter 2006–2007 = 2.1%, $n = 1119$, Lebarbenchon et al., 2007b). This raises the concern of potential transmission and establishment of avian IAVs in wild boars, as has been shown to happen with domestic pigs (Pensaert et al., 1981). Furthermore, human populations have increased continuously in the region of focus over the last decade, which has led to increased contact between humans and wildlife and a large number of humans (e.g., hunters, farmers, naturalists) frequent the habitats used by wild boars. Humans are commonly infected by IAVs especially during winter and could potentially be a source of IAV infection in wild boars. Indeed, human IAVs have been shown to transmit back from humans to domestic pigs and to establish in the pig population Considering these informations, the Camargue area provides unique opportunities for influenza A virus transmission from waterfowl, and possibly humans, to wild boars. Therefore, to gain insights into the circulation of IAVs in the wild boar population, we conducted a virological and serological survey in the Camargue between September 2009 and November 2010.

2. Materials and methods

2.1. Animals and sample collections

Because our aims were to test if wild boars are commonly infected by IAVs and to investigate the origin of circulating strains, the analysis was based on nasal swabs with a target set to be able to detect a 1% prevalence with a 95% confidence interval. In case of a positive detection, contrary to serological samples, nasal swabs would also allow isolation of IAV strains for analysis of phylogenetic and molecular characteristics. Samples were taken from wild boars supplied by a network of hunters throughout the Camargue during the 2009–2010 hunting season (September 2009 to January 2010) and at the beginning of the 2010–2011 season (August 2010 to November 2010). Nasal swabs were collected and placed in universal transport media (Copan Diagnostics Inc.) following Offlu recommendations (OIE/FAO, 2009), usually within 1 h of death. In addition, samples were taken from animals caught in the Tour du Valat estate using walk-in drop door traps from December 2009 to November 2010. All traps were baited with soured corn and used a hook attached to a string to trigger the door. Wild boars were marked before a nasal swab and, when possible, a blood sample (≈8 ml collected with a sterile needle) were taken. The animal was then released. Nasal swabs were kept at 4 °C during transport to the laboratory at the Tour du Valat research centre and then frozen at −80 °C. Serum (≈2 ml) was obtained from whole blood by centrifugation and stored frozen at −80 °C.

2.2. Viral analysis

All nasal swabs were analysed for the presence of IAVs at the Molecular Genetics of RNA Viruses unit (Pasteur Institute, Paris). RNA extraction was carried out on 140 µl sample using the Nucleospin 96 Virus Core Kit (Macherey–Nagel) following the manufacturer's instructions with minor modifications, and RNA was eluted in 60 µl of elution buffer. The presence of IAVs was detected by a one-step TaqMan real-time reverse transcription polymerase chain reaction (RT-PCR) targeting the matrix gene segment using a LightCycler 480 (Roche). We chose this method since it is one of those that have been validated and recommended by the FAO to detect H1N1pdm09 and other influenza viruses in swine (FAO, 2010). Amplification was performed on 2.5 µl of RNA combined with the Superscript III Platinum kit reagents (Invitrogen) in a total reaction volume of 10 µl. The primers and probe were previously described by Duval et al., 2010. The following cycling conditions were used: 15 min at 45 °C, 3 min at 95 °C, 50 cycles of 10 s at 95 °C, 10 s at 55 °C, 20 s at 72 °C, and finally 30 s at 40 °C.

2.3. Serological examination

All sera were analysed at Anses (Ploufragan), the national reference laboratory for swine influenza. Sera were tested for antibodies

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against the nucleoprotein of influenza A viruses using the ID Screen® Antibody Influenza A Competition ELISA kit (reference FLUACA) from ID-Vet (Montpellier, France) according to the manufacturer's instructions.

3. Results and discussion

During the study, a total of 315 nasal swabs were collected, of which 176 were from hunted wild boars and 139 were collected from 105 trapped individuals (34 were captured twice). Samples tested using real-time RT-PCR targeting the M gene gave negative results, indicating that no IAV could be detected in these animals. Our sampling for virological examination extended over the whole year and covered a significant portion of the targeted population (approximately 9–14%, depending on the population estimates). Our results should reflect the epidemiological situation in the Camargue since our sample size would permit to detect a 1% prevalence with 95% confidence. However, it is important to note that the swine viral excretion pattern is variable. Pigs typically excrete influenza A viruses for 7–10 days (Brown, 2000). Also, excretion can be intermittent as shown by Lange et al. (2009) in a study implying pigs experimentally infected with H1N1 swine virus (A/Regensburg/D6/09/H1N1). Moreover excretion timing and duration may differ between domestic pigs and wild boars. Therefore, we cannot exclude that a low and/or occasional IAV circulation took place in the population of focus during the study period. As a complementary analysis, serological tests were performed. A total of 20 sera samples were obtained from 19 trapped animals (one recapture). All ELISA tests for IAV-specific antibodies as markers of past infection were negative. These results, although limited in number, support the idea that few, if any, IAV infections occur in wild boars in the Camargue.

Wild boars could have been infected by water contaminated by viruses shed by infected birds (contact or ingestion) or through direct ingestion of dead infected ducks. Indeed, during the study period, wild waterfowl had IAV infection rates varying from 4% to 15%, depending on the month (Vittecoq et al. unpubl. data). In addition, mallards (*Anas platyrhynchos*) reared and released for hunting in the Camargue during the study period (>30,000 each year; Champagnon et al. unpubl. data) have also been shown to be infected by avian IAVs at variable rates before their release (Vittecoq et al. in preparation), Avian-like viruses have frequently been detected in domestic pigs but to our knowledge have never been reported in wild boars or in feral swine. This absence could reflect a higher probability of avian-to-swine viral transmission in high-density farming conditions: industrial rearing practices create new ecosystems where pathogen transmission is different compared to what is observed in natural habitats (e.g., Lebarbenchon et al., 2010b). Furthermore, as is the case in other mammals including humans, transmission in swine usually implies either animal-to-animal contact via the nasopharyngeal route, with the virus being shed in nasal secretions, or air-born infection through disseminated droplets or aerosols (Brown, 2000). Such a transmission route is greatly affected by population density and further limits the possibilities of establishing new IAVs in wild boars.

Influenza A virus transmission to wild boars could also originate from humans that share with them the use of wetlands and were infected by the H1N1pdm09 virus throughout our sampling period in this area (GROG, 2010; INVS, 2010). The ability of the H1N1pdm09 virus to transmit back from humans to pigs has been documented and the H1N1pdm09 virus is believed to have become endemic in the pig population in many regions (e.g., Forgie et al., 2011; Hofshagen et al., 2009). However, no human-like virus was observed in wild boars during our study. To our knowledge, no human-like influenza viruses have been observed in other wild boar populations. This likely illustrates that closer and more frequent contact between humans and swine as in intensive pig farming conditions is necessary for virus transmission. Indeed, antibodies specific to swine influenza are frequently detected in people who work in close contact with domestic pigs (e.g., Krumbholz et al., 2010; Kuntz-Simon and Madec, 2009; Terebuh et al., 2010).

4. Conclusions

No influenza A virus was detected in the wild boar population of the Camargue between September 2009 and November 2010, suggesting a low or null viral circulation in this animal population. This finding does not contradict previous serological studies led on wild boars in France and other European countries. Indeed, Payne et al. (2011) recently highlighted very variable seroprevalence in French wild boars populations (0/109 to 31/351 positive individuals) with very low IAV seroprevalence in the two departments from Southern France, Pyrénées-Atlantiques and Aveyron with 0/109 and 1/110 positive individuals, respectively. In the same way, a large serological study underlined very variable seroprevalences in Germany (0/120 to 21/81 positive individuals; Kaden et al., 2009). However, our results are in contrast to the fact that a substantial proportion of the human and waterfowl populations, that share habitats with the wild boars population in the Camargue, were infected by IAVs during the study period. Thus, viral transmission from both waterfowl and humans to wild boars appears to be rare, if any, and wild boars did not seem to be a key element in the dispersion dynamics of IAVs in the Camargue during our study. More studies on feral swine and wild boar infections are needed to determine whether our observations reflect the normal situation or an exception. In the context of the continued circulation of highly pathogenic IAVs such as H5N1 and of the worldwide circulation of the new H1N1pdm09 virus in both humans and domestic pigs, such studies should be integrated into multidisciplinary projects involving research on both the avian, swine and human compartments.

Acknowledgments

M. Vittecoq was supported by an AXA Ph.D. research fellowship. We are grateful to hunting managers and hunters who authorized us to collect samples from wild boars. We gratefully acknowledge O. Pineau, the estate management team of the Tour du Valat, and all the students for their great help with trapping and sampling live wild boars. We also thank Nicolas Barbier (Anses-Ploufragan) for his technical support. Finally, we thank the Institut de Microbiologie et de Maladies Infectieuses, the Centre National de la Recherche Scientifique, and the Agence inter-établissements de recherche pour le développement and the MAVA fondation for their financial support.

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Chapitre 3

Importance de l'environnement abiotique dans la dynamique des pathogènes : l'exemple des virus Influenza A

Caricature de Cham parue en 1852 dans l'album « Croquis Parisiens » publié par le bureau du Charivari. Au XIXème siècle, comme aujourd'hui, la méconnaissance des paramètres qui conditionnaient le début et la fin des épidémies de grippe conduisait à de multiples interprétations relayées par les médias incluant la mise en cause du corps médical (voir Dievoet & Lara 2009).

I. Persistance des virus Influenza A dans l'environnement

La transmission d'un virus implique son passage d'un hôte à un autre. Ce passage peut s'effectuer sans passer par une phase libre dans l'environnement. C'est le cas pour les agents pathogènes à transmission sexuelle tels que le virus du SIDA . L'existence d'un hôte vecteur évite aussi le passage par l'environnement comme dans les cas du virus West Nile évoqué précédemment. Cependant, la majorité des pathogènes à l'origine d'infections gastro–intestinales ou respiratoires, chez l'Homme comme chez les autres mammifères et les oiseaux, se transmettent via un passage par une phase libre dans l'environnement (air, eau ou surface de dépôt ; Li *et al.* 2009). Pour ces micro-organismes la persistance dans l'environnement est un paramètre crucial pour la transmission.

Les VIA causent, en fonction des hôtes et des sous-types, des infections gastro-intestinales ou respiratoires et se transmettent comme la plupart des autres maladies de ce type via un passage dans l'environnement. Ils présentent la particularité de pouvoir être transmis à la fois via l'air, l'eau et les surfaces contaminées (appelées fomites) en fonction des souches, des hôtes et des conditions environnementales. Au sein de l'avifaune sauvage les VIA se transmettent principalement par la voie oro-fécale impliquant l'ingestion d'eau contaminée par des fèces ou le contact avec les fèces eux-mêmes (Wallensten 2007). La persistance dans l'eau des VIA apparaît donc jouer un rôle majeur dans leur dynamique (Roche *et al.* 2009). Elle permettrait notamment leur maintien dans des plans d'eau d'une année à l'autre, entre deux périodes de présence des espèces hôtes, influençant ainsi leur capacité d'invasion (Rohani *et al.* 2009; Breban 2012). Il a également été proposé que la capacité de persistance dans l'environnement pourrait jouer un rôle majeur dans la co-existence des différentes souches de VIA circulant chez les oiseaux (Roche & Rohani 2010), ainsi que sur l'évolution de leur virulence (Roche, Drake & Rohani 2011). Les virus ayant une faible capacité de persistance doivent se maintenir continuellement au sein de la population d'hôtes et seraient donc moins virulents que ceux qui peuvent se maintenir longtemps entre la mort de leur hôte et l'arrivée de nouveaux individus sensibles. La détection d'excrétion de VIA au niveau de la trachée chez les Laridés (Bahl & Pomeroy 1977; Costa *et al.* 2011), et, dans une moindre mesure, chez les Anatidés (Ellström *et al.* 2009; Kleijn *et al.* 2010; Jourdain *et al.* 2010), suggère par ailleurs l'existence d'une transmission aérienne de certaines souches aviaires dont l'importance est méconnue (cf. **article 6**).

Chez les humains et les autres mammifères terrestres tels que le porc la transmission aérienne joue un rôle important (Brown 2000; Tellier 2006). Elle implique le contact avec des gouttelettes projetées par un individu infecté soit directement soit après que ces dernières aient persisté en suspension dans l'air (jusqu'à plusieurs heures pour les microgouttelettes les plus petites ; Tellier 2006). Le contact avec les surfaces contaminées par le dépôt de gouttelettes contenant des particules virales pourrait aussi jouer un rôle important qui fait encore débat (Brankston *et al.* 2007; Lowen & Palese 2009). Il a notamment été suggéré que la transmission aérienne serait prépondérante en milieu tempéré, tandis que la transmission par contact avec les fomites aurait plus d'importance en milieu tropical du fait des conditions climatiques défavorables à la persistance des VIA dans l'air (Lowen & Palese 2009).

Chez les oiseaux, comme chez les humains et les autres mammifères, la persistance environnementale apparaît donc comme un paramètre clé de la dynamique des VIA. Pourtant, les études portant sur cette persistance sont encore peu nombreuses. Les travaux ayant visé à faire le bilan des données disponibles s'étaient jusque là intéressés aux VIA circulant soit chez les oiseaux (Stallknecht *et al.* 2009), soit chez les humains (Weber & Stilianakis 2008). Or, nous avons souligné tout au long des chapitres précédents les liens étroits entre les VIA circulant dans les compartiments aviaire, porcin et humain illustrant l'absence de barrière nette entre ces différents types de souches. Nous avons donc souhaité rassembler l'ensemble des connaissances disponibles concernant la persistance des VIA dans l'environnement en incluant les travaux les plus récents. L'objectif de cette synthèse est de disposer d'une base solide permettant de dégager les principales lacunes existantes et les axes à développer en priorité pour les combler **[Article 10].**

Le bilan des résultats des études existantes montre qu'en milieu aquatique les virus persistent d'autant plus longtemps que la salinité est faible, la température basse et le pH proche d'un optimum qui varie autour de 8 en fonction des souches (Stallknecht *et al.* 1990, 1991; Zarkov 2006; Brown *et al.* 2007, 2009; Dublineau *et al.* 2011). Des épisodes de gel répétés détruisent rapidement les VIA (Lebarbenchon *et al.* 2011, 2012). Dans l'air les particules virales ont une stabilité maximale lorsque l'humidité et la température sont faibles et le rayonnement modéré (Wells & Brown 1936; Tamm & Fluke 1950; Harper 1961; Schaffer, Soergel & Straube 1976; Lowen *et al.* 2007, 2008; Shaman & Kohn 2009). Déposés sur des surfaces, les VIA persistent d'autant plus longtemps que celles-ci sont poreuses et leur stabilité est maximisée s'ils sont inclus dans des sécrétions nasales (Bean *et al.* 1982; Tiwari *et al.* 2006; Noyce, Michels & Keevil 2007; Thomas *et al.* 2008).

Notre connaissance des paramètres influençant la persistance des VIA dans l'environnement demeure cependant très superficielle. Tout d'abord, les mécanismes qui régissent ces influences sont mal compris, si l'on excepte l'influence du pH dont on connaît le rôle dans l'activation de l'hémagglutinine (Reed *et al.* 2010). Par ailleurs, l'impact des variations des paramètres en jeu a fait l'objet de très peu d'analyses. Des études telles que celles publiées par Lebarbenchon et al. (2010 & 2012) concernant l'impact des fluctuations de température devraient être menées sur l'ensemble des

paramètres impliqués qui varient en conditions naturelles (salinité, UV, humidité etc.). De plus, le rôle de certaines variables peu étudiées, telles que les espèces et la densité de bactéries présentes dans l'eau, devrait être exploré.

Au-delà de la compréhension des mécanismes influençant la persistance des VIA dans l'environnement, les données manquent pour comprendre le rôle de cette persistance dans la dynamique de ces pathogènes, chez les humains, comme chez les autres mammifères et les oiseaux. Cette compréhension passe notamment par des études expérimentales des modes de transmission à l'exemple de celles menées par Lowen et al. chez le cochon d'Inde (*Cavia porcellus)* (Lowen *et al.* 2007, 2008). Une évaluation de l'impact des conditions environnementales sur l'épidémiologie des VIA est par ailleurs nécessaire.

II. Influence des conditions météorologiques sur la présence du virus H1N1pdm09 en France

Suite à cette conclusion, nous avons mis en place une étude visant à déterminer si les conditions environnementales (i.e. température, humidité et rayonnement UV) avaient joué un rôle dans la dynamique du virus H1N1pdm09 au sein de la population française d'août 2009 à avril 2010 **[Article 11].** En effet, de nombreuses études attestent du lien entre le début des épidémies de grippe chez l'Homme et des conditions météorologiques favorables telles qu'une humidité faible, une température basse et une faible insolation (voir Tamerius 2011 pour une synthèse). Cependant, ces études se limitent en général à une ou plusieurs métropoles (e.g. Tang *et al.* 2010; Davis, Rossier & Enfield 2012) si l'on excepte celles menées aux Etats-Unis (Shaman & Kohn 2009; Stark *et al.* 2012). De plus, elles traitent principalement de la dynamique des épidémies saisonnières, tandis que celle des pandémies n'a été jusqu'alors que peu abordée. Les seuls travaux existant à notre connaissance sur ce point ont montré que les phases froides prononcées ou prolongées du phénomène El Niño favorisaient la survenue des pandémies (Mazzarella, Giuliacci & Pregliasco 2011; Shaman & Lipsitch 2012). A une échelle plus fine un lien a été établi entre la dynamique du virus pandémique H1N1pdm09 et les conditions météorologiques. A Niamey (Niger) en zone désertique la transmission de ce virus apparait favorisée par des températures faibles et une grande visibilité (liée aux jours de pluie ; Jusot, Adamou & Collard 2011). Tandis qu'une étude menée à Brisbane (Australie) a montré que, sous ce climat subtropical humide, une

faible température et de faibles précipitations favorisent la présence des cas de grippe causés par le virus H1N1pdm09 (Hu *et al.* 2012).

Nous avons souhaité tester l'influence des conditions environnementales sur la dynamique d'un virus de grippe pandémique dans un pays tempéré et à une échelle plus grande. Nous avons utilisé les données de présence de cas de grippe dus au virus H1N1pdm09 récoltées par le réseau des GROG[4] au sein de 235 villes françaises pour tester l'association entre la présence du virus dans la population et la température, l'humidité et l'ensoleillement extérieur. Nous avons par ailleurs intégré des données démographiques incluant l'effectif des communes et des départements, ainsi que leur densité, qui peuvent influer sur les taux de contacts entre les hôtes. Les périodes de congés scolaires ont également été prises en compte car il a été montré que ces congés diminuaient l'incidence de la grippe en France (Cauchemez *et al.* 2008).

Les résultats de cette étude mettent en évidence une corrélation entre la présence des cas de grippe et un ensoleillement faible, une température faible et une humidité relative faible. Ces trois paramètres étant corrélés entre eux il est difficile de déterminer leurs rôles respectifs, cependant la corrélation la plus marquée est celle liant l'ensoleillement et les cas de grippe. Par ailleurs la présence de la grippe apparaît favorisée dans les grandes villes. Ces relations sont moins marquées lorsque les enfants sont en congés que pendant les périodes scolaires.

Ces résultats confirment à l'échelle d'un pays l'influence des conditions environnementales sur la transmission du virus H1N1pdm09. Cependant, les mécanismes impliqués restent à explorer. En effet, les paramètres climatiques peuvent déterminer la persistance des VIA dans l'air ou les autres milieux comme évoqué plus haut. Cependant, ils influencent également certainement les taux de contact entre les hôtes, notamment en faisant varier le temps passé en intérieur, même si aucune étude n'a pu en apporter la preuve pour le moment (Tamerius 2011). Ils peuvent également modifier la sensibilité des hôtes à l'infection. Un faible ensoleillement prolongé diminue le taux de vitamine D dans l'organisme ce qui réduit les réponses immunitaires (Cannell *et al.* 2008), tandis que des températures basses et l'humidité faible rendent les voies

[4] *Le réseau des Groupes Régionaux d'Observation de la Grippe a été crée en 1984 dans le but de détecter le début des épidémies de grippe et de suivre leur évolution chaque année. Plus de 500 médecins exerçant dans 21 des 22 régions de France métropolitaine (la Corse n'est pas incluse) participent à ce réseau qui s'appuie sur des cas confirmés en laboratoire pour suivre la progression des épidémies de grippe ainsi que la circulation d'autres agents infectieux (VRS, rhinovirus, parainfluenzae etc.).*

respiratoires supérieures plus sensibles aux infections du fait de différents mécanismes (synthétisés par Tamerius 2011). Des études de transmissions expérimentales sont nécessaires pour comprendre les mécanismes impliqués. Cette compréhension apparaît indispensable à la mise en œuvre de moyens de contrôle appropriés dans un contexte pandémique.

Article 10. Persistance des virus influenza A en fonction des paramètres environmentaux.

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Publié dans *Virologie.*

revue

Virologie

Virologie 2011, 15 (6) : 371-9

Persistance des virus influenza A en fonction des paramètres environnementaux

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Résumé. Les populations d'oiseaux aquatiques constituent le réservoir naturel des virus influenza A. La transmission entre individus a principalement lieu par la voie orofécale, comprenant l'ingestion d'eau contaminée. Au sein des populations humaines, les virus influenza A se transmettent, soit par inhalation de gouttelettes porteuses de virus, soit par contact direct avec des sécrétions. Ainsi, dans les compartiments aviaires et humains, les virus doivent passer par une phase libre pour être transmis. Afin de mieux comprendre la dynamique virale, il importe de mieux connaître la persistance virale durant cette étape clé au sein de ces deux compartiments interconnectés. C'est pourquoi nous présentons un bilan des connaissances actuelles concernant la persistance environnementale des virus influenza A aviaires et humains. Les données synthétisées ici montrent en milieu aquatique l'influence prépondérante de la température, du pH et de la salinité sur la stabilité virale ; en milieu aérien, l'importance de la température, des rayonnements ultraviolets et de l'humidité de l'air. Les mécanismes qui sous-tendent l'influence des facteurs considérés et le rôle potentiel d'autres paramètres sont discutés. Nous soulignons la nécessité de multiplier les études pour améliorer notre connaissance de la stabilité des virus influenza A et donc de leur transmission.

Mots clés : virus influenza A, stabilité, transmission

Abstract. Wild waterfowl represents the natural reservoir of influenza A viruses. Transmission within bird populations occurs through an indirect fecal-oral route implying contaminated water. Within human populations, influenza A viruses can be transmitted through large droplets, aerosols, or direct contact with secretions. Thus, in the human compartment as in the avian one, influenza A viruses have to experiment a free living stage. The knowledge of factors influencing viral persistence during that key step is needed to understand their transmission dynamic. Data gathered here describe the major role played by temperature, pH and salinity on viral persistence in aquatic environment and the importance of UV radiations, humidity and temperature in mid air. We discuss mechanisms underlying these roles and the potential influence of other factors. We point out the need to develop researches to improve our understanding on influenza A virus stability and thus transmission.

Key words: influenza A virus, stability, transmission

Introduction

Les virus influenza de type A sont des virus enveloppés à ARN monocaténaire de polarité négative, appartenant à la famille des *Orthomyxoviridae*. Ils se distinguent des virus influenza de type B et C par des critères génétiques et antigéniques, notamment au niveau de deux protéines internes : la nucléoprotéine (NP) et la protéine de la matrice (M1) [1]. Leur génome est constitué de huit segments codant pour 11 protéines. Deux de ces protéines présentes à la surface du virus jouent un rôle majeur dans l'infection et la réplication virale : l'hémagglutinine (HA) assurant la fixation et la pénétration du virus à l'intérieur de la cellule et

Tirés à part : M. Vittecoq

doi:10.1684/vir.2011.0425

Pour citer cet article : Vittecoq M, Ottmann M, Renaud F, Thomas F, Gauthier-Clerc M. Persistance des virus influenza A en fonction des paramètres environnementaux. *Virologie* 2011; 15(6) : 371-9 doi:10.1684/vir.2011.0425

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la neuraminidase (NA) permettant la libération des nouveaux virions après la phase de multiplication virale. À ce jour, 16 HA (H1 à H16) et neuf NA (N1 à N9) antigéniquement distinctes ont été décrites [1]. Un sous-type de virus, désigné par sa combinaison HxNy des deux glycoprotéines virales, peut rassembler de nombreuses souches génétiquement distinctes.

Les virus influenza A peuvent infecter une large gamme d'hôtes dont de nombreux oiseaux, les humains, les chevaux, les porcs ainsi que certains carnivores et cétacés [2] *(figure 1)*. Les oiseaux de l'ordre des Ansériformes (canards, oies, cygnes) et des Charadriiformes (mouettes, goélands, limicoles) constituent le réservoir naturel des virus influenza aviaires [1]. À partir de ce réservoir, de nouvelles souches peuvent être transmises aux oiseaux domestiques. Les virus influenza aviaires représentent une importante cause de morbidité et de mortalité pour les élevages de volaille au sein desquels les fortes densités et la faible diversité génétique favorisent l'évolution de formes plus virulentes [3]. Les sous-types H5 et H7 font ainsi partie des agents infectieux à déclaration obligatoire selon le code sanitaire pour les animaux terrestres de l'OIE (World Organization for Animal Health) car ils présentent la particularité de pouvoir passer d'une forme faiblement pathogène à une forme hautement pathogène [4]. Les virus H5N1 hautement pathogènes qui circulent actuellement chez les volailles principalement en Asie représentent notamment un problème économique important, ainsi qu'un enjeu de santé public. Ils sont parfois transmis à l'homme et ont causé depuis 2003 la mort de 332 personnes, alors même qu'ils ne possèdent pas la capacité de se transmettre d'homme à homme [5]. À intervalles irréguliers, de nouvelles souches issues du réservoir aviaire acquièrent cette capacité, ce qui entraîne parfois des pandémies. On sait par exemple à présent que la pandémie grippale de 1918 qui fit plus de 50 millions de morts était due à un virus dont la NA et l'HA étaient issues de souches aviaires [6]. De même, la pandémie de 2009 due au nouveau variant H1N1 (H1N1pdm09) résulte d'un réassortiment multiple entre des souches aviaires, humaines et porcines [7]. Ces exemples illustrent les relations étroites existant entre les virus influenza A circulant dans les populations aviaires et dans les populations humaines. La compréhension de leurs dynamiques ne peut donc être envisagée qu'en considérant conjointement les données concernant le compartiment humain et le compartiment aviaire.

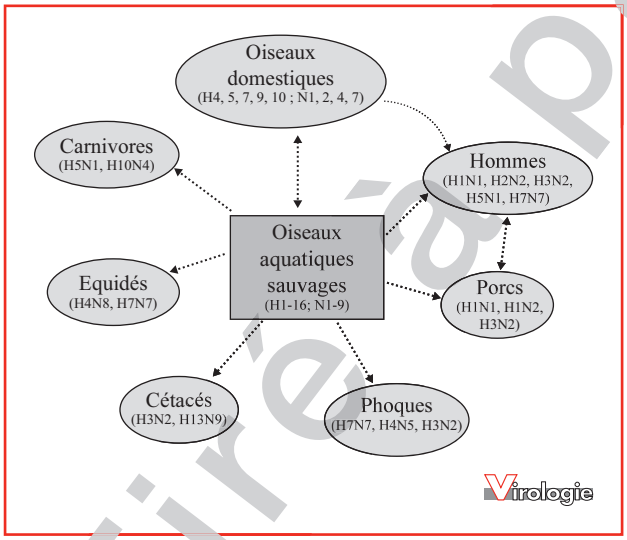

Figure 1. Liens entre le réservoir naturel des virus influenza A et les autres hôtes de ces agents pathogènes. Dans chaque compartiment les sous-types présents sont indiqués. Au sein de la population humaine, il est important de distinguer les sous-types à transmission interhumaine (H1N1, H2N2 et H3N2) de ceux qui ne se transmettent que d'oiseau à homme. Différents sous-types appartiennent à cette seconde catégorie, mais seuls les virus H5N1 et H7N7 hautement pathogènes sont responsables d'infections humaines parfois fatales.

Une saisonnalité est observée au sein de ces deux compartiments. Chez les oiseaux sauvages, les pics d'infection par les virus influenza A ont habituellement lieu à la fin de l'été et au début de l'automne, lorsque les hôtes migrent de leurs zones de reproduction vers leurs quartiers d'hivernage [8]. Ce schéma temporel est attribué à l'afflux d'individus juvéniles immunitairement naïfs et donc sensibles aux virus influenza A circulants. Cependant, le rôle des réservoirs abiotiques tels que les étangs et leurs sédiments reste méconnu. Différents modèles ont montré l'effet primordial de la persistance des particules virales dans l'eau sur les capacités de transmission et de dispersion des virus influenza A [9, 10]. En effet, chez les oiseaux aquatiques, la transmission est principalement orofécale. Celle-ci peut donc avoir lieu, soit par contact, *via* les sécrétions d'un individu infecté, soit par l'eau contaminée *(figure 2)*. Au sein des populations humaines, les épidémies de grippe ont généralement lieu en hiver dans les zones tempérées, tandis que des infections ont lieu toute l'année en zone tropicale où une saisonnalité est néanmoins observée puisqu'un plus grand nombre de cas est enregistré durant la saison des pluies [11]. Ces schémas temporels, connus de longue date, demeurent mal compris. Les facteurs influant sur l'immunité des hôtes et leurs contacts apparaissent déterminant quoique méconnus [12]. Les paramètres influant sur la persistance virale dans l'environnement semblent également devoir jouer un rôle important [11]. En effet, pour infecter un nouvel hôte, les virus influenza A passent généralement par une phase libre dans l'environnement. Celle-ci peut être très brève, comme dans le cas de la projection de gouttelettes contaminantes directement inhalables ou plus longues si les virus se déposent sur des surfaces. Leur

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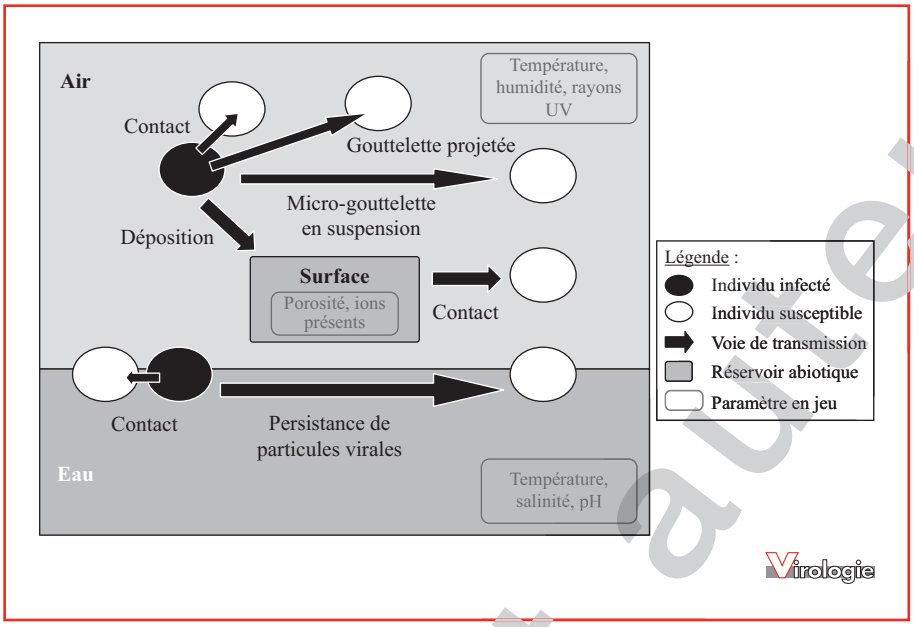

Figure 2. Voies de transmission des virus influenza A en milieu aquatique et aérien et facteurs environnementaux, influant sur la persistance virale dans l'environnement.

stabilité dans cet environnement représente nécessairement un déterminant essentiel de leur capacité de transmission. Dans la population humaine, la transmission peut avoir lieu selon trois modes : soit par inhalation de particules virales en suspension dans l'air, soit *via* des gouttelettes projetées par une personne infectée, soit par contact direct avec des surfaces contaminées *(figure 2)*. L'importance relative de ces trois modes d'infection reste sujette à controverse [13-15]. Connaître les facteurs influençant la persistance des virus influenza A dans l'air et sur les surfaces sur lesquelles ils se déposent est indispensable à la compréhension de l'épidémiologie du virus au sein des populations humaines. Si l'infection est majoritairement acquise par inhalation de virus, le port de masque représente une protection efficace [16]. Le nettoyage des surfaces et le lavage des mains ne sont utiles que si le contact avec des surfaces contaminantes est considéré comme une voie de transmission importante. La connaissance des voies de transmission privilégiées en fonction des paramètres environnementaux permet ainsi la mise en place de moyens de prévention efficaces en cas d'épidémie.

Il apparaît ainsi que la persistance des virus influenza A en milieu abiotique est un élément clé de leur dynamique au sein des compartiments aviaire et humain. Ces derniers, étant étroitement connectés, nous avons souhaité présenter et discuter de façon conjointe les connaissances actuelles de la persistance dans l'environnement des virus influenza A circulant au sein des populations humaines et aviaires. Notre objectif est ainsi de compléter les précédents travaux de synthèse ayant abordé cette problématique [14, 17] en apportant une vision globale et actualisée des données disponibles ainsi que de leur signification en termes de dynamique virale.

Persistance virale en milieu aquatique

L'eau constitue un réservoir abiotique pour de nombreux pathogènes dont les virus influenza A, en particulier ceux circulant chez les oiseaux. Ces derniers sont fréquemment isolés à partir des plans d'eau fréquentés par les oiseaux, en particulier par les canards [18, 19]. Les virus excrétés dans l'eau demeurent infectieux plus ou moins longtemps en fonction de différents paramètres, dont les principaux identifiés à ce jour sont la température, le pH et la salinité.

Plusieurs études du même laboratoire ont démontré le rôle de ces trois facteurs pour la stabilité virale. L'équipe de Stallknecht a ainsi testé la persistance des virus influenza aviaires à 17 ou 28 °C pour une salinité de 0 ou 2 % NaCl et un pH allant de 6,2 à 8,2 [20, 21]. Ces travaux ont montré que dans des conditions de salinité modérée (2 % NaCl), la persistance du virus, maximale à pH 6,2, décroît aux pH plus élevés (7,2 et *a fortiori* 8,2). L'effet du pH est plus important à 28 qu'à 17 °C, ces deux températures représentant les moyennes estivales et hivernales de l'eau d'un étang en pays tempéré (plus exactement dans cette étude

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en Louisiane). Cependant, à salinité nulle, l'effet du pH est inversé, la stabilité maximale étant observée à pH 8,2, ce qui témoigne du lien étroit entre salinité et pH. Stallknecht *et al*. ont complété ces résultats en étendant la gamme des valeurs des paramètres testés [22, 23]. Ils ont ainsi testé un pH allant de 5,8 à 8,6, une température de 4 à 37 °C et une salinité de 0 à 3 % NaCl. Leurs résultats montrent une meilleure persistance virale à 17 °C ; en revanche, à cette température, l'effet négatif de la salinité est plus marqué qu'à 28 °C. Par ailleurs, la persistance virale, maximale à pH 7,8, décroît au-delà et *en deçà* de cette valeur. Il est important de noter que les réactions aux paramètres testés étaient très variables en fonction des souches considérées, les souches faiblement pathogènes apparaissant plus stables que les souches hautement pathogènes. L'ensemble de ces résultats expérimentaux est étayé par une étude sur la persistance virale au sein d'échantillons d'eau prélevée en milieu naturel avec une persistance nulle pour un pH de 9,34 [24]. Globalement, la persistance des virus influenza aviaires dans l'eau apparaît donc maximale à faible température et à faible salinité ainsi qu'à pH légèrement basique (*tableau 1*). Cependant, il existe peu de données sur l'effet des fluctuations de ces paramètres qui adviennent en milieu naturel. L'étude de ces effets apparaît d'autant plus importante que les premières recherches sur les conséquences de variations de température sur la persistance des virus influenza aviaires dans l'eau ont montré que celles-ci avaient un impact important [19, 25]. Plus précisément, un passage régulier de l'eau

Tableau 1. Articles relatant la persistance des virus influenza aviaires en milieu aquatique selon le pH, la salinité et la température.

Études	Paramètres	Valeurs(s) testée(s)	Relation avec la persistance virale
Stallknecht *et al.*, 1990a	Température	17 et 28 °C	Persistance plus courte à 17 qu'à 28 °C
Stallknecht *et al.*, 1990b	Température	17 et 28 °C	Persistance plus courte à 17 qu'à 28 °C
	Salinité	0 et 2 % NaCl	Persistance plus courte quand la salinité augmente, effet plus marqué à 28 qu'à 17 °C
	pH	5,8 à 8,2	Valeur optimale variable en fonction de la salinité de la température (T, °C) et de la souche
Brown *et al.*, 2007	Température	17 et 28 °C	Persistance plus courte quand T, °C augmente
	Salinité	0 ; 15 ; 30 % NaCl	Persistance plus courte quand la salinité augmente
Brown *et al.*, 2009	Température	4 à 37 °C	Persistance plus courte quand la T, °C augmente
	Salinité	0 à 30 % NaCl	Persistance plus courte quand la salinité augmente
	pH	5,8 à 8,6	Persistance optimale pour un pH de 7,8 (variable selon les souches)
Stallknecht *et al.*, 2010	Température	- 20 et 4 °C	Persistance longue à 4 °C quand la T, °C est constante Rapide réduction de la qualité de virus dans l'eau lorsqu'on alterne les 2 T, °C
Lebarbenchon *et al.*, 2011	Température	- 20 et 28 °C	T, °C constante : persistance plus courte quand la T, °C augmente Alternance entre 2 T,°C : 17/23 °C : persistance intermédiaire entre celles observées pour les 2 valeurs - 20/4 °C : persistance très faible (inférieure à celle observée à 28 °C)

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de 17 à 23 °C conduit à une durée de persistance proche de celle observée à 17 °C [19]. À l'inverse, des cycles de gel et dégel successifs (passage de 4 à - 20 °C) écourtent considérablement la persistance des particules virales par rapport aux valeurs observées à une température constante de 4 ou - 20 °C (*tableau 1*) [19, 25]. Les mécanismes pouvant expliquer les effets des paramètres testés sont partiellement connus. On considère que le pH influe sur la stabilité virale du fait du changement conformationnel irréversible que subit l'HA à partir d'une certaine acidité favorisant la fusion membranaire [26]. Si cette activation se produit après l'attachement à une cellule cible, l'infection peut avoir lieu. Dans le cas contraire, le virus est définitivement inactivé. Les seuils d'activation varient en fonction des souches. Un faible pH de fusion peut engendrer une activation tardive dans le lysosome ne permettant pas l'infection. À l'inverse, un pH de fusion élevé entraînera généralement un plus grand risque d'inactivation dans certains organes de l'hôte, en particulier le gésier chez les oiseaux [27]. L'inactivation pourra également avoir lieu dans l'eau où le virus est excrété. Ainsi, pour pouvoir se propager efficacement, un virus influenza A doit posséder un pH d'activation optimal en fonction de son hôte et du milieu au sein duquel il est propagé. Ce seuil d'activation dépend de l'HA portée par le virus, mais aussi de la NA dont l'activité peut augmenter le pH d'activation de la HA [28]. Les rôles de la température et de la salinité sont moins bien compris. La stabilité accrue du virus à faible température pourrait résulter de l'ordonnancement croissant des phospholipides présents à la surface des virus influenza A avec la diminution de la température ou d'une absence de dégradation du génome viral [29].

Les études se sont jusqu'alors principalement concentrées sur le pH, la salinité et la température, laissant de côté d'autres facteurs potentiellement importants comme les rayonnements ultraviolets (UV) ou la constitution même des particules virales. L'effet inactivant des rayonnements UV a été observé sur différents virus en milieu marin [30], tout comme celui des protéases et des nucléases produites par certaines bactéries [31]. De même, il a été montré que les bivalves filtreurs pouvaient diminuer la quantité et l'infectiosité des virus influenza aviaires présents dans l'eau [32]. Ces résultats soulignent l'importance de prendre en compte l'effet des différents organismes et micro-organismes aquatiques sur la persistance des virus influenza dans l'eau.

Persistance virale en milieu aérien

Chez les humains, les virus influenza A sont projetés dans l'air au sein de micro-gouttelettes rejetées par les individus infectés lors de quintes de toux ou d'éternuements (*figure 2*). Les plus grosses de ces gouttelettes (> 20 µm) se déposent directement sur les surfaces environnantes. L'individu peut alors s'infecter *via* ses mains portées au visage après avoir été contaminées au contact d'une surface. Les gouttelettes de taille intermédiaire (entre 5 et 20 µm) peuvent être inhalées par une personne se trouvant à faible distance de l'individu infecté. Enfin, les plus petites (< 5 µm) peuvent rester en suspension dans l'air et être inhalées à plus grande distance [33]. Pour que de nouveaux hôtes soient infectés, la stabilité des virus au sein des gouttelettes doit être suffisante pour qu'un des trois modes de transmission au moins soit efficace. Or, comme c'est le cas en milieu aquatique, la persistance des virus influenza A en milieu aérien est fonction de nombreux paramètres interconnectés.

Le rôle de l'humidité de l'air apparaît important. Deux expressions de cette variable peuvent être prises en compte, l'humidité absolue (AH) représente la quantité de vapeur d'eau présente dans l'air et l'humidité relative (RH), représente le rapport de cette quantité sur la valeur pour laquelle l'air est saturé. L'RH varie en fonction de la température et de la pression contrairement à l'AH. Hemmes *et al*. montraient ainsi dès 1962 que la survie de virus influenza A humains au sein de micro-gouttelettes produites artificiellement était favorisée par une RH faible [34]. De même, la transmission aérienne d'une souche humaine chez le cochon d'Inde à 20 °C est plus efficace pour une RH faible (20 à 35 %) que pour des valeurs plus élevées (50 et 80 %) (*figure 3*) [35]. Néanmoins, certains isolats semblent être plus stables à RH élevée (70 %) que pour des valeurs intermédiaires (50 %) [36]. Des modèles ont été conçus pour estimer la corrélation de la température, l'RH et l'AH avec le succès de transmission à partir des données de Lowen

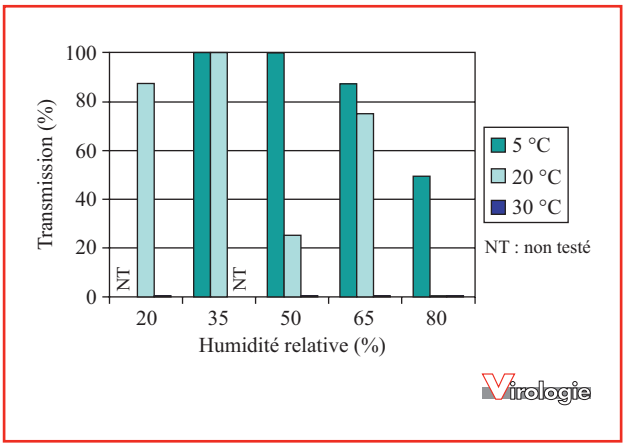

Figure 3. Efficacité de la transmission chez le cochon d'Inde en fonction de la température (5, 20 et 30 °C) et de l'humidité relative (données Lowen *et al.*, 2007 et 2008). Les pourcentages de transmission indiqués correspondent aux proportions d'individus initialement sains qui excrétaient des virus influenza A après et/ou durant leur exposition par voie aérienne à des individus infectés.

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et al. [35]. Selon l'analyse de l'ensemble de ces données, l'hypothèse la plus robuste est que la transmission du virus est favorisée par des valeurs faibles d'AH [37]. En d'autres termes, plus l'air est sec, moins le virus se transmet. Shaman et Kohn [37] confirment ce point en incluant dans leurs modèles les résultats supplémentaires présentés par Lowen *et al*. [38] à l'issue d'une seconde étude de transmission expérimentale chez le cochon d'Inde *(figure 3)*. Ils reprennent en outre les données de Harper [39] concernant la stabilité des virus influenza A dans l'air en fonction de la température et de l'RH. En calculant à partir des deux paramètres testés l'AH correspondante, ils montrent, par des modèles de régression, une forte corrélation non linéaire entre l'AH et la survie des virus. Il apparaît ainsi que l'AH favorise la transmission principalement en augmentant la stabilité du virus et non par la modification de la taille des micro-gouttelettes en suspension dans l'air. La stabilité du virus dépendrait donc de la quantité absolue de vapeur d'eau présente dans l'air environnant, représentée par l'AH plutôt que du taux de saturation en eau de l'air que représente l'RH.

L'influence de la température sur la stabilité des virus influenza A est aussi importante en milieu aérien. En effet, à RH constante, la transmission aérienne (d'une souche influenza humaine) est plus efficace chez le cochon d'Inde à 5 °C qu'à 20 °C *(figure 3)*, bien que la réponse immunitaire innée des individus testés soit peu affectée par cette variation de température [35]. La transmission serait donc bien influencée par une plus grande stabilité virale à faible température. Néanmoins, le taux d'excrétion virale était plus fort chez les hamsters testés à 5 °C que chez les autres, suggérant un rôle de l'hôte dont les muqueuses, cibles des virus influenza A, peuvent être affectées par la température. Bien que le taux d'excrétion virale ne différait pas entre les individus élevés à 20 et 30 °C au cours de l'étude suivante [38] aucune transmission aérienne n'a eu lieu à 30 °C quelle que soit l'RH, tandis qu'à 20 °C, seule une RH de 80 % inhibait totalement la transmission. Ces résultats confirment le rôle de la température sur la stabilité virale. Les modèles cités plus haut incorporant ces données montrent donc une meilleure transmission à faible température [37].

En outre, l'action des rayons UV sur les virus influenza A est connue de longue date. Dès 1936, Wells et Brown [40] montraient au cours d'une expérience d'infection expérimentale chez le furet utilisant une souche humaine, qu'aucun individu n'était infecté par un flux d'air issu des cages d'animaux porteurs de virus influenza A, si ce flux d'air subissait une irradiation aux rayons UV. À l'inverse, tous les individus testés étaient infectés si ce traitement n'était pas appliqué. Cette inactivation semble directement liée à la dégradation de l'ARN viral sous l'action des UV. Celle-ci est maximale lorsque les rayons utilisés sont des UV-C, 265 nm apparaissant comme la longueur d'onde optimale pour dégrader des ARN [41]. L'action virucide des UV-C, moins bien connue que leur action bactéricide, a récemment été étudiée par Walker et Ko [42]. Leurs résultats révèlent une relation positive entre l'RH et la capacité d'inactivation virale des UV-C pour le bactériophage MS2 et l'adenovirus [42]. Un modèle a par ailleurs été dernièrement développé pour évaluer le taux quotidien d'inactivation potentielle de virus influenza A humains en suspension dans l'air dû aux rayons UV dans diverses métropoles [43], en se basant sur les taux d'inactivation calculés expérimentalement par Hemmes *et al*. [34]. Leurs résultats montrent de grandes disparités en fonction des latitudes et des saisons, la durée de persistance des virus allant de quelques heures à quelques jours selon l'ensoleillement. Ces auteurs concluaient à un rôle de l'action des rayons UV sur la stabilité des virus influenza A beaucoup plus important que ceux de l'RH ou de la température. Cependant, certaines erreurs commises dans cette étude ont été soulignées, ramenant l'influence des rayons UV sur la stabilité virale au même ordre de grandeur que celles de l'RH et de la température [14].

À ces trois facteurs s'ajoute le type de substrat de dépôt lorsque les virus ne sont plus en suspension dans l'air (*tableau 2*). La porosité de cette surface a alors un rôle déterminant dans le maintien de l'infectiosité virale. Globalement, la stabilité virale est meilleure sur des surfaces de faible porosité [44]. Des virus influenza A humains actifs peuvent ainsi être isolés sur les pages d'un magazine après 48 heures alors qu'on n'en retrouve plus sur les pyjamas des patients après 24 heures [45]. Le même type de résultats s'applique à des virus influenza aviaires qui subsistent moins de 24 heures sur des boîtes à œufs en carton alors qu'on peut encore en isoler au bout de six jours sur des gants en latex [46]. Cependant, cette différence pourrait être inhérente au protocole expérimental, l'élution ne permettant pas de récupérer les virus une fois fixés dans les pores des surfaces testées, ce qui ne signifierait pas que ceux-ci sont inactivés [46]. Toutefois, selon cette hypothèse, les virus qui persisteraient ne joueraient plus de rôle dans la transmission puisque, bloqués au sein de la surface, ils ne contamineraient pas les individus entrant en contact avec cette dernière. D'autres propriétés des surfaces peuvent avoir leur rôle. Ainsi, le cuivre inactive les virus influenza humains en moins de six heures alors que ceux-ci restent actifs plus de 24 heures sur de l'acier inoxydable [47]. Le mécanisme expliquant cette inactivation est encore mal compris, il pourrait être expliqué par l'action des ions cuivre sur le génome des micro-organismes suggérés par des résultats obtenus par la même équipe sur *Staphylococcus aureus* [47]. En outre, le milieu dans lequel le virus est déposé peut plus ou moins favoriser sa persistance. Les sécrétions nasales au sein desquelles les virus influenza circulant au sein des populations humaines sont naturel-

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Tableau 2. Articles présentant la durée de persistance moyenne des virus influenza A selon le type de substrat de dépôt.

Études	Virus	Température (°C)	Humidité relative (%)	Surface	Persistance moyenne (h)
Bean *et al.*, 1982	Humain H3N2	27,8-28,3	35-40	Acier inoxydable	72
				Magazine, plastique	48
				Pyjama, mouchoir	24
Tiwari *et al.*, 2006	Aviaire H13N7	« Température ambiante »	Non précisée	Acier	72
				Latex	144
				Coton	24
				Plume	144
Noyce *et al.*, 2007	Humain H1N1	20-24	50-60	Cuivre	6
				Acier inoxydable	24
Thomas *et al.*, 2008	Humain H1N1	21-28	30-50	Billet de banque	1
	Humain H3N2				24

lement excrétés maximisent leur survie sur des billets de banque [48]. L'infectiosité peut persister jusqu'à 17 jours lorsque l'isolat est déposé sur un billet de banque suisse, peu poreux car recouvert de résine, au sein de sécrétions, alors que sa survie est limitée à deux jours en l'absence de ce milieu. Il est par ailleurs important de noter que ces résultats varient considérablement en fonction des souches testées et de la concentration initiale de l'isolat déposé.

Conclusion et perspectives

L'ensemble des résultats cités précédemment laisse apparaître des tendances globales concernant l'influence de quelques paramètres sur la stabilité des virus influenza aviaires et humains. Ils persistent plus longtemps dans une eau froide, légèrement basique et faiblement salée. Rejetés dans l'air, leur stabilité est maximale avec une faible température et une faible humidité (qu'elle soit relative ou absolue) ainsi qu'un rayonnement UV modéré. Déposés sur une surface, ils y demeurent infectieux d'autant plus longtemps que celle-ci est peu poreuse et qu'ils sont inclus dans des sécrétions nasales. Les valeurs optimales pour chaque facteur pris en compte varient considérablement en fonction de la souche considérée et de l'interaction existant entre les différents facteurs.

Ces résultats soulignent cependant les nombreuses faiblesses qui subsistent encore dans notre connaissance du devenir des virus influenza A au sein de l'environnement. En premier lieu, les mécanismes moléculaires expliquant les effets observés des différents paramètres environnementaux demeurent méconnus. Le rôle du pH dans l'activation de l'HA fait figure d'exception, notamment grâce à des études comme celles de Reed *et al*. [28] qui devraient être multipliées afin de mieux appréhender le rôle de la salinité de l'eau ou l'humidité de l'air. En outre, les études testant expérimentalement la stabilité des virus influenza A en fonction des différents paramètres environnementaux se bornent actuellement à une gamme limitée de valeurs discrètes pour chacune des variables prises en compte. Il apparaît donc primordial d'élargir à l'avenir cette gamme et d'expérimenter des valeurs continues, en testant par exemple l'effet d'une montée de la salinité de l'eau ou de ses fluctuations, telles qu'elles peuvent se produire en milieu naturel. Le nombre de variables testées à l'heure actuelle apparaît également limité. Les espèces et les densités de bactéries présentes dans l'eau devraient ainsi être également prises en compte. Cependant, la multiplication des paramètres testés dans une même étude complique l'interprétation des résultats obtenus du fait des interactions pouvant exister entre eux. En outre, étant donnée la complexité d'une telle étude « exhaustive » pour un virus, il faudrait sans doute disposer de moyens puissants permettant un titrage viral à « haut débit ». Il pourrait alors être intéressant de comparer la persistance d'un virus humain saisonnier, d'un virus aviaire issu de la faune domestique et d'un virus aviaire circulant dans la faune sauvage. De plus, comme l'ont récemment souligné Irwin *et al*. [49], la présentation des résultats s'avère très disparate entre

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les études, ce qui complique leur comparaison. Il serait préférable de traduire uniformément la stabilité observée des virus en termes de taux de décroissance et/ou de demi-vie virale.

En outre, il convient de replacer l'étude de la stabilité des virus influenza A dans le contexte plus large de leur transmission dont elle n'est qu'un élément. Cette transmission dépend en effet de nombreuses autres composantes telles que le taux de contact entre les hôtes ou leur comportement. Les capacités prédictives des modèles de transmission utilisant uniquement les données de persistance virale sont ainsi limitées par la nécessité de prendre en compte ces autres facteurs. La multiplication des études de transmission expérimentale semble ainsi indispensable à la progression de notre connaissance des diverses composantes de la transmission des virus influenza A. Elle est cependant limitée par des raisons tant éthiques qu'économiques. Parmi les populations d'oiseaux sauvages, les modèles de transmission permettent d'appréhender la dispersion potentielle de différentes souches au gré des mouvements des animaux, dont leurs migrations, en fonction des capacités de persistance des virus dans les différents plans d'eau qu'ils fréquentent [10]. Au sein des populations humaines, ils pourraient expliquer la saisonnalité des épidémies de grippe A en zone tempérée comme l'existence d'une transmission continue en zone tropicale. Shaman *et al*. [50] ont ainsi récemment mis en évidence une corrélation entre les anomalies négatives d'HA et le début des épidémies de grippe A aux États-Unis, quoique ce lien soit variable en fonction des régions considérées [14]. D'après les données de stabilité virale, Lowen et Palese [15] ont émis l'hypothèse d'une prépondérance de la transmission aérienne dans les pays tempérés contrastant avec une transmission par contact plus importante en milieu tropical qui expliquerait, au moins partiellement, les différences de circulation virale entre les deux zones.

En conclusion, la limitation des impacts des virus influenza A sur la santé publique et vétérinaire nécessite la mise en place de moyens de lutte adéquats, qui ne peuvent être efficacement conçus que si la circulation virale est comprise au sein de chaque compartiment concerné, oiseaux sauvages, animaux domestiques et population humaine. Or, nous venons de voir que la persistance virale au sein de l'environnement est multifactorielle et un élément clé de cette dynamique, c'est pourquoi sa connaissance apparaît primordiale. Cette connaissance est pourtant encore très parcellaire, sa progression nécessite une approche pluridisciplinaire qui reste à développer, alliant virologistes, biochimistes, écologues et épidémiologistes.

Conflits d'intérêts : aucun.

Remerciements. Cet article est issu d'un travail bibliographique réalisé pour le diplôme universitaire de virologie médicale de l'université Lyon 1 intitulé : « Tout ce que vous avez toujours voulu savoir sur le virus influenza » dirigé par le professeur Bruno Lina au cours de l'année universitaire 2009-2010.

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Article 11. Does the weather play a role in pandemic influenza spread ? A study of H1N1pdm09 infections in France in 2009-2010.

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En préparation.

Does the weather play a role in pandemic influenza spread ? A study of H1N1pdm09 infections in France in 2009-2010.

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Introduction

Influenza is one of the most significant infectious diseases in humans, considered to be associated with approximately 250 000 to 500 000 deaths worldwide annually (WHO 2009). The economic burden of influenza is also huge, notably due to absenteeism at work, treatment costs and hospitalizations (Simonsen 1999). As an example, in the United States the total economic burden of influenza epidemics is estimated to reach $87.1 billion annually (Molinari *et al.* 2007). In temperate regions annual influenza dynamics follow a clear seasonal pattern marked by an important infection peak during winter months, while this seasonality is less defined in tropical areas, where infections occur throughout the year (Lipsitch & Viboud 2009). Episodically, new influenza variants, for which no immunity exists, infect the human population and cause pandemics. The four pandemics of the last century alone claimed more than 50 millions lives (Stohr 2002).

A growing number of studies is documenting the link between seasonal influenza infections and particular weather conditions. Low temperature, low humidity (either absolute or relative) and low insolation have been associated with influenza infections onset and spread at different scales (see Tamerius 2011 for a review). Yet, as these factors can influence contact rates, immunity and virus survival in the environment, the mechanisms underlying these relations have still to be determined. To our knowledge the only studies focusing on the role of meteorogical parameters on pandemics apparition and dynamics investigated the

influence of El Nino events (ENSO). Strong or prolonged ENSO cold phase appeared to favor influenza pandemics (Mazzarella, Giuliacci & Pregliasco 2011; Shaman & Lipsitch 2012). ENSO cold phases have also been linked to increased seasonal influenza mortality and morbidity in France and California (Flahault *et al.* 2004; Viboud *et al.* 2004; Choi, Christakos & Wilson 2006).

The last pandemic was due to a novel avian/swine/human reassortant that was first detected in Mexico in March 2009 (Neumann, Noda & Kawaoka 2009). This new variant rapidly spread worldwide, notably thanks to the intense air traffic existing between Mexico and a number of big cities in the other continents (Khan *et al.* 2009). A year later, 214 countries and overseas territories had reported laboratory confirmed cases of pandemic influenza H1N1 2009 including over 18449 deaths (WHO 2010). Despite the role of air travel in viral transmission, two studies led on two important cities (Brisbane, Australia and Niamey, Niger) showed at a local scale that meteorogical conditions, including temperature and precipitation, had an important role in pandemic H1N1 transmission (Jusot, Adamou & Collard 2011; Hu *et al.* 2012).

Here we aimed at exploring this association between weather conditions and pandemic H1N1 spread at a broader scale and in a temperate country. We chose to investigate France where 13 to 24% of the population was infected as two complementary surveillance networks collected detailed data on influenza infections (Vaux 2010). We used presence absence data of H1N1 laboratory confirmed cases between August 2009 and April 2010 provided by the GROG network other 235 French cities distributed throughout metropolitant France (Fig. 1). We wanted to test three hypothesis: i) At the national scale the virus has spread from a few sites of emergence to the rest of the country ii) H1N1 was more likely to spread and remain present in places showing low insolation, low humidity and low temperature iii) Socio-demographic factors also shaped H1N1 transmission, which is supposed to be more frequent in large cities that are highly connected to a number of various geographical points through, train, air traffic and roads and present more meeting points such as hospitals, administrations and schools.

We chose to study temperature, humidity and UV radiations because these parameters have been extensively linked with influenza transmission (see Tamerius 2011 for a review). We also included school schedules because it has been shown that school closures have a negative effect on influenza transmission in several countries including France (Cauchemez *et al.* 2008). We added demographic factors as a proxy of contact rate patterns as big cities are more likely to possess concentration places (hospitals, highschools etc.) and transport hubs

(airports, important train stations etc.) that favor both influenza amplification and geographical spread.

Methods

- Epidemiological data

We used presence/absence data collected by the GROG network during 33 weeks (S36 2009 to S15 2010) in 235 French cities by 286 physicians (see Fig.1). The H1N1pdm09 virus was considered to be present in a city if at least one infection case was laboratory confirmed during the focus week in that place (see http://www.grog.org/ for more details on diagnostic methods). The virus was considered to be absent if at least one sample had been collected in the city during the focus week and no positive sample has been observed. When no sample was made we do not considered the data. Indeed, we could not know if no sample was made because the virus was absent or due to other factors such as doctor absence or infected patients that failed to consult their doctor.

- Climatic and socio-demographic variables

We used Meteo-France weekly climatic data including temperature (mean, maximum and minimum), relative humidity (mean, maximum and minimum) and mean insolation (see Table 1). Additionally, air pressure was used along with relative humidity and altitude to calculate mean absolute humidity following the formulas used in the conversion software available at: http://www.cactus2000.de, and based on Lowe & Ficke (1974)

Each virological sampling site was associated to the nearest meteorogical station that recorded temperature, humidity and air pressure. Insolation data was added only when these stations also recorded this parameter (in 151 cities). Indeed, systematically searching for station recording insolation data would have greatly increased the mean distance between meteorological and epidemiological sampling sites (see Fig1. b), thus reducing the accuracy of climatic data. Statistical analyses were led on both the dataset containing 235 cities but no insolation data and the dataset containing insolation data but only 151 cities.

Population sizes and densities were included at the city scale and the department scale using the last data recorded by the INSEE (INSEE 2008; Table 1). Finally, school schedules were obtained from the French Ministry of Education website (http://www.education.gouv.fr/cid197/les-archives-calendrier-scolaire-partir-1960.html; Table 1).

- Statistical analysis

To test the spatial autocorrelation between epidemiological data we used two Mantel tests. The first one tested the correlation between the distance separating cities and the similarity of epidemiological data. The second tested the correlation between the distance separating cities and the date of local epidemic start (first case occurrence, see Fig. 2).

To investigate the role of climatic and socio-demographic variables on H1N1pdm09 presence we used GEEGLM models because they allowed us to modelize binomial data according to both quantitative and qualitative variables while taking into account their temporal autocorrelation through an AR-1 correlation matrix. We first assessed the correlation between our variables using a pairwise correlation test. We then selected in each group of highly correlated variables the one that was the most significant while being not highly correlated with other groups of variables. Finally we used a backward step selection to select the best model.

Results

- Spatial autocorrelation

The correlation between the matricx of distances between sampling sites and the week of the first case detection was not significant (Mantel; 1000 iterations; $P = 0.395$). In the same way, the correlation between the distance matricxand the matricx including all the virological presence/absence data was not significant (Mantel; 1000 iterations; $P = 0.938$). Besides, the date of detection of first influenza case was negatively correlated to both density and population size of city with city density having the strongest effect, which means that cases were detected earlier in the most densely populated cities (glm, family Poisson, $\beta = -4.70.10^{-5}$; S.E. $= 9.65.10^{-6}$; $P = 1.08.10^{-6}$).

- Role of weather and socio-demographic conditions

Correlations between the different variables ranged from 0.05 to 1. We selected one variable in each group of highly correlated ones, based on the significance of its effect on viral presence in an univariate GEE model with AR-1 correlation structure (See Table 1). Correlations between the variables we selected were ≤ 0.4. The values that are presented in Table 1 were obtained using the dataset containing insolation values (151 sampling sites). We also led that first step of our analysis on the dataset containing 235 cities (but no insolation data). The same variables were selected, following this approach. All further steps of our

analysis were led on the dataset containing insolation values since insolation had a highly significant effect on H1N1pdm09 presence.

We used a backward selection process to select the model that fitted best to our data. The model that was selected and the corresponding parameters are presented in Table 3. According to that model both insolation and humidity had a negative effect on pandemic influenza cases (temperature effect could not be included since temperature and humidity are highly correlated). Besides, the population size of the cities had a positive effect on that presence. Furthermore, H1N1pdm09 were more likely to be observed during school time and the impact of meteorogical conditions was lessened during child holidays. Finally, the highly significant temporal autocorrelation observed (α = 0.327, S.E. = 0.178) showed that infections were more likely to occur in a city if cases had been observed there during the previous weeks.

Discussion

- Spatial spread

First, our results did not highlight any spatial pattern in the spread of H1N1pdm09 across France. This result contrasts with those observed in USA, where seasonal influenza infections seem to be synchronized in highly populated states and then spread to neighboring states that have a smaller population (Viboud *et al.* 2006). Yet, our findings are in accordance with the conclusion of a study previously led on seasonal influenza spread in France, which showed that distance between regions had no effect on viral spread contrary to different types of transports flows (road, train and plane traffic ; Crepey & Barthelemy 2007). According to these results, we can suggest that French regions are highly interconnected, which abolishes any distance effect in viral spread between cities. This connectedness is higher in large cities, which could explain why pandemic influenza was more likely to be present in such cities and reach them earlier, following the same way as seasonal influenza that first reaches states with a large population in USA (Viboud *et al.* 2006).

- Role of weather conditions

Second, our findings underline the role of weather conditions on pandemic influenza spread at the scale of a temperate country. The effects of humidity and insolation we

highlighted are in accordance with the results of experimental transmission studies as well as viral persistence ones. Indeed, on one hand, human influenza A virus survival in the environment is favored by low humidity, low temperature and low insolation (reviewed in Weber & Stilianakis 2008). On the other hand, experimental transmission studies led on mammals (guinea pigs; *Cavia porcellus* and ferrets ; *Mustela putorius*) have shown that these conditions also favored aerial transmission (Wells & Browns 1936; Lowen *et al.* 2007, 2008)

Yet, further experimental transmission studies are needed to determine which mechanisms underly the effects we evidenced. Indeed, weather conditions may affect influenza infections through multiple ways (see Lofgren *et al.* 2007 for a review). First, they can favor viral survival in the environment as discussed above. Second, they can influence host susceptibility to the infection. For example, a prolonged underexposition to UV radiations decreases the levels of vitamin D in the organism, which impairs immunity (Cannell *et al.* 2008). Third, weather conditions can influence contact rates between hosts and time spent indoors.

- Limitations of our study

Our study was limited by the sampling protocol of epidemiological data we used. Indeed, the sampling was done on patients who had decided to consult their physician and that presented characteristic symptoms, which did not allowed detecting asymptomatic cases. Additionally, physicians may have been absent during the study period, thus we could not regard missing data as an influenza absence proof. Furthermore, we did not include case number estimations in our analysis, since those that are collected in France by the Sentinel network are based on « influenza-like cases » numbers rather than on laboratory confirmed cases, which do not allow to distinguish influenza infections from some other respiratory infections. This point appears especially important knowing that some pathogens that can cause influenza-like symptoms, such as rhinovirus, were circulating in France before the beginning of the H1N1pdm09 pandemic and are thought to have influenced its timing (Casalegno *et al.* 2010).

- Perspectives

It appears difficult to improve the sampling protocol described above since virological analysis are limited by their cost as well as by the willingness of the members of the GROG network to provide samples, while they have less and less time to deal with their patients. Influenza surveillance needs to be urgently improved at a global scale, since influenza virus

spread extremely rapidly from one country to another and many countries worldwide, including some European ones, lack an efficient influenza surveillance network.

This need of surveillance appears especially acute if we consider that global changes will potentially increase the frequency of influenza pandemics. Indeed, rearing practise intensification coupled with exponential increase of goods and people flows create new opportunities for new viral variants to emerge and spread. Besides, as highlighted by our results, weather conditions seem to influence pandemic influenza dynamics, which means that climatic changes may have important impacts on future pandemics. To meet the challenge of pandemic influenza prevention, surveillance should be improved in both humans and domestic animals, since pandemic influenza viruses generally originate in reassortment between human, pig and/or avian viruses. Besides, experimental infection studies exploring the mechanisms involved in influenza virus transmission are needed to build up efficient control measures.

Aknowledgements

We sincerely acknowledge Eric Elguero and Simon Daoust for their valuable advices during the preparation of this manuscript. M. Vittecoq is supported by an AXA research fund Ph.D. fellowship. This work was funded by the Agence inter-établissements de recherche pour le développement, the MAVA foundation and the Région Provence-Alpes-Côte d'Azur.

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Variable	Mean		Median		Range	
	D1	D2	D1	D2	D1	D2
Weekly minimum temperature (C°)	4.7	4.3	5.1	4.7	-8.4 – 19.1	-8.4 – 19.1
Weekly maximum temperature (C°)	12.4	11.9	12.4	11.9	-2.5 – 30.4	-2.3 – 30.4
Weekly average temperature (C°)	8.3	7.9	8.7	8.2	-5.2 – 24.1	-5.2 – 22.8
Weekly minimum relative humidity (%)	61.1	61.9	63.8	64.1	20.9 – 96.7	23 – 96.7
Weekly maximum relative humidity (%)	93.7	93.9	94.7	95.0	61.7 – 100.0	63.2 – 100.0
Weekly average relative humidity (%)	80.7	80.9	82.7	83.1	44.4 – 99.1	49.7 – 99.1
Weekly average absolute humidity ($g.kg^{-1}$)	7.1	6.9	7.0	6.8	2.6 – 14.1	2.6 – 13.6
Weekly mean insolation ($J.cm^{-2}$)	NA	675.2	NA	489.6	NA	91.3 – 2 282.3
City population size 2008	56 698	72 131	10 883	9 935	318 – 2 211 297	462 - 2 211 297
Department population 2008	846 313	833 717	710 034	647 420	123 907 – 2 564 969	123 907– 2 564 969
City population density 2008 (pop. per km^2)	1763.7	2009.2	652.2	595.7	15.8 – 25 192.7	15.8 – 25 192.7
Department population density 2008 (pop. per km^2)	509.5	681.9	122.2	99.9	22.3 – 20 980.0	22.3 – 20 980.0
School holidays	D1 : 5/33 Yes (27 cities) ; 6 Yes (110) ; 7 Yes (98)		D2 : 5/33 Yes (19 cities) ; 6 Yes (65) ; 7 Yes (67)		Yes /No	

Table 1 : General statistics for all the variables we studied. General statistics are given for both datasets (D1 : 235 cities ; D2 : 151 cities), except for insolation, which was available in 151 cities only.

Variable	Effect in an univariate model (GEE glm AR-1 correlation structure)			
	B (S.E.)		P-value	
	D1	D2	D1	D2
Weekly minimum temperature	$-1.22.10^{-2}$ ($7.17.10^{-3}$)	$-7.42.10^{-3}$ ($9.40.10^{-3}$)	$8.80.10^{-2}$	$4.30.10^{-1}$
Weekly maximum temperature	$-2.60.10^{-2}$ ($5.87.10^{-3}$)	$-1.99.10^{-2}$ ($7.46.10^{-3}$)	$\underline{9.3.10^{-6}}$	$\underline{7.70.10^{-3}}$
Weekly average temperature	$-2.19.10^{-2}$ ($6.72.10^{-3}$)	$-1.58.10^{-2}$ (0.00862)	$\underline{1.10.10^{-3}}$	$\underline{6.70.10^{-2}}$
Weekly minimum relative humidity	$2.60.10^{-2}$ ($2.80.10^{-3}$)	$3.05.10^{-2}$ (0.00337)	$\underline{< 2.10^{-16}}$	$\underline{< 2.10^{-16}}$
Weekly maximum relative humidity	$2.29.10^{-2}$ ($7.16.10^{-3}$)	$3.41.10^{-2}$ (0.00924)	$\underline{1.40.10^{-3}}$	$\underline{2.2.10^{-4}}$
Weekly average relative humidity	$3.37.10^{-2}$ ($4.10.10^{-3}$)	$4.16.\ 10^{-2}$ (0.00505)	$\underline{2.2.10^{-16}}$	$\underline{2.2.10^{-16}}$
Weekly average absolute humidity	$-2.83.10^{-2}$ ($1.52.10^{-2}$)	$-8.49.10^{-3}$ ($1.99.10^{-2}$)	$6.20.10^{-2}$	$6.70.10^{-1}$
Weekly mean insolation	NA	$-2.39.10^{-3}$ ($1.92.10^{-4}$)	NA	$\underline{< 2.10^{-16}}$
City population size 2008	$3.71.10^{-7}$ ($6.12.10^{-8}$)	$3.58.10^{-7}$ ($5.03.10^{-8}$)	$\underline{1.40.10^{-9}}$	$\underline{10^{-12}}$
Department population 2008	$1.64.10^{-7}$ ($7.84.10^{-8}$)	$1.43.10^{-7}$ ($1.15.\ 10^{-7}$)	$3.60.10^{-2}$	$2.10.10^{-1}$
City population density 2008	$3.28.10^{-5}$ ($1.68.10^{-8}$)	$3.79.10^{-5}$ ($1.77.10^{-5}$)	$5.10.10^{-2}$	$\underline{3.2.10^{-2}}$
Department population density 2008	$2.85.10^{-5}$ ($1.40.10^{-5}$)	$3.39.10^{-5}$ ($1.25.10^{-5}$)	$4.10.10^{-2}$	$\underline{7.10^{-3}}$
School holidays	$3.02.10^{-2}$ (0.104)	$1.64.10^{-1}$ ($1.27.10^{-1}$)	$7.70.10^{-1}$	$2.00.10^{-1}$

Table 2 : Effect of each variable on H1N1pdm09 case presence in an univariate model. The parameters and P-values of univariate models are given for both datasets (D1 : 235 cities ; D2 : 151 cities), except for insolation, which was available in 151 cities only. Significative P-values are underlined.

Variable	B (S.E.)	Wald	P-value
Weekly maximum relative humidity	$-2.26.10^{-2}$ ($1.28.10^{-2}$)	3.09	$7.89.10^{-2}$
Weekly mean insolation	$-2.69.10^{-3}$ ($2.32.10^{-4}$)	134.48	$< 2.10^{-16}$
City population size 2008	$4.71.10^{-7}$ ($1.32.10^{-7}$)	12.73	$3.60.10^{-4}$
School holidays	-14.6 (4.04)	13.08	$3.00.10^{-4}$
Weekly maximum relative humidity / School holidays (Yes)	$1.46.10^{-1}$ ($4.15.10^{-2}$)	12.35	$4.40.10^{-4}$
Weekly mean insolation / School holidays (Yes)	$1.46.10^{-3}$ ($4.13.10^{-4}$)	12.55	$4.00.10^{-4}$

Table 3 : Results of the generalized estimating equations model of variable associated to pandemic influenza presence in 151 french cities

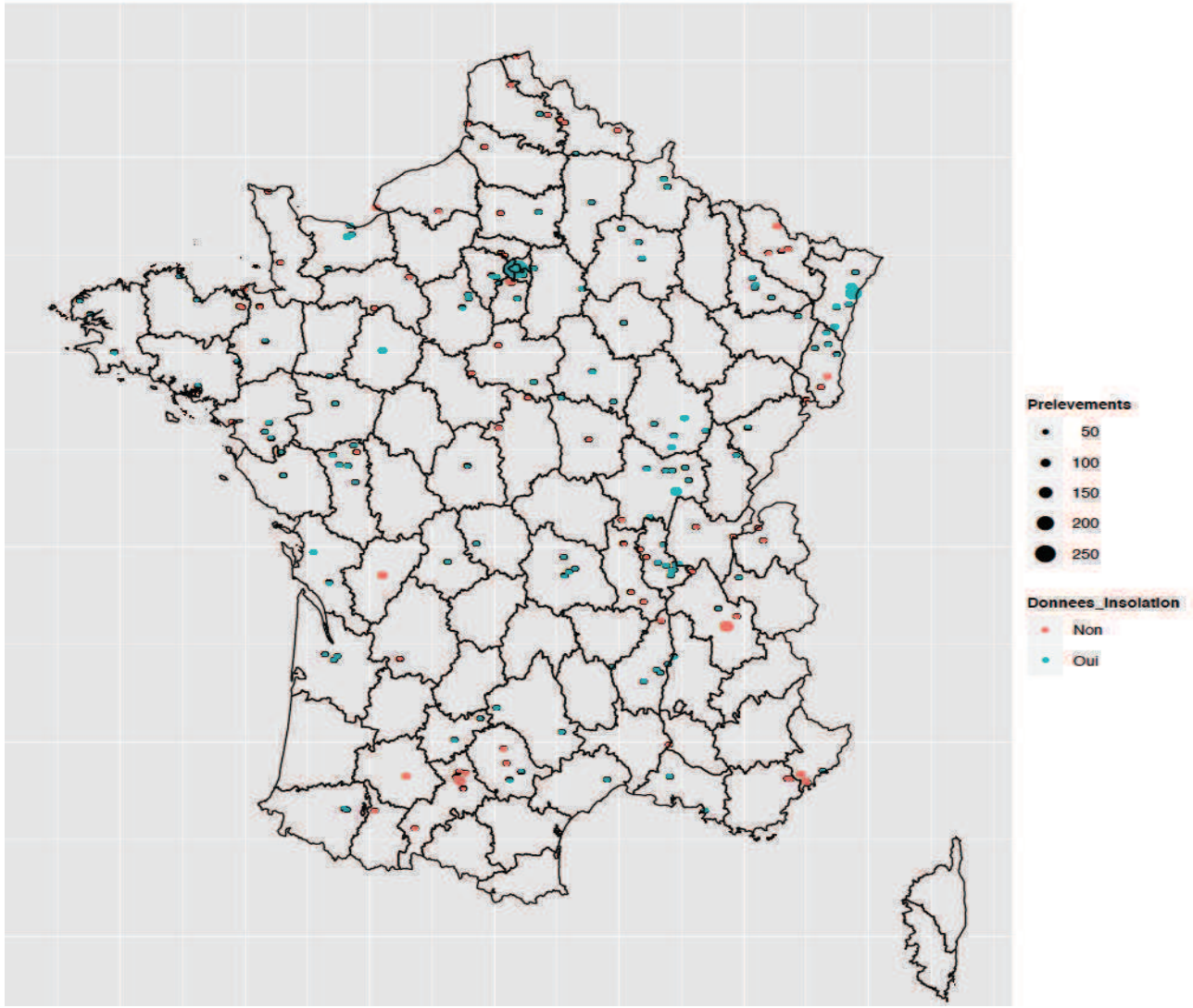

Figure 1 : Sampling sites. The size of the points is proportional to the total number of samples collected in the city. The cities for which insolation data were available are indicated in blue, the other are indicated in red.

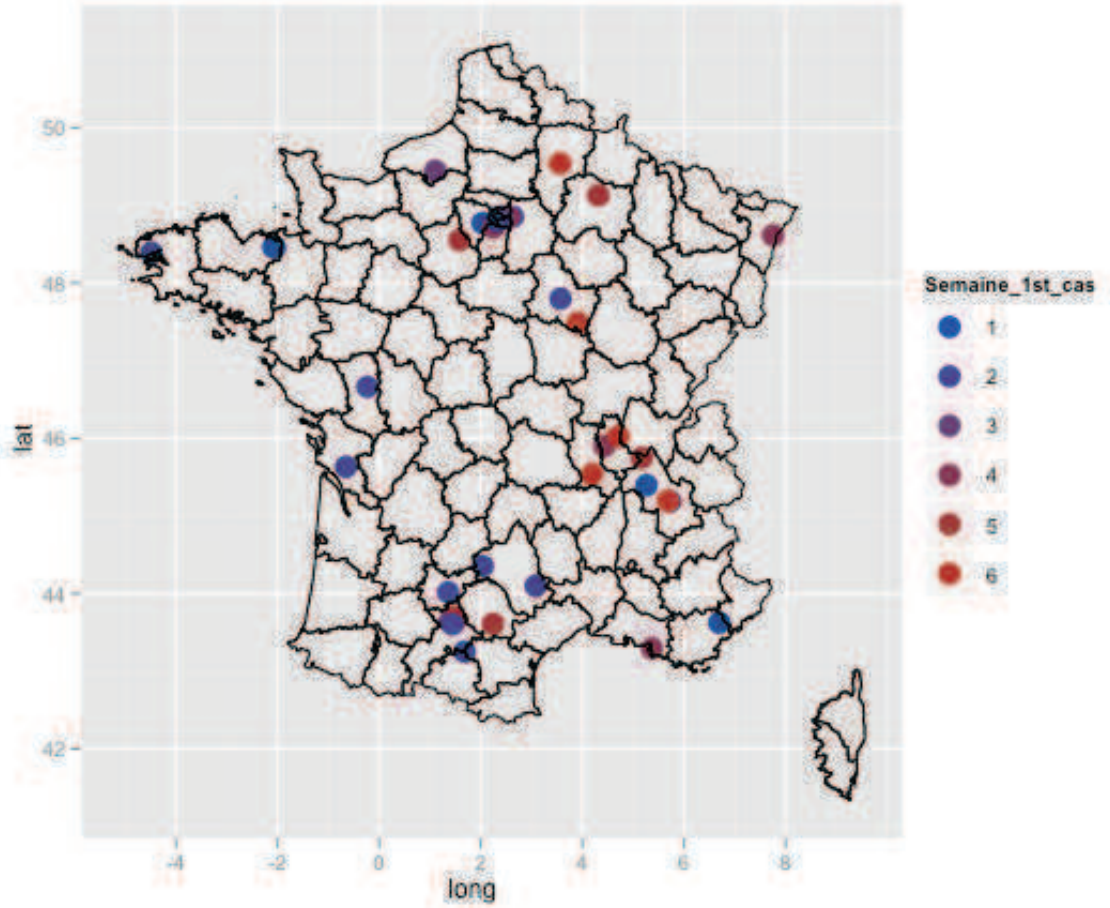

Figure 2 : Map of french cities where H1N1pdm09 was detected during the 6 weeks following the first national case detection. The colour of the points indicate the week of first local case detection.

Discussion

Le Nid, Nils Udo, 1978, Lande de Lunebourg, Allemagne. L'émergence de maladies issues de la faune sauvage a participé à la remise en question de la place de l'Homme dans l'environnement en montrant que la santé des écosystèmes, des animaux et des Hommes étaient étroitement liées. De nombreux artistes contemporains expriment cette prise de conscience, notamment à travers le mouvement du Land art dont Nils Udo est un représentant.

I. Importance de la surveillance de la faune sauvage

1) Des suivis qui doivent être menés à long terme

Nos travaux soulignent l'importance de la surveillance épidémiologique de la faune sauvage en tant que première étape de l'étude de la dynamique des pathogènes émergents. La surveillance répond à la question primaire « le pathogène recherché est-il présent au sein de la population étudiée ? » et n'apporte une information intéressante en termes de compréhension de la circulation d'un agent infectieux que si elle est menée sur le long terme.

Nous avons tout d'abord mis en évidence la circulation récente (2009 et/ou 2010) du virus West Nile au sein de l'avifaune sauvage en Camargue, alors que le dernier indice

de la présence du virus dans la région (séroconversion d'un moineau ; *Passer domesticus* ;(Balança *et al.* 2009) datait de 2007. Ce résultat souligne la nécessité de reprendre la surveillance active du virus West Nile, arrêtée en France depuis 2007. Cette surveillance apparaît nécessaire pour détecter précocement la présence du virus et anticiper d'éventuels cas humains ou équins, ainsi que pour déterminer si les dons de sang peuvent être effectués sans risque dans la région. En effet, la transmission du virus West Nile par la transfusion sanguine est avérée (CDC 2002). En France, le dépistage des anticorps spécifiques à ce virus n'est pas systématique chez les donneurs, les personnes ayant séjourné dans une « région présentant des cas, au moment du séjour, de transmission du virus à l'Homme » étant exclues des dons pour une période de 28 jours (Ministère de la Santé et des sports 2009). Or, la majorité des infections humaines par le virus West Nile étant asymptomatiques (Samuel & Diamond 2006), cette mesure pourrait être insuffisante pour garantir la sécurité des dons de sang dans le cas d'une circulation humaine non détectée. La surveillance active de l'avifaune sauvage, et en particulier de la pie bavarde dont nous confirmons l'efficacité en tant qu'espèce sentinelle, apparaît dans ce cadre, avec la surveillance active des chevaux, un moyen efficace de définir les zones de circulation du virus pour éviter toute transmission associée aux transfusions.

Par ailleurs, la reprise de la surveillance du virus West Nile sur le long terme permettrait de confirmer (ou non) que le virus se maintient d'une année sur l'autre en Camargue. Les indices d'une circulation régulière du virus dans la région de 2000 à 2007 tendent à confirmer cette hypothèse. Ce maintien pourrait être expliqué par la survie hivernale de vecteurs du genre *Culex* infectés par le virus West Nile (Balenghien et al. 2008). A contrario, une approche mathématique de la dynamique du virus West Nile au sein de l'avifaune camarguaise suggère que l'introduction régulière du virus par des oiseaux migrateurs pourrait également expliquer les données de circulation disponibles sans qu'un processus d'hivernage du pathogène ne soit mis en jeu (Durand *et al.* 2010). L'isolement des souches de virus West Nile circulant dans la faune sauvage au cours d'années successives permettrait, via une approche phylogénétique, de déterminer si de nouveaux virus sont apportés par les oiseaux migrateurs, ou si une ou des souches locales se maintiennent et se transmettent plusieurs années durant, les deux mécanismes n'étant pas mutuellement exclusifs. De tels isolements complèteraient les données issues d'un isolement précédent sur la pie en Camargue (Jourdain *et al.* 2007c).

Une telle approche a déjà été mise en œuvre, notamment grâce à l'isolement de souches issues de pies infectées, dans plusieurs pays européens dont la Grèce (Valiakos *et al.* 2011), l'Italie (Savini *et al.* 2008, 2012) et l'Espagne (Sotelo *et al.* 2009). Là encore, des données sur le long terme sont nécessaires pour préciser le schéma observé. Cependant, il semble qu'après une introduction à partir du nord de l'Afrique deux clades distincts de virus (lignées 1 et 2) évoluent et se maintiennent en Europe. Le rôle de la réintroduction potentielle de virus par les oiseaux migrateurs reste cependant mal compris.

2) Des méthodes innovantes pour minimiser les coûts et maintenir le suivi des pathogènes émergents

La surveillance active de l'avifaune sauvage a été abandonnée en France du fait de son coût (Hars *et al.* 2008). Le budget étant très fréquemment un facteur limitant des programmes de surveillance de la faune sauvage (cf. Chap. 1. I. 3.), l'exploration de méthodes de surveillance innovantes peu couteuses apparaît primordiale. L'étude menée dans ce cadre sur le goéland leucophée à l'ouest du bassin méditerranéen montre qu'il ne s'agit pas, à priori, d'une espèce à retenir en tant que sentinelle pour le suivi du virus West Nile. En revanche, la détection d'anticorps spécifiques au virus Meaban au sein d'une colonie espagnole confirme l'intérêt de la méthode de suivi des anticorps maternels dans les œufs pour la surveillance d'autres flavivirus. Dans le contexte de la détection récente de circulation du virus Bagaza au sein de l'avifaune espagnole (Agüero *et al.* 2011) et de la découverte d'autres flavivirus jusqu'alors non décrits transmis par des moustiques en Espagne également (Sánchez-Seco *et al.* 2010; Vázquez *et al.* 2012), cette méthode pourrait être utilisée pour développer la connaissance de la circulation des flavivirus dans la région. Par ailleurs, certains mammifères chez lesquels il est relativement facile d'établir un suivi sérologique, comme le chien ou le sanglier, ont été récemment identifiés comme des espèces sentinelles potentielles pour le suivi du virus West Nile (Resnick *et al.* 2008; Gutiérrez-Guzmán *et al.* 2012; Boadella *et al.* 2012). Cependant, des études pilotes sont nécessaires pour déterminer l'efficacité de ces nouveaux types de suivis envisageables.

II. Les réservoirs naturels de pathogènes des compartiments complexes et hétérogènes

1) Hétérogénéité interspécifique

Contrairement à certains élevages au sein desquels les animaux ont le même âge, des génotypes très proches et le même historique de vie, les réservoirs naturels des pathogènes émergents sont des ensembles très hétérogènes. Nos travaux sur la saisonnalité des infections par les VIA chez les canards colverts et les goélands leucophées illustrent cette complexité au niveau interspécifique. Notre étude montre en effet que la dynamique temporelle des infections peut être très différente entre deux espèces qui vivent pourtant en sympatrie une grande partie de l'année. Les mécanismes impliqués dans cette différence restent à explorer. En ce sens, des études expérimentales semblent nécessaires pour comprendre dans quelle mesure la réponse immunitaire d'une part et les voies de transmission des VIA d'autre part sont différentes entre les deux groupes d'hôtes en question.

Par ailleurs, l'étude phylogénétique des souches circulant chez chacun des groupes semble cruciale pour comprendre les échanges entre les deux ensembles et le rôle épidémiologique de chacun. Une hypothèse envisageable serait que les goélands leucophées, abondants en Camargue toute l'année, contribuent au maintien des VIA via une faible circulation continue au sein des zones humides de la région au printemps et en été, périodes durant lesquelles les canards sont peu nombreux. On trouverait alors une grande similitude entre les souches circulant chez les goélands en été et chez les canards à l'automne. A l'inverse, sachant que la majorité des VIA détectés chez les Charadriiformes sont de type H13 ou H16 et que ces sous-types sont principalement maintenus chez ce groupe (Olsen *et al.* 2000; Yamnikova *et al.* 2003; Fouchier *et al.* 2005; Toennessen *et al.* 2011) une autre hypothèse serait que les infections détectées chez les canards et les goélands font intervenir des souches différentes aux dynamiques indépendantes. Les études montrant l'existence d'un pool commun de souches circulant à la fois chez les canards et les goélands se multiplient, ce qui tend à invalider cette dernière hypothèse (Chen & Holmes 2009; Ramey *et al.* 2010; Van Borm *et al.* 2012). En accord avec ces études, nos travaux portant sur les anticorps maternels dans les œufs de goéland leucophée montrent que, les virus H13 et H16 ne représentent qu'une faible proportion des VIA présents chez cette espèce dans le sud de la France comme dans le

reste de la zone d'étude (Espagne et Algérie). Cependant, les sous-types qui circulent restent à déterminer pour pouvoir comparer les pools de VIA circulant chez les deux groupes étudiés.

Par ailleurs, nous n'avons détecté aucun virus H5 chez les goélands leucophées ni aucun anticorps qui leur soit spécifique dans les œufs de cette espèce. A contrario, ce sous-type représente une proportion non négligeable des VIA détectés chez les canards colverts en Camargue. Bien que ces virus soient tous FP, le risque de leur passage vers des élevages et de leur possible évolution vers une forme HP pousse à chercher pourquoi leur présence s'accroit d'année en année en Camargue et pourquoi on ne les retrouve que chez les Anatidés dans la région. Là encore des études phylogénétiques permettraient de savoir s'ils se maintiennent dans les écosystèmes camarguais d'une année à l'autre ou s'ils sont apportés par les migrateurs chaque automne (depuis le nord et l'est de l'Europe) ou chaque printemps (depuis l'Afrique).

2) Hétérogénéité intraspécifique

Notre exploration de la variabilité entre les hôtes s'est limitée aux différences entre espèces dans le cadre de cette thèse. Cependant de nombreuses études soulignent que les différences interindividuelles influent également sur l'épidémiologie des VIA au sein de chaque espèce d'hôtes. L'âge est depuis longtemps connu pour influer sur les prévalences observées chez les canards, les juvéniles, au système immunitaire naïf, étant plus souvent infectés que les adultes (Munster *et al.* 2007; Ip *et al.* 2008; Gunnarsson *et al.* 2012). D'autres facteurs, tels que les habitats fréquentés, semblent également jouer un rôle important dans l'exposition aux VIA (Hoye, Fouchier & Klaassen 2012). De plus, au sein d'une même espèce, les individus empruntant des voies migratoires différentes pour se rendre sur un même lieu d'hivernage semblent porteurs de VIA distincts (Gunnarsson *et al.* 2012). De telles différences semblent difficiles à prendre en compte via les méthodes d'échantillonnage utilisées au cours de cette thèse chez les goélands leucophées En effet, les œufs ne reflètent que l'exposition des femelles matures et il est difficile de connaître les caractéres individuels des femelles ayant pondu chaque œuf analysé (Hammouda *et al.* 2011), tandis que l'individu ayant excrété chaque fiente récoltée au sol ne peut être identifié. En revanche, la généralisation du baguage des individus pourrait permettre de mieux connaître les mouvements des individus des

différentes colonies (Sadoul & Pin 2009; Baaloudj *et al.* 2012). Ces informations pourraient apporter des éléments de réponse quant aux variations observées entre les prévalences des différentes colonies étudiées. Elles pourraient permettre de tester l'hypothèse selon laquelle les individus s'infectent durant leurs premières années au cours desquelles ils vont hiverner loin de leur colonie d'origine. Chez les canards colverts, l'accès direct à l'individu prélevé lors de l'échantillonnage pourrait permettre, via l'observation des bagues et l'analyse isotopique des plumes, de récolter des informations précises sur l'historique de vie des oiseaux et de le relier directement aux souches détectées. Cependant le baguage et/ou l'observation des canards sur leurs zones de reproduction ainsi que la détermination des signatures isotopiques de ces différentes zones sont actuellement limitées, notamment dans les vastes étendues russes, alors qu'elles apparaissent indispensables pour compléter ces informations.

III. Les compartiments abiotiques : des ensembles à ne pas négliger

1) La persistance dans les milieux abiotiques permet la survie lors de l'absence d'hôtes sensibles

Pour comprendre la dynamique des pathogènes émergents au sein de leur réservoir naturel comme des populations humaines le rôle des compartiments abiotiques et plus généralement des conditions environnementales doit être considéré.

Notre synthèse portant sur la persistance des VIA dans l'environnement montre qu'il s'agit d'un paramètre clé de leur transmission. Au sein de l'avifaune sauvage cette persistance détermine en particulier si les VIA peuvent se maintenir dans les écosystèmes d'une année sur l'autre en traversant des périodes où les hôtes sont rares voire absents. En Camargue des travaux préliminaires associant données de salinité et de températures mensuelles des plans d'eau et connaissances quant à la persistance des VIA dans l'eau ont montré que les VIA pourraient se maintenir dans une grande partie des zones humides tout au long de l'année (Bournez 2010). Ces travaux pourraient être repris pour en affiner les conclusions au vu des données récentes montrant notamment l'influence primordiale du gel (Lebarbenchon *et al.* 2011, 2012) ainsi qu'en tirant parti des nouveaux modèles hydrologiques disponibles pour prédire l'évolution des salinités dans les zones humides locales au cours des futures décennies (Loubet 2012).

Ce type d'approche devrait être complétée par l'étude des VIA présents dans les compartiments abiotiques. De telles études menées récemment aux Etats-Unis ont montré que des virus ayant persisté plusieurs mois dans des lacs sont capables d'infecter ensuite des canards colverts (Lebarbenchon *et al.* 2011).

2) Les conditions environnementales influencent la dynamique des vecteurs et des maladies qu'ils transmettent

Les conditions environnementales influent sur la dynamique des vecteurs et donc sur la dynamique des pathogènes à transmission vectorielle. De fait, il a été suggéré que les changements climatiques seraient favorables à l'expansion des maladies vectorielles dans les zones tempérées notamment car ils faciliteraient l'hivernage de vecteurs infectés (Patz *et al.* 1996; Purse *et al.* 2005). Ainsi, si la surveillance de l'avifaune sauvage confirme que le virus West Nile se maintient en France d'une année sur l'autre, les conditions environnementales seraient cruciales pour la continuité de sa transmission. L'analyse des conditions climatiques et des habitats des vecteurs a précédemment permis d'établir des cartes de présence des vecteurs du virus West Nile en fonction des saisons en Camargue (Tran *et al.* 2007). Couplées à la projection de leur évolution utilisant les prédictions climatiques (IPCC 2007) et de changement d'utilisation des terres locales (Mathevet *et al.* 2003; Delmotte *et al.* 2010) ces cartes permettraient de localiser la réapparition précoce de la circulation du virus à la fin de la diapause des vecteurs, actuellement et dans les prochaines décennies. La surveillance de l'avifaune pourrait ainsi être ciblée dans ces zones à risque en constante évolution afin d'optimiser la détection précoce de la présence du virus.

3) Les conditions environnementales influencent la dynamique des pathogènes au sein des populations humaines

Les données qui s'accumulent quant à l'influence du climat sur les maladies infectieuses au sein des populations humaines incitent à vouloir directement prédire l'incidence et la distribution future des maladies au vu des changements climatiques prévus. Notre étude de la présence du virus H1N1pdm09 en France montre encore une fois le lien entre une maladie (de surcroit un virus pandémique) et les conditions climatiques. Cependant, nos travaux soulignent également l'absence de compréhension

des mécanismes sous-jacents à cette association. Ainsi, il est difficile de créer des cartes des risques futurs de présence de ce virus en utilisant uniquement la définition des conditions favorables déterminées par notre étude associée à la prédiction des conditions attendues. D'abord car le statut immunitaire de la population a changé (beaucoup moins d'individus sont sensibles à l'infection). Ensuite, car les prédictions climatiques actuelles sont à l'échelle des saisons alors que nous montrons ici, comme d'autres études (e.g. Shaman *et al.* 2010; Davis *et al.* 2012; Hu *et al.* 2012), que les conditions jouent à une échelle plus fine (hebdomadaire) sur la présence de la grippe. De plus, parmi les paramètres impliqués ici, seule la température est actuellement prise en compte dans les prédictions de l'IPCC (IPCC 2007). Mais surtout, de telles cartes ne seraient valides que dans l'hypothèse que ces paramètres ont un rôle direct sur la dynamique virale, la survie du virus par exemple. En revanche, si les conditions environnementales jouent indirectement, par exemple en faisant varier les taux de contact entre les hôtes, l'association que nous avons observée pourrait être modifiée par une adaptation comportementale au nouveau type de climat. De façon intéressante un lien entre la saisonnalité du chômage et celle de la grippe a été mis en évidence aux Etats-Unis, notamment du fait du plus faible taux de vaccination des personnes sans emploi (Cornwell 2011). De tels mécanismes sociologiques indirects doivent être pris en compte pour être à même de comprendre le rôle des conditions environnementales sur la dynamique des pathogènes au sein des populations humaines. Ce rôle ne doit pas être minimisé, cependant il doit être considéré en parallèle de celui des autres composantes des changements globaux, ainsi que des évolutions socio-économiques actuelles. Ce principe a été illustré par l'étude de l'expansion de la maladie de Lyme en Europe. Celle-ci est favorisée par le réchauffement du climat, favorable à la multiplication des tiques vecteurs à des latitudes et des altitudes plus hautes que précédemment (Bennet, Halling & Berglund 2006; Lindgren, Jaenson & Europe 2006). Cependant, d'autres facteurs, tels que les changements socio-économiques associés à la chute de l'URSS, ont également joué un rôle important. En effet ces changements ont augmenté le contact entre humains et vecteurs à travers l'augmentation de la fréquentation des milieux boisés pour la chasse ou l'exploitation du bois (Randolph 2001, 2004). Ainsi, l'expansion de la maladie de Lyme ne peut être comprise qu'en prenant en compte l'ensemble des facteurs en jeu.

IV. Rôle des activités humaines dans la dynamique des maladies émergentes

1) Les activités humaines influent sur la démographie des hôtes et des vecteurs

Les activités humaines influencent l'évolution des pathogènes à travers de multiples mécanismes incluant le changement d'utilisation des terres, le prélèvement par la chasse ou la pêche, ou encore la présence de ressources alimentaires liées à ces activités (Lebarbenchon *et al.* 2008). Nos différents travaux portant sur la circulation des VIA chez le goéland leucophée ont montré que cette espèce semblait avoir un rôle à ne pas négliger, distinct de celui des Anatidés, dans la dynamique des VIA. Or, la démographie du goéland leucophée est intimement liée à la disponibilité des ressources anthropiques (Duhem *et al.* 2003, 2008; Ramos *et al.* 2009). C'est principalement la présence de déchetteries à ciel ouvert, ainsi que de déchets de pêche et d'ordures dans les milieux urbains, qui a induit l'explosion démographique de cette espèce durant les dernières décennies (Vidal, Medail & Tatoni 1998). Il en va de même pour plusieurs autres espèces de Laridés dans le monde chez lesquelles des VIA ont également été détectés (e.g. Goéland dominicain, *Larus dominicanus ;* Pereda *et al.* 2008; Lisnizer, Garcia-Borboroglu & Yorio 2011). La disponibilité des ressources anthropiques favorisent probablement la transmission des VIA au sein de ces espèces via le maintien de fortes densités d'hôtes. Elle favorise également potentiellement le contact des VIA circulant chez cette espèce avec l'Homme du fait de la colonisation des milieux urbains. Ce dernier point est à prendre en compte au vu des études expérimentales qui montrent que les virus H16 spécifiques aux Laridés sont capables de se lier aux cellules humaines de l'appareil respiratoire supérieur (Lindskog *et al.* In Prep.). Il serait dans ce cadre intéressant d'essayer de comprendre les différences d'exposition aux VIA détectées via l'étude des anticorps maternels dans les œufs en intégrant le mode d'alimentation privilégié dans chaque colonie étudiée (déchetteries, milieux urbains, invertébrés aquatiques etc.). A ce facteur pourrait être également ajoutés les effectifs des populations locales et, dans la mesure du possible, une estimation des contacts avec les autres espèces hôtes.

2) La gestion cynégétique conditionne la santé du gibier mais aussi celle des espèces sympatriques

Les populations d'Anatidés sont quant à elles impactées par la chasse. Notre étude du rôle des élevages de canards à but cynégétique dans la dynamique des VIA en Camargue souligne l'importance de prendre en compte les conséquences sanitaires des mesures de gestion cynégétique. En l'occurrence, d'appliquer les mesures de contrôle sanitaire des oiseaux avant leur lâcher prévues par la loi (Ministère français de l'agriculture et de la pêche 2006). Ces mesures sont essentielles du fait qu'à la suite des lâchés les animaux domestiques sont amenés à vivre directement dans le milieu occupé par les populations sauvages. Les conséquences de tels lâchés ont déjà été soulignées en Espagne où les lapins lâchés favorisent la dispersion de la gale sarcoptique (Navarro-Gonzalez *et al.* 2010), tandis que les lâchés de perdrix rouges *(Alectoris rufa)* augmentent les taux d'infection par différents helminthes chez l'ensemble des perdrix vivant dans les zones concernées (Villanua *et al.* 2008). Cependant, il semble important de noter qu'au-delà des lâchés toutes les mesures de gestion de la faune sauvage ou semi-sauvage peuvent avoir des conséquences sanitaires qu'il convient de prendre en compte. Ainsi, l'augmentation des densités d'animaux peut favoriser les épizooties, qu'elle soit induite par la mise en place de clôtures aux frontières des territoires de chasse (Vicente Baños *et al.* 2007), ou au contraire par le rassemblement d'animaux au sein d'aires protégées (Lebarbenchon *et al.* 2006). De même, les rassemblements dus au nourrissage augmentent les taux de contacts entre les hôtes et favorisent la transmission des pathogènes, qu'ils aient lieu suite à des mesures cynégétiques (Vicente Baños *et al.* 2007; Castillo *et al.* 2011), ou de protection de la faune (Robinson *et al.* 2010).

Conclusion

Nos travaux illustrent les enjeux de la compréhension de la dynamique des pathogènes émergents au sein de l'ensemble des compartiments où ils circulent. Ils soulignent l'intérêt des études menées sur le long terme et à plusieurs échelles. En particulier, l'identification des risques liés aux maladies émergentes dans le bassin Méditerranéen incite à uniformiser les systèmes de suivi dans la région en renforçant les collaborations internationales. Par ailleurs l'étude des VIA comme des flavivirus montre que ces virus sont communs dans la faune sauvage au sein de laquelle ils ne constituent pas un problème sanitaire majeur. La gestion des risques sanitaires associés à la circulation de tels agents pathogènes ne passe pas nécessairement pas leur éradication mais par de nouvelles formes de contrôle et de surveillance prenant en compte le rôle des activités anthropiques. Ces nouvelles formes émergent suite à la constatation de l'échec du contrôle systématique des agents pathogènes, notamment illustré par l'émergence d'antibio-résistances et de résistances aux antiviraux qui constituent un des problèmes sanitaires majeurs au niveau mondial (WHO 2012*c*). Ces nouvelles formes de gestion du risque sanitaire nécessitent une connaissance fine de l'épidémiologie des agents pathogènes émergents impliquant une étroite collaboration entre médecins, vétérinaires, écologues et acteurs de la gestion de la faune, des filières d'élevage et de la santé publique. L'initiative One Health (http://www.onehealthinitiative.com/) est un exemple de ce genre d'approche multidisciplinaire qui permet d'apporter un nouvel éclairage sur les maladies infectieuses émergentes.

Les approches couplant écologie évolutive et médecine humaine et vétérinaire, telles que celles portées par « Ecohealth Alliance » (http://www.ecohealthalliance.org/) permettent en outre d'identifier le rôle d'agents pathogènes dans des maladies jusqu'alors jugées non infectieuses, en particulier les cancers. On estime à l'heure actuelle qu'au moins 20% des cancers ont une origine infectieuse chez l'Homme (Zur Hausen 2009). Parmi les agents infectieux associés à la formation de tumeurs chez l'Homme certains sont zoonotiques ce qui illustre encore une fois les liens entre santé animale et santé humaine. Les études des indices pouvant révéler le lien entre pathogènes et cancer, telle que la corrélation spatiale de leur occurrence, amènent à identifier de nouveaux agents infectieux potentiellement associés à des cancers chez l'Homme. L'identification de tels agents pourrait amener à redéfinir la pathogénicité de parasites tels que *Toxoplasma gondii*. Ce parasite omniprésent au sein de la faune domestique et sauvage ainsi que dans les populations humaines (plus d'un tiers de

l'humanité y serait exposé; Tenter, Heckeroth & Weiss 2000) semble en effet lié aux cas de cancer du cerveau (corrélation spatiale) (Annexes 1 & 2; Thomas *et al.* 2012).

Enfin, la transmission des virus étudiés au cours de cette thèse est intimement liée aux zones humides. Du fait de ce lien avec les maladies infectieuses ces dernières ont été depuis l'antiquité considérées comme une source de miasmes à assécher. L'essor des approches multidisciplinaires permettant d'étudier l'écologie de la transmission des pathogènes associés aux zones humides permet à présent de concilier prévention des maladies infectieuses et préservation des zones humides qui ont une valeur culturelle, et économique forte à travers des méthodes de gestion adaptées (Horwitz & Finlayson 2011; Dale & Connelly 2012).

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Annexes

Annexe 1. Brain cancer mortality rates increase with *Toxoplasma gondii* seroprevalence in France.

Vittecoq M., Elguero E., Lafferty K.D., Roche B, Brodeur J., Gauthier-Clerc M., Missé D. & Thomas F.

Publié dans *Infection, Genetics and Evolution.*

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Infection, Genetics and Evolution 12 (2012) 496–498

Contents lists available at SciVerse ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Short communication

Brain cancer mortality rates increase with *Toxoplasma gondii* seroprevalence in France

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ARTICLE INFO

Article history:
Received 6 January 2012
Received in revised form 12 January 2012
Accepted 13 January 2012
Available online 25 January 2012

Keywords:
Encephalon tumors
Medical geography
Malignancy
Nervous system
Latent toxoplasmosis

ABSTRACT

The incidence of adult brain cancer was previously shown to be higher in countries where the parasite *Toxoplasma gondii* is common, suggesting that this brain protozoan could potentially increase the risk of tumor formation. Using countries as replicates has, however, several potential confounding factors, particularly because detection rates vary with country wealth. Using an independent dataset entirely within France, we further establish the significance of the association between *T. gondii* and brain cancer and find additional demographic resolution. In adult age classes 55 years and older, regional mortality rates due to brain cancer correlated positively with the local seroprevalence of *T. gondii*. This effect was particularly strong for men. While this novel evidence of a significant statistical association between *T. gondii* infection and brain cancer does not demonstrate causation, these results suggest that investigations at the scale of the individual are merited.

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1. Introduction

More than 237 000 central nervous system cancers are diagnosed each year worldwide and survival remains low even in the most developed countries (GLOBOCAN, 2008). Despite considerable research, the causes of these malignancies are largely unknown. Some studies have highlighted associations between brain tumors and environmental factors including ionizing radiation, diverse chemical products and electromagnetic fields (Fisher et al., 2007). In animals, infections by different viruses have experimentally led to brain tumors (Wrensch et al., 2002). Persistent infections may promote cancer by increasing mutation rates through the inflammation they induce. Moreover, intracellular pathogens may disrupt cell barriers to cancer. However, there are very few studies investigating the link between pathogens and brain cancer in humans. Recently, using a dataset including 37 countries, Thomas et al. (2012) detected a positive relationship between the national seroprevalence of the protozoan parasite *Toxoplasma gondii* and the national incidence of brain cancer.

T. gondii is an apicomplexan parasite that is transmitted to its final host, a felid, through the consumption of an intermediate host, which can be any warm-blooded vertebrate, including humans (Dubey, 1998). This parasite is very common and infects more than one-third of the world human population. Humans are infected by eating tissue cysts in contaminated meat or by contact with soil contaminated with oocysts from cat feces. Within Europe and North America, most strains isolated from humans and domestic/farm animals belong to three clonal types: genotypes I, II and III (Boothroyd, 2009). Type I strains are lethal and do not readily give rise to chronic infections in mice. An increased frequency of type I strains in severe congenital toxoplasmosis in humans suggests that this lineage may also be more pathogenic for humans than lineage II and III (e. g. Howe and Sibley, 1995). By contrast, type II and III strains are considerably less virulent and characterized by long-term infections (Su et al., 2002). In Europe, human infections are most commonly associated with Type II strains (Sibley et al., 2009). Latent *T. gondii* infections are traditionally considered benign by conventional medicine, but evidence is accumulating that the bradyzoite stages encysted in the brain during the latent phase are responsible for diverse neurological pathologies (see Flegr, 2010 for a recent review). *T. gondii* is sufficiently common in humans that it could lead to a large proportion of brain cancer cases (Thomas et al., 2012).

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doi:10.1016/j.meegid.2012.01.013

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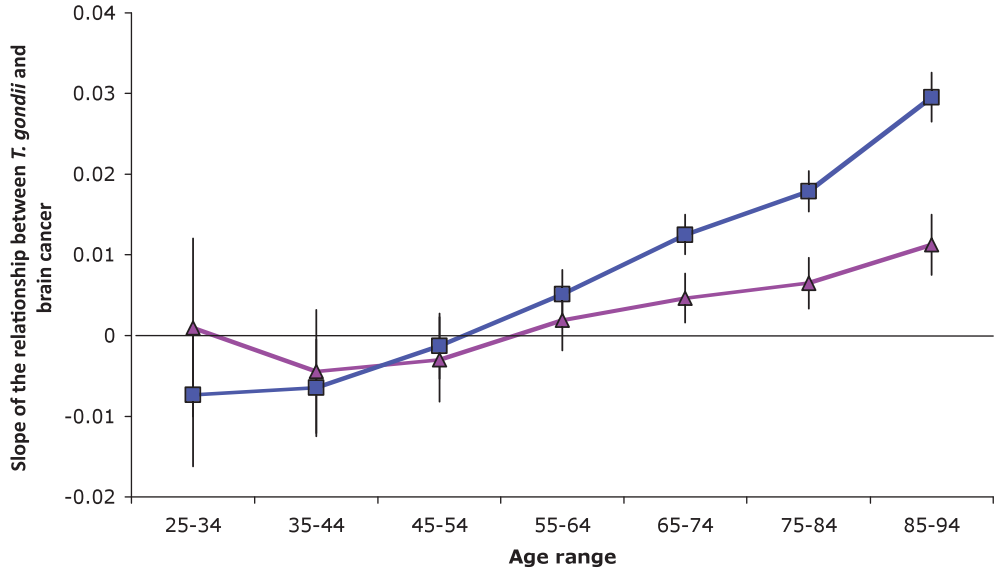

Fig. 1. Relationship between *Toxoplasma gondii* seroprevalence and mortality rates due to brain cancers. Slopes (+/− 95% confidence intervals) of the relationship between *Toxoplasma gondii* prevalence in a region in France (range 34–62) and mortality rates due to brain cancers in adults (per 1,00,000 individuals), separated by gender and age class (after controlling for year). Slopes > 0 indicate a positive relationship between regional brain cancer mortality and *T. gondii* prevalence for males (squares) and females (triangles).

2. Materials and methods

In France, mortality causes, including brain cancer, are available for each of the 22 administrative regions since 1979 (until 2007), and regional data on *T. gondii* seroprevalence is available for women of childbearing age for 1995 and 2003 (Berger et al., 2007). We asked whether the prevalence of *T. gondii* in a region was positively associated with the mortality due to brain cancer in that region.

National statistics on brain cancer mortalities in men and women for each of the 29 years (1979–2007) were obtained from an Inserm database available at http://www.cepidc.vesinet.inserm.fr/. These data do not include the incidence of brain cancer. They concern mortality from malignant primary encephalon tumors, those classified 191 (until 1999) and C71 (from 2000) in the International Classification of Disease (ICD, 9th and 10th revision, available at: http://apps.who.int/classifications/apps/icd/icd10online/). Because sample sizes were too small among the C71/191 sub-categories (0–9, depending on localization in the brain), data were pooled prior to analyses. Mortality data due to malignant C70 and C72 tumors were anecdotal over the entire period and were not analyzed. The 22 regions of metropolitan France served as replicates. Mortality data are crude rates for 100 000 inhabitants. Because the reliability of these estimations may depend on the number of inhabitants per region, rates were weighted for the analysis by the relative regional population size in 1995 (near the midpoint of the dataset) available at http://www.insee.fr/fr/bases-de-donnees/default.asp?page=recensements.htm. National statistics on *T. gondii* seroprevalence for the 22 regions were from two successive studies on pregnant women in 1995 ($n = 13\ 459$) and 2003 ($n = 15\ 108$) (Berger et al., 2007). We analyzed age-standardized seroprevalence, which were obtained through direct standardization using the total population sampled in 2003 as the reference (for both 1995 and 2003 data) and six age groups (<20, 20–24, 25–29, 30–34, 35–39, >39; see Berger et al., 2007 for details). For each region, the estimates for the 2 years were highly correlated ($r = 0.83$; $P < 0.0001$), though prevalence in 2003 was lower than in 1995 ($P < 0.0001$). At first, we used the mean seroprevalence value across the 2 years for each region. Consistent with other studies (e.g., Jones et al., 2003; Studeničová et al., 2006) we assumed that this mean for pregnant women adequately represents the infection status of men and women. We also analyzed the years separately, which led to similar results. In the statistical models, brain cancer mortality was the dependent variable and *T. gondii* seroprevalence, year of reported death, sex, and their interactions were predictors. It was not possible to directly test a *T. gondii* effect in a generalized linear model as residuals were not normally distributed. Hence, we used a generalized linear model with Poisson Distribution and a Log-link function. These results were consistent with general linear models and non-parametric analyses. Because there was a strong interaction between age class, gender, and *T. gondii* prevalence, we opted to analyze age classes and genders separately.

3. Results and discussion

T. gondii prevalence in pregnant women was lower in 2003 than in 1995 (probably due to changes in meat production that have led to less contamination with cysts) and brain cancer related mortalities increased over time (probably due to improvements in detection of brain cancer). Both *T. gondii* prevalences and brain cancer mortalities were highly variable across regions. As an example in 2003 prevalence varied from 29% to 56.3% and mortality varied from 3.15 to 5.44 deaths per 100 000 inhabitants (mean mortality rate for the whole population of a region). Brain cancer mortality was consistently higher for males than for females.

Once mortality data were corrected for year effects, a moderate but significant effect of *T. gondii* was detected for men from 55 years and older (*P*-values: 55–64 years = 0.0282, 65–74 to 85–94 years < 0.0001), for women from 65 years (*P*-values: 65 years = 0.0023, 75–84 and 85–94 years < 0.0001). This effect increased with age and was stronger for men than for women (Fig. 1). Both results are consistent with the fact that *T. gondii* exposure increases with age and has different neurological effects on men and women (Flegr, 2010). While this novel evidence of a significant statistical association between *T. gondii* infection and brain cancer does not indicate causation, it is consistent with a similar pattern at the global level for the incidence of brain cancer (Thomas et al., 2012). Although we cannot exclude the existence of confounding variables, these results, together with those by Thomas et al. (2012), suggest it would be worth investigating the prediction

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that individuals with brain cancer are more likely to have been infected with *T. gondii*. Finding a causal link between *T. gondii* and brain cancer would radically change the way we consider *T. gondii* infections, and provide a means to reduce the risk of brain cancer, particularly in countries like France where the incidence of brain cancer and *T. gondii* are both high.

Acknowledgments

This work was supported by the French Consortium Evolution et cancer (CNRS).

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Annexe 2. Cat ownership is neither a strong predictor of *Toxoplasma gondii* infection nor a risk factor for brain cancer.

Vittecoq M., Lafferty K.D., Elguero E., Brodeur J., Gauthier-Clerc M., Missé D., Roche B.

Publié dans *Biology Letters.*

Downloaded from rsbl.royalsocietypublishing.org on September 28, 2012

biology letters
Pathogen biology

Biol. Lett.
doi:10.1098/rsbl.2012.0625
Published online

Invited reply

Cat ownership is neither a strong predictor of *Toxoplasma gondii* infection nor a risk factor for brain cancer

Using a dataset including 37 countries, we recently reported a positive correlation between the national seroprevalence of the protozoan parasite *Toxoplasma gondii* and the national incidence of brain cancer [1]. We further established the significance of this association in a second paper showing that in France, regional mortality rates owing to brain cancer correlated positively with the local seroprevalence of *T. gondii* [2]. These results do not demonstrate causation but suggest that *T. gondii* should be investigated further as a possible oncogenic pathogen of humans.

Benson *et al.* [3] used data from a large UK prospective cohort of middle-aged women among whom 18 per cent owned at least one cat. Comparing brain cancer incidence in women living with a cat or without any pet, they found no correlation between cat ownership and brain cancer. This is an important finding because the popular press is drawn to the headline that pet cats are a health risk to their owners. Our disagreement with Benson *et al.* is that their findings do not inform how *T. gondii* is linked to brain cancer, which was the main finding of our research.

Although cats are a necessary part of the life cycle of *T. gondii*, multiple studies have shown that cat ownership is not a strong predictor of risk of *T. gondii* infection [4,5]. Contact with oocysts from cat faeces can occur through contaminated soil or vegetables [6]. Contact with contaminated soil (combined with poor hygiene) and eating unwashed vegetables are significant risk factors [6]. In addition, undercooked meat consumption has been identified as the chief risk factor for human toxoplasmosis in a number of European countries, including the UK ([7]; see [6] for a review). Thus, cat ownership is not a good proxy of the probability of being infected by *T. gondii*, either through oocysts originating from cats or through *T. gondii* asexual stages. So, while cats are a key host for *T. gondii* and *T. gondii* seems to be a risk factor for brain cancer, cat ownership is not necessarily a significant risk factor for brain cancer, at least not unless other risk factors are accounted for statistically.

To conclude, the study of Benson *et al.* should be reassuring to cat owners, but it does not test whether *T. gondii* affects risks of brain cancer. Clearly, what is now needed to assess the role of *T. gondii* in brain cancer risk are studies that compare the seroprevalence of *T. gondii* in individuals with brain cancer with matched controls.

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The accompanying comment can be viewed at http://dx.doi.org/doi:10.1098/rsbl.2012.0511.

Received 3 July 2012
Accepted 1 August 2012

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<u>Annexe 3</u> : Liste des communications

- Ecologie de la santé et biodiversité: les gestionnaires face aux maladies émergentes. **Vittecoq M**. & Gauthier-Clerc M. Forum des gestionnaires de l'ATEN, Nantes, France, 12-13 décembre 2011. (Communication orale)

- Influenza A virus infection rates in Mallards reared for shooting in the Camargue. **Vittecoq M**., Grandhomme V., Champagnon, J., Guillemain M., Renaud F., Thomas F., Gauthier-Clerc M. & van der Werf S. Conservation sciences in the Mediterranean region : Conference for Young Scientists, Arles, 08-10 décembre 2011. (Communication orale)

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<u>Annexe 4</u> : Album photos: Prélèvements biologiques

Quelques images des différents prélèvements et captures : a) Observation des ailes d'une pie pour déterminer son âge. b) Prélèvement d'une fiente de goéland leucophée au sol. c) Regroupement de canards d'élevage dans leur enclos avant les prélèvements. d) Prélèvements cloacaux sur canards tués à la chasse. e) Manipulation d'un sanglier avant collecte d'un écouvillon nasal.

Résumé

L'émergence de maladies telles que le SRAS et le SIDA au cours des dernières décennies a fait prendre conscience des liens étroits existant entre santé animale, santé humaine et santé des écosystèmes. En effet, les pathogènes émergents ont pour la plupart une origine zoonotique (i.e. ils circulaient à l'origine au sein des populations animales). Les risques sanitaires associés à ces émergences sont en constante évolution sous l'influence des changements globaux qui modifient les écosystèmes et les contacts entre les hôtes. La prévention et le contrôle des maladies infectieuses émergentes nécessitent la compréhension de leur dynamique dans l'ensemble des compartiments dans lesquels elles circulent. Le travail présenté ici avait pour objectif d'améliorer cette compréhension au sein des zones humides méditerranéennes en ce concentrant sur deux pathogènes émergents : les virus Influenza A (VIA) et le virus West Nile. Il a été structuré selon trois axes de recherche : i) Utiliser la surveillance épidémiologique de l'avifaune sauvage pour comprendre la circulation du virus West Nile dans le bassin méditerranéen ii) Comprendre la dynamique des VIA au sein des différents compartiments où ils circulent et à leur interface iii) Comprendre le rôle des conditions environnementales dans la dynamique des VIA notamment au sein des populations humaines. Nos résultats mettent en évidence l'intérêt de mener des études multidisciplinaires sur le long terme pour comprendre l'épidémiologie des maladies émergentes. Ils soulignent également le rôle des activités anthropiques et des conditions environnementales dans la dynamique de ces maladies. Nos études apportent des éléments de réflexion pour allier gestion des risques d'émergence et gestion des écosystèmes et des populations. Elles encouragent à développer ce type d'approche afin de relever le défi de la prévention et du contrôle des pathogènes émergents.

Mots clés : écologie de la santé, maladies infectieuses émergentes, changements globaux, zones humides, Méditerranée, Camargue, virus influenza A, virus West Nile.

Abstract

During the last decades, the emergence of numerous infectious diseases such as SARS and AIDS has raised awareness of the close links that exist between animal health, human health and ecosystem health. Many of the emerging pathogens have a zoonotic origin (i.e. they originally circulated among animal populations). The health risks associated with the emergence of these diseases are progressing under the influence of global changes that affect ecosystems and contacts between hosts. The prevention and control of emerging infectious diseases require an in-depth understanding of their dynamics in all the compartments in which they occur. The aim of the present work is to improve our understanding of these phenomena within the context of Mediterranean wetlands by focusing on two emerging pathogens: Influenza A viruses (IAV) and West Nile virus. The thesis is structured around three research axes i) Using epidemiological surveillance of wild birds to investigate the circulation of West Nile virus in the Mediterranean Basin ii) Exploring IAV dynamics in the different compartments in which they circulate and at their interface iii) Determining the role of environmental conditions in IAV dynamics, especially within human populations. Our results highlight the value of long-term interdisciplinary studies for the understanding of the epidemiology of emerging diseases. They also emphasize the role of human activities and environmental conditions in the dynamics of these diseases. Our studies open up perspectives for combining emerging disease risk management and the management of ecosystems and populations. They also argue in favour of further developing this type of approach in order to meet the challenge of emerging pathogen prevention and control.

Keywords: health ecology, emerging infectious diseases, global changes, wetlands, Mediterranean, Camargue, influenza A virus, West Nile virus.