- UNIVERSITE MONTPELLIER II-
SCIENCES ET TECHNIQUES DU LANGUEDOC

THESE

En vue d'obtenir le grade de
Docteur de l'Université Montpellier II

Discipline : Biologie des Populations et Ecologie
Ecole Doctorale : Systèmes Intégrés en Biologie, Agronomie, Géosciences, Hydrosciences, Environnement (SIBAGHE)

Présentée et soutenue publiquement par
Audrey ARNAL
le 30 octobre 2012

Circulation d'agents pathogènes en populations naturelles : approches éco-épidémiologiques chez le Goéland leucophée (*Larus michahellis*)

Membres du jury :

Julien Gasparini Maitre de Conférence, Université Pierre & Marie Curie Paris	Rapporteur
Philippe Christe Directeur de recherche, Dép. d'Ecologie et d'Evolution, Lausanne	Rapporteur
Bernard Godelle Professeur, Université Montpellier II	Examinateur
Marc Artois Professeur d'infectiologie, VetAgro Sup Lyon	Examinateur
Elsa Jourdain Chercheur INRA, Theix	Invité
Thierry Boulinier Directeur de recherche, CEFE-CNRS, Montpellier	Directeur de thèse
Michel Gauthier-Clerc Directeur de recherche, Tour du Valat, Camargue	Directeur de thèse

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Une vision plus ample de ce qu'est une épidéme… tient compte de l'existence d'autres organismes qui en constituent le réservoir naturel, et des transformations dans l'environnement écologique ou le comportement des populations humaines qui sont susceptibles de créer de nouvelles niches pour la bactérie ou le virus, et de nouvelles voies de propagation.

M. Morange, *Les secrets du vivant : contre la pensée unique en biologie*

Merci…

This is the end!
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La thèse ce n'est que le début…The show must go on !

Liste des publications scientifiques

Publications présentées dans la thèse

Article 1
Audrey Arnal*, Marion Vittecoq*, Jessica Pearce-Duvet, Michel Gauthier-Clerc, Thierry Boulinier and Elsa Jourdain **Laridae species constitute a neglected reservoir of avian influenza virus?** In prep.
*Equal contribution

Article 2
Abdesslem Hammouda, Jessica Pearce-Duvet, Mohamed Ali Chokri, **Audrey Arnal**, Michel Gauthier-Clerc, Thierry Boulinier and Slaheddine Selmi (2011) **Prevalence of influenza A antibodies in Yellow-Legged Gull (*Larus michahellis*) eggs and adults in southern Tunisia.** *Vector-Borne and Zoonotic Diseases* 11: 1583-1590

Article 3
Jessica Pearce-Duvet, **Audrey Arnal**, Romain Garnier and Thierry Boulinier **Counting eggs before they hatch: Yolk antibodies reveal avian influenza prevalence patterns in the Mediterranean basin.** In prep.

Article 4
Audrey Arnal, Jessica Pearce-Duvet, Michel Gauthier-Clerc, Thierry Boulinier and Elsa Jourdain **Spatial patterns of influenza A antibodies in yellow-legged gull (*Larus michahellis*) eggs in the Mediterranean basin**. To be submitted to *Virology Journal*

Article 5
Audrey Arnal, Elena Gómez-Díaz, Marta Cerdà-Cuéllar, Sylvie Lecollinet, Jessica Pearce-Duvet, Núria Busquets, Ignacio García-Bocanegra, Nonito Pagès Martinez, Marion Vittecoq, Abdesslem Hammouda, Boudjéma Samraoui, Romain Garnier, Raül Ramos, Slaheddine Selmi, Jacob González-Solís, Michel Gauthier-Clerc, Elsa Jourdain and Thierry Boulinier **Meaban flavivirus in Yellow-Legged Gulls (*Larus michahellis*) and Seabird Ticks (*Ornithodoros maritimus*) in the Mediterranean Basin**. To be submitted to *Emerging Infectious Diseases*

Article 6
Marion Vittecoq, Sylvie Lecollinet, Elsa Jourdain, Frédéric Thomas, Thomas Blanchon, **Audrey Arnal**, Steeve Lowenski and Michel Gauthier-Clerc **Recent circulation of West Nile and USUTU virus in Southern France.** To be submitted to *Vector-Borne and Zoonotic Diseases*

Article 7
Abdesslem Hammouda, Slaheddine Selmi, Jessica Pearce-Duvet, Mohamed Ali Chokri, **Audrey Arnal**, Michel Gauthier-Clerc, Thierry Boulinier (2012) **Maternal antibody transmission in relation to mother fluctuating asymmetry in a long-lived colonial seabird: the Yellow-Legged Gull *Larus michahellis.*** *Plos One* 7(5)

Publications présentées en annexe

Audrey Arnal, Michèle Ottmann, Romain Garnier (2011) **Circulation des virus Influenza A: apports de la modélisation.** *Virologie* 15(6):363-370

Autres publications

Elodie Chapuis, **Audrey Arnal** and Jean-Baptiste Ferdy (2012) **Trade-offs shape the evolution of the vector-borne insect pathogen *Xenorhabdus nematophila***. *Proceedings Of The Royal Society B-Biological Sciences* 279(1738): 2672-2680

Pierre Kengne, **Audrey Arnal**, Cecile Brengues, Abdoulaye Diabaté, Hubert Bassene, Didier Fontenille, Frederic Simard and Koumbobr Roch Dabire **Genetic polymorphism of odor receptors (AgOr38) genes among incipient species of the African malaria vector *Anopheles gambiae* (Diptera: Culicidae) in Senegal.** In prep.

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Chapitre 3

Figure 3.1 *Carte des colonies échantillonnées dans l'ouest méditerranéen*
France (n = 8), Espagne (n = 4), Algérie (n = 3) et Tunisie (n = 2)
Les croix représentent les colonies ou aucun anticorps contre les flavivirus n'est détecté ; les ronds signalent les colonies qui présentent des anticorps contre les falvivirus.

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Synthèse

Circulation d'agents pathogènes en populations naturelles :
approches éco-épidémiologique chez le Goéland leucophée
(*Larus michahellis*)

Dans les populations humaines, animales et végétales, la diversité spécifique des agents pathogènes n'est pas distribuée de manière aléatoire (Guernier *et al.* 2004). Elle montre une distribution géographique assez proche de celle que l'on connaît pour de nombreux groupes d'animaux et de végétaux en biogéographie (Holt *et al.* 2005) : les agents pathogènes sont plus diversifiés dans les zones intertropicales. Cette diversité maximale s'explique sans doute par la richesse exceptionnelle en espèces animales, représentant autant d'hôtes et de vecteurs pour des microorganismes potentiellement pathogènes pour l'Homme (Guernier *et al.* 2004). Mais de nombreuses perturbations souvent liées aux activités humaines, ont provoqué le déplacement géographique de certains agents pathogènes, qui se retrouvent aujourd'hui au sein de populations démunies de toutes formes de résistances (Harvell *et al.* 1999; Patz *et al.* 2004; Lebarbenchon *et al.* 2008). L'industrialisation, le développement des transports (Colizza *et al.* 2007) ou l'intensification des modes d'élevage (Martin *et al.* 2011), ne sont que quelques exemples de l'étendue des actions de l'Homme sur les écosystèmes (Gauthier-Clerc *et al.* 2007; Lebarbenchon *et al.* 2008).

De telles perturbations ne sont pas nouvelles à l'échelle de l'humanité. En effet, depuis le Néolithique, le développement de l'agriculture et des premiers élevages d'animaux sauvages ont induit la transformation de nombreux habitats (Weiss 2001; Pearce-Duvet 2006). La domestication a favorisé le contact étroit entre les animaux et les populations humaines, créant ainsi un environnement favorable à la transmission d'agents pathogènes (Diamond 2002). Ce qui est en revanche totalement inédit, c'est l'ampleur de ces perturbations pouvant favoriser l'évolution de la circulation des agents pathogènes (Daszak *et al.* 2000;

Gortazar *et al.* 2007). Pour comprendre leur circulation, il est donc nécessaire de prendre en compte les relations étroites qui existent entre les agents responsables de maladies infectieuses, les hôtes, éventuellement les vecteurs et l'environnement (Figure 1 ; Aron *et al.* 2001).

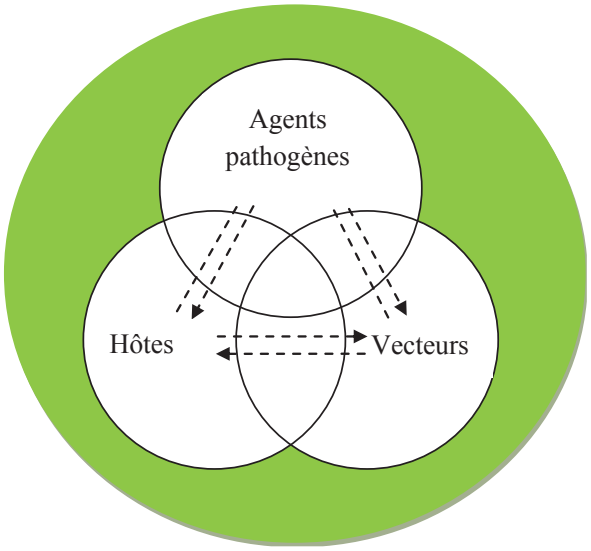


Figure 1 : *Relations étroites entre les agents pathogènes, les hôtes, les vecteurs dans l'environnement.*

Illustration schématique montrant les liens possibles entre les agents responsables de maladies infectieuses, les hôtes, les vecteurs dans l'environnement (en vert) et toutes les composantes interagissent les unes avec les autres. (Modifié de Thomas *et al.* 2007)

Pour prendre en compte ces relations, la synthèse épidémiologie/écologie est indispensable, notamment parce que les événements d'épidémies évoqués par le premier champ disciplinaire

peuvent être expliqués par le second (Smith *et al.* 2005). Cette approche transdisciplinaire est en effet obligatoire pour répondre aux problèmes de santé des populations en déterminant les facteurs, voire les interactions entre les facteurs, qui favorisent la circulation d'agents pathogènes. Dans cette introduction, nous allons présenter les différents facteurs, nommés éco-épidémiologiques, qui sont liés aux interactions étroites entre l'agent pathogène et l'hôte dans l'environnement, ou uniquement liés à l'écologie de l'hôte, indépendamment de l'agent pathogène considéré.

Facteurs éco-épidémiologiques liés aux interactions entre les agents pathogènes, les hôtes et l'environnement

Le cycle de vie d'un agent pathogène est un système complexe, mais qui peut être schématisé par trois étapes majeures et nécessaires : la transmission et la réplication qui conditionnent la propagation d'un agent pathogène (Figure 2 Choisy 2010).

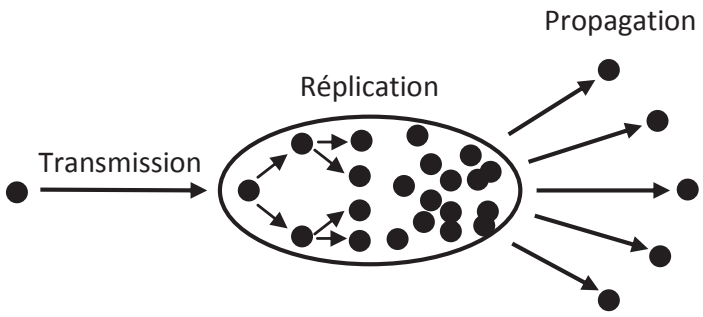
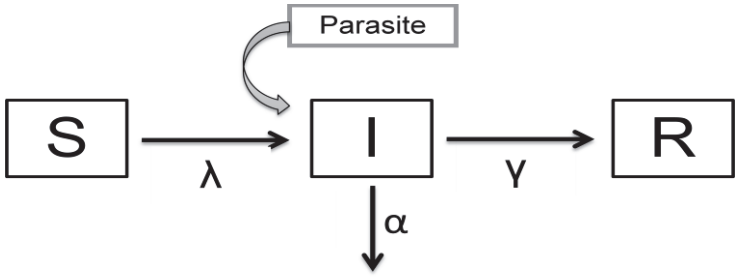


Figure 2 : *Cycle de vie d'un agent pathogène.*

L'agent pathogène (points noirs) infecte un individu hôte (transmission), se multiplie au sein de cet individu (réplication) et se propage à d'autres individus hôtes (propagation). (Modifié de Guégan *et al.* 2009)

Pour mieux comprendre les trois étapes du cycle de vie d'un agent pathogène, il est possible de se placer aux différents compartiments qui constituent le modèle SIR (**Encadré 1**).

Encadré 1 : *Modèle SIR*



Ce modèle décrit l'état de la population d'hôtes en la compartimentant en groupes d'individus différenciés selon leur statut vis-à-vis de l'agent pathogène : sensibles (S), infectieux (I) et guéris (R, *recovered* en anglais).

Les individus sensibles deviennent infectieux (contaminés par un agent pathogène et pouvant le transmettre) au taux λ, communément appelé force d'infection. La force d'infection dépend en partie de la capacité des individus à transmettre l'infection (β : taux de transmission).

Ces individus infectieux peuvent présenter un taux de mortalité accrue, traduisant l'effet généralement négatif de la présence de l'agent pathogène. Ce taux α correspond donc à la virulence de l'agent pathogène.

Dans ce modèle, certains individus infectieux guérissent, c'est-à-dire produisent des anticorps spécifiques, au taux γ.

Un tel modèle permet, par exemple, de déterminer si un agent pathogène peut apparaître et se propager (*cf.* Etape 3, Figure 2) dans une population d'individus sensibles. Pour cela, il est possible de calculer le nombre reproducteur de base R_0, qui représente le nombre moyen d'infections secondaires dues à l'introduction d'un individu infecté dans une population entièrement sensible (Keeling *et al.* 2008). Ce paramètre clef en épidémiologie est défini par l'équation suivante :

$$R0 = \frac{N\,\beta}{\gamma+\alpha}$$

Pour qu'une épidémie survienne, il faut qu'un individu infecté transmette son infection au minimum à un autre individu ($R_0>1$), sinon l'agent pathogène est éliminé de la population ($R_0<1$). Ce nombre reproducteur de base R_0 dépend de plusieurs paramètres :

$$R0 = \frac{\textcircled{N}\,\beta}{\gamma+\alpha}$$

Le nombre d'individu de la population hôte (N)
La densité d'individu est un facteur éco-épidémiologique intrinsèque à l'hôte et sera donc présenté dans la partie suivante.

$$R0 = \frac{N\beta}{\gamma+\alpha}$$

Le taux de transmission (β)

Ce taux dépend en partie du mode de transmission qui peut être direct (comme présenté dans le modèle SIR, *cf.* **Encadré 1**) ou indirect (Woolhouse 2002). Comme son nom l'indique, la transmission directe nécessite un contact rapproché entre un individu infectieux (contaminé par l'agent pathogène et pouvant le transmettre) et un individu sain. Par exemple, la transmission de l'agent viral responsable de la varicelle se fait par contact avec un individu infectieux (Silhol *et al.* 2010). Les écoles, favorisant le contact entre les enfants, constituent l'environnement idéal pour la transmission de ce virus (Silhol *et al.* 2011). *A contrario*, la transmission par contact indirect nécessite la survie de l'agent pathogène dans l'environnement via l'intervention d'un vecteur inerte (*e.g.* objet contaminé). A titre d'exemple, les Staphylocoques doré (*Staphylococcus aureus*) peuvent persister jusqu'à quelques semaines sur des surfaces contaminées (Boyce 2007). La transmission indirecte peut également nécessiter un vecteur biologique (*e.g.* arthropode piqueur). C'est le cas du paludisme, maladie infectieuse du à un agent pathogène du genre *Plasmodium*, qui est transmis par certaines espèces de moustiques (White *et al.* 2011).

La transmission d'un agent pathogène dépend aussi de sa capacité à infecter un nouvel hôte (Woolhouse *et al.* 2001). Les agents pathogènes ont en effet une gamme d'hôtes très variable : certains infectent naturellement une seule espèce d'hôte (par exemple, *Plasmodium falciparum* chez l'Homme ; Liu *et al.* 2010), tandis que d'autres peuvent infecter des hôtes d'ordres taxonomiques différents (par exemple le virus de la rage qui peut infecter l'Homme, le chien ou encore les

chauves souris ; Holmes *et al.* 2002). Les raisons de cette variation sont souvent mal comprises bien que certains facteurs, comme la voie de transmission indirecte, soient connues pour être associées à un spectre d'hôtes très large (Woolhouse *et al.* 2001). Pour les virus, l'un de ces facteurs est l'utilisation de récepteurs cellulaires évolutivement stables (Woolhouse *et al.* 2005). En effet, pour pouvoir infecter l'hôte, les virus ont besoin de récepteurs cellulaires appropriés. Lorsque ces récepteurs sont conservés chez une large gamme d'hôte, ils sont alors prédisposés à l'infection par les virus qui ciblent ces récepteurs (Woolhouse *et al.* 2005). Parmi les hôtes, certaines espèces ou groupes d'espèces ont la capacité d'assurer l'amplification et la transmission de l'agent pathogène, on parle alors de réservoir (**Encadré 2 ;** Haydon *et al.* 2002).

Encadré 2 : *La notion de réservoir*

Il ne suffit pas d'identifier ou d'isoler un agent pathogène chez un hôte pour que celui-ci soit considéré comme réservoir. La définition la plus pertinente est donc qu'un réservoir correspond à une ou plusieurs populations ou encore à un milieu, dans lequel l'agent pathogène persiste de manière pérenne et peut être transmis à d'autres populations. Suivant les agents pathogènes, des espèces vectrices peuvent être associées à ce concept (Haydon *et al.* 2002).

Cette notion de réservoir peut varier dans le temps et dans l'espace. En effet, un schéma qui se réalise à un endroit donné avec certaines espèces ne sera peut-être pas reproductible ailleurs.

Enfin, la pérennité du cycle de vie d'un agent pathogène peut parfois reposer sur une combinaison de réservoirs principaux, bien connus, et de réservoirs secondaires, minoritaires donc moins bien documentés, mais dont l'existence, peut permettre le maintien de l'agent pathogène dans l'environnement sur le long terme (Haydon *et al.* 2002).

$$R0 = \frac{N\,\beta}{\gamma+\alpha}$$

Enfin, la virulence de l'agent pathogène (α) et le taux de guérison de l'hôte (γ) peuvent influencer la capacité de réplication de l'agent pathogène.

La réplication

La réplication d'un agent pathogène à l'intérieur de son hôte est une étape essentielle qui détermine sa propagation à d'autres individus hôtes. Elle est fonction de la virulence de l'agent pathogène (α) (Gandon *et al.* 2000). L'un des exemples les mieux documentés est le cas du protozoaire *Ophryocystis elektroscirrha*, parasite obligatoire du papillon monarque (de Roode *et al.* 2008). Les auteurs de cette étude ont montré que le nombre de spores produites par ce protozoaire est positivement corrélé à sa virulence. Mais bien qu'un agent pathogène vive aux dépens de son hôte, il a aussi besoin de lui pour survivre (Alizon *et al.* 2009). Se pose alors la notion d'infection de l'hôte : (*i*) Etre très virulent, utiliser au maximum les ressources de l'hôte et produire un grand nombre de formes infectantes ce qui entrainera rapidement la mort de l'hôte ? Ou au contraire (*ii*) évoluer vers l'avirulence (*i.e.* l'agent pathogène a aucun effet ou un effet limité sur la valeur sélective de l'hôte) et ainsi peut maintenir son hôte plus longtemps en vie, ce qui favorise alors sa dispersion géographique ? La virulence joue donc un rôle déterminant dans l'évolution du statut de l'interaction entre l'agent pathogène et son hôte.

Cependant la réplication d'un agent pathogène pourra également être influencée par la défense de l'hôte, qui détermine son taux de guérison

(γ). Cette défense repose en grande partie sur l'induction d'une réponse immunitaire (Frost 1999) qui est une combinaison de mécanismes innés rapides, généralement non spécifiques et de mécanismes acquis d'expression plus tardive, mais aussi plus spécifique de l'agent pathogène (Wakelin 1996). Une partie de cette réponse immunitaire acquise se traduit par la production de composés immunoactifs, les anticorps ou immunoglobulines. La persistance des anticorps, autrement dit la durée de protection qu'elle confère, dépend de l'agent pathogène et a des conséquences importantes sur sa dynamique épidémiologique (Frank 2002; Boulinier *et al.* 2008). En effet, le maintien d'un agent pathogène dans une nouvelle population est lié au nombre d'individus qualifiés de « susceptibles », c'est-à-dire n'ayant jamais été exposés à cet agent et donc ne possédant pas d'anticorps correspondants. Ainsi, une arrivée massive de nouveaux individus dans une population correspond à une arrivée massive d'individus susceptibles, ce qui peut favoriser la transmission d'agents pathogènes (Begon *et al.* 2009).

Facteurs éco-épidémiologiques liés à l'écologie et aux propriétés intrinsèques de l'hôte

Les facteurs éco-épidémiologiques intrinsèques aux hôtes peuvent être évalués à différentes échelles spatiales (Figure 3. Kneitel *et al.* 2004).

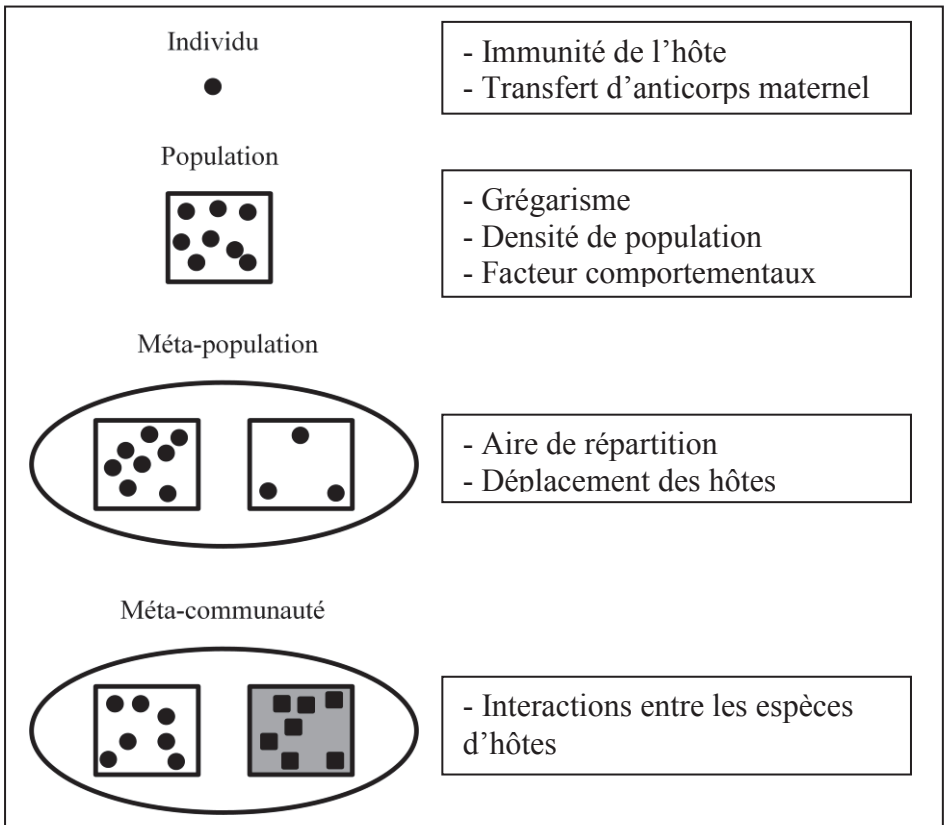


Figure 3. *Types d'habitats et facteurs éco-épidémiologiques associés.* (Modifié d'après Kneitel *et al.* 2004)

A l'échelle de l'individu, l'immunité de l'hôte peut varier indépendamment de l'agent pathogène, du fait des facteurs génétiques propres à chaque individu (Grindstaff *et al.* 2003). Cependant, relativement peu de travaux en population naturelle ont abordé ce sujet (Sorci *et al.* 1997). Pour donner un exemple, chez un oiseau colonial comme la mouette tridactyle (*Rissa tridactyla*), l'existence d'une relation positive entre le nombre de tiques *Ixodes uriae* trouvées sur les poussins d'une génération et celles trouvées sur les poussins de la génération suivante (*i.e.* nombre mesuré sur les poussins élevés plusieurs années après) a aussi suggéré une variation génétique de la susceptibilité aux agents pathogènes (Boulinier *et al.* 1997). De surcroit, les conditions environnementales affectent clairement le développement du système immunitaire (Lochmiller *et al.* 1993). Ainsi, des contraintes nutritives fortes peuvent altérer la capacité des individus à répondre aux agressions des agents pathogènes : certains nutriments, tels que les caroténoïdes, peuvent directement affecter l'immunité des individus (Chew *et al.* 2004).

Parmi les conditions environnementales, les effets parentaux, c'est-à-dire les effets non génétiques du à l'un ou aux deux parents, peuvent avoir des conséquences importantes sur la dynamique épidémiologique d'un agent pathogène dans une population (Boulinier *et al.* 2008). Chez les mammifères et les oiseaux, les anticorps peuvent être transmis de la mère à sa descendance, ce qui permet une protection temporaire des jeunes lors de la phase critique de maturation de leur propre système immunitaire (Wallach *et al.* 1992; Smith *et al.* 1994; Al-Natour *et al.* 2004; Kariyawasam *et al.* 2004; Pravieux *et al.* 2007). La durée de protection des anticorps maternels est un facteur qui peut contribuer à modifier la structure de sensibilité de la population en protégeant un nombre variable de nouveau-nés sur une durée plus ou moins étendue (Boulinier *et al.* 2008). Mais le transfert d'anticorps maternels est-il forcément synonyme de protection ? Certaines études ont montré l'effet

positif des anticorps maternels sur le taux de croissance (Heeb *et al.* 1998) ou le développement de la réponse immunitaire des juvéniles (Anderson 1995; Gasparini *et al.* 2006; Grindstaff *et al.* 2006). D'autres, en revanche, ont mis en évidence un « effet bloquant » des anticorps maternels sur la réponse immunitaire des juvéniles limitant ainsi l'efficacité d'un vaccin (Al-Natour *et al.* 2004). Cependant, cet « effet bloquant » pourrait aussi avoir un rôle protecteur en limitant l'investissement des juvéniles dans leurs propres réponses immunitaires à une période critique pour leurs développements(Boulinier *et al.* 2008).

A l'échelle de la population, la structure spatiale des hôtes conditionne les contacts entre les individus et ainsi la transmission des agents pathogènes (Messinger *et al.* 2009). Le grégarisme ou la forte densité des hôtes sont donc des facteurs importants qui conditionnent la transmission d'agents pathogènes en multipliant les contacts potentiels entre individus (Adler *et al.* 2008; Smith *et al.* 2009; Bagamian *et al.* 2012). Prenons l'exemple des virus H5N1 hautement pathogènes qui circulent actuellement chez les volailles principalement en Asie (Ito *et al.* 2001; Lebarbenchon *et al.* 2010). Le scénario de ces processus épidémiques pouvait être prévisible d'un point de vue écologique. En effet, d'énormes densités d'individus (ici plusieurs milliards de volailles estimées en Asie du Sud-Est) sont des hôtes potentiels des virus H5N1 hautement pathogènes. La densité favorise ainsi la sélection de variants plus virulents (Martin *et al.* 2011) qui ne pourraient peut être pas se propager dans des configurations de moindre densité.

Toujours à l'échelle de la population, les facteurs comportementaux tel que le comportement alimentaire, influencent l'exposition des hôtes aux sources potentielles d'agents pathogènes. C'est le cas des salmonelles capables de persister sur des aliments souillés et qui peuvent être ingérées par des espèces d'oiseaux, tel que le goéland argenté (*Larus argentatus*) ou le goéland leucophée (*Larus michahellis*), se nourrissant

dans les décharges à ciel ouvert (Monaghan *et al.* 1985; Ramos *et al.* 2010).

Au sein d'une méta-population, l'aire de répartition d'un hôte et les déplacements qu'il y effectue conditionnent directement la capacité de propagation d'un agent pathogène (Altizer *et al.* 2011). Ainsi l'épidémie de virus Ebola, qui en 2007 a causé la mort de 186 personnes en République Démocratique du Congo, était liée à la migration de plusieurs espèces de chauves-souris (Leroy *et al.* 2009).

Enfin, à l'échelle d'une méta-communauté, les interactions entre les espèces d'hôtes conditionnent directement la capacité de propagation d'un agent pathogène (Buckee *et al.* 2007). Les zones d'interface, entre la faune sauvage, les populations humaines et les animaux domestiques, sont des environnements propices à la circulation d'agents pathogènes (Jones *et al.* 2008; Caron *et al.* 2011). Prenons l'exemple de la bactérie responsable de la tuberculose bovine (Tschopp *et al.* 2010). Au sud de l'Afrique, la transmission de cet agent bactérien s'effectue au niveau de points d'eau, zone d'interface entre le bétail bovin et la faune sauvage : buffle d'Afrique (*Syncerus caffer*) et cobe de Lechwe (*Kobus leche*) (Tschopp *et al.* 2010). La faune sauvage infectée représente alors un risque de dispersion de la bactérie au travers des frontières nationales (Tschopp *et al.* 2010). L'Homme peut également être infecté par cet agent bactérien par contact avec le bétail ou la faune sauvage, aussi bien par voie aérogène qu'alimentaire (Addo *et al.* 2007; Oloya *et al.* 2008).

Ainsi, en mettant en évidence ces facteurs éco-épidémiologiques intrinsèques aux hôtes et en prenant en compte leurs variations saisonnières (Altizer *et al.* 2006), il est possible d'améliorer notre compréhension de la circulation des agents pathogènes entre et au sein des populations d'hôtes et ainsi établir une évaluation du risque d'émergence.

Qu'est ce que l'émergence d'un agent pathogène ?

L'émergence d'un agent pathogène se définit par (*i*) son apparition dans une population, ou lorsque (*ii*) son aire de répartition géographique ou l'incidence de la maladie (nombre de nouveaux cas au sein d'une population en un temps donné) augmentent rapidement. Quels sont les points communs entre le SIDA, le paludisme, la peste noire, la fièvre Ebola, la dengue, ou encore la grippe aviaire H5N1 ? En plus d'être considérées comme des maladies émergentes à risque en termes de santés humaine et animale, elles ont toutes une origine animale, sauvage ou domestique (Taylor *et al.* 2001). On estime aujourd'hui que 60,3% des maladies infectieuses émergentes chez l'Homme ont une origine zoonotique (Jones *et al.* 2008), c'est-à-dire causées par des agents pathogènes se transmettant naturellement entre l'Homme et d'autres espèces d'animaux vertébrés (Taylor *et al.* 2001; Woolhouse *et al.* 2001; Woolhouse 2002). Enfin, parmi ces agents pathogènes zoonotiques, 71,8% proviennent d'animaux sauvages (Daszak *et al.* 2000; Woolhouse 2002; Gortazar *et al.* 2007; Jones *et al.* 2008) et la plupart sont d'origine virale (Figure 4 ; Taylor *et al.* 2001).

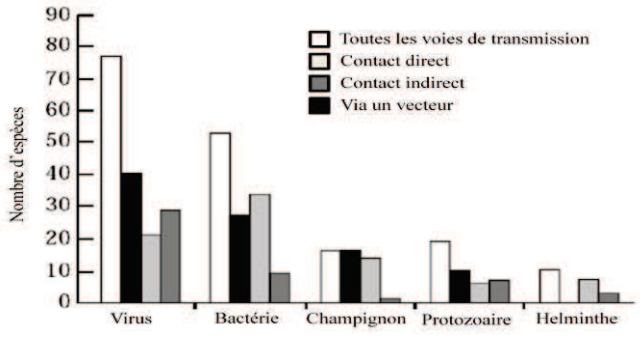


Figure 4. *Nombre d'agents pathogènes considérés comme émergents.* (Modifié d'après Taylor *et al.* 2001)

Enjeux de la surveillance en population naturelle

La surveillance des agents pathogènes dans les populations sauvages est donc cruciale (Morner *et al.* 2002; Butler 2006) et répond à plusieurs enjeux :

i- Un enjeu patrimonial, car un agent pathogène virulent peut constituer un obstacle à la conservation de la biodiversité. Prenons comme exemple le cas du loup d'Ethiopie (*Canis simensis*), dont les populations comptent moins de 500 individus dans le monde (Randall *et al.* 2004). Le loup d'Ethiopie est particulièrement sensible aux virus de la rage, responsable de la mort d'un nombre considérable d'individus, favorisant ainsi le déclin de cette espèce (SilleroZubiri *et al.* 1996; Randall *et al.* 2004).

ii- Un enjeu économique, quand l'agent pathogène est transmis aux animaux d'élevages. Par exemple les buffles africains sauvages (*Syncerus caffer*) hébergent certaines souches du virus de la fièvre aphteuse (sérotypes SAT1, SAT2 et SAT3). Ils peuvent alors les transmettre aux bovins domestiques (Ayebazibwe *et al.* 2010) ce qui constitue un danger pour les élevages (Sutmoller *et al.* 2000).

iii- Un enjeu de santé publique, lorsque l'agent pathogène est transmissible à l'Homme. C'est le cas du virus West Nile, un flavivirus dont les réservoirs naturels sont les oiseaux sauvages (Calistri *et al.* 2010). Ce virus, le plus souvent transmis par des moustiques du genre *Culex*, peut aussi infecter l'Homme et le cheval qui peuvent développer des symptômes cliniques allant d'un simple syndrome fébrile à des encéphalites graves (Jourdain *et al.* 2007; Calistri *et al.* 2010).

iv- Enfin, un enjeu scientifique, car l'objectif est de comprendre au mieux la circulation des agents pathogènes, leurs modes de transmissions, leurs virulences ou encore leurs répartitions géographiques, afin de prédire une éventuelle menace de pandémie ou d'évaluer l'impact des différentes politiques de santé publique sur la propagation des maladies infectieuses (Artois *et al.* 2001; Morner *et al.* 2002; Butler 2006).

Il est reconnu que les pays qui mettent en place une surveillance des agents pathogènes en population naturelle ont une meilleure compréhension de leur circulation et sont donc plus à même de protéger la faune sauvage, les animaux domestiques et les populations humaines (Morner *et al.* 2002). L'effort de surveillance en population naturelle est donc crucial pour être capable de décrire la circulation des agents pathogènes dans l'espace et le temps (Woolhouse 2002; Murphy 2008; Hoye *et al.* 2010; Knight-Jones *et al.* 2010; Krauss *et al.* 2010; Lee *et al.* 2010).

Rôle des oiseaux sauvages dans la dynamique des agents pathogènes : cas du Goéland leucophée (Larus michahellis)

Les oiseaux sauvages peuvent être infectés par une grande diversité d'agents pathogènes transmissibles à l'Homme et/ou aux animaux domestiques (Reed *et al.* 2003). Les virus influenza aviaires (Webster *et al.* 1992; Alexander 2000), les bactéries entéropathogènes tel que les salmonelles (Hilbert *et al.* 2012) ou les campylobacters (Broman *et al.* 2002), les agents pathogènes à transmission vectorielle comme le virus du West Nile (Jourdain *et al.* 2007) ou encore les bactéries antibio-résistantes (Bonnedahl *et al.* 2009) ne sont que quelques exemples des agents pathogènes détectés dans les populations naturelles d'oiseaux (Reed *et al.* 2003).

Les oiseaux sauvages effectuent, tout au long de l'année, des mouvements plus ou moins importants, allant de la prospection alimentaire aux migrations sur de longues distances (Reed *et al.* 2003). Lors de ces mouvements, les oiseaux transportent et dispersent à la fois des agents pathogènes (Reed *et al.* 2003; Altizer *et al.* 2011) et des arthropodes vecteurs d'agent pathogènes (Gomez-Diaz *et al.* 2012), favorisant ainsi leurs circulations à différentes échelle spatiales : locale, continentale ou intercontinentale.

La surveillance des populations naturelles d'oiseaux n'est donc pas à négliger, en particulier dans les zones où les interfaces entre les oiseaux sauvages et l'Homme sont fortes (Jones *et al.* 2008; Caron *et al.* 2011).

Certaines espèces de Laridés, comme le goéland leucophée (*Larus michahellis*), sont en contact étroit avec les milieux urbains (Sol *et al.* 1995; Raven *et al.* 1997; Duhem *et al.* 2008; Lisnizer *et al.* 2011) ce qui accroit les risques de transmission et de propagation de maladies

zoonotiques aux populations humaines (Bradley *et al.* 2007). Cette espèce est, en effet, connue pour s'alimenter de ressources anthropiques, faciles d'accès et régulièrement renouvelées (essentiellement les ordures ménagères déposées dans des sites à ciel ouvert, mais également les rebuts de la pêche industrielle, jetés à la mer ; Sol *et al.* 1995; Ramos *et al.* 2009). Le goéland leucophée peut également partager divers habitats avec d'autres espèces d'oiseaux aquatiques (Cramp *et al.* 1983) comme par exemple le canard colvert (*Anas platyrhynchos*), principal réservoir des virus influenza aviaires (Stallknecht *et al.* 2007b) et sur lequel il a une action de prédation (Tamisier *et al.* 1999) ou encore la pie bavarde (*Pica pica*) espèce sentinelle du virus West Nile (Jourdain *et al.* 2008).

Du fait de son caractère opportuniste et anthropophile, le goéland leucophée connait une forte expansion démographique dans le bassin méditerranéen (Beaubrun 1993). Le goéland leucophée est aussi perçu comme une menace pour des espèces sympatriques, tel que le goéland d'Audouin (*Ichthyaetyus audouinii*) sur lequel il a une action de prédation importante (Oro *et al.* 1994; GonzalezSolis *et al.* 1997). La régulation des populations de goéland leucophée, comprenant les campagnes de stérilisation d'œufs, facilite donc l'échantillonnage à des fins scientifiques (Pearce-Duvet *et al.* 2009; Hammouda *et al.* 2011).

L'ensemble de ces caractéristiques, sa consommation de ressources anthropiques ou encore la facilité d'échantillonnage, font du goéland leucophée un modèle pertinent pour étudier la dynamique des agents pathogènes en populations naturelles.

L'objectif général de cette thèse est de comprendre, par des approches éco-épidémiologiques, la circulation d'agents pathogènes dans des populations sauvages de goéland leucophée, espèce en contact étroit avec l'Homme.

Les virus influenza aviaires ont un impact économique très important sur l'aviculture et représentent une réelle menace en termes de santé humaine. Les oiseaux aquatiques sauvages (notamment les Anatidés (canards, oies et cygnes) et les Laridés (goélands et sternes)) sont considérés comme les réservoirs naturels de ces virus. Cependant, peu de données sont disponibles sur la circulation des virus influenza aviaires chez les Laridés car la majorité des études se penchent sur les Anatidés, réservoir naturel principal. L'**Article 1** met donc en lumière les connaissances actuelles sur les virus influenza aviaires chez les Laridés et explique en quoi c'est un réservoir à ne pas négliger dans la surveillance des agents pathogènes en population naturelle. L'un des objectifs de la surveillance des agents pathogènes est de déterminer la distribution des populations exposées, donc potentiellement à risque. Le premier chapitre vise donc à mettre en exergue les différentes méthodes de surveillance des agents pathogènes, ainsi que leurs limites lorsqu'elles sont menées en populations naturelles. La plupart des études se basent sur l'isolement du virus ou encore sur des enquêtes sérologiques. Au travers de l'**Article 2**, portant sur les virus influenza aviaires, nous mettons en évidence que la quantification des anticorps maternels dans les œufs est un outil efficace, qui apporte des informations intéressantes et nécessaires à la compréhension de la circulation des agents pathogènes.

La surveillance des maladies infectieuses a besoin d'outils d'analyses spatiales à large échelle afin de mettre en évidence les facteurs éco-épidémiologiques qui peuvent influencer la circulation des agents pathogènes dans et entre les populations d'hôtes. L'objectif du chapitre 2 est donc d'améliorer notre compréhension de la dynamique des virus influenza aviaires en étudiant les patrons de prévalence à l'échelle de l'ouest méditerranéen (**Article 3 et 4**). En utilisant les œufs comme un indicateur de l'exposition des adultes à un agent pathogène (*cf.* chapitre 1), nous déterminons les patrons d'exposition aux virus influenza aviaires sur plusieurs colonies méditerranéennes, dans le but de mettre en évidence les facteurs éco-épidémiologiques impliqués dans la circulation de ces virus.

Les flavivirus, tel que le virus West Nile qui peut être responsable de syndromes fébriles ou d'encéphalites graves chez l'Homme et le cheval, représentent une menace en termes de santé humaine et animale. Le goéland leucophée a d'ailleurs été identifié comme potentiellement impliqué dans introduction du virus West Nile en France. Contrairement aux virus influenza aviaires (*cf.* chapitre 2), les flavivirus nécessitent la présence d'un arthropode hématophage pour être transmis d'un hôte vertébré à un autre. Il ne suffit plus de prendre en compte les facteurs éco-épidémiologiques de l'hôte qui favorisent la circulation de l'agent pathogène ; il faut aussi intégrer les facteurs éco-épidémiologiques intrinsèques aux vecteurs. L'objectif du chapitre 3 est donc d'améliorer notre compréhension de la dynamique des flavivirus (**Article 5**) en déterminant leurs patrons d'exposition dans l'ouest de la Méditerranée, puis en identifiant le(s) flavivirus correspondant aux anticorps maternels détectés. Nous déterminons ensuite le ou les vecteurs potentiellement impliqués dans la circulation des flavivirus. Ce résultat et la prise en compte des données sur l'écologie du goéland leucophée et du vecteur nous permettrons alors de définir les facteurs

éco-épidémiologiques potentiellement impliqués dans la circulation et la propagation des flavivirus.

Enfin, les résultats présentés dans les chapitres précités génèrent de nouvelles questions et permettent d'envisager de futurs axes de recherche, présentés dans la partie conclusion et perspective qui clôturera cette thèse.

Chapitre 1 : La quantification des anticorps maternels comme outil de surveillance des agents pathogènes

Introduction

Les virus influenza aviaires (IA) sont une préoccupation mondiale en raison de leurs impacts économiques et de la menace qu'ils représentent en terme de santé humaine (Li *et al.* 2004; Chen *et al.* 2005; Ferguson *et al.* 2005; De Wit *et al.* 2008). Ces virus présentent des dynamiques épidémiologiques complexes (Webster *et al.* 1992), caractérisées par leur capacité à être transmis de la faune sauvage aux animaux domestiques, ou des mammifères domestiques/sauvages à l'Homme (Olsen *et al.* 2006; Gauthier-Clerc *et al.* 2007).

Les virus IA se distinguent par la forte diversité génétique de deux de leurs protéines de surface, l'hémagglutinine et la neuraminidase. Ces deux protéines ont un rôle clé dans l'infection et la réplication du virus et, de ce fait, représentent les principales cibles du système immunitaire de l'hôte (Suarez *et al.* 2000; Subbarao *et al.* 2007). Différents sous-types (notés HxNy) sont identifiés selon la combinaison de ces deux protéines et, pour chaque sous-type, plusieurs souches génétiquement différentes peuvent être distinguées. A ce jour, seize sous-types d'hémagglutinine (H1 à H16) et neuf de neuraminidase (N1 à N9) ont été décrits (Webster *et al.* 1992; Fouchier *et al.* 2005). Le sous-type H17 a récemment été détecté chez des chauves souris (Tong *et al.* 2012).
Les oiseaux aquatiques sauvages, en particulier les Ansériformes (canards, oies et cygnes (Anatidés)) et Charadriiformes (limicoles (Charadriidés et Scolopacidés) ; goélands et sternes (Laridés)), sont considérés comme des réservoirs naturels des virus IA (Webster *et al.*

1992). Deux formes de virus IA peuvent se distinguer en fonction de leur pathogénicité chez les volailles. Les virus IA faiblement pathogènes (IAFP) circulent naturellement dans les populations d'oiseaux aquatiques sauvages, dans lesquelles ils semblent causer peu ou pas de symptômes (Webster *et al.* 1992; Alexander 2000). Cependant, les virus IAFP dans les populations de volailles domestiques peuvent évoluer en une forme plus virulente dite hautement pathogène (Ito *et al.* 2001; Lebarbenchon *et al.* 2010). La circulation de virus influenza aviaire hautement pathogène (IAHP) peut alors causer une forte mortalité dans les élevages, pouvant avoisiner les 100% (Swayne 2008). À ce jour, chez les oiseaux, seul les sous-types H5 et H7 sont connus pour évoluer vers des formes hautement pathogènes (Alexander 2000; Fouchier *et al.* 2007). Mais la grande variété de sous-types circulant chez les oiseaux sauvages (Webster *et al.* 1992; Alexander 2000) et les contact étroit entre les populations naturelles, les animaux domestiques et l'Homme (Caron *et al.* 2011) peuvent conduire à des réassortiments entre virus qui permettent l'émergence de nouveaux sous-types viraux (Chen *et al.* 2006a; Van Poucke *et al.* 2010).

Pour diverses raisons, notamment leur abondance à travers le monde (Cramp *et al.* 1983) et le fait qu'elles représentent le réservoir naturel principal des virus IA (Stallknecht *et al.* 2007b), la plupart des études ont mis l'accent sur la circulation de ces virus chez les espèces d'Anatidés (Figure 1.1.). Cependant, afin d'anticiper l'émergence de nouveaux sous-types, il est très important de ne négliger aucun réservoir (Haydon *et al.* 2002). La première étape de mon travail de thèse a été de faire la revue des connaissances actuelles sur les virus IA chez les Laridés (**Article 1**) et d'expliquer en quoi les Laridés représentent un réservoir à ne pas négliger.

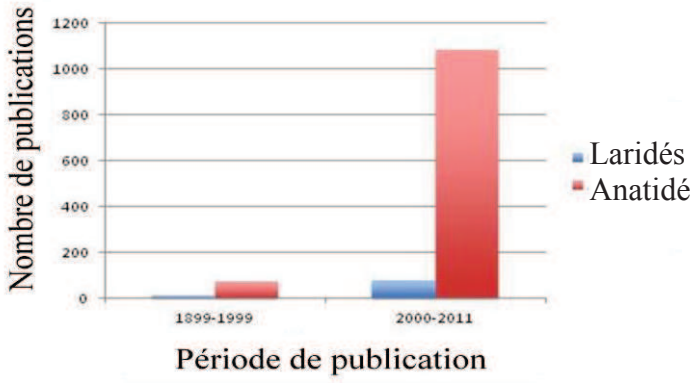
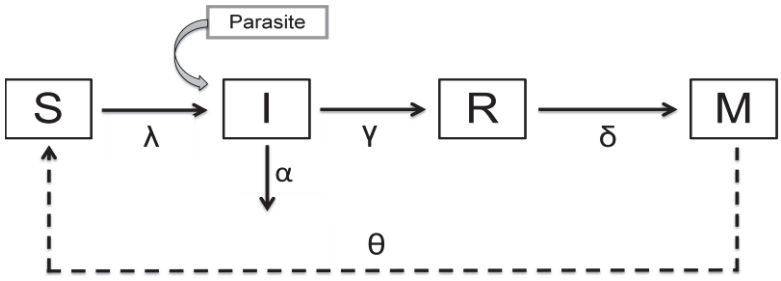


Figure 1.1. *Nombre de publications traitant des virus influenza aviaires chez les Anatidés et les Laridés entre 1899 à 2011*

L'effort de surveillance des virus IA pour l'ensemble des réservoirs est donc crucial pour être capable de décrire leurs circulation dans l'espace et le temps (Haydon *et al.* 2002; Woolhouse 2002; Murphy 2008; Hoye *et al.* 2010; Knight-Jones *et al.* 2010; Krauss *et al.* 2010; Lee *et al.* 2010). Pour comprendre les méthodes de surveillances des agents pathogènes et ainsi mieux appréhender leurs limites, il est possible de se placer aux différents compartiments qui constituent le modèle SIRM (**Encadré 3**). Actuellement, les efforts de surveillance sont le plus souvent concentrés au niveau du compartiment des individus infectieux (I). En effet, la plupart des études actuelles se basent sur l'isolement de l'agent pathogène ou sur la détection directe du génome viral/bactérien, ce qui fournit des informations sur la probabilité d'occurrence des maladies et permet le suivi phylogéographique des souches (Monaghan *et al.* 1985; Munster *et al.* 2007; Pereda *et al.* 2008; Germundsson *et al.* 2010). Cette approche s'est avérée utile lorsque la proportion des individus infectieux est élevée sur une longue période de temps. Cependant, ce n'est pas le cas, par exemple, des virus IA qui n'ont été

détectés que dans seulement 0,9% de plus de 200 goélands leucophées échantillonnés en Camargue (France) de Septembre 2006 à Juillet 2007 (Lebarbenchon *et al.* 2010).

Encadré 3 : modèle SIRM



Comme le modèle SIR (*cf.* Encadré 1), ce modèle décrit l'état de la population d'hôtes par groupes d'individus en ajoutant un nouveau compartiment : sensibles (S), infectieux (I), guéris (R, *recovered* en anglais) et enfin protégés par des anticorps maternels (M).
Les individus sensibles deviennent infectieux au taux λ. Le taux α traduit la virulence du pathogène, c'est à dire le taux de mortalité des hôtes du fait de la présence de l'agent infectieux.
Dans ce modèle, certains individus infectieux guérissent en produisant des anticorps spécifiques, au taux γ. Les femelles guéries peuvent alors transmettre une partie de leur anticorps à leur descendance au taux δ. Cette protection n'étant que temporaire, les individus retourne à l'état sensible au taux θ.

Lorsque le niveau de prévalence est trop faible pour mener des suivis fondés sur ces méthodes directes, il est possible de se placer au niveau du compartiment des individus guéris (R). Des enquêtes sérologiques

peuvent alors être mises en place, afin de détecter la réponse immunologique spécifique de l'exposition à un agent pathogène (Kuno 2001). La détection d'anticorps spécifiques à un agent pathogène n'est utile que si la réponse immunitaire de l'hôte est détectable pendant une période de temps relativement longue (Kuno 2001). En effet, si la persistance des anticorps est courte, les tests sérologiques ne permettront pas de détecter l'exposition aux agents pathogènes. Cependant, les données concernant la persistance des réponses immunitaires en populations naturelles sont très peu disponibles (Garnier *et al.* in prep.). L'une des raisons de la faible disponibilité est probablement la difficulté à estimer la décroissance des réponses immunitaires, notamment car cela nécessite d'échantillonner de manière répétée les mêmes individus, ce qui peut être compliqué en milieu naturel. Cependant, la durée de vie des Laridés (environ 30 ans ; Cramp *et al.* 1983) peut favoriser le développement de réponses immunitaires acquises qui perdurent longtemps (Lee 2006). La plupart des échantillonnages d'enquêtes sérologiques, sont réalisés sur des adultes, ce qui complique l'acquisition de données. En effet, certains oiseaux sauvages sont difficiles à capturer et sont sensibles au stress qu'engendre la capture (Arnold *et al.* 2008). C'est le cas du goéland leucophée, pour lequel la détection d'anticorps maternels dans les œufs peut alors se révéler un outil utile. L'objectif de ce chapitre est de mettre en évidence l'intérêt de la quantification des anticorps maternels au travers d'un exemple portant sur les virus IA. Pour cela, dans l'**Article 2**, nous avons déterminé l'exposition aux virus IA de deux colonies de goéland leucophée en mesurant les niveaux de prévalence en anticorps présents dans les œufs et en les comparant aux niveaux d'anticorps circulant dans le plasma des femelles.

Matériel et méthode

Echantillonnage

L'échantillonnage a eu lieu dans le golfe de Gabès, au sud-est de la Tunisie, sur deux colonies, à Sfax et sur l'île de Djerba (Figure 1.2.). Afin de comparer le niveau d'anticorps maternels entre les œufs d'une même couvée, certain nids choisis aléatoirement ont été visités régulièrement afin de collecter l'ensemble des œufs de la couvée. L'ordre d'échantillonnage des œufs a définit le rang de la ponte.

Afin de déterminer si la quantité d'anticorps détectée dans le sang des mères était corrélée positivement à la quantité d'anticorps dans ses œufs, le plus grand nombre possible de parent a été capturé lors de l'incubation des œufs en utilisant un piège placé sur le nid. Un échantillon de sang (1 ml) a été prélevé pour chaque adulte dans des tubes d'héparine maintenu dans une glacière à 4°C. Chaque individu a ensuite été « sexé » par analyse du plasma selon le protocole décrit par Griffiths *et al.* 1998.

Analyses sérologiques

Les jaunes des œufs récoltés ont été séparés de l'albumen et les anticorps contenus dans le jaune extraits au chloroforme et conservés à -20°C (Gasparini *et al.* 2001).

Les extraits d'œufs et les plasmas ont été testés pour détecter la présence en anticorps dirigés contre la nucléoprotéine des virus IA en utilisant un kit ELISA commercial (ID Screen® Influenza A Antibody Competition Multi-Species, ID VET, Montpellier, France). Les résultats ont été interprétés selon les instructions du kit.

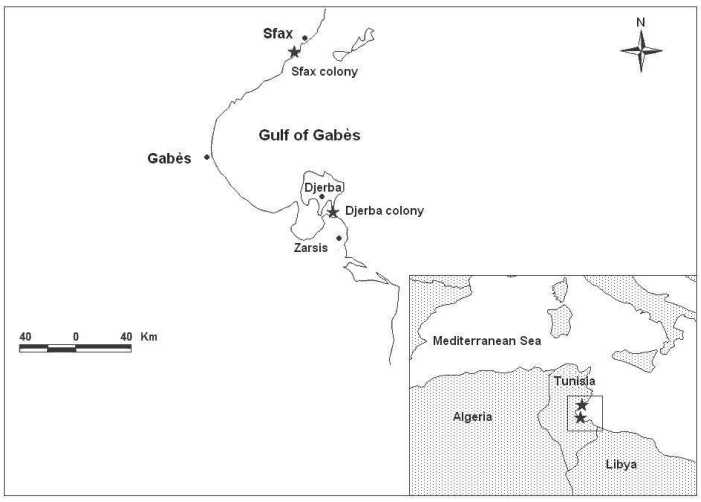


Figure 1.2. *Carte du Golfe de Gabès.*

Les étoiles déterminent la localisation des deux colonies échantillonnées.

Analyses des données

Toutes les analyses ont été effectuées avec le logiciel SAS (SAS 1998). La comparaison des niveaux de prévalence en anticorps entre les deux colonies (Sfax *vs.* Djerba) et entre mâles et femelles ont été effectués par un test du $\chi 2$. Une approche de capture-recapture (MacKenzie *et al.* 2002; MacKenzie *et al.* 2003) a été utilisée pour vérifier si la probabilité de détection des anticorps maternels dans un nid varie avec le rang de ponte (ordre de prélèvement des œufs d'un même nid). Enfin, en utilisant les résultats du sexage moléculaire des adultes, nous avons réalisé une régression linéaire entre la quantité d'anticorps circulant dans le sang des mères et la quantité d'anticorps maternels dans leurs œufs. Pour normaliser les données, une transformation logarithmique leur a été appliquée. Lorsque plusieurs œufs avaient été récoltés pour une même femelle, la valeur moyenne de la couvée a été utilisée.

Résultats et discussion

Intérêt de la quantification d'anticorps maternels…

L'objectif principal de cette étude était de déterminer si la quantification d'anticorps maternels dans les œufs pouvait être un outil rapide et efficace de surveillance des agents pathogènes. Nous avons constaté qu'il y avait une forte corrélation entre la quantité d'anticorps contre les virus IA présente dans le plasma des mères et la quantité d'anticorps maternels présent dans le jaune d'œuf (Figure 1.3.). Cette corrélation positive en anticorps mère/œuf avant déjà était mise en évidence pour l'agent responsable de la maladie de Lyme (*Borrelia burgdorferi*) chez une autre espèce de Laridés, la mouette tridactyle (Gasparini *et al.* 2002). Cependant, la détection de cette corrélation dans notre étude montre qu'elle peut être transposable à d'autres agents pathogènes et que la quantité d'anticorps détectée dans les œufs reflète bien l'exposition présente et passée des femelles à un agent pathogène.

Cependant, nous avons constatés des différences de prévalence en anticorps contre les virus IA entre le sérum des adultes et leurs œufs (Tableau 1.1.). En effet, le niveau de prévalence détecté chez les mères était significativement plus élevé que dans les œufs. Cependant, la détection d'anticorps maternels reste une bonne estimation de l'exposition des mères à un agent pathogène car la quantité en anticorps contre les virus IA détectée chez la mère est corrélée positivement à la quantité détectée dans les œufs.

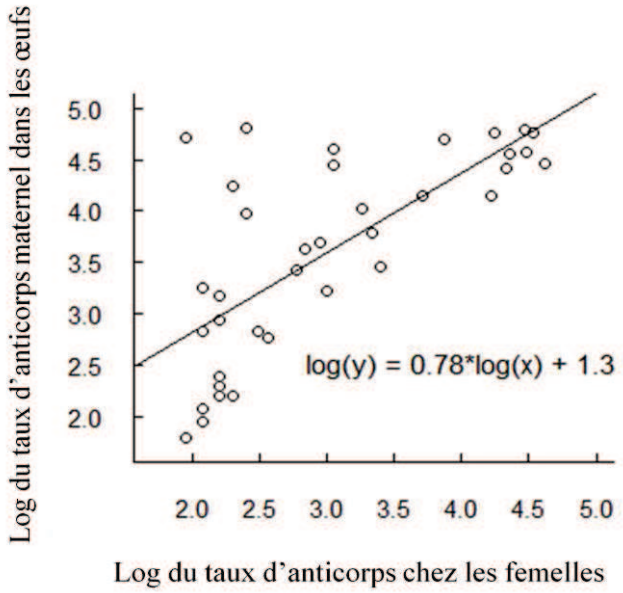


Figure 1.3. *Régression linéaire entre la quantité d'anticorps circulant dans le sang des femelles et la quantité d'anticorps maternels dans leurs œufs.*

Tiré de l'**Article 3**, cette régression présente les résultats pour les 36 femelles et leurs œufs.

Tableau 1.1. *Prévalences en anticorps contre les virus influenza aviaires détectées dans le plasma des adultes, dans les œufs et dans les œufs de la même couvée.*

	Sfax (N ; %)	Djerba (N ; %)	Total (N ; %)
Plasma d'oiseaux	20 (22 ; 91)	28 (42 ; 67)	48(64; 75)
Mâles	5(6 ; 83)	14(22 ; 63)	19(28 ; 68)
Femelles	15(16 ; 94)	14(20 ; 70)	29(36 ; 81)
Echantillons d'œufs	84(127 ; 66)	87 (206 ; 42)	171 (333 ; 51)
Echantillons d'œufs du même nid	32 (45 ; 71)	43 (90 ; 48)	75 (135 ; 56)

Cette étude a également permis de détecter une prévalence élevée en anticorps contre les virus IA dans les deux colonies de goélands échantillonnées (Tableau 1.1.). Qu'une forte proportion de la population soit exposée aux virus IA n'est pas surprenant puisque les goélands ont une durée de vie relativement longue (survie annuelle des adultes > 0,8, durée de vie ≈ 30 ans ; Cramp *et al.* 1983) ce qui favorise le développement de réponses immunitaires acquises (Lee 2006). De surcroit, ce résultat concorde avec ceux précédemment détectés chez les goélands adultes au Canada (Velarde *et al.* 2010), en Norvège (Toennessen *et al.* 2011) et au États-Unis (Graves 1992). Cette concordance suggère que notre technique donne des résultats biologiquement cohérents.

Enfin, l'approche de capture-recapture utilisée dans cette étude permet de mettre en évidence que la probabilité de détection des anticorps ne diffère pas significativement selon le rang de ponte. Ce résultat suggère qu'échantillonner un seul œuf par nid, de quelque rang qu'il soit, est suffisant pour étudier la prévalence en anticorps au sein de la nichée.

… pour mettre en évidence les facteurs éco-écologiques qui peuvent influencer la circulation des virus influenza aviaires.

La transmission des virus IA peut se faire par l'intermédiaire de réservoirs abiotiques. En effet, les virus excrétés dans l'eau demeurent infectieux plus ou moins longtemps, en fonction de différents paramètres, tels que la température, le pH ou le niveau de salinité (Stallknecht *et al.* 1990; Brown *et al.* 2009). Or, les deux colonies étudiées sont des îles entourées d'eau salée, peu favorable à la persistance des virus IA (Brown *et al.* 2009). En supposant que l'infection se fait au sein du site de reproduction et sachant que les goélands leucophées nichent en colonies très denses (Sol *et al.* 1995), la transmission des virus IA serait alors plutôt favorisée par le contact

direct entre individus, ou via les fientes (Ellstrom *et al.* 2008; Costa *et al.* 2011). Cependant, les fortes densités de population ne se retrouvent pas qu'au sein des colonies. Les goélands leucophées se nourrissent dans les décharges à ciel ouvert (Sol *et al.* 1995) sur lesquels l'agrégation des individus est susceptible de conduire à des taux élevés de contact favorable à la transmission des virus IA (McCallum *et al.* 2001). Ceci peut également expliquer les niveaux de prévalence élevés observés pour les deux colonies (Bin Muzaffar *et al.* 2006). D'autres investigations sont donc nécessaires notamment pour déterminer si la transmission se fait sur le site de reproduction. Il serait alors intéressant de tester une éventuelle auto-corrélation spatiale des œufs positifs (par analyses ELISA). Enfin, une étude à plus large échelle permettrait d'évaluer les patrons d'exposition aux virus IA au sein et entre les sites de reproduction, afin de mettre en exergue les différents facteurs favorisant la circulation des virus IA (présence d'eau douce à proximité de la colonie, de décharge à ciel ouvert ou encore d'autres espèces d'oiseaux réservoir de virus IA).

Nos résultats montrent également que la prévalence en anticorps ne diffère pas entre sexes (femelles : 81% ; mâles : 73% ; χ^2=0,4830 ; p=0,4871). Chez cette espèce, mâle et femelle incubent les œufs, partagent les mêmes aires de reproduction et d'alimentation. Ils pourraient donc partager les mêmes risques d'expositions aux virus IA. Les prévalences en anticorps détectées à la fois dans le plasma et dans les œufs étaient significativement plus élevées à Sfax (95%) qu'à Djerba (68%) (χ^2=5,7676 ; p=0,0163). Ce résultat pourrait être une conséquence des différences de densité de population entre les deux colonies. En effet, sur la colonie de Djerba, le goéland leucophée est la seule espèce de Laridés présente, qui partage ce site de reproduction avec une petite colonie d'aigrettes (*Egretta spp.*). *A contrario*, la colonie de Sfax est considérée comme l'une des plus importantes d'Afrique, en terme de diversité et de nombre d'oiseaux (Fishpool *et al.* 2001). Elle se

compose en effet d'une riche et dense communauté d'oiseaux aquatiques partageant les mêmes habitats, dont plus de 30 000 individus de 49 espèces migratrices et 8 500 individus de 9 espèces résidentes (Chokri *et al.* 2008). Cette densité et cette diversité d'espèces pourrait ainsi favoriser la transmission des virus IA.

Conclusion

Nos résultats mettent en évidence que la quantification d'anticorps maternels est un outil pertinent pour étudier la circulation d'agents pathogènes et ouvre ainsi de futurs axes de recherche. Dans cet article, les analyses immunologiques permettent ainsi de détecter les anticorps spécifiques aux virus IA en ciblant la nucléoprotéine commune à tous les sous-types. En conséquence, nous ne connaissons pas l'identité des sous-types auxquels les goélands ont été exposés, et nous ne savons pas si des infections simultanées avec plusieurs sous-types pourraient se produire. Il serait donc intéressant d'identifier les différents sous-types qui circulent au sein de ses colonies, et tout particulièrement les sous-types H13 et H16 détectés presque exclusivement dans les populations de Laridés (Fouchier *et al.* 2005; Olsen *et al.* 2006; Fouchier *et al.* 2007). Les goélands sont également infectés par d'autres sous-types. Ainsi, la première épizootie de virus IAHP documentée en faune sauvage était due au sous-type H5N3, détecté chez les sternes pierregarins (*Sterna hirundo*) (Becker 1966; Alexander 2000), un groupe taxonomique (la famille Sternidae) proche des goélands (famille des Laridés) (Van Tuinen *et al.* 2004).

Chapitre 2 : Etude à large échelle des virus influenza aviaires circulant dans l'ouest du bassin méditerranéen

Introduction

La surveillance en faune sauvage a pour but de déterminer la répartition des populations exposées aux agents pathogènes, donc potentiellement à risque (*cf*. Chapitre 1 ; Morner *et al.* 2002). Ces populations à risque n'étant, *a priori*, pas réparties de façon homogène, il est primordial de disposer de la distribution et du statut des individus dans l'espace (Hudson *et al.* 2002). L'une des premières études à avoir analysée la répartition des cas portait sur l'épidémie de choléra survenue à Londres au XIX^{e} siècle (Snow 1855). Le simple fait de reporter les cas sur un plan de quartier a permis à John Snow (*i*) d'identifier le lieu de la contamination : l'une des pompes à eau du quartier, (*ii*) d'améliorer les connaissances sur le mode de transmission de l'agent pathogène : *Vibrio cholerae* est transmis via l'eau (*iii*) et enfin, d'intervenir efficacement pour arrêter l'épidémie : en retirant la poignée de la pompe à eau (Newsom 2006). Cet exemple, illustre parfaitement le besoin d'intégrer les outils d'épidémiologie spatiale dans la surveillance de maladies infectieuses.

L'épidémiologie spatiale se définie comme l'analyse de la distribution et de la dynamique spatiale des prévalences d'un agent pathogène (Real *et al.* 2007). Cette discipline se concentre sur les facteurs éco-écologiques qui peuvent influencer la circulation des agents pathogènes dans et entre les populations d'hôtes (Real *et al.* 2007; Messinger *et al.* 2009). En utilisant les œufs comme un indicateur de l'exposition des adultes à un agent pathogène (*cf*. Chapitre 1), il est possible de mettre en évidence les patrons de prévalence au sein d'un grand nombre de

populations d'hôtes. Ceci permet de déterminer, rapidement et à moindre coût, les zones de fortes prévalences et d'y établir des analyses complémentaires pour définir les facteurs éco-épidémiologiques influençant la circulation d'agents pathogènes (Rutten *et al.* 2012).

L'objectif de ce chapitre est donc d'améliorer notre compréhension de la dynamique des virus influenza aviaires (IA) en étudiant les patrons de prévalence à une échelle spatiale plus large : l'ouest du bassin méditerranéen (**Article 3 et 4**). Pour ce faire, nous avons étudié la variation spatiale de la présence en anticorps contre les virus IA dans les œufs de goéland leucophée échantillonnés en 2009 (**Article 3**) et 2009/2010 (**Article 4**). Deux études antérieures, menées chacune sur deux colonies de goélands leucophées, en France (Pearce-Duvet *et al.* 2009) et en Tunisie (*cf.* Chapitre 1), ont permis de détecter une prévalence élevée en anticorps maternels contre les virus IA dans les œufs. Cependant, les résultats de ces deux études sont contradictoires en ce qui concerne la variation spatiale des prévalences entre colonies. Une étude menée dans l'ouest du bassin méditerranéen, nous permettra donc de déterminer les niveaux de prévalence à plus large échelle et de clarifier la variation spatiale entre colonies.

Les connaissances actuelles sur l'écologie des virus IA semblent indiquer que les plus forts taux d'infections chez les oiseaux aquatiques sont observés principalement en périodes de regroupement, par exemple lors des rassemblements pré-migratoires (Krauss *et al.* 2004; Lebarbenchon *et al.* 2007; Wallensten *et al.* 2007; Hanson *et al.* 2008). Sachant que les Goélands leucophées nichent souvent en colonies dense (Sol *et al.* 1995), le taux de contact entre les oiseaux peut favoriser la transmission virale entre les individus de la population. Nous avons donc étudié l'agrégation spatiale en anticorps contre les virus IA au sein des sites de reproduction.

Pour finir, afin d'évaluer le rôle joué par les goélands leucophées dans la circulation des virus IA, nous avons caractérisé certains des sous-types correspondant aux anticorps maternels détectés. Sachant que des études antérieures avaient montrées de fortes prévalences des sous-types H13 et H16 dans des populations de Laridés (Fouchier *et al.* 2005; Olsen *et al.* 2006; Fouchier *et al.* 2007), nous nous attendions à un résultat similaire. Nous avons également cherché la présence d'anticorps spécifiques aux sous-types H5 et H7, seuls sous-types connus pour évoluer en forme hautement pathogènes chez les volailles.

Matériel et méthodes

Echantillonnage

En 2009 et 2010, au cours de la saison de reproduction (Mars/Avril), 1568 œufs (entre 8 et 127 œufs par colonie et par année) ont été échantillonnés. Ce prélèvement a été effectué sur 14 sites de reproduction en 2009/2010 situés en France (n = 7), en Espagne (n = 3), en Algérie (n = 1) et sur 3 colonies en 2009 situés en Tunisie (Sfax et Djerba) et en France (Grau du roi) (Figure 2.1.). Un seul œuf par nid a été échantillonné (*cf.* Chapitre 1).

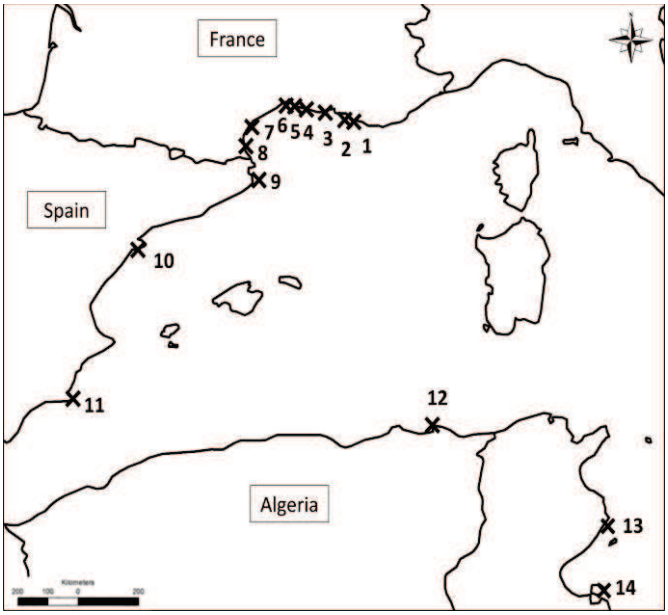


Figure 2.1. *Carte des colonies échantillonnées dans l'ouest de la Méditerranée*

France (n = 8), Espagne (n = 3), Algérie (n = 1) et Tunisie (n = 2)

Le nombre de couples reproducteurs par colonie a été estimé en examinant la littérature et en discutant avec des chercheurs ou des responsables gouvernementaux qui étudient le goéland leucophée (Tableau 2.1.). Dans 3 colonies en 2009 (îles Riou, Villeneuve et Corrège) et dans l'ensemble des colonies de 2010, l'emplacement des nids prélevés a été enregistré par GPS afin de vérifier l'auto-corrélation spatiale des échantillons porteurs d'anticorps maternels au sein des colonies.

Tableau 2.1. *Données disponibles sur le nombre de couple reproducteur (taille de colonie) des sites échantillonnés.*

Colonie	Taille de colonie	Source d'information
1-Riou	8000	Duhem 2004
2-Frioul	8000	P. Mayet comm. pers.
3-Carteau	400	P. Mayet comm. pers.
6-Villeneuve	400	P. Mayet comm. pers.
7-Gruissan	350	P. Mayet comm. pers.
8-Corrège	4000	P. Mayet comm. pers.
9-Medes	6500	Ramos *et al.* 2009
10-Ebro Delta	6000	Ramos *et al.* 2009
11-Isla Grosa	1400	D. Oro comm. pers.
13-Sfax	100	Hammouda comm. pers.
14-Djerba	2500	Hammouda comm. pers.

Analyses sérologiques

La longueur et la largeur des œufs prélevés en 2009 ont été mesurées afin d'estimer leurs volumes, indicateur de la qualité des parents (Hoyt 1979). Les jaunes d'œufs ont ensuite été séparés de l'albumen et les anticorps contenus dans le jaune extraits au chloroforme (Gasparini *et al.* 2001) et conservés à -20°C.

Les extraits d'œufs ont été testés pour détecter la présence des anticorps dirigés contre la nucléoprotéine des virus IA en utilisant un kit ELISA commercial (ID Screen® Influenza A Antibody Competition Multi-Species, ID VET, Montpellier, France). Les résultats ont été exprimés selon les instructions du kit.

Identification de certains sous-types de virus IA

À l'exception de quelques échantillons, l'ensemble des œufs porteur d'anticorps maternels contre les virus IA ont été testés pour définir si ces anticorps sont spécifiques aux sous-types H5 et H7 (ELISA commerciaux ; ID Screen® Influenza H5 and H7 Antibody Competition, ID VET, Montpellier, France). Comme précédemment, les résultats ont été interprétés selon les instructions du kit.

Ces échantillons ont aussi été testés pour chercher la présence en anticorps hémagglutinants spécifiques aux sous-types H13 et H16 par test d'inhibition d'hémagglutination (TIH) en suivant la procédure standard (Kuiken *et al.* 1998; WHO manual on animal influenza diagnosis and surveillance 2002). Les titres de TIH $\geq$ 1:20 ont été considérés comme positif.

Analyses statistiques

Toutes les analyses ont été effectuées à l'aide R.2.15.0 (R Development Core Team 2012). L'importance des facteurs dans nos modèles a été

déterminée en utilisant une comparaison de modèles basée sur l'élimination de la dernière variable.

Nous avons utilisé des modèles linéaires généralisés (GLM) pour déterminer si les prévalences en anticorps contre les virus IA étaient différant entre les colonies.

Nous avons utilisé un modèle linéaire généralisé mixte (GLMMPQL avec distribution binomiale, avec comme effet aléatoire la variable « colonie », bibliothèque MASS) afin d'évaluer si les niveaux de prévalence diffèrent au fil du temps.

Un test de Mantel (bibliothèque ade4 dans R) a été utilisé pour évaluer si les échantillons positifs en anticorps IA sont répartis de façon homogène (Vicente *et al.* 2007) sur les trois colonies choisies aléatoirement : Delta de l'Ebre (Espagne), Corrège (France) et le Frioul (France). Ce test a été utilisé ici pour calculer la corrélation linéaire entre deux matrices de distance (géographique et de prévalence en anticorps contre les virus IA). Nous avons contrôlé les comparaisons multiples en utilisant la correction du « false discovery rate » $q^* = 0,05$ (Benjamini *et al.* 1995). Cette approche contrôle la proportion des hypothèses qui seraient rejetées par erreur, ce qui augmente la puissance statistique du test par rapport aux méthodes traditionnelles de comparaisons multiples (Garcia 2003).

Enfin, les relations entre le volume des œufs et la prévalence de chaque œuf ont été déterminés avec une régression logistique binomiale. La différence du volume moyen des œufs entre colonies a été testée en utilisant un modèle linéaire avec l'effet colonie comme effet prédictif et le volume des œufs comme effet réponse.

Résultats et discussion

Circulation des virus IA chez le goéland leucophée dans l'ouest du bassin méditerranéen

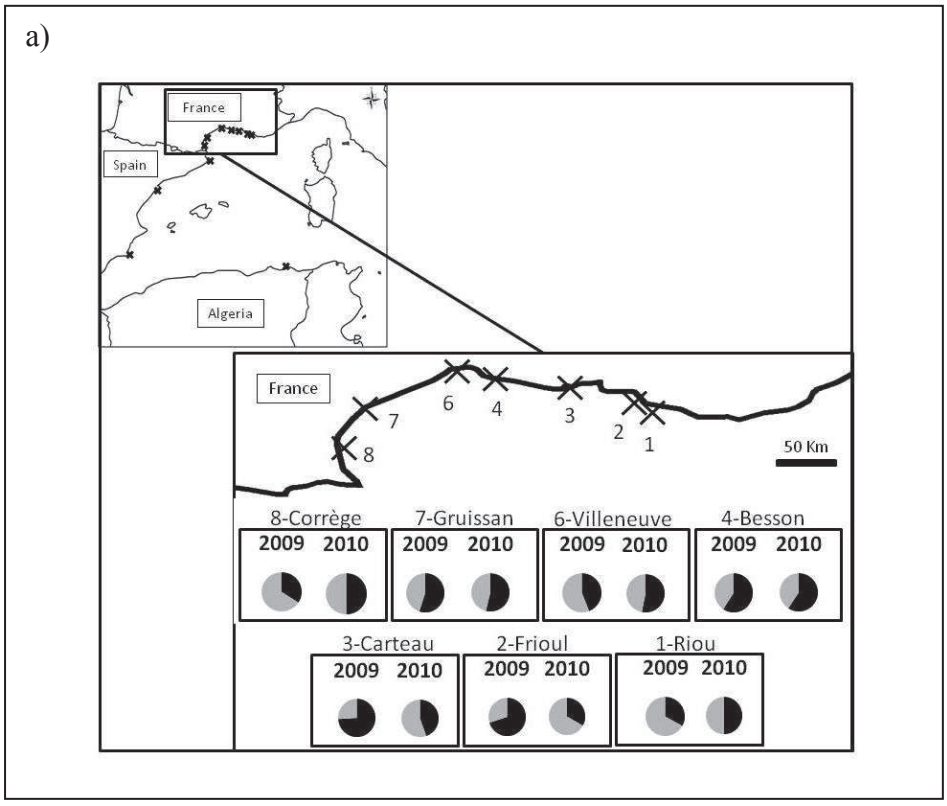
Au cours des deux années d'études, des niveaux de prévalence élevés ont été détectés dans chaque colonie (Tableau 2.2.). Les goélands leucophées sont donc exposés aux virus IA. Les niveaux élevés de prévalence confirment les résultats antérieurs obtenus sur les œufs de goéland leucophée (*cf.* chapitre 1 ; Pearce-Duvet *et al.* 2009). Cependant, notre étude à l'échelle de l'ouest méditerranéen, permet de clarifier la variation spatiale entre les colonies.

Tableau 2.2. *Prévalences en anticorps contre les virus influenza aviaires détectées dans les œufs de goéland leucophée.*

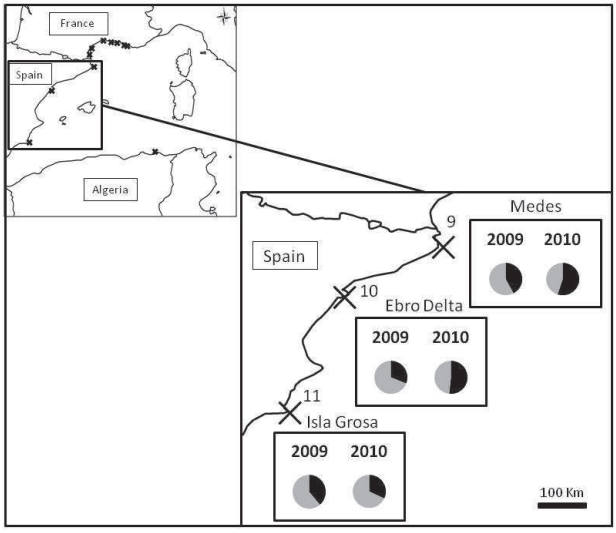
Pays	Colonie	Année	Taille d'échantillonnage	Nombre d'échantillons positifs
France	1-Riou	2009	127	42 (33%)
		2010	8	4 (50%)
	2-Frioul	2009	79	38 (48%)
		2010	51	17 (33%)
	3-Carteau	2009	102	57 (56%)
		2010	56	25 (45%)
	4-Besson	2009	79	34 (43%)
		2010	59	35 (59%)
	5-Grau du Roi	2009	50	17(34%)
	6-Villeneuve	2009	70	20 (29%)
		2010	64	34 (53%)
	7-Gruissan	2009	99	33 (33%)
		2010	91	49 (54%)
	8-Corrège	2009	70	15 (21%)
		2010	54	27 (50%)
Espagne	9-Medes	2009	38	11 (29%)
		2010	49	27 (55%)
	10-Ebro delta	2009	55	12 (22%)
		2010	29	15 (52%)
	11-Isla Grosa	2009	66	13 (20%)
		2010	50	16 (32%)
Algerie	12-Chetaïbi	2009	55	17 (31%)
		2010	33	11 (33%)
Tunisie	13-Sfax	2009	45	21 (47%)
	14-Djerba	2009	89	23 (26%)

Différence de prévalence entre les colonies

Les différences significatives des niveaux de prévalence entre colonies suggèrent que le risque d'exposition aux virus IA n'est pas homogène. Ces différences sont positivement corrélées à un gradient Sud/Nord (Figure 2.2. ; modèle linéaire généralisé mixte: Estimation = 0,1, Erreur std. = 0,5, t = 2,8, p = 0,021). Ce résultat confirme la variation de prévalence précédemment détectée dans les deux colonies en Tunisie (*cf.* Chapitre 1). Bien que nous n'ayons pas récolté de données détaillées sur les conditions environnementales ou sur l'écologie des hôtes, nos données préliminaires permettent néanmoins de poser certaines hypothèses explicatives.



b)



c)

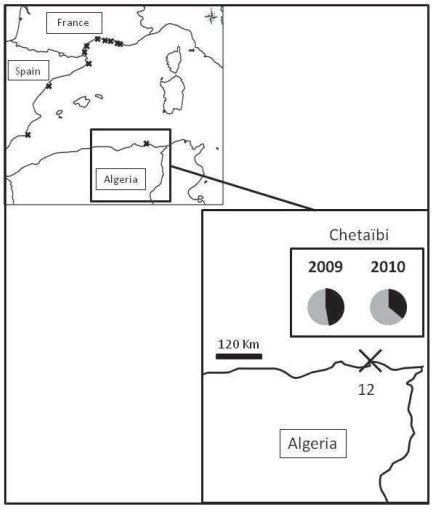


Figure 2.2. *Prévalences des anticorps contre les virus influenza aviaires.* Pour les colonies échantilonnées en 2009/2010 en a) France ; b) Espagne et c) Algérie

Ces différences significatives de prévalence pourraient être dues à des différences entre des facteurs environnementaux qui suivent un gradient Sud/Nord. En particulier, les canards colverts, principal réservoir naturel des virus IA (Stallknecht *et al.* 2007b), ne sont que marginalement présents en Algérie (Cramp *et al.* 1983; Samraoui *et al.* 2008; Samraoui *et al.* 2011). En revanche, ils sont de plus en plus abondants vers le Nord, notamment dans les zones humides telles que la Camargue (Lebarbenchon *et al.* 2010) ou le delta de l'Ebre (Mateo *et al.* 1998). La différence de proximité avec les canards colverts pourrait expliquer les différences de prévalence entre les colonies.

Ces patrons d'exposition pourraient également être liés à l'âge moyen des individus. En effet, chez les oiseaux marins, les femelles âgées et expérimentées ont tendance à pondre de plus gros œufs (Amundsen *et al.* 1996; Christians 2002) et à nicher sur des sites de meilleure qualité (accès à la nourriture facilité ; Pugesek *et al.* 1983). Les différences significatives du volume moyen des œufs entre les colonies, détectées en 2009, pourraient donc suggérer une variation de qualité des sites de reproduction et donc un investissement plus important dans la production d'anticorps (Grindstaff *et al.* 2003). Toutefois, la quantité d'anticorps détectée dans les œufs et le volume de l'œuf ne sont pas corrélés : ce qui signifie que même si un œuf est gros, il n'est pas plus susceptible de contenir des anticorps maternels contre les virus IA qu'un petit œuf. Par ailleurs, le volume moyen des œufs au sein d'une colonie et la prévalence en anticorps contre les virus IA de cette colonie ne sont pas corrélés. Cela pourrait suggérer que :

i- l'exposition est indépendante de l'âge, sachant que des anticorps contre les virus IA ont été détectés aussi bien chez des adultes que chez des poussins de Goéland à bec cerclé (*Larus delawarensis)* âgés de 3 à 5 semaines (Velarde *et al.* 2010). A cet âge, les anticorps sont peu susceptibles d'être d'origine maternelle sachant que la quantité

d'anticorps maternels décroit rapidement chez les espèces de Laridés (Garnier *et al.* 2012).

ii- le patron d'exposition lié à l'âge est masqué par la longue persistance des anticorps chez les femelles. La persistance des anticorps spécifiques aux virus IA n'a jamais été évaluée chez les Laridés. C'est donc un axe de recherche qui reste à développer.

Un autre facteur qui pourrait expliquer la différence des patrons d'exposition, est taille des colonies. Le nombre important d'individus dans les grandes colonies (Cramp *et al.* 1983) et l'espace limité sur les petits sites de reproduction (Duhem *et al.* 2007) favorisent l'augmentation de la densité d'individu. Les virus IAFP sont excrétés par les selles (Olsen *et al.* 2006) ou les voies respiratoires (Costa *et al.* 2011) des individus infectieux et transmis, à des hôtes sensibles, par ingestion des fèces (Olsen *et al.* 2006) ou par contact rapproché (Costa *et al.* 2011). La taille des colonies pourrait augmenter le risque d'exposition aux virus IA soit en favorisant la rencontre entre individu, soit par l'accumulation de matières fécales, donc de virus (Muller *et al.* 2004; Fouchier *et al.* 2007).

Différences de prévalence au sein des colonies

Cette hypothèse est supportée par les résultats obtenus sur la répartition spatiale des œufs contenant des anticorps au sein de la colonie. En effet, le fait qu'aucune auto-corrélation spatiale n'ait été détectée dans les colonies testées (Villeneuve Corrège et Riou en 2009, Delta de l'Ebre, Corrège et Frioul en 2010) suggère que les oiseaux ne sont pas infectés au sein des site de reproduction (Keeling *et al.* 2002). Les goélands leucophées peuvent s'infecter durant l'hiver, lorsque les conditions de température sont plus favorables à la persistance de virus IA dans l'environnement (Brown *et al.* 2009). Le goéland leucophée peut aussi

être infecté dans les zones où la densité des individus peut favoriser la transmission des virus IA (Krauss *et al.* 2004; Lebarbenchon *et al.* 2007; Hanson *et al.* 2008), sur les sites d'alimentation (notamment les décharges à ciel ouvert ; Duhem *et al.* 2003) ou encore les sites de repos (McCallum *et al.* 2001; Bin Muzaffar *et al.* 2006).

Patrons temporels

Les niveaux de prévalence augmentent significativement entre 2009 et 2010 (modèle linéaire généralisé mixte: Estimation = 0,6 ; Erreur std. = 0,2 ; t = 3,3 ; p = 0,009). Ce résultat suggère que le goéland leucophée est régulièrement exposé à ces virus. En effet, si ce patron était dû à la transmission passée de virus IA, il faudrait s'attendre à ce que les facteurs démographiques (mortalité naturelle, recrutement d'individus sensibles…) conduisent à une stabilisation ou à une diminution des niveaux de prévalence, ce qui n'est pas le cas ici. Bien que ce résultat ne soit que sur deux années et doit donc être confirmé par une étude à plus long terme il démontre l'importance fondamentale de mettre en œuvre des études épidémiologiques sur le long terme, afin de mieux comprendre la dynamique virale au sein des populations. Des axes de recherche restent donc à développer comme, par exemple, déterminer le taux de transmission au sein des populations, nécessaire pour maintenir de tels niveaux de prévalence.

Sous-types spécifiques des anticorps détectés chez les goélands leucophées dans le bassin méditerranéen

Aucun des anticorps spécifiques aux sous-types H5 et H7 n'est détectés, résultat cohérent avec d'autres études suggérant que ces sous-types ne sont que ponctuellement détectés chez les espèces de Laridés (Muzinic *et al.* 2010; Savic *et al.* 2010). Au contraire, sachant que les sous types H13 et H16 circulent fréquemment dans les populations de Laridés

(Fouchier *et al.* 2005; Olsen *et al.* 2006; Fouchier *et al.* 2007), il était attendu que la détection de fortes prévalences en anticorps contre ces sous-types soit observée, ce qui n'est pas le cas (Tableau 2.3.). En effet, en comparaison avec les prévalences établies en Norvège sur les mouettes tridactyles (Toennessen *et al.* 2011), nos deux années d'études ne permettent de détecter que de faibles niveaux de prévalence en anticorps pour l'ensemble des colonies échantillonnées. Ces différences de prévalence peuvent être liées au fait que le goéland leucophée et la mouette tridactyle sont deux espèces avec des écologies qui leurs sont propres.

Tableau 2.3. *Prévalences en anticorps contre les sous-types H13 et H16 détectées dans les œufs de goéland leucophée par inhibition d'hémagglutination.*

Pays	Colonies	Année	Positif en IA (N;%)	H13 (%)	H16 (%)
France	1-Riou	2009	40 (127 ; 31%)	9 (22.5%)	0
		2010	4 (8 ; 50%)	2 (50%)	1 (25%)
	2-Frioul	2009	37 (79 ; 47%)	11 (30%)	0
		2010	17 (51 ; 33%)	2 (12%)	1 (6%)
	3-Carteau	2009	55 (102 ; 54%)	8 (15%)	0
		2010	25 (56 ; 45%)	8 (32%)	2 (8%)
	4-Besson	2009	34 (79 ; 43%)	12 (35%)	0
		2010	35 (59 ; 59%)	12 (34%)	2 (6%)
	6-Villeneuve	2009	20 (70 ; 29%)	10 (50%)	0
		2010	34 (64 ; 53%)	22 (65%)	3 (9%)
	7-Gruissan	2009	32 (99 ; 32%)	5 (16%)	1 (3%)
		2010	49 (91 ; 54%)	21 (43%)	4 (8%)
	8-Corrège	2009	14 (70 ; 20%)	12 (86%)	1 (7%)
		2010	27 (54 ; 50%)	7 (26%)	0
Espagne	9-Medes	2009	10 (38 ; 26%)	5 (50%)	1 (10%)
		2010	27 (49 ; 55%)	6 (22%)	2 (7%)
	10-Ebro delta	2009	12 (55 ; 22%)	3 (25%)	0
		2010	15 (29 ; 52%)	9 (60%)	2 (13%)
	11-Isla Grosa	2009	13 (66 ; 20%)	7(54%)	4 (31%)
		2010	16 (50 ; 32%)	4 (25%)	4 (25%)
Algerie	12-Chetaïbi	2009	17 (55 ; 31%)	6 (35%)	1 (6%)
		2010	11 (33 ; 36%)	5 (45%)	0

Cette variabilité de prévalence peut également s'expliquer par des différences de protocole entre notre étude et celle de Tonnessen *et al.* (2011). Le protocole mis en place par Tonnessen *et al.* (2011) utilise des antigènes H13/H16 et le même seuil de positivité (titre ≥ 1:20) que dans notre étude. Par conséquent, la seule différence notoire est que l'étude en Norvège porte sur du sérum prélevé sur des adultes et des poussins de 3 semaines (assez âgés pour éliminer la présence potentielle d'anticorps d'origine maternelle ; Garnier *et al.* 2012). Le fait d'échantillonner les adultes et les poussins permet de détecter à la fois des immunoglobulines de type M (IgM) et de type Y (IgY). Chez les oiseaux, la réponse immunitaire acquise est principalement médiée par les IgY (qui est un équivalent fonctionnel des IgG chez les mammifères ; West *et al.* 2004). Il s'agit de la seule immunoglobuline capable d'être transférée dans le jaune d'œuf (West *et al.* 2004). Par conséquent, les tests d'inhibition d'hémagglutination effectués sur des œufs prélevés dans notre étude ne peuvent déceler les anticorps IgM, ce qui peut expliquer les différences de prévalence observée entre Tonnessen *et al.* (2011) et notre étude. Cette différence de prévalence entre sérum et œuf avait déjà été observée dans l'**Article 2** (*cf.* Chapitre 1) mais comme la quantité d'anticorps contre les virus IA détectée chez la mère était corrélée positivement à la quantité détectée dans les œufs, la détection d'anticorps maternels reste une bonne estimation de l'exposition à un agent pathogène.

Enfin, seulement 1% de tous les échantillons testés en inhibition d'hémagglutination (TIH), contenaient des anticorps spécifiques aux sous-types H13 et H16 (Tableau 2.4.).

Tableau 2.4. *Titres d'inhibition d'hémagglutination observés pour les œufs contenant des anticorps contre les deux sous-types H13 et H16.*

Pays	Colonie	Année	Titre H13	Titre H16
France	1-Riou	2010	1:80	1:40
	3-Carteau	2010	1:320	1:80
			1:40	1:40
	4-Besson	2010	1:40	1:20
			1:20	1:20
	6-Villeneuve	2010	1:80	1:20
			1:320	1:20
			1:320	1:160
	7-Gruissan	2010	1:80	1:20
			1:40	1:20
			1:80	1:20
	8-Corrège	2009	1:40	1:20
Espagne	9-Medes	2009	1:20	1:80
		2010	1:160	1:40
			1:20	1:20
	10-Ebro Delta	2010	1:40	1:80
	11-Isla Grossa	2009	1:20	1:20
			1:80	1:80
Algérie	12-Chetaïbi	2009	1:160	1:20

Ce pourcentage est relativement faible en comparaison des 37,5% détectés en Norvège (Toennessen *et al.* 2011). Ce très faible pourcentage pourrait être lié à un problème de détectabilité des anticorps maternels : comme des réactions croisées entre anticorps de sous-types différents (Sharp *et al.* 1997). Pour vérifier la détectabilité de la quantification des anticorps maternels, nous avons testé, par TIH, 12 échantillons de jaune d'œufs de mouettes tridactyles récoltés sur la même colonie de Norvège (Hornøya) que Toennessen *et al.* (2011). Les anticorps dirigés contre les sous-types H13 et H16 ont été observés respectivement pour 7/12 et 2/12 des échantillons. Même si la quantité d'échantillon est insuffisante pour conclure, les résultats obtenus montrent une prévalence en H13 relativement élevée, comme trouvé en Norvège. Pour H16, les prévalences sont plus faibles ce qui pourrait

s'expliquer par le fait que les 12 œufs testés n'ont pas été échantillonnés la même année que dans l'étude de Toennessen *et al.* (2011). Ces résultats suggèrent que les faibles prévalences détectées dans notre étude pour les sous-types H13 et H16 ne sont pas liées à un manque de détectabilité par les anticorps maternels.

Conclusion

Cette étude rigoureuse permet la surveillance simultanée de l'exposition aux virus IA dans différentes colonies d'oiseaux sauvages de l'ouest méditerranéen. Nos résultats montrent que les goélands leucophées sont exposés à certains sous-types de virus IA tel que H13 et H16 qui circulent donc en Méditerranée. Ce résultat est intéressant car la grande diversité de sous-types circulant chez les oiseaux sauvages (Webster *et al.* 1992; Alexander 2000) associé aux contact étroit de cette espèce avec l'Homme et les animaux domestiques peuvent conduire à des réassortiments entre virus qui permettent l'émergence de nouveau sous-types viraux (Chen *et al.* 2006a; Van Poucke *et al.* 2010). Cependant, sachant qu'on détecte de fortes prévalence en anticorps dans les œufs mais que relativement peu de ces échantillons ont des anticorps spécifiques aux sous-types H13 et H16, on peut présumer que d'autre sous-types, qui n'ont pas été identifiés dans ces travaux, circulent dans la population de goélands leucophées. Sachant que les goélands leucophées partagent certains habitats avec les canards (Cramp *et al.* 1983), ils peuvent peut être s'infecter avec des sous-types circulant chez les Anatidés. Leur indentification pourrait alors améliorer nos connaissances sur les sous-types circulant en Méditerranée. Enfin, il serait intéressant d'étendre cette étude à d'autres agents pathogènes d'intérêt en santé humaine et animale, aux modes de transmission différents et pour lesquels les oiseaux sauvages représentent le principal réservoir.

Chapitre 3 : Circulation d'agents pathogènes à transmission vectorielle : les flavivirus

Introduction

Au cours des dernières décennies, les changements climatiques et les activités humaines ont conduit à l'émergence de nombreux arbovirus chez l'Homme et les animaux (Gould *et al.* 2009). Les arbovirus (« arbo » pour *arthropod-borne* en anglais) sont des virus nécessitant la présence d'un arthropode hématophage pour être transmis d'un hôte vertébré à un autre (Gubler 1996). Ils sont classés en cinq familles (Bunyaviridae, Flaviviridae, Reoviridae, Rhabdoviridae et Togaviridae) en fonction de leurs morphologies, leurs mécanismes de réplication et leurs relations antigéniques (Porterfield 1975). Chez l'Homme, la plupart des arboviroses sont asymptomatiques mais certains flavivirus peuvent causer des maladies du système nerveux pouvant entrainer la mort (Weaver *et al.* 2004).

Le genre flavivirus comprend environ 70 virus antigéniquement apparentés, largement distribués dans le monde (Gaunt *et al.* 2001). Une parfaite compréhension du rôle des réservoirs naturels de ces virus est essentielle pour évaluer et prédire les risques de transmission des flavivirus à l'Homme ou aux animaux domestiques. Pour les flavivirus, dont certains circulent en Europe (Chastel *et al.* 1985a; Hubalek *et al.* 2008), les oiseaux sauvages représentent l'un des plus important réservoir naturel (Weaver *et al.* 2004). C'est le cas du virus West Nile (WN), transmis par des arthropodes hématophages, le plus souvent des moustiques du genre *Culex*, et dont les oiseaux sauvages sont le réservoir naturel (Calistri *et al.* 2010). Si l'Homme et le cheval peuvent être infectés, ils ne sont habituellement pas capables de transmettre le

virus aux moustiques et sont donc considérés comme des « culs-de-sac » épidémiologiques (Kulasekera *et al.* 2001). Ils peuvent cependant développer des symptômes cliniques allant d'un simple syndrome fébrile à des troubles neurologiques mortels (Calistri *et al.* 2010). En Europe, depuis les années 1960, la circulation du virus WN chez l'Homme et les chevaux a été signalée à intervalles irréguliers (Calistri *et al.* 2010). En 2010, de multiples foyers ont réapparus dans plusieurs pays européens, en particulier dans le pourtour méditerranéen (EpiSouth 2010). De même, le virus Usutu (USU), un flavivirus phylogénétiquement proche du virus WN, est transmis par des moustiques et a pour réservoir naturel des oiseaux sauvages (Calzolari *et al.* 2010). La circulation des virus USU a été rapportée pour la première fois en 2001 en Autriche, causant la mort de plusieurs espèces d'oiseaux, en particulier des passereaux (Weissenbock *et al.* 2003). Le virus est ensuite détecté dans différents pays européens (Calzolari *et al.* 2010) mais très peu de données sont disponibles sur l'impact de ce virus en santé humaine. En 2009, des symptômes liés à la présence du virus USU ont été signalés chez deux patients immunodéprimés dans la région d'Emilia-Romagna en Italie (Cavrini *et al.* 2009). Toujours en Italie, le virus USU a également été détecté dans le sérum de deux donneurs d'organes testés pour un dépistage du virus WN (Capobianchi *et al.* 2010).

D'autres flavivirus transmis par les tiques sont connus pour circuler en Europe dans les populations d'oiseaux sauvages. Ainsi, le virus de Tyulieniy a été isolé pour la première fois en 1969, chez des tiques *Ixodes uriae* échantillonnées dans une colonie d'oiseaux de mer à l'Est de la Russie (Karabatsos 1985; Dobler 2010). Ce virus a ensuite été détecté et isolé chez les mêmes espèces de tiques sur la côte atlantique en France (Chastel *et al.* 1983; Chastel *et al.* 1985b), sur les îles Lofoten en Norvège (Saikku *et al.* 1980) et sur la côte ouest des Etats-Unis (Clifford *et al.* 1971). Les données sérologiques suggéraient alors

que le virus Tyuleniy serait bénin pour l'Homme (Chastel *et al.* 1983). D'autres flavivirus moins connus ont également pu être détectés, tels que le virus Meaban isolé en 1985 sur la côte atlantique française, à partir de tiques *Ornithodoros maritimus* échantillonnées dans des nids de goélands argentés (*Larus argentatus*) (Chastel *et al.* 1985a). Des anticorps dirigés contre ce virus ont également été détectés dans ces colonies (Chastel *et al.* 1985b). Ce flavivirus n'a jamais été isolé nul part ailleurs (Dobler 2010) et très peu de données sont disponibles sur sa pathogénicité chez l'Homme ou chez d'autres espèces animales. *A contrario*, le virus de l'encéphalite à tiques (VET) est un flavivirus hautement pathogène, qui circule également en Europe (Mansfield *et al.* 2009). Le VET peut affecter l'Homme et causer des infections neurologiques fatales (Mansfield *et al.* 2009). Le principal vecteur du VET est la tique *Ixodes ricinus*, largement distribuée en Europe (Mansfield *et al.* 2009). Cette liste, loin d'être exhaustive, démontre l'intérêt de mettre en évidence les facteurs éco-épidémiologiques affectant la circulation d'une telle diversité de flavivirus pour certains agents pathogènes et pour d'autres dont la pathogénicité n'est pas bien définie.

L'objectif de ce chapitre est d'améliorer notre compréhension de la dynamique des flavivirus en étudiant les patrons de prévalence à l'échelle de l'ouest méditerranéen (**Article 5**). Pour cela, nous avons recherché la présence d'anticorps maternels spécifiques aux flavivirus dans les jaunes d'œufs de goélands leucophées sur 19 colonies de 4 pays méditerranéens. Certaines espèces de goéland, dont le goéland leucophée, ont été identifiés comme potentiellement impliqués dans introduction du virus WN en France (Jourdain *et al.* 2007). En supposant que cette espèce est effectivement sensible au virus WN et sachant que ce virus a récemment circulé dans le bassin méditerranéen comme en Espagne (Figuerola *et al.* 2007b; EpiSouth 2010), en Afrique du Nord (Amraoui *et al.* 2012) et dans le Sud de la France (Calistri *et*

al. 2010), notre prédiction était de détecter des prévalences élevées des anticorps contre ce flavivirus dans les colonies de goéland proches des cas équins ou humains, notamment dans le delta de l'Ebre en Espagne (Bofill *et al.* 2006) et en Camargue (Jourdain *et al.* 2007).

Dans une seconde étape, afin de documenter plus précisément la circulation des flavivirus, un échantillonnage de vecteurs potentiels, tiques et moustiques a été réalisé dans l'ensemble des colonies où les anticorps contre les flavivirus ont été détectés. Certaines colonies comme en Camargue ou dans le delta de l'Ebre sont relativement proches de points d'eau douce ou d'eau saumâtre, nécessaires au développement des moustiques. D'autres colonies comme Isla Grossa ou Medes en Espagne sont entourées d'eau de mer et ne présentent pas de points d'eau douce à proximité, limitant ainsi le développement des moustiques. Si le vecteur de flavivirus s'avère être une espèce de moustique, on pourrait donc s'attendre à des patrons d'exposition relativement hétérogènes. En revanche, les tiques sont présentes dans les nids de nombreuses colonies de goélands échantillonnées (EstradaPena *et al.* 1996 ; K.D. McCoy et R. Garnier, observations personnelles) ce qui augmente la probabilité de rencontre avec l'hôte. Des patrons d'exposition plus homogènes entre les colonies devraient être observés. Dans le cas contraire, des facteurs environnementaux, intrinsèques aux colonies où de fortes expositions en flavivirus sont détectées, pourraient favoriser le développement viral.
Après avoir identifié le vecteur potentiel, nous nous sommes penché sur l'écologie du goéland et du vecteur afin de définir les facteurs éco-épidémiologiques impliqués dans la circulation et la propagation des flavivirus. Sachant que le goéland leucophée est présent en milieu urbain (Sol *et al.* 1995; Duhem *et al.* 2008), il est d'autant plus important de connaître la propagation des flavivirus si ce virus est pathogène de l'Homme ou si sa pathogénicité n'est pas bien définie.

Pour finir, nous avons identifié par test de neutralisation les flavivirus correspondant aux anticorps maternels détectés. Sachant que des études antérieures ont montré que des espèces de Laridés sont porteur du virus WN (Komar *et al.* 2003; Marra *et al.* 2004), notamment en Espagne (Figuerola *et al.* 2007a), du virus Meaban (Chastel *et al.* 1985a) ou encore du virus Tyuleniy (Chastel *et al.* 1983; Chastel *et al.* 1985b) notre prédiction était de détecter un ou plusieurs de ces flavivirus.

Matériel et méthodes

Echantillonnage

De 2009 à 2011, au cours de la saison de reproduction (Mars/Avril), un œuf par couvée a été échantillonné dans chacun des 19 sites de reproduction situés en France (n = 9), Espagne (n = 5), Tunisie (n = 2) et Algérie (n = 3). Au total, 1 098 œufs (de 8 à 50 œufs par colonie) ont été analysés. Un échantillonnage supplémentaire a également été réalisé dans les colonies où les anticorps spécifiques aux flavivirus avaient été détectés. Ainsi, du sérum de poussins de goéland leucophée a été échantillonné pendant les trois années consécutives (2009 à 2011). Pour cela, jusqu'à 2 ml de sang ont été prélevés par poussin afin de déceler la présence d'anticorps spécifiques aux flavivirus. Comme la concentration en anticorps maternels diminue avec l'âge (Garnier *et al.* 2012), chaque poussin a été pesé et mesuré afin d'estimer son âge.
En parallèle, des tiques *Ornithodoros maritimus* ont été échantillonnées sur les poussins de goéland leucophée. Enfin, 34 pièges lumineux à CO_2 ont été mis en place pendant 24h en août 2011, afin de capturer des moustiques de la famille des *Culicidae*.

Analyses sérologiques

Les jaunes d'œufs sont séparés de l'albumen et les anticorps contenus dans le jaune extraits au chloroforme (Gasparini *et al.* 2001) et conservés à -20°C.
Les extraits d'œufs et le plasma de poussin ont été testés pour détecter la présence d'anticorps dirigés contre la protéine d'enveloppe (protéine E) du virus WN. Le kit ELISA commercial (ID Screen® West Nile Competition, ID VET, Montpellier, France) a été utilisé conformément aux instructions du fabricant.

Des tests de neutralisation des anticorps ont été réalisés par Sylvie Lecollinet (ANSES, UMR1161 INRA, Maisons-Alfort, France) pour détecter :

i- la présence d'anticorps neutralisants spécifiques aux virus WN dans certains des œufs positifs en ELISA choisis aléatoirement (souche IS-98-ST1 ; n = 74) et dans tous les échantillons de plasma positifs en ELISA (souche Eg101 ; n = 13) (Figuerola *et al.* 2007b).

ii- la présence d'anticorps neutralisants spécifiques aux virus USU (souche SAAR-1776) dans certains échantillons d'œufs positifs en ELISA choisis aléatoirement (n = 15).

iii- la présence d'anticorps neutralisants spécifiques aux VET (souche PRNT90) pour les mêmes 15 œufs que pour le virus USU.

iv- Et enfin la présence d'anticorps neutralisants spécifiques aux virus Meaban sur un échantillon de 61 œufs choisis aléatoirement.

Analyses génétiques

Ces analyses ont été effectuées par Elena Gómez-Díaz (Institut de Biologia Evolutiva CSIC UPF, Barcelona, Spain). Les tiques et les moustiques ont été testés par « reverse-transcriptase nested polymerase chain reaction » (RT-nested PCR) conçu pour détecter le génome à acide ribonucléique (ARN) (Sanchez-Seco *et al.* 2005). L'ARN a été extrait à l'aide d'un kit (Qiagen, Valencia, CA, USA) suivant le protocole du fabricant. La transcription inverse de l'ARN en ADNc et l'amplification ont été réalisées en utilisant Access RT-PCR System (Promega, Madison, WI, USA). Un fragment du gène viral NS5 (143bp) a été amplifié en utilisant des amorces dégénérées dans les conditions décrites précédemment (Sanchez-Seco *et al.* 2005). Les produits de PCR ont été soumis à une électrophorèse sur gel d'agarose 2% et révélés au bromure d'éthidium. Les produits d'amplification ont été séquencés par un sous-traitant (Macrogen, Inc.).

Les chromatogrammes obtenus par séquençage ont été vérifiés manuellement, puis assemblés à l'aide du logitiel Geneious v 5.3.6 (Biomatters Ltd). Enfin, une analyse de similitude a été effectuée avec l'outil Basic Local Alignment Search Tool (BLAST), afin de trouver la meilleure correspondance avec les séquences publiées dans GenBank (http://www.ncbi.nlm.nih.gov/).

Analyses statistiques

Les analyses ont été effectuées en utilisant le logiciel R 2.15.0 (R Development Core Team 2012). L'importance des facteurs dans nos modèles a été déterminée en utilisant une comparaison de modèle basée sur l'élimination de la dernière variable.

Nous avons utilisé des modèles linéaires généralisés (GLM) pour déterminer si la prévalence des anticorps contre les flavivirus diffère entre colonies et entre années.

Résultats et discussion

Au cours des trois années d'étude, nous avons constaté que les goélands leucophées ont été exposés à des flavivirus, mais que de fortes différences de prévalence des anticorps existaient entre les colonies (Figure 3.1.).

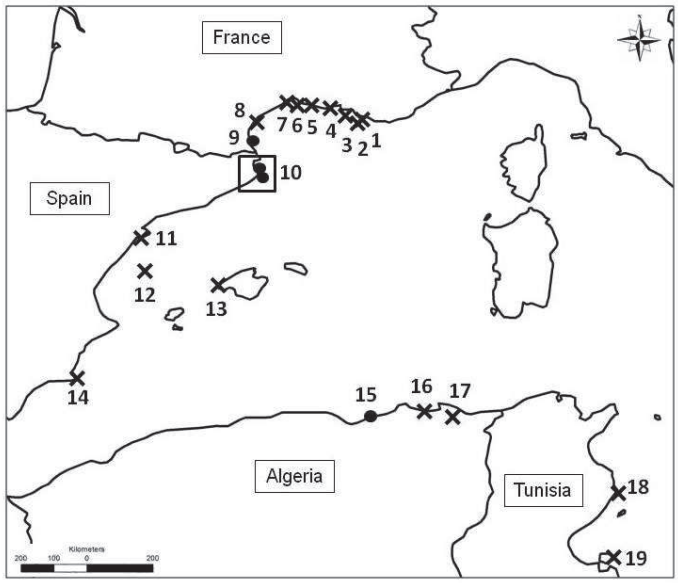


Figure 3.1. *Carte des colonies échantillonnées dans l'ouest méditerranéen*

France (n = 8), Espagne (n = 4), Algérie (n = 3) et Tunisie (n = 2)

Les croix représentent les colonies ou aucun anticorps contre les flavivirus n'est détecté ; les ronds signalent les colonies qui présentent des anticorps contre les falvivirus.

À l'exception de la colonie de Medes en Espagne (colonie numéro 10 sur la Figure 3.1.), les niveaux de prévalence observés des anticorps dirigés contre la protéine E des flavivirus étaient toujours faibles, voire nuls (Tableau 3.1.).

Tableau 3.1. *Prévalences en anticorps contre les flavivirus détectées dans les œufs de goéland leucophée.*

Pays	Colonie	Année	Taille échantillonnage	Nombre de postif
France	1-Plane	2010	20	0
	2-Riou	2009	32	0
		2010	8	0
	3-Frioul	2009	32	0
		2010	32	0
	4-Carteau	2009	32	0
		2010	32	0
		2011	30	0
	5-Besson	2009	32	0
		2010	32	0
	6-Grau-du- roi	2009	32	0
	7-Villeneuve	2009	32	0
		2010	32	0
	8-Gruissan	2009	32	0
		2010	32	0
	9-Corrège	2009	32	1
		2010	32	1
Espagne	10-Escala	2010	6	4
	10-Medes	2009	38	14
		2010	49	24
		2011	49	33
	11-Ebro delta	2009	32	0
		2010	29	0
		2011	30	0
	12-Columbretes	2010	50	0
	13-Dragonera	2010	39	0
	14-Isla Grosa	2009	32	0
		2010	32	0
		2011	30	0
Algérie	15-Jijel	2010	31	1
	16-Skikda	2010	17	0
	17-Chetaïbi	2009	32	0
		2010	32	0
Tunisie	18-Djerba	2009	32	0
	19-Sfax	2009	32	0

Sur l'ensemble des 19 sites de reproduction, seule l'île de Medes en Espagne présente un niveau de prévalence élevé des anticorps contre les flavivirus. En effet, nous avons détecté un grand nombre d'œufs positifs en ELISA sur cette île soit 37%, 49% et 67% respectivement pour 2009, 2010 et 2011. Le niveau de prévalence des anticorps augmente entre 2009 et 2011 (modèle linéaire généralisé: Pente = 0,6 ; Erreur std. = 0,2 ; Z = 2,8 ; p = 0,004) ce qui suggère une circulation locale des flavivirus au sein de la colonie.

Les anticorps dirigés contre des flavivirus ont également été détectés dans 13 des 256 échantillons de plasma de poussin échantillonnés sur l'île de Medes. Comme la plupart des poussins étaient âgés d'au moins 15 jours, les anticorps détectés sont peu susceptibles d'être d'origine maternelle car la concentration de ces derniers décroit rapidement chez les poussins de Laridés (Garnier *et al.* 2012). Bien que les tests de neutralisation n'aient pas détecté d'anticorps contre WN, ces résultats suggèrent une exposition des poussins aux flavivirus après leur naissance.

L'ensemble de ces tests ELISA reposent sur la détection d'anticorps dirigés contre la protéine E des flavivirus. En raison de sa capacité antigénique à déclencher une réponse immunitaire à l'infection, cette protéine est couramment utilisée comme antigène cible pour les sérodiagnostiques du virus WN (Setoh *et al.* 2011). Cependant, un des inconvénients important des tests utilisant la détection de cette protéine E, est le degré élevé de réactivité croisée observée avec d'autres protéines de flavivirus antigéniquement proches (Cardosa *et al.* 2002). Du fait de ces réactions croisées, il est possible de confondre deux flavivirus antigéniquement proches et il est même souvent impossible d'identifier précisément le virus infectant sans test de neutralisation (Cardosa *et al.* 2002).

Afin d'identifier le(s) flavivirus, des tests de neutralisation ont été réalisés sur les échantillons d'œufs pour lesquels la circulation d'anticorps avait été révélée par les tests ELISA. Or, contrairement à nos prédictions, 1 seul des 74 jaunes d'œuf testés (Tableau 3.2.) présentait un effet de neutralisation faible contre le virus WN sur l'île de Medes (au titre de 1:10). Ce résultat suggère, soit une exposition passée de la femelle reproductrice à ce virus, soit plus probablement à une réaction croisée avec un autre flavivirus, sachant que le titre détecté est faible. Enfin, aucun échantillon ne présente d'effets neutralisants contre les virus USU et VET. Ces résultats montrent donc que les goélands leucophées adultes de l'ouest méditerranéen n'ont pas été récemment exposés aux virus du WN, USU et VET.

Tableau 3.2. *Identification des anticorps par test de neutralisation contre les virus West Nile, Usutu, Encéphalite à tique et Meaban.*

Pays	Colonie	Année	West Nile N (positifs)	Usutu N (positifs)	Encéphalite à tique N (positifs)	Meaban N (positifs)
France	9-Corrège	2009	1(0)	1(0)	1(0)	1(1)
		2010	1(0)	1(0)	1(0)	
Espagne	10-Escala	2010	2(0)	2(0)	2(0)	2(2)
	10-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)

Afin d'identifier le flavivirus circulant sur la colonie de Medes, nous avons échantillonné des arthropodes potentiellement vecteurs. L'analyse des séquences d'ARN extraites des tiques d'oiseaux de mer, *Ornithodoros maritimus*, a montré la présence d'une séquence d'ARN ayant 95% d'homologie avecle gène NS5 décrit comme le virus Meaban. A partir de ces résultats, des tests de neutralisation contre le virus Meaban ont été effectués sur 61 échantillons d'œufs de l'île de Medes. Ce test a montré qu'un effet neutralisant contre le virus Meaban

est détecté pour l'ensemble des 61 échantillons d'œufs (Tableau 3.2.), confirmant que ce virus est bien celui détecté par le test ELISA.

Ce flavivirus n'avait jusqu'alors été détecté et isolé que sur la même espèce de tique *O. maritimus* échantillonnée sur la côte atlantique française (Chastel *et al.* 1985a). Cette tique infeste 90% des nids de goélands dans sur l'île de Medes (EstradaPena *et al.* 1996), elle est aussi présente sur plusieurs autres colonies échantillonnées, et pour lesquelles aucun anticorps spécifiques aux flavivirus n'a été détecté (par exemple, Riou, Porquerolles et Leucate; K.D. McCoy et R. Garnier, observations personnelles). Par conséquent, d'autres facteurs environnementaux spécifiques aux îles Medes pourraient favoriser l'exposition des goélands leucophées au virus Meaban. La détection ciblée des niveaux de prévalence d'anticorps détecté sur l'île de Medes ne peut être expliquée que par les mouvements limités des vecteurs infectés et/ou les goélands virémiques. De récentes études ont montré que la dispersion des *O. maritimus* peut se faire sur de longues distances via les mouvements des oiseaux marins (Gomez-Diaz *et al.* 2012). Bien que le goéland leucophée soit relativement fidèle à son site de reproduction (Oro 2008), les adultes peuvent effectuer des mouvements hivernaux le long de la côte atlantique et dans le bassin méditerranéen (Cramp *et al.* 1983). Ils peuvent également effectuer des mouvements plus limités de prospection alimentaire ou entre colonies (Oro *et al.* 1995). L'ensemble de ces mouvements peuvent permettre la dispersion des *O. maritimus* et du virus Meaban (McCoy *et al.* 2005; Gomez-Diaz *et al.* 2012). La dispersion du virus Meaban n'est donc pas à négliger sachant que sur les trois échantillons détectés positifs en anticorps contre les flavivirus : un échantillon sur la colonie de Jijel en Algérie en 2010 et les deux autres sur la colonie de Corrège en France (un en 2009 et un en 2010 Tableau 3.1.), les tests de neutralisation ont permis de détecter la présence d'anticorps neutralisant contre le virus Meaban pour l'un des échantillons de Corrège(Tableau 3.2.).

Enfin, en ce qui concerne les quatre échantillons de l'Escala détectés positifs en anticorps contre les flavivirus, deux d'entre eux (les deux seuls testés) présentaient des anticorps neutralisant contre le virus Meaban. Les œufs échantillonnés au village de l'Escala pourraient donc refléter la colonisation urbaine par les goélands originaires de l'île de Medes (à seulement 11km). Cependant, si la persistance des anticorps maternels contre le virus Meaban est longue (plus d'une année), les œufs positifs détectés dans le village de l'Escala pourrait être liée à une exposition passé des femelles sur l'île de Medes et non pas à la présence du vecteur. Dans ce cas de figure, le virus ne circulerait peut-être pas en milieu urbain. Dans les deux cas, des axes de recherche restent à explorer, comme déterminer le temps de persistance des anticorps contre le virus Meaban ou encore mettre en place des échantillonnages de tiques dans les nids de goéland de l'Escala afin de comprendre les implications de la circulation des agents pathogènes dans les habitats urbains. En effet, il est crucial de comprendre les implications de la dispersion en milieu urbain sachant que peu de données sont disponibles sur le potentiel zoonotique du virus Meaban (Chastel *et al.* 1983). L'étude de Chastel *et al.* 1983 n'a pas permis de détecter d'anticorps neutralisant contre le virus Meaban dans l'ensemble des 562 sérums collectés chez des humains vivant en Bretagne. Cependant, ces données ne sont pas suffisantes pour conclure sur la capacité zoonotique du virus Meaban.

Conclusion

Notre étude rapporte la première détection du virus Meaban dans le bassin méditerranéen. En dépit d'un effort d'échantillonnage à large échelle (19 colonies dans 4 pays), les anticorps contre ce flavivirus n'ont été détectés que sur l'île de Medes en Espagne. Nos résultats suggèrent donc que les goélands leucophées peuvent localement favoriser la circulation des flavivirus, y compris le virus Meaban dont le potentiel

zoonotique est inconnu (Chastel *et al.* 1985a). Afin de déterminer les hôtes potentiellement impliqués dans la circulation du virus Meaban et d'évaluer plus précisément sa dispersion en Méditerranée, de futures investigations pourraient cibler d'autres espèces d'oiseaux nichant à Medes et la structure génétique des populations de tique.

Les anticorps maternels, un outil de surveillance efficace

Le premier objectif de ma thèse était de mettre en exergue la quantification des anticorps maternels dans les œufs comme un outil efficace pour comprendre la circulation des agents pathogènes (**Article 2**). Pour optimiser la surveillance en population naturelle, cet outil pourrait être utilisé en complément de méthodes de détection directe ou d'enquêtes sérologiques (Pearce-Duvet *et al.* 2009). Cette approche pourrait, par exemple, être intégrée aux études portant sur des espèces sentinelles comme le suivit du canard colvert en France, espèce sentinelle des virus IA (Hars *et al.* 2011) ou de la pie bavarde, espèce sentinelle du virus WN (Hars *et al.* 2005; Jourdain *et al.* 2007; Jourdain *et al.* 2008). L'enquête sérologique présentée dans l'**Article 6** met en évidence la circulation récente chez la pie bavarde du virus WN et potentiellement d'autres flavivirus étroitement apparentés, bien qu'aucun cas humain ou équin n'ait été signalé en Camargue depuis 2004 (Calistri *et al.* 2010). Cette étude démontre l'importance fondamentale de mettre en œuvre des suivis épidémiologiques sur le long terme, afin de mieux comprendre la dynamique virale au sein des populations naturelles (Krauss *et al.* 2012; Rutten *et al.* 2012). Cependant, les suivis sur le long terme impliquent un travail et des coûts importants. La quantification d'anticorps maternels pourrait alors permettre de cibler, rapidement et à moindre coût, les populations à risque et d'y établir des analyses complémentaires pour appréhender les facteurs éco-épidémiologiques influençant la circulation d'agents pathogènes.

Dynamique des agents pathogènes en population naturelle

Le second objectif de ma thèse était d'améliorer notre compréhension de la circulation des agents pathogènes en mettant en évidence les facteurs éco-épidémiologiques pouvant influencer leurs dynamiques. Pour cela, nous avons déterminé les patrons d'exposition à l'échelle de l'ouest méditerranéen pour les virus IA (**Article 3 et 4**) et le virus Meaban (**Article 5**).

Les virus IA présentent des patrons d'exposition élevés sur l'ensemble des colonies. La densité de population ou la proximité avec les canards colverts sont potentiellement des facteurs favorisant la circulation des virus IA (**Articles 3 et 4).** Pour ce qui est de l'âge des individus de la population, aucune corrélation entre le volume moyen des œufs au sein des colonies (indicateur de l'âge et de la qualité de la mère) et les prévalences d'anticorps n'a été détectée, ce qui suggère que (*i*) l'exposition est indépendante de l'âge, ou que (*ii*) le patron d'exposition lié à l'âge est masqué par la persistance relativement longue des anticorps au cours du temps. La persistance des anticorps spécifiques aux virus IA n'a jamais été évaluée chez les Laridés. Ceci est sans doute lié à la difficulté de contrôler l'exposition des individus en population naturelle (Staszewski *et al.* 2004). Des expériences de vaccination des goélands pourraient donc être mises en place sur des populations captives. Pour déterminer le temps de persistance des anticorps, il faudrait alors, après vaccination, échantillonner de façon répétée les individus sur un temps relativement long. Les individus pourraient également être vaccinés avec différents sous-types de virus IA pour déterminer si le temps de persistance varie en fonction du sous-type.

Pour le virus Meaban, sachant que la tique *O. maritimus* est présente dans les nids de nombreuses colonies de goélands leucophées

échantillonnées (EstradaPena *et al.* 1996 ; K.D. McCoy et R. Garnier, observations personnelles), notre prédiction était donc de détecter des patrons d'exposition homogènes entre les colonies. Or, sur l'ensemble des sites de reproduction échantillonnés, seule l'île de Medes présente une forte prévalence en anticorps contre le virus Meaban. Il serait donc nécessaire de déterminer s'il existe des facteurs physico-chimiques (*e.g.* la température, l'hygrométrie, *etc.*), intrinsèques à cette colonie, qui pourraient favoriser le développement du virus sur l'île de Medes. La présence d'autres réservoirs pourraient aussi être impliqués dans la circulation localisée du virus. La tique *O. maritimus* a été détectée sur d'autres espèces aviaires tel que le cormoran huppé d'Europe (*Phalacrocorax aristotelis*) qui niche sur les côtes de la péninsule ibérique (Nuttall *et al.* 1984). Ces investigations sur les hôtes potentiellement impliqués dans la circulation du virus Meaban permettraient d'évaluer plus précisément la dispersion de ce virus en Méditerranée.

Dans les études menées sur les virus IA et Meaban, les prévalences en anticorps augmentent significativement entre les années d'échantillonnage ce qui suggère que le goéland leucophée est régulièrement exposé à ces virus. Cependant, ces résultats se basent sur deux à trois années d'échantillonnage, et restent donc à confirmer sur le long terme (Krauss *et al.* 2012; Rutten *et al.* 2012). De telles investigations permettraient notamment de déterminer, à l'aide d'outils de modélisation mathématique, le taux de transmission nécessaire pour maintenir les niveaux de prévalence observés. Pour cela, il faudrait connaitre au préalable le temps de persistance des anticorps contre les virus IA et Meaban.

Plus spécifiquement pour les virus IA, sachant qu'on détecte de fortes prévalences des anticorps dans les œufs mais que relativement peu de ces échantillons ont des anticorps spécifiques aux sous-types H13 et

H16, on peut présumer que d'autre sous-types, qui n'ont pas été identifiés dans ces travaux, circulent dans la population de goélands leucophées. Leur identification pourrait améliorer nos connaissances sur les sous-types circulant en Méditerranée. Le sous-type H10N7 a, par exemple, été détecté chez les canards colverts en Camargue (Vittecoq *et al.* 2012). L'une des souches de ce sous-type est responsable d'une épizootie dans des élevages de dindes aux Etats-Unis (Karunakaran *et al.* 1983) et d'infections bénignes chez l'Homme notamment en Egypte et en Australie (Alvarado de la Barrera *et al.* 2005). Cette souche fait partie du même cluster que celle détectée chez les canards en Camargue (Vittecoq *et al.* 2012). Or, le goéland leucophée est connu pour partager les même habitats que le canard colvert (Cramp *et al.* 1983) sur lequel il a une action de prédation (Tamisier *et al.* 1999). Le goéland peut donc être infecté par le sous-type H10N7 ou tout autre sous-type circulant chez les Anatidés, via l'eau douce contaminée par les fèces (Hinshaw *et al.* 1982; Brown *et al.* 2009), par contact direct avec un canard (Costa *et al.* 2011) ou par la consommation de canards contaminés (Brown *et al.* 2008).

La mise en évidence des facteurs éco-épidémiologiques permet d'améliorer notre compréhension de la circulation des agents pathogènes entre et au sein des populations de goélands et ainsi établir une évaluation du risque d'émergence. Ces connaissances sont d'autant plus importantes que le goéland leucophée est une espèce en contact avec l'Homme (Sol *et al.* 1995; Duhem *et al.* 2003).

Le goéland leucophée, modèle pertinent pour étudier la dynamique des agents pathogènes en population naturelle

Le goéland leucophée est très présent dans les milieux urbains (Sol *et al.* 1995; Duhem *et al.* 2003). Dans l'**Article 4**, nous avons détecté la présence d'anticorps maternels contre les flavivirus dans les œufs échantillonnés au village de l'Escala. Cependant, détecter la présence d'anticorps maternels contre ce virus ne reflète peut-être pas sa circulation en milieu urbain. En effet, suivant le temps de persistance des anticorps chez la mère, elle pourra transférer des anticorps maternels pendant une durée plus ou moins longue. Les anticorps maternels contre le virus Meaban pourraient donc provenir d'une exposition passée des femelles sur l'île de Medes, qui auraient ensuite colonisé le milieu urbain. Le virus ne circulerait alors pas dans le village de l'Escala. Pour mettre en évidence la circulation du virus Meaban en milieu urbain et l'implication du goéland leucophée dans cette circulation, il faudrait donc déterminer le temps de persistance des anticorps chez la mère contre ce virus. La mise en place un échantillonnage de tique dans les nids de goéland du village de l'Escala pourrait également permettre de confirmer la présence du vecteur. Ces tiques pourront ensuite être analysées afin de rechercher le virus Meaban. Sachant que la capacité zoonotique du virus Meaban n'est pas bien documentée (Chastel *et al.* 1983), il est d'autant plus intéressant de rechercher sa présence en milieu urbain.

Le goéland leucophée peut également être porteur de bactéries résistantes aux antibiotiques (Dolejska *et al.,* 2007; Gionechetti *et al*., 2008; Bonnedahl *et al.,* 2009). Dans la région méditerranéenne, dont la France, l'utilisation des antibiotiques en médecine humaine et vétérinaire est à un niveau relativement élevé (Goosens *et al.,* 2005). L'étude de Bonnedahl *et al.* (2009) a permis notamment de détecter un

niveau important de résistance aux antibiotiques chez des *E. coli* isolés de goéland leucophée, notamment sur la colonie de Carteau échantillonnée dans notre étude. Les goélands peuvent alors jouer un rôle non seulement de réservoir mais aussi de vecteurs disséminant des bactéries antibio-résistantes au gré de leurs déplacements (Reed *et al.* 2003; Bonnedahl *et al.* 2009). D'autres bactéries zoonotiques, telles que les salmonelles ou les campylobacters, connues pour persister sur les aliments souillés dans les décharges à ciel ouvert (Ramos *et al.* 2010) et résistantes suite à une exposition prolongée à des antibiotiques dans les élevages intensifs (Perron *et al.* 2008) pourraient également être recherchées chez le goéland leucophée. Le goéland pourrait alors être utilisé comme un bio-indicateur d'agents pathogènes et/ou de gènes de résistance (Bonnedahl *et al.,* 2009).

Enfin, le goéland leucophée est une espèce connue pour s'alimenter dans les décharges à ciel ouvert ou par les rebuts de la pêche industrielle (Sol *et al.* 1995). L'abondance et la nature des ressources alimentaires d'origine anthropique peuvent avoir des impacts variés sur les populations de goélands. En effet, ces ressources déterminent souvent le choix des sites de nidification (Scarton *et al.* 1999) et conduisent donc à une augmentation des taux de recrutement liés à un meilleur succès reproducteur (Pons *et al.* 1995; Duhem *et al.* 2008). Ce recrutement de nouveaux individus correspond à l'arrivée d'individus susceptibles ou porteurs de nouveaux pathogènes, favorisant ainsi la circulation d'agents pathogènes (Begon *et al.* 2009). Le nombre d'individus susceptibles au sein d'une population va donc déterminer la capacité du parasite à circuler. L'immunité est, par conséquent, un paramètre important à ne pas négliger.

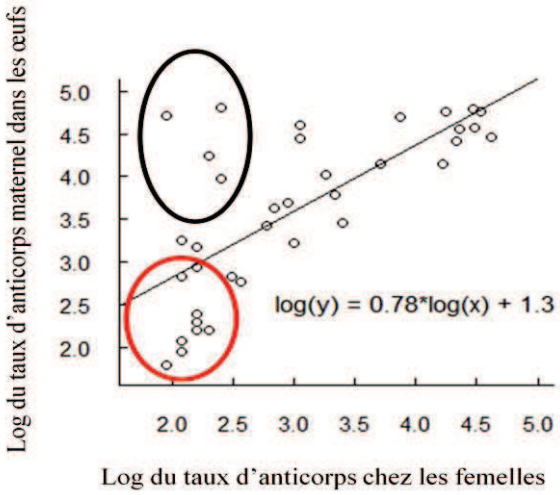
Rôle des facteurs intrinsèques de l'immunité

La variabilité du système immunitaire est une notion clé pour comprendre la circulation des agents pathogènes car elle explique en partie les différences de charge et de susceptibilité entre les individus (Frank 2002; Boulinier *et al.* 2008).

Le transfert d'anticorps maternels est probablement coûteux car il est associé à la production d'une réponse immunitaire chez la mère (Lochmiller *et al.* 2000). Son évolution pourrait donc conduire à des stratégies d'allocations différentes entre les individus (Ricklefs *et al.* 2002). C'est peut être pour cela que sur la régression linéaire présenté dans l'**Encadré 4**, certaines femelles qui ont beaucoup d'anticorps circulants en transmettent beaucoup dans les œufs (points entourés d'un cercle rouge) tandis que d'autres n'en transmettent que très peu (points entourés d'un cercle noir). La variabilité génétique entre les femelles pourrait en partie expliquer ces différences de tendance à transférer des anticorps maternels. De tels travaux en population naturelle sont difficiles car ils doivent être réalisés sur le long terme (Charmantier *et al.* 2008) pour suivre et échantillonner des individus sur plusieurs générations et qu'il faut connaitre l'apparentement entre les individus (Kruuk 2004). Cependant, aucun programme de sélection n'a encore été mené pour déterminer si la capacité de la mère à transmettre ses anticorps au jaune d'œuf a bien une base génétique.

Encadré 4. *Régression linéaire entre la quantité d'anticorps circulant dans le sang des femelles et la quantité d'anticorps maternels dans leurs œufs.*

Les femelles qui ont un fort taux d'anticorps circulant, peuvent en transférer beaucoup (cercle rouge) ou très peu (cercle noir).



Enfin, les conditions environnementales affectent clairement le développement du système immunitaire (Lochmiller *et al.* 1993). Outre les besoins quantitatifs en termes d'énergie associée à sa mise en place, à son maintien en activité et au développement de réponses spécifiques (Lochmiller *et al.* 2000), des besoins qualitatifs en termes de nutriments vont aussi rendre le système immunitaire tributaire de l'environnement dans lequel vivent les individus. Les contraintes nutritives peuvent altérer la qualité du système immunitaire mais aussi modifier la quantité d'anticorps maternels transmis à la descendance (Lochmiller *et al.* 1993). Comparons une femelle qui a accès à une grande quantité de nourriture à une autre femelle vivant dans un environnement qui ne lui fournit pas les apports nutritionnels suffisants. Contrairement à la

femelle dite de mauvaise qualité, celle qui peut avoir un accès à une grande quantité de nourriture devraient avoir l'énergie nécessaire à la synthèse d'une plus grande quantité d'immunoglobuline et en transmettra plus à sa descendance (Grindstaff *et al.* 2003; Grindstaff *et al.* 2005). La qualité des femelles est souvent estimée par le niveau d'asymétrie fluctuante c'est-à-dire l'incapacité de l'individu à se développer normalement du fait d'un accès plus limité aux ressources alimentaires (Moller 2006). Dans l'**Article 6**, nous avons mis en évidence que la quantité d'anticorps maternels détectés dans les œufs de goéland leucophée dépend de la qualité de la mère, estimée par son asymétrie fluctuante.

Cette thèse apporte des éléments pour comprendre la circulation de certains virus en populations naturelles et le rôle potentiel du goéland leucophée dans la dynamique virale. Les approches éco-épidémiologiques abordées dans les différents chapitres ne sont pas limitées aux virus IA ou au virus Meaban mais peuvent être transposables à d'autres agents pathogènes. Les résultats obtenus dans cette thèse permettent d'envisager d'autres axes de recherche nécessaires pour évaluer plus précisément la dispersion de ces virus en Méditerranée. Ces travaux futurs pourront alors être optimisés par différentes disciplines : écologie, épidémiologie, immunologie ou encore ornithologie. En effet, seule une approche intégrée des problèmes de santé permettra une parfaite compréhension de la dynamique des agents pathogènes.

Addo, K., K. Owusu-Darko, D. Yeboah-Manu, P. Caulley, M. Minamikawa, F. Bonsu, C. Leinhardt, P. Akpedonu and D. Ofori-Adjei (2007). "Mycobacterial species causing pulmonary tuberculosis at the korle bu teaching hospital, accra, ghana." Ghana medical journal **41**(2): 52-57.

Adler, F. R., J. M. C. Pearce-Duvet and M. D. Dearing (2008). "How host population dynamics translate into time-lagged prevalence: An investigation of Sin Nombre virus in deer mice." Bulletin of Mathematical Biology **70**(1): 236-252.

Al-Natour, M. Q., L. A. Ward, Y. M. Saif, B. Stewart-Brown and L. D. Keck (2004). "Effect of different levels of maternally derived antibodies on protection against infectious bursal disease virus." Avian Diseases **48**(1): 177-182.

Alexander, D. J. (2000). "A review of avian influenza in different bird species." Veterinary Microbiology **74**(1-2): 3-13.

Alizon, S., A. Hurford, N. Mideo and M. Van Baalen (2009). "Virulence evolution and the trade-off hypothesis: history, current state of affairs and the future." Journal Of Evolutionary Biology **22**(2): 245-259.

Altizer, S., R. Bartel and B. A. Han (2011). "Animal Migration and Infectious Disease Risk." Science **331**(6015): 296-302.

Altizer, S., A. Dobson, P. Hosseini, P. Hudson, M. Pascual and P. Rohani (2006). "Seasonality and the dynamics of infectious diseases." Ecology Letters **9**(4): 467-484.

Alvarado de la Barrera, C. and G. Reyes-Teran (2005). "Influenza: Forecast for a pandemic." Archives of Medical Research **36**(6): 628-636.

Amraoui, F., G. Krida, A. Bouattour, A. Rhim, J. Daaboub, Z. Harrat, S.-C. Boubidi, M. Tijane, M. Sarih and A.-B. Failloux (2012). "*Culex pipiens*, an Experimental Efficient Vector of West Nile and Rift Valley Fever Viruses in the Maghreb Region." PLoS One **7**(5).

Amundsen, T., S. H. Lorentsen and T. Tveraa (1996). "Effects of egg size and parental quality on early nestling growth: An

experiment with the Antarctic petrel." Journal of Animal Ecology **65**(5): 545-555.
Anderson, R. W. (1995). "On the maternal transmission of immunity: a 'molecular attention' hypothesis." Biosystems **34**(1-3): 87-105.
Arnold, J. M., S. A. Oswald, C. C. Voigt, R. Palme, A. Braasch, C. Bauch and P. H. Becker (2008). "Taking the stress out of blood collection: comparison of field blood-sampling techniques for analysis of baseline corticosterone." Journal of Avian Biology **39**(5): 588-592.
Aron, J. L. and J. A. Patz (2001). Ecosystem Change and Public Health, Johns Hopkins University Press, Baltimore, Usa.
Artois, M., R. Delahay, V. Guberti and C. Cheeseman (2001). "Control of infectious diseases of wildlife in Europe." Veterinary Journal **162**(2): 141-152.
Ayebazibwe, C., F. N. Mwiine, K. Tjornehoj, S. N. Balinda, V. B. Muwanika, A. R. A. Okurut, G. J. Belsham, P. Normann, H. R. Siegismund and S. Alexandersen (2010). "The role of African buffalos (*Syncerus caffer*) in the maintenance of foot-and-mouth disease in Uganda." Bmc Veterinary Research **6**.
Bagamian, K. H., R. J. Douglass, A. Alvarado, A. J. Kuenzi, B. R. Amman, L. A. Waller and J. N. Mills (2012). "Population Density and Seasonality Effects on Sin Nombre Virus Transmission in North American Deermice (Peromyscus maniculatus) in Outdoor Enclosures." PLoS One **7**(6).
Beaubrun, P. C. (1993). "Status of Yellow-legged Gull (*Larus cachinnans*) in Morocco and in the western Mediterranean." In Status and Conservation of seabirds (Aguilar, J.S., Monbailliu, X. & Paterson, A.M. eds.). Proceedings of the 2nd Medmaravis, SEO, Madrid.
Becker, W. B. (1966). "The isolation and classification of Tern virus: Influenza Virus A/Tern/South Africa/1961." Journal of Hygiene-Cambridge **64**(3): 309-&.
Begon, M., S. Telfer, M. J. Smith, S. Burthe, S. Paterson and X. Lambin (2009). "Seasonal host dynamics drive the timing of recurrent epidemics in a wildlife population." Proceedings Of

The Royal Society B-Biological Sciences **276**(1662): 1603-1610.

Benjamini, Y. and Y. Hochberg (1995). "Controlling the false discovery rate a practical and powerful approach to multiple testing." Journal of the Royal Statistical Society Series B-Methodological **57**(1): 289-300.

Bin Muzaffar, S., R. C. Ydenberg and I. L. Jones (2006). "Avian influenza: An ecological and evolutionary perspective for waterbird scientists." Waterbirds **29**(3): 243-257.

Bofill, D., C. Domingo, N. Cardenosa, J. Zaragoza, F. de Ory, S. Minguell, M. P. Sanchez-Seco, A. Dominguez and A. Tenorio (2006). "Human West Nile virus infection, Catalonia, Spain." Emerging Infectious Diseases **12**(7): 1163-1164.

Bonnedahl, J., M. Drobni, M. Gauthier-Clerc, J. Hernandez, S. Granholm, Y. Kayser, A. Melhus, G. Kahlmeter, J. Waldenstrom, A. Johansson and B. Olsen (2009). "Dissemination of Escherichia coli with CTX-M Type ESBL between Humans and Yellow-Legged Gulls in the South of France." PLoS One **4**(6).

Boulinier, T., G. Sorci, J. Y. Monnat and E. Danchin (1997). "Parent-offspring regression suggests heritable susceptibility to ectoparasites in a natural population of kittiwake Rissa tridactyla." Journal Of Evolutionary Biology **10**(1): 77-85.

Boulinier, T. and V. Staszewski (2008). "Maternal transfer of antibodies: raising immuno-ecology issues." Trends In Ecology & Evolution **23**(5): 282-288.

Boyce, J. M. (2007). "Environmental contamination makes an important contribution to hospital infection." Journal of Hospital Infection **65**: 50-54.

Bradley, C. A. and S. Altizer (2007). "Urbanization and the ecology of wildlife diseases." Trends In Ecology & Evolution **22**(2): 95-102.

Broman, T., H. Palmgren, S. Bergstrom, M. Sellin, J. Waldenstrom, M. L. Danielsson-Tham and B. Olsen (2002). "*Campylobacter jejuni* in black-headed gulls (*Larus ridibundus*): Prevalence,

genotypes, and influence on *C. jejuni* epidemiology." Journal Of Clinical Microbiology **40**(12): 4594-4602.

Brown, J. D., G. Goekjian, R. Poulson, S. Valeika and D. E. Stallknecht (2009). "Avian influenza virus in water: Infectivity is dependent on pH, salinity and temperature." Veterinary Microbiology **136**(1-2): 20-26.

Brown, J. D., D. E. Stallknecht and D. E. Swayne (2008). "Experimental infections of herring gulls (*Larus argentatus*) with H5N1 highly pathogenic avian influenza viruses by intranasal inoculation of virus and ingestion of virus-infected chicken meat." Avian Pathology **37**(4): 393-397.

Buckee, C., L. Danon and S. Gupta (2007). "Host community structure and the maintenance of pathogen diversity." Proceedings Of The Royal Society B-Biological Sciences **274**(1619): 1715-1721.

Butler, D. (2006). "Disease surveillance needs a revolution." Nature **440**(7080): 6-7.

Calistri, P., A. Giovannini, Z. Hubalek, A. Ionescu, F. Monaco, G. Savini and R. Lelli (2010). "Epidemiology of West Nile in Europe and in the Mediterranean basin." Open Virol J **4**: 29-37.

Calzolari, M., P. Bonilauri, R. Bellini, A. Albieri, F. Defilippo, G. Maioli, G. Galletti, A. Gelati, I. Barbieri, M. Tamba, D. Lelli, E. Carra, P. Cordioli, P. Angelini and M. Dottori (2010). "Evidence of simultaneous circulation of West Nile and Usutu viruses in mosquitoes sampled in Emilia-Romagna region (Italy) in 2009." PLoS One **5**(12): e14324.

Capobianchi, M. R., V. Sambri, C. Castilletti, A. M. Pierro, G. Rossini, P. Gaibani, F. Cavrini, M. Selleri, S. Meschi, D. Lapa, A. Di Caro, P. Grossi, C. De Cillia, S. Venettoni, M. P. Landini, G. Ippolito, A. N. Costa and N. Italian Transplant (2010). "Retrospective screening of solid organ donors in Italy, 2009, reveals unpredicted circulation of West Nile virus." Eurosurveillance **15**(34).

Cardosa, M. J., S. M. Wang, M. S. H. Sum and P. H. Tio (2002). "Antibodies against prM protein distinguish between previous infection with dengue and Japanese encephalitis viruses." Bmc Microbiology **2**.

Caron, A., M. de Garine-Wichatitsky and S. Morand (2011). "Parasite Community Ecology and Epidemiological Interactions at the Wildlife/Domestic/Human Interface: Can We Anticipate Emerging Infectious Diseases in Their Hotspots?" Ecohealth **7**: S24-S24.

Cavrini, F., P. Gaibani, G. Longo, A. M. Pierro, G. Rossini, P. Bonilauri, G. E. Gerundi, F. Di Benedetto, A. Pasetto, M. Girardis, M. Dottori, M. P. Landini and V. Sambri (2009). "Usutu virus infection in a patient who underwent orthotropic liver transplantation, Italy, August-September 2009." European communicable disease bulletin **14**(50).

Charmantier, A., R. H. McCleery, L. R. Cole, C. Perrins, L. E. B. Kruuk and B. C. Sheldon (2008). "Adaptive phenotypic plasticity in response to climate change in a wild bird population." Science **320**(5877): 800-803.

Chastel, C., C. Guiguen, G. Lelay, J. Y. Monnat, E. Hardy, G. Kerdraon and J. C. Beaucournu (1985b). "Arbovirus serosurvey among seabirds and landbirds in Brittany." Bulletin De La Societe De Pathologie Exotique **78**(5): 594-605.

Chastel, C., C. Guiguen, G. Lelay, J. Y. Monnat, M. C. Quillien and J. C. Beaucournu (1983). "Are the Arboviruses from seabirds colonies in Brittany able to infect man." Revue d'épidemiologie et de sante publique **31**(4): 445-457.

Chastel, C., A. J. Main, C. Guiguen, G. Lelay, M. C. Quillien, J. Y. Monnat and J. C. Beaucournu (1985a). "The isolation on Meaban virus, a new *Flavivirus* from the seabird tick *Ornithodoros* (*Alectorobius*) *maritimus* in France." Archives of Virology **83**(3-4): 129-140.

Chen, H., G. J. D. Smith, K. S. Li, J. Wang, X. H. Fan, J. M. Rayner, D. Vijaykrishna, J. X. Zhang, L. J. Zhang, C. T. Guo, C. L. Cheung, K. M. Xu, L. Duan, K. Huang, K. Qin, Y. H. C. Leung, W. L. Wu, H. R. Lu, Y. Chen, N. S. Xia, T. S. P. Naipospos, K. Y. Yuen, S. S. Hassan, S. Bahri, T. D. Nguyen, R. G. Webster, J. S. M. Peiris and Y. Guan (2006a). "Establishment of multiple sublineages of H5N1 influenza virus in Asia: Implications for

pandemic control." Proceedings Of The National Academy Of Sciences Of The United States Of America **103**(8): 2845-2850.

Chen, H., G. J. D. Smith, S. Y. Zhang, K. Qin, J. Wang, K. S. Li, R. G. Webster, J. S. M. Peiris and Y. Guan (2005). "H5N1 virus outbreak in migratory waterfowl." Nature **436**(7048): 191-192.

Chew, B. P. and J. S. Park (2004). "Carotenoid action on the immune response." Journal of Nutrition **134**(1): 257S-261S.

Choisy, M. (2010). Modélisation mathématique en épidémiologie. Ecologie de la santé et biodiversité. D. B. Supérieur.

Chokri, M. A., N. Sadoul, K. Medhioub and A. Béchet (2008). "Analyse comparative de la richesse avifaunistique du salin de Sfax dans le contexte tunisien et méditerranéen." Rev Ecol Terre Vie **63**: 4.

Christians, J. K. (2002). "Avian egg size: variation within species and inflexibility within individuals." Biological Reviews **77**(1): 1-26.

Clifford, C. M., C. E. Yunkeir, L. A. Thomas, E. R. Easton and D. Corwin (1971). "Isolation of a group B arbovirus from Ixodes uriae collected in Three Arch Rocks National Wildlife Refuge, Oregon." The American Journal of Tropical Medicine and Hygiene **20**: 461--468.

Colizza, V., A. Barrat, M. Barthelemy, A. J. Valleron and A. Vespignani (2007). "Modeling the worldwide spread of pandemic influenza: Baseline case and containment interventions." Plos Medicine **4**(1): 95-110.

Costa, T. P., J. D. Brown, E. W. Howerth and D. E. Stallknecht (2011). "Variation in viral shedding patterns between different wild bird species infected experimentally with low-pathogenicity avian influenza viruses that originated from wild birds." Avian Pathology **40**(2): 119-124.

Cramp, S., K. E. L. Simmons, D. J. Brooks, N. J. Collar, E. Dunn, R. Gillmor, P. A. D. Hollom, R. Hudson, E. M. Nicholson, M. A. Ogilvie, P. J. S. Olney, C. S. Roselaar, K. H. Voous, D. I. M. Wallace, J. Wattel and M. G. Wilson (1983). Handbook of the Birds of Europe, the Middle East and North Africa: The Birds of the Western Palearctic, . Oxford.

Daszak, P., A. A. Cunningham and A. D. Hyatt (2000). "Wildlife ecology - Emerging infectious diseases of wildlife - Threats to biodiversity and human health." Science **287**(5452): 443-449.

de Roode, J. C., A. J. Yates and S. Altizer (2008). "Virulence-transmission trade-offs and population divergence in virulence in a naturally occuring butterfly parasite." Proceedings Of The National Academy Of Sciences Of The United States Of America **105**(21): 7489-7494.

De Wit, E. and R. A. M. Fouchier (2008). "Emerging influenza." Journal Of Clinical Virology **41**(1): 1-6.

Diamond, J. (2002). "Evolution, consequences and future of plant and animal domestication." Nature **418**(6898): 700-707.

Dobler, G. (2010). "Zoonotic tick-borne flaviviruses." Veterinary Microbiology **140**(3-4): 221-228.

Duhem, C. (2004). Goélands surabondants et ressources alimentaires anthropiques : Cas des colonies insulaires de Goélands leucophées du littoral provençal. Docteur de l'Université Paul Cezanne, Université Paul Cezanne (Aix Marseille III).

Duhem, C., P. Roche, E. Vidal and T. Tatoni (2007). "Distribution of breeding sites and food constrains size and density of yellow-legged gull colonies." Ecoscience **14**(4): 535-543.

Duhem, C., P. Roche, E. Vidal and T. Tatoni (2008). "Effects of anthropogenic food resources on yellow-legged gull colony size on Mediterranean islands." Population Ecology **50**(1): 91-100.

Duhem, C., E. Vidal, J. Legrand and T. Tatoni (2003). "Opportunistic feeding responses of the Yellow-legged Gull *Larus michahellis* to accessibility of refuse dumps." Bird Study **50**: 61-67.

Duhem, C., E. Vidal, P. Roche and J. Legrand (2005). "How is the diet of yellow-legged gull chicks influenced by parents' accessibility to landfills?" Waterbirds **28**(1): 46-52.

Ellstrom, P., N. Latorre-Margalef, P. Griekspoor, J. Waldenstrom, J. Olofsson, J. Wahlgren and B. Olsen (2008). "Sampling for low-pathogenic avian influenza A virus in wild Mallard ducks: Oropharyngeal versus cloacal swabbing." Vaccine **26**(35): 4414-4416.

EpiSouth (2010). "West Nile virus circulation in the EpiSouth countries and neighbouring areas (Hungary and Russia)." www.episouth.org/download.php?&id=12182.

EstradaPena, A., M. Bosch and V. Pedrocchi (1996). "First record of *Ornithodoros (Alectorobius) maritimus* Vermeil & Marguet (Acari: Argasidae) in Spain." Acarologia **37**(1): 19-21.

Ferguson, N. M., D. A. T. Cummings, S. Cauchemez, C. Fraser, S. Riley, A. Meeyai, S. Iamsirithaworn and D. S. Burke (2005). "Strategies for containing an emerging influenza pandemic in Southeast Asia." Nature **437**: 209-214.

Figuerola, J., M. A. Jimenez-Clavero, G. Rojo, C. Gomez-Tejedor and R. Soriguer (2007b). "Prevalence of West Nile virus neutralizing antibodies in colonial aquatic birds in southern Spain." Avian Pathology **36**(3): 209-212.

Figuerola, J., R. Soriguer, G. Rojo, C. G. Tejedor and M. A. Jimenez-Clavero (2007a). "Seroconversion in wild birds and local circulation of West Nile virus, Spain." Emerging Infectious Diseases **13**(12): 1915-1917.

Fishpool, L. D. C. and M. I. Evans (2001). Important Bird Areas in Africa and Associated Islands: Priority for Conservation Newbury and Cambridge, United Kingdom: Pisces Publications and Birdlife international (Birdlife Conservation Series No. 11).

Fouchier, R. A. M., V. Munster, A. Wallensten, T. M. Bestebroer, S. Herfst, D. Smith, G. F. Rimmelzwaan, B. Olsen and A. Osterhaus (2005). "Characterization of a novel influenza a virus hemagglutinin subtype (H16) obtained from black-headed gulls." Journal Of Virology **79**(5): 2814-2822.

Fouchier, R. A. M., V. J. Munster, J. Keawcharoen, A. D. M. E. Osterhaus and T. Kuiken (2007). "Virology of avian influenza in relation to wild birds." Journal Of Wildlife Diseases **43**(3): S7-S14.

Frank, S. A. (2002). Immunology and Evolution of Infectious Disease. New Jersey.

Frost, S. D. W. (1999). The immune system as an inducible defense. The ecology and evolution of inducible defenses (eds. Tollrian

R., Harvell C.D.), Princeton, NJ, Princeton University Press**:** 104-126.

Gandon, S. and Y. Michalakis (2000). "Evolution of parasite virulence against qualitative or quantitative host resistance." Proceedings Of The Royal Society Of London Series B-Biological Sciences **267**(1447): 985-990.

Garcia, L. V. (2003). "Controlling the false discovery rate in ecological research." Trends In Ecology & Evolution **18**(11): 553-554.

Garnier, R., B. Boulinier and S. Gandon (in prep.). "Evolution of the persistence of immune protection."

Garnier, R., R. Ramos, V. Staszewski, T. Militao, E. Lobato, J. Gonzalez-Solis and T. Boulinier (2012). "Maternal antibody persistence: a neglected life-history trait with implications from albatross conservation to comparative immunology." Proceedings Of The Royal Society B-Biological Sciences **279**(1735): 2033-2041.

Gasparini, J., K. D. McCoy, C. Haussy, T. Tveraa and T. Boulinier (2001). "Induced maternal response to the Lyme disease spirochaete *Borrelia burgdorferi* sensu lato in a colonial seabird, the kittiwake *Rissa tridactyla*." Proceedings Of The Royal Society Of London Series B-Biological Sciences **268**(1467): 647-650.

Gasparini, J., K. D. McCoy, T. Tveraa and T. Boulinier (2002). "Related concentrations of specific immunoglobulins against the Lyme disease agent Borrelia burgdorferi sensu lato in eggs, young and adults of the kittiwake (Rissa tridactyla)." Ecology Letters **5**(4): 519-524.

Gasparini, J., A. Roulin, V. A. Gill, S. A. Hatch and T. Boulinier (2006). "In kittiwakes food availability partially explains the seasonal decline in humoral immunocompetence." Functional Ecology **20**(3): 457-463.

Gaunt, M. W., A. A. Sall, X. de Lamballerie, A. K. I. Falconar, T. I. Dzhivanian and E. A. Gould (2001). "Phylogenetic relationships of flaviviruses correlate with their epidemiology, disease association and biogeography." Journal of General Virology **82**: 1867-1876.

Gauthier-Clerc, M., C. Lebarbenchon and F. Thomas (2007). "Recent expansion of highly pathogenic avian influenza H5N1: a critical review." Ibis **149**(2): 202-214.

Germundsson, A., K. I. Madslien, M. J. Hjortaas, K. Handeland and C. M. Jonassen (2010). "Prevalence and subtypes of Influenza A Viruses in Wild Waterfowl in Norway 2006-2007." Acta Veterinaria Scandinavica **52**.

Gomez-Diaz, E., J. A. Morris-Pocock, J. Gonzalez-Solis and K. D. McCoy (2012). "Trans-oceanic host dispersal explains high seabird tick diversity on Cape Verde islands." Biology letters.

GonzalezSolis, J., D. Oro, V. Pedrocchi, L. Jover and X. Ruiz (1997). "Bias associated with diet samples in Audouin's gulls." Condor **99**(3): 773-779.

Gortazar, C., E. Ferroglio, U. Hoefle, K. Froelich and J. Vicente (2007). "Diseases shared between wildlife and livestock: a European perspective." European Journal of Wildlife Research **53**(4): 241-256.

Gould, E. A. and S. Higgs (2009). "Impact of climate change and other factors on emerging arbovirus diseases." Transactions of the Royal Society of Tropical Medicine and Hygiene **103**(2): 109-121.

Graves, I. L. (1992). "Influenza-viruses in birds of the Atlantic flyway." Avian Diseases **36**(1): 1-10.

Griffiths, R., M. C. Double, K. Orr and R. J. G. Dawson (1998). "A DNA test to sex most birds." Molecular Ecology **7**(8): 1071-1075.

Grindstaff, J. L., E. D. Brodie and E. D. Ketterson (2003). "Immune function across generations: integrating mechanism and evolutionary process in maternal antibody transmission." Proceedings Of The Royal Society Of London Series B-Biological Sciences **270**(1531): 2309-2319.

Grindstaff, J. L., G. E. Demas and E. D. Ketterson (2005). "Diet quality affects egg size and number but does not reduce maternal antibody transmission in Japanese quail *Coturnix japonica*." Journal of Animal Ecology **74**(6): 1051-1058.

Grindstaff, J. L., D. Hasselquist, J. A. Nilsson, M. Sandell, H. G. Smith and M. Stjernman (2006). "Transgenerational priming of immunity: maternal exposure to a bacterial antigen enhances offspring humoral immunity." Proceedings Of The Royal Society B-Biological Sciences **273**(1600): 2551-2557.

Gubler, D. J. (1996). "The global resurgence of arboviral diseases." Transactions of the Royal Society of Tropical Medicine and Hygiene **90**(5): 449-451.

Guégan, J. F. and M. Choisy (2009). Introduction à l'épidémiologie intégrative des maladies infectieuses et parasitaires, De Boack Université.

Guernier, V., M. E. Hochberg and J. F. O. Guegan (2004). "Ecology drives the worldwide distribution of human diseases." Plos Biology **2**(6): 740-746.

Hammouda, A., J. Pearce-Duvet, M. Chokri, A. Arnal, M. Gauthier-Clerc, T. Boulinier and S. Selmi (2011). "Prevalence of Influenza A antibodies in yellow-legged gull (*Larus michahellis*) eggs and adults in southern Tunisia." Vector-Borne and Zoonotic Diseases

Hanson, B. A., M. P. Luttrell, V. H. Goekjian, L. Niles, D. E. Swayne, D. A. Senne and D. E. Stallknecht (2008). "Is the occurrence of avian influenza virus in Charadriiformes species and location dependent?" Journal Of Wildlife Diseases **44**(2): 351-361.

Hars, J., P. Augé, J. Pradel, M. Mortamais, D. Chavernac, G. Balença, S. Zientara, I. Shuffenecker and H. G. Zeller (2005). "Surveillance of West Nile virus in the avifauna of Southern France." In: 8th European Association of Avian Veterinarians Conference **Arles,France**.

Hars, J., A. L. Brochet, E. Niqueux and V. Jestin (2011). "FRIA 08-010: Mieux connaître la circulation des virus IA FP et HP chez les oiseaux sauvages et leur statut immunitaire en France." ONCFS, ANSES: 75 p.

Harvell, C. D., K. Kim, J. M. Burkholder, R. R. Colwell, P. R. Epstein, D. J. Grimes, E. E. Hofmann, E. K. Lipp, A. Osterhaus, R. M. Overstreet, J. W. Porter, G. W. Smith and G. R. Vasta (1999). "Review: Marine ecology - Emerging marine diseases - Climate

links and anthropogenic factors." Science **285**(5433): 1505-1510.

Haydon, D. T., S. Cleaveland, L. H. Taylor and M. K. Laurenson (2002). "Identifying reservoirs of infection: A conceptual and practical challenge." Emerging Infectious Diseases **8**(12): 1468-1473.

Heeb, P., I. Werner, M. Kolliker and H. Richner (1998). "Benefits of induced host responses against an ectoparasite." Proceedings Of The Royal Society B-Biological Sciences **265**(1390): 51-56.

Hilbert, F., F. J. M. Smulders, R. Chopra-Dewasthaly and P. Paulsen (2012). "Salmonella in the wildlife-human interface." Food Research International **45**(2): 603-608.

Hinshaw, V. S., G. M. Air, A. J. Gibbs, L. Graves, B. Prescott and D. Karunakaran (1982). "Antigenic and Genetic Characterization of a Novel Hemagglutinin Subtype of Influenza A Viruses from Gulls." Journal Of Virology **42**(3): 865-872.

Holmes, E. C., C. H. Woelk, R. Kassis and H. Bourhy (2002). "Genetic constraints and the adaptive evolution of rabies virus in nature." Virology **292**(2): 247-257.

Holt, R. and T. Boulinier (2005). Ecosystems and parasitism: the spatial dimension. Parasitism and ecosystems (eds. Thomas F, Renaud F & Guégan J-F). UK, Oxford University Press Oxford**:** 68-84.

Hoye, B. J., V. J. Munster, H. Nishiura, M. Klaassen and R. A. M. Fouchier (2010). "Surveillance of Wild Birds for Avian Influenza Virus." Emerging Infectious Diseases **16**(12): 1827-1834.

Hoyt, D. F. (1979). "Practical methods of estimating volume and fresh weight of bird eggs." Auk **96**(1): 73-77.

Hudson, P., A. Rizzoli, B. T. Grenfell, B. T. Heerterbeek and A. P. Dobson (2002). Spatial aspects of disease dynamics. The Ecology of Wildlife Diseases, Owford University Press**:** 102-118.

Ito, T., H. Goto, E. Yamamoto, H. Tanaka, M. Takeuchi, M. Kuwayama, Y. Kawaoka and K. Otsuki (2001). "Generation of a highly pathogenic avian influenza A virus from an avirulent

field isolate by passaging in chickens." Journal Of Virology **75**(9): 4439-4443.

Jones, K. E., N. G. Patel, M. A. Levy, A. Storeygard, D. Balk, J. L. Gittleman and P. Daszak (2008). "Global trends in emerging infectious diseases." Nature **451**(7181): 990-U994.

Jourdain, E., M. Gauthier-Clerc, P. Sabatier, O. Grege, T. Greenland, A. Leblond, M. Lafaye and H. G. Zeller (2008). "Magpies as hosts for West Nile virus, southern France." Emerging Infectious Diseases **14**(1): 158-160.

Jourdain, E., Y. Toussaint, A. Leblond, D. J. Bicout, P. Sabatier and M. Gauthier-Clerc (2007). "Bird species potentially involved in introduction, amplification, and spread of West Nile virus in a Mediterranean wetland, the Camargue (Southern france)." Vector-Borne and Zoonotic Diseases **7**(1): 15-33.

Karabatsos, N. (1985). International Catalogue of Arboviruses. Washington DC, U.S.A.

Kariyawasam, S., B. N. Wilkie and C. L. Gyles (2004). "Resistance of broiler chickens to Escherichia coli respiratory tract infection induced by passively transferred egg-yolk antibodies." Veterinary Microbiology **98**(3-4): 273-284.

Karunakaran, D., V. Hinshaw, P. Poss, J. Newman and D. Halvorson (1983). "Influenza-A outbreaks in Minnesota turkeys due to subtype-H10N7 and possible transmission by waterfowl." Avian Diseases **27**(2): 357-366.

Keeling, M. J. and P. Rohani (2002). "Estimating spatial coupling in epidemiological systems: a mechanistic approach." Ecology Letters **5**(1): 20-29.

Keeling, M. J. and P. Rohani (2008). Modeling Infectious Diseases in humans and animals, Press, Princeton University.

Kneitel, J. M. and J. M. Chase (2004). "Trade-offs in community ecology: linking spatial scales and species coexistence." Ecology Letters **7**(1): 69-80.

Knight-Jones, T. J. D., R. Hauser, D. Matthes and K. D. C. Staerk (2010). "Evaluation of effectiveness and efficiency of wild bird surveillance for avian influenza." Veterinary Research **41**(4).

Komar, N., S. Langevin, S. Hinten, N. Nemeth, E. Edwards, D. Hettler, B. Davis, R. Bowen and M. Bunning (2003). "Experimental infection of north American birds with the New York 1999 strain of West Nile virus." Emerging Infectious Diseases **9**(3): 311-322.

Krauss, S., D. Walker, S. P. Pryor, L. Niles, C. H. Li, V. S. Hinshaw and R. G. Webster (2004). "Influenza A viruses of migrating wild aquatic birds in North America." Vector-Borne and Zoonotic Diseases **4**(3): 177-189.

Krauss, S. and R. G. Webster (2010). "Avian Influenza Virus Surveillance and Wild Birds: Past and Present." Avian Diseases **54**(1): 394-398.

Krauss, S. and R. G. Webster (2012). "Predicting the next influenza virus." Science (New York, N.Y.) **337**(6095): 644-644.

Kruuk, L. E. B. (2004). "Estimating genetic parameters in natural populations using the 'animal model'." Philosophical Transactions Of The Royal Society Of London Series B-Biological Sciences **359**(1446): 873-890.

Kuiken, T., F. A. Leighton, G. Wobeser, K. L. Danesik, J. Riva and R. A. Heckert (1998). "An epidemic of Newcastle disease in double-crested cormorants from Saskatchewan." Journal Of Wildlife Diseases **34**(3): 457-471.

Kulasekera, V. L., L. Kramer, R. S. Nasci, F. Mostashari, B. Cherry, S. C. Trock, C. Glaser and J. R. Miller (2001). "West Nile Virus infection in mosquitoes, birds, horses, and humans, Staten Island, New York, 2000." Emerging Infectious Diseases **7**(4): 722-725.

Kuno, G. (2001). "Persistence of arboviruses and antiviral antibodies in vertebrate hosts: its occurrence and impacts." Reviews in Medical Virology **11**(3): 165-190.

Lebarbenchon, C., S. P. Brown, R. Poulin, M. Gauthier-Clerc and F. Thomas (2008). "Evolution of pathogens in a man-made world." Molecular Ecology **17**(1): 475-484.

Lebarbenchon, C., C.-M. Chang, V. Grandhomme, M. Dietrich, Y. Kayser, E. Elguero, F. Renaud, F. Thomas, S. van der Werf and M. Gauthier-Clerc (2010). "Avian Influenza Circulation in the

Camargue (South of France) During the 2006-07 Season." Avian Diseases **54**(1): 446-449.

Lebarbenchon, C., C.-M. Chang, S. van der Werf, J.-T. Aubin, Y. Kayser, M. Ballesteros, F. Renaud, F. Thomas and M. Gauthier-Clerc (2007). "Influenza A virus in birds during spring migration in the Camargue, France." Journal Of Wildlife Diseases **43**(4): 789-793.

Lee, D.-H., H.-J. Lee, Y.-J. Lee, H.-M. Kang, O.-M. Jeong, M.-C. Kim, J.-S. Kwon, J.-H. Kwon, C.-B. Kim, J.-B. Lee, S.-Y. Park, I.-S. Choi and C.-S. Song (2010). "DNA Barcoding Techniques for Avian Influenza Virus Surveillance in Migratory Bird Habitats." Journal Of Wildlife Diseases **46**(2): 649-654.

Lee, K. A. (2006). "Linking immune defenses and life history at the levels of the individual and the species." Integrative and Comparative Biology **46**(6): 1000-1015.

Leroy, E. M., A. Epelboin, V. Mondonge, X. Pourrut, J.-P. Gonzalez, J.-J. Muyembe-Tamfum and P. Formenty (2009). "Human Ebola Outbreak Resulting from Direct Exposure to Fruit Bats in Luebo, Democratic Republic of Congo, 2007." Vector-Borne and Zoonotic Diseases **9**(6): 723-728.

Li, K. S., Y. Guan, J. Wang, G. J. D. Smith, K. M. Xu, L. Duan, A. P. Rahardjo, P. Puthavathana, C. Buranathai, T. D. Nguyen, A. T. S. Estoepangestie, A. Chaisingh, P. Auewarakul, H. T. Long, N. T. H. Hanh, R. J. Webby, L. L. M. Poon, H. Chen, K. F. Shortridge, K. Y. Yuen, R. G. Webster and J. S. M. Peiris (2004). "Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia." Nature **430**(6996): 209-213.

Lisnizer, N., P. Garcia-Borboroglu and P. Yorio (2011). "Spatial and temporal variation in population trends of Kelp Gulls in northern Patagonia, Argentina." Emu **111**(3): 259-267.

Liu, W., Y. Li, G. H. Learn, R. S. Rudicell, J. D. Robertson, B. F. Keele, J.-B. N. Ndjango, C. M. Sanz, D. B. Morgan, S. Locatelli, M. K. Gonder, P. J. Kranzusch, P. D. Walsh, E. Delaporte, E. Mpoudi-Ngole, A. V. Georgiev, M. N. Muller, G. M. Shaw, M. Peeters, P. M. Sharp, J. C. Rayner and B. H. Hahn

(2010). "Origin of the human malaria parasite *Plasmodium falciparum* in gorillas." Nature **467**(7314): 420-U467.

Lochmiller, R. L. and C. Deerenberg (2000). "Trade-offs in evolutionary immunology: just what is the cost of immunity?" Oikos **88**(1): 87-98.

Lochmiller, R. L., M. R. Vestey and J. C. Boren (1993). "Relationship between protein nutritional-status and immunocompetence in northern Bobwhite chicks." Auk **110**(3): 503-510.

MacKenzie, D. I., J. D. Nichols, J. E. Hines, M. G. Knutson and A. B. Franklin (2003). "Estimating site occupancy, colonization, and local extinction when a species is detected imperfectly." Ecology **84**(8): 2200-2207.

MacKenzie, D. I., J. D. Nichols, G. B. Lachman, S. Droege, J. A. Royle and C. A. Langtimm (2002). "Estimating site occupancy rates when detection probabilities are less than one." Ecology **83**(8): 2248-2255.

Mansfield, K. L., N. Johnson, L. P. Phipps, J. R. Stephenson, A. R. Fooks and T. Solomon (2009). "Tick-borne encephalitis virus - a review of an emerging zoonosis." Journal of General Virology **90**: 1781-1794.

Marra, P. P., S. Griffing, C. Caffrey, A. M. Kilpatrick, R. McLean, C. Brand, E. Saito, A. P. Dupuis, L. Kramer and R. Novak (2004). "West Nile virus and wildlife." Bioscience **54**(5): 393-402.

Martin, V., X. Zhou, E. Marshall, B. Jia, F. Guo, M. A. FrancoDixon, N. DeHaan, D. U. Pfeiffer, R. J. S. Magalhaes and M. Gilbert (2011). "Risk-based surveillance for avian influenza control along poultry market chains in South China: The value of social network analysis." Preventive Veterinary Medicine **102**(3): 196-205.

Mateo, R., A. Gamez and R. Guitart (1998). "Organochlorine residues in hunted wild mallards in the Ebro Delta, Spain." Bulletin of Environmental Contamination and Toxicology **60**(1): 134-141.

McCallum, H., N. Barlow and J. Hone (2001). "How should pathogen transmission be modelled?" Trends In Ecology & Evolution **16**(6): 295-300.

McCoy, K. D., T. Boulinier and C. Tirard (2005). "Comparative host-parasite population structures: disentangling prospecting and dispersal in the black-legged kittiwake *Rissa tridactyla*." Molecular Ecology **14**(9): 2825-2838.

Messinger, S. M. and A. Ostling (2009). "The Consequences of Spatial Structure for the Evolution of Pathogen Transmission Rate and Virulence." American Naturalist **174**(4): 441-454.

Moller, A. P. (2006). "A review of developmental instability, parasitism and disease infection, genetics and evolution." Infection Genetics and Evolution **6**(2): 133-140.

Monaghan, P., C. B. Shedden, K. Ensor, C. R. Fricker and R. W. A. Girdwood (1985). "Salmonella carriage by Herring-Gulls in the clyde area of Scotland in relation to their feeding ecology." Journal of Applied Ecology **22**(3): 669-680.

Morner, T., D. L. Obendorf, M. Artois and M. H. Woodford (2002). "Surveillance and monitoring of wildlife diseases." Revue Scientifique Et Technique De L Office International Des Epizooties **21**(1): 67-76.

Muller, W., T. G. G. Groothuis, C. Dijkstra, H. Siitari and R. V. Alatalo (2004). "Maternal antibody transmission and breeding densities in the Black-headed Gull Larus ridibundus." Functional Ecology **18**(5): 719-724.

Munster, V. J., C. Baas, P. Lexmond, J. Waldenstrom, A. Wallensten, T. Fransson, G. F. Rimmelzwaan, W. E. P. Beyer, M. Schutten, B. Olsen, A. D. M. E. Osterhaus and R. A. M. Fouchier (2007). "Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds." Plos Pathogens **3**(5): 630-638.

Murphy, F. A. (2008). "Emerging zoonoses: The challenge for public health and biodefense." Preventive Veterinary Medicine **86**(3-4): 216-223.

Muzinic, J., C. J. Feare, M. Pavlak, D. Cvitkovic and M. Tadic (2010). "The Epidemiological Environment of Avian Influenza H5N1 Outbreaks in Wild Birds in Croatia." Polish Journal of Environmental Studies **19**(2): 379-385.

Newsom, S. W. B. (2006). "Pioneers in infection control: John Snow, Henry Whitehead, the Broad Street pump, and the beginnings of geographical epidemiology." Journal of Hospital Infection **64**(3): 210-216.

Nuttall, P. A., T. C. Kelly, D. Carey, S. R. Moss and K. A. Harrap (1984). "Mixed Infections with Tick-Borne Viruses in a Seabird Colony in Eire." Archives of Virology **79**(1-2): 35-44.

Oloya, J., J. Opuda-Asibo, R. Kazwala, A. B. Demelash, E. Skjerve, A. Lund, T. B. Johansen and B. Djonne (2008). "Mycobacteria causing human cervical lymphadenitis in pastoral communities in the Karamoja region of Uganda." Epidemiology and Infection **136**(5): 636-643.

Olsen, B., V. J. Munster, A. Wallensten, J. Waldenstrom, A. Osterhaus and R. A. M. Fouchier (2006). "Global patterns of influenza A virus in wild birds." Science **312**(5772): 384-388.

Oro, D. (2008). "Living in a ghetto within a local population: An empirical example of an ideal despotic distribution." Ecology **89**(3): 838-846.

Oro, D., M. Bosch and X. Ruiz (1995). "Effects of a trawling moratorium on the breeding success of the Yellow-legged Gull *Larus cachinnans*." Ibis **137**(4): 547-549.

Oro, D. and A. Martinezvilalta (1994). "Factors affecting kleptoparasitism and predation rates upon a colony of Audouins Gull (*Larus audouinii*) by Yellow-legged Gulls (*Larus cachinnans*) in Spain." Colonial Waterbirds **17**(1): 35-41.

Patz, J. A., P. Daszak, G. M. Tabor, A. A. Aguirre, M. Pearl, J. Epstein, N. D. Wolfe, A. M. Kilpatrick, J. Foufopoulos, D. Molyneux, D. J. Bradley and D. Working Grp Land Use Change (2004). "Unhealthy landscapes: Policy recommendations on land use change and infectious disease emergence." Environmental Health Perspectives **112**(10): 1092-1098.

Pearce-Duvet, J. M. C. (2006). "The origin of human pathogens: evaluating the role of agriculture and domestic animals in the evolution of human disease." Biological Reviews **81**(3): 369-382.

Pearce-Duvet, J. M. C., M. Gauthier-Clerc, E. Jourdain and T. Boulinier (2009). "Maternal antibody transfer in yellow-legged gulls." Emerging Infectious Diseases **15**(7): 1147-1149.

Pereda, A. J., M. Uhart, A. A. Perez, M. E. Zaccagnini, L. La Sala, J. Decarre, A. Goijman, L. Solari, R. Suarez, M. I. Craig, A. Vagnozzi, A. Rimondi, G. Konig, M. V. Terrera, A. Kaloghlian, H. Song, E. M. Sorrell and D. R. Perez (2008). "Avian influenza virus isolated in wild waterfowl in Argentina: Evidence of a potentially unique phylogenetic lineage in South America." Virology **378**(2): 363-370.

Perron, G. G., S. Quessy and G. Bell (2008). "A Reservoir of Drug-Resistant Pathogenic Bacteria in Asymptomatic Hosts." PLoS One **3**(11).

Pons, J. M. and P. Migot (1995). "Life-history strategy of the Herring gull: Changes in survival and fecundity in a population subjected to various feeding conditions." Journal of Animal Ecology **64**(5): 592-599.

Porterfield, J. S. (1975). "Basis of arbovirus classification." Medical biology **53**(5): 400-405.

Pravieux, J. J., H. Poulet, C. Charreyre and V. Juillard (2007). "Protection of newborn animals through maternal immunization." Journal of Comparative Pathology **137**: S32-S34.

Pugesek, B. H. and K. L. Diem (1983). ". A Multivariate Study of the Relationship of Parental Age to Reproductive Success in California Gulls." Ecology **64**(4): 829-839.

Ramos, R., M. Cerda-Cuellar, F. Ramirez, L. Jover and X. Ruiz (2010). "Influence of Refuse Sites on the Prevalence of *Campylobacter spp*. and *Salmonella* Serovars in Seagulls." Applied And Environmental Microbiology **76**(9): 3052-3056.

Ramos, R., F. Ramirez, C. Sanpera, L. Jover and X. Ruiz (2009). "Diet of Yellow-legged Gull (Larus michahellis) chicks along the Spanish Western Mediterranean coast: the relevance of refuse dumps." Journal of Ornithology **150**(1): 265-272.

Randall, D. A., S. D. Williams, I. V. Kuzmin, C. E. Rupprecht, L. A. Tallents, Z. Tefera, K. Argaw, F. Shiferaw, D. L. Knobel, C.

Sillero-Zubiri and M. K. Laurenson (2004). "Rabies in endangered Ethiopian wolves." Emerging Infectious Diseases **10**(12): 2214-2217.

Raven, S. J. and J. C. Coulson (1997). "The distribution and abundance of Larus gulls nesting on buildings in Britain and Ireland." Bird Study **44**: 13-34.

Real, L. A. and R. Biek (2007). "Spatial dynamics and genetics of infectious diseases on heterogeneous landscapes." Journal Of The Royal Society Interface **4**(16): 935-948.

Reed, K. D., J. K. Meece, J. S. Henkel and S. K. Shukla (2003). "Birds, migration and emerging zoonoses: west nile virus, lyme disease, influenza A and enteropathogens." Clinical medicine & research **1**(1): 5-12.

Ricklefs, R. E. and M. Wikelski (2002). "The physiology/life-history nexus." Trends In Ecology & Evolution **17**(10): 462-468.

Rutten, N., J. L. Gonzales, A. R. W. Elbers and A. G. J. Velthuis (2012). "Cost Analysis of Various Low Pathogenic Avian Influenza Surveillance Systems in the Dutch Egg Layer Sector." PLoS One **7**(4).

Saikku, P., A. J. Main, I. Ulmanen and M. Brummer-Korvenkontio (1980). "Viruses in *Ixodes uriae* (Acari: Ixodidae) from seabird colonies at Rost Islands, Lofoten, Norway." Journal of Medical Entomology **17**: 360--366.

Samraoui, B. and F. Samraoui (2008). "An ornithological survey of Algerian wetlands: Important Bird Areas, Ramsar sites and threatened species." Wildfowl **58**: 71–96.

Samraoui, F., A. H. Alfarhan, K. A. S. Al-Rasheid and B. Samraoui (2011). "Aa appraisal of the status and distribution of waterbirds of Algeria: indicators of global changes?" Ardeola **58**(1): 137-163.

Sanchez-Seco, M. P., D. Rosario, C. Domingo, L. Hernandez, K. Valdes, M. G. Guzman and A. Tenorio (2005). "Generic RT-nested-PCR for detection of flaviviruses using degenerated primers and internal control followed by sequencing for specific identification." Journal Of Virological Methods **126**(1-2): 101-109.

Savic, V., A. Labrovic, T. A. Zelenika, M. Balenovic, S. Separovic and L. Jurinovic (2010). "Multiple Introduction of Asian H5N1 Avian Influenza Virus in Croatia by Wild Birds During 2005-2006 and Isolation of the Virus from Apparently Healthy Black-Headed Gulls (Larus ridibundus)." Vector-Borne and Zoonotic Diseases **10**(9): 915-920.

Setoh, Y. X., J. Hobson-Peters, N. A. Prow, P. R. Young and R. A. Hall (2011). "Expression of recombinant West Nile virus prM protein fused to an affinity tag for use as a diagnostic antigen." Journal Of Virological Methods **175**(1): 20-27.

Sharp, G. B., Y. Kawaoka, D. J. Jones, W. J. Bean, S. P. Pryor, V. Hinshaw and R. G. Webster (1997). "Coinfection of wild ducks by influenza a viruses: Distribution patterns and biological significance." Journal Of Virology **71**(8): 6128-6135.

Silhol, R., F. P. Alvarez, C. Arena, J. P. Amoros, A. Flahault, T. Hanslik and P. Y. Boelle (2010). "Micro and macro population effects in disease transmission: the case of varicella." Epidemiology and Infection **138**(4): 482-490.

Silhol, R. and P.-Y. Boelle (2011). "Modelling the Effects of Population Structure on Childhood Disease: The Case of Varicella." Plos Computational Biology **7**(7).

SilleroZubiri, C., A. A. King and D. W. Macdonald (1996). "Rabies and mortality in Ethiopian wolves (Canis simensis)." Journal Of Wildlife Diseases **32**(1): 80-86.

Smith, K. F., A. P. Dobson, F. E. McKenzie, L. A. Real, D. L. Smith and M. L. Wilson (2005). "Ecological theory to enhance infectious disease control and public health policy." Frontiers in Ecology and the Environment **3**(1): 29-37.

Smith, M. J., S. Telfer, E. R. Kallio, S. Burthe, A. R. Cook, X. Lambin and M. Begon (2009). "Host-pathogen time series data in wildlife support a transmission function between density and frequency dependence." Proceedings Of The National Academy Of Sciences Of The United States Of America **106**(19): 7905-7909.

Smith, N. C., M. Wallach, M. Petracca, R. Braun and J. Eckert (1994). "Maternal transfer of antibodies induced by infection with

Eimeria maxima partially protects chickens against challenge with Eimeria tenella." Parasitology **109**: 551-557.

Snow, J. (1855). "On the Mode of Communication of Cholera." John Churchill, London, UK http://mines.humanoriented.com/classes/2010/fall/csci568/papers/On_the_mode_of_communication_of_cholera.pdf.

Sol, D., J. M. Arcos and J. C. Senar (1995). "The influence of refuse tips on the winter distribution of Yellow-legged gulls *Larus cachinnans*." Bird Study **42**: 216-221.

Sorci, G., A. P. Moller and T. Boulinier (1997). "Genetics of host-parasite interactions." Trends In Ecology & Evolution **12**(5): 196-200.

Stallknecht, D. E. and D. J. Brown (2007b). "Wild birds and the epidemiology of avian influenza." Journal of Wildlife Diseases **43**(3): 15-20.

Stallknecht, D. E., M. T. Kearney, S. M. Shane and P. J. Zwank (1990). "Effects of pH, temperature and salinity on persistence of avian influenza-viruses in water." Avian Diseases **34**(2): 412-418.

Staszewski, V. and T. Boulinier (2004). "Vaccination: a way to address questions in behavioral and population ecology?" Trends in Parasitology **20**(1): 17-22.

Suarez, D. L. and S. Schultz-Cherry (2000). "Immunology of avian influenza virus: a review." Developmental and Comparative Immunology **24**(2-3): 269-283.

Subbarao, K. and T. Joseph (2007). "Scientific barriers to developing vaccines against avian influenza viruses." Nature Reviews Immunology **7**(4): 267-278.

Sutmoller, P., G. R. Thomson, S. K. Hargreaves, C. M. Foogin and E. C. Anderson (2000). "The foot-and-mouth disease risk posed by African buffalo within wildlife conservancies to the cattle industry of Zimbabwe." Preventive Veterinary Medicine **44**(1-2): 43-60.

Swayne, D. E. (2008). Avian Influenza, Blackwell Publishing.

Tamisier, A., O. Dehorter and M. Jay (1999). Camargue, canards et foulques : Fonctionnement et devenir d'un prestigieux quartier d'hiver, Broché.

Taylor, L. H., S. M. Latham and M. E. J. Woolhouse (2001). "Risk factors for human disease emergence." Philosophical Transactions Of The Royal Society Of London Series B-Biological Sciences **356**(1411): 983-989.

Thomas, F., J. F. Guégan and F. Renaud (2007). Ecologie et évolution des systèmes parasités.

Toennessen, R., A. Germundsson, C. M. Jonassen, I. Haugen, K. Berg, R. T. Barrett and E. Rimstad (2011). "Virological and serological surveillance for type A influenza in the black-legged kittiwake (*Rissa tridactyla*)." Virology Journal **8**.

Tong, S., Y. Li, P. Rivailler, C. Conrardy, D. A. Alvarez Castillo, L.-M. Chen, S. Recuenco, J. A. Ellison, C. T. Davis, I. A. York, A. S. Turmelle, D. Moran, S. Rogers, M. Shi, Y. Tao, M. R. Weil, K. Tang, L. A. Rowe, S. Sammons, X. Xu, M. Frace, K. A. Lindblade, N. J. Cox, L. J. Anderson, C. E. Rupprecht and R. O. Donis (2012). "A distinct lineage of influenza A virus from bats." Proceedings Of The National Academy Of Sciences Of The United States Of America **109**(11): 4269-4274.

Tschopp, R., A. Aseffa, E. Schelling, S. Berg, E. Hailu, E. Gadisa, M. Habtamu, K. Argaw and J. Zinsstag (2010). "Bovine Tuberculosis at the Wildlife-Livestock-Human Interface in Hamer Woreda, South Omo, Southern Ethiopia." PLoS One **5**(8).

Tschopp, R., S. Berg, K. Argaw, E. Gadisa, M. Habtamu, E. Schelling, D. Young, A. Aseffa and J. Zinsstag (2010). "Bovine tuberculosis in Ethiopian wildlife." Journal Of Wildlife Diseases **46**(3): 753-762.

Van Poucke, S. G. M., J. M. Nicholls, H. J. Nauwynck and K. Van Reeth (2010). "Replication of avian, human and swine influenza viruses in porcine respiratory explants and association with sialic acid distribution." Virology Journal **7**.

Van Tuinen, M., D. Waterhouse and G. J. Dyke (2004). "Avian molecular systematics on the rebound: a fresh look at modern shorebird phylogenetic relationships." Journal of Avian Biology **35**(3): 191-194.

Velarde, R., S. E. Calvin, D. Ojkic, I. K. Barker and E. Nagy (2010). "Avian Influenza Virus H13 Circulating in Ring-Billed Gulls (*Larus delawarensis*) in Southern Ontario, Canada." Avian Diseases **54**(1): 411-419.

Vicente, J., U. Hofle, J. M. Garrido, I. G. Fernandez-de-Maria, P. Acevedo, R. Juste, M. Barral and C. Gortazar (2007). "Risk factors associated with the prevalence of tuberculosis-like lesions in fenced wild boar and red deer in south central Spain." Veterinary Research **38**(3): 451-464.

Vittecoq, M., V. Grandhomme, J. Champagnon, M. Guillemain, B. Crecenzo-Chaigne, F. Thomas, M. Gauthier-Clerc and S. van der Werf (2012). "High Influenza A virus infection rates in Mallards bred for hunting in the Camargue, south of France." Plos One **7**(8).

Wakelin, D. (1996). Immunity to parasites: How parasitic infections are controlled., 2nd Edition Cambridge University Press, Cambridge, UK.

Wallach, M., A. Halabi, G. Pillemer, O. Sarshalom, D. Mencher, M. Gilad, U. Bendheim, H. D. Danforth and P. C. Augustine (1992). "Maternal Immunization With Gametocyte Antigens As A Means Of Providing Protective Immunity Against Eimeria-Maxima In Chickens." Infection and Immunity **60**(5): 2036-2039.

Wallensten, A., V. J. Munster, N. Latorre-Margalef, M. Brytting, J. Elmberg, R. A. M. Fouchier, T. Fransson, P. D. Haemig, M. Karlsson, A. Lundkvist, A. D. M. E. Osterhaus, M. Stervander, J. Waldenstrom and B. Olsen (2007). "Surveillance of influenza A virus in migratory waterfowl in northern Europe." Emerging Infectious Diseases **13**(3): 404-411.

Weaver, S. C. and A. D. T. Barrett (2004). "Transmission cycles, host range, evolution and emergence of arboviral disease." Nature Reviews Microbiology **2**(10): 789-801.

Webster, R. G., W. J. Bean, O. T. Gorman, T. M. Chambers and Y. Kawaoka (1992). "Evolution And Ecology Of Influenza-A Viruses." Microbiological Reviews **56**(1): 152-179.

Weiss, R. A. (2001). "The Leeuwenhoek Lecture 2001. Animal origins of human infectious disease." Philosophical Transactions Of The Royal Society B-Biological Sciences **356**(1410): 957-977.

Weissenbock, H., J. Kolodziejek, K. Fragner, R. Kuhn, M. Pfeffer and N. Nowotny (2003). "Usutu virus activity in Austria, 2001-2002." Microbes and Infection **5**(12): 1132-1136.

West, A. P., A. B. Herr and P. J. Bjorkman (2004). "The chicken yolk sac IgY receptor, a functional equivalent of the mammalian MHC-related Fc receptor, is a phospholipase A(2) receptor homolog." Immunity **20**(5): 601-610.

White, B. J., F. H. Collins and N. J. Besansky (2011). Evolution of *Anopheles gambiae* in Relation to Humans and Malaria. Annual Review of Ecology, Evolution, and Systematics, Vol 42. D. J. Futuyma, H. B. Shaffer and D. Simberloff. **42:** 111-132.

WHO manual on animal influenza diagnosis and surveillance (2002). Available from http://whqlibdoc.who.int/hq/2002/WHO_CDS_CSR_NCS_2002.5.pdf. [cited 2012 June 9].

Woolhouse, M. E. J. (2002). "Population biology of emerging and re-emerging pathogens." Trends in Microbiology **10**(10): S3-S7.

Woolhouse, M. E. J. and S. Gowtage-Sequeria (2005). "Host range and emerging and reemerging pathogens." Emerging Infectious Diseases **11**(12): 1842-1847.

Woolhouse, M. E. J., L. H. Taylor and D. T. Haydon (2001). "Population biology of multihost pathogens." Science **292**(5519): 1109-1112.

Partie II

Articles

Article 1

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.

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 *et al.* 2004; Peiris *et al.* 2004; Wang *et al.* 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 (Li *et al.* 2004; Chen *et al.* 2005; Ferguson *et al.* 2005; De Wit *et al.* 2008). 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 Webster *et al.* 1992; Alexander 2000. 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 (Ellstrom *et al.* 2009; 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 (Webster *et al.* 1978; Ellstrom *et al.* 2008), even if oropharyngeal excretion is also observed (Ellstrom *et al.* 2008; Fereidouni *et al.* 2010; Jourdain *et al.* 2010; Costa *et al.* 2011). 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 (Stallknecht *et al.* 1990; Brown *et al.* 2009).

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 *et al.* 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.

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References

Altwegg R., Crawford R.J.M., Underhill L.G., Martin A.P. & Whittington P.A. (2007) Geographic variation in reproduction and survival of kelp gulls Larus dominicanus vetula in southern Africa. *Journal of Avian Biology* 38, 580–586.

Bahl A.K. & Pomeroy B.S. (1977) EXPERIMENTAL EXPOSURE OF FRANKLINS'GULLS (Larvus pipixcan) AND MALLARDS (Anas platyrhynchos) TO A TURKEY INFLUENZA A VIRUS A/Turkey/Minn/BF/72 (Hav6Neq2). *Journal of Wildlife Diseases* 13, 420–426.

Becker W.B. (1966) The isolation and classification of tern virus: influenza virus A/tern/South Africa/1961. *Journal of Hygiene* 64, 309–320.

Blokpoel H. & Spaans A.L. (1991) Introductory remarks: superabundance in gulls: causes, problems and solutions. *Acta XX congressus internationalis ornithologici*, 2361–2363.

Boere G. & Dodman T. (2011) Understanding the Flyway Approach to Conservation. *WOW training resources*.

Bonnedahl J., Drobni M., Gauthier-Clerc M., Hernandez J., Granholm S., Kayser Y., *et al.* (2009) Dissemination of Escherichia coli with CTX-M type ESBL between humans and yellow-legged gulls in the south of France. *PLoS One* 4.

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

Breton A.R., Fox G.A. & Chardine J.W. (2008) Survival of Adult Herring Gulls (Larus argentatus) from a Lake Ontario Colony Over Two Decades of Environmental Change. *Waterbirds* 31, 15–23.

Brown J.D., Goekjian G., Poulson R., Valeika S. & Stallknecht D.E. (2009) Avian influenza virus in water: Infectivity is dependent on pH, salinity and temperature. *Veterinary Microbiology* 136, 20–26.

Brown J.D., Stallknecht D.E., Beck J.R., Suarez D.L., Swayne D.E. & others (2006) Susceptibility of North American ducks and gulls

to H5N1 highly pathogenic avian influenza viruses. *Emerg Infect Dis* 12, 1663–70.

Brown J.D., Stallknecht D.E. & Swayne D.E. (2008) Experimental infections of herring gulls (Larus argentatus) with H5N1 highly pathogenic avian influenza viruses by intranasal inoculation of virus and ingestion of virus-infected chicken meat. *Avian Pathology* 37, 393–397.

Chen H.-X., Shen H.-G., Li X.-L., Zhou J.-Y., Hou Y.-Q., Guo J.-Q., *et al.* (2006) Seroprevalance and identification of influenza A virus infection from migratory wild waterfowl in China (2004-2005). *Journal of veterinary medicine. B, Infectious diseases and veterinary public health* 53, 166–170.

Chen R. & Holmes E.C. (2009) Frequent inter-species transmission and geographic subdivision in avian influenza viruses from wild birds. *Virology* 383, 156–161.

Costa T.P., Brown J.D., Howerth E.W. & Stallknecht D.E. (2011) Variation in viral shedding patterns between different wild bird species infected experimentally with low-pathogenicity avian influenza viruses that originated from wild birds. *Avian Pathology: Journal of the W.V.P.A* 40, 119–124.

Dolejska M., Cizek A. & Literak I. (2007) High prevalence of antimicrobial-resistant genes and integrons in Escherichia coli isolates from Black-headed Gulls in the Czech Republic. *Journal of applied microbiology* 103, 11–19.

Duhem C., Roche P., Vidal E. & Tatoni T. (2008) Effects of anthropogenic food resources on yellow-legged gull colony size on Mediterranean islands. *Population Ecology* 50, 91–100.

Earn D.J.D., Dushoff J. & Levin S.A. (2002) Ecology and evolution of the flu. *Trends in ecology & evolution* 17, 334–340.

Ellis T.M., Bousfield R.B., Bissett L.A., Dyrting K.C., Luk G.S.M., Tsim S.T., *et al.* (2004) Investigation of outbreaks of highly pathogenic H5N1 avian influenza in waterfowl and wild birds in Hong Kong in late 2002. *Avian Pathology* 33, 492–505.

Elphick J. (2007) *The atlas of bird migration*. Struik.

Fereidouni S.R., Grund C., Häuslaigner R., Lange E., Wilking H., Harder T.C., *et al.* (2010) Dynamics of specific antibody

responses induced in mallards after infection by or immunization with low pathogenicity avian influenza viruses. *Avian diseases* 54, 79–85.

Fouchier R.A.., Munster V., Wallensten A., Bestebroer T.M., Herfst S., Smith D., *et al.* (2005) Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. *Journal of virology* 79, 2814–2822.

Fouchier R.A.M., Olsen B., Bestebroer T.M., Herfst S., Van der Kemp L., Rimmelzwaan G.F., *et al.* (2003) Influenza A virus surveillance in wild birds in Northern Europe in 1999 and 2000. *Avian Diseases* 47, 857–860.

Gaidet N., Cappelle J., Takekawa J.Y., Prosser D.J., Iverson S.A., Douglas D.C., *et al.* (2010) Potential spread of highly pathogenic avian influenza H5N1 by wildfowl: dispersal ranges and rates determined from large-scale satellite telemetry. *Journal of Applied Ecology* 47, 1147–1157.

Gaidet N., Dodman T., Caron A., Balança G., Desvaux S., Goutard F., *et al.* (2007) Influenza surveillance in wild birds in Eastern Europe, the Middle East, and Africa: Preliminary results from an ongoing FAO-led survey. *Journal of Wildlife Diseases* 43, S22–S28.

Gasparini J., McCoy K.D., Haussy C., Tveraa T. & Boulinier T. (2001) Induced maternal response to the Lyme disease spirochaete Borrelia burgdorferi sensu lato in a colonial seabird, the kittiwake Rissa tridactyla. *Proceedings of the Royal Society of London. Series B: Biological Sciences* 268, 647–650.

GenBank (2012) GenBank Home.

Germundsson A., Madslien K.I., Hjortaas M.J., Handeland K. & Jonassen C.M. (2010) Prevalence and subtypes of Influenza A Viruses in Wild Waterfowl in Norway 2006-2007. 52, 28–28.

Gionechetti F., Zucca P., Gombac F., Monti-Bragadin C., Lagatolla C., Tonin E., *et al.* (2008) Characterization of antimicrobial resistance and class 1 integrons in Enterobacteriaceae isolated from Mediterranean herring gulls (Larus cachinnans). *Microbial Drug Resistance* 14, 93–99.

Graves I.L. (1992) Influenza viruses in birds of the Atlantic flyway. *Avian diseases* 36, 1–10.

Gresikova M., Sekeyova M., Tŭmová B. & Stumpa A. (1979) Isolation of an influenza A virus strain from a bird embryo (Larus ridibundus) collected in Slovakia. *Acta virologica* 23, 89.

Hanson B.A., Luttrell M.P., Goekjian V.H., Niles L., Swayne D.E., Senne D.A., *et al.* (2008) IS THE OCCURRENCE OF AVIAN INFLUENZA VIRUS IN CHARADRIIFORMES SPECIES AND LOCATION DEPENDENT? *Journal of Wildlife Diseases* 44, 351 –361.

Harvell C.D., Kim K., Burkholder J.M., Colwell R.R., Epstein P.R., Grimes D.J., *et al.* (1999) Emerging marine diseases–climate links and anthropogenic factors. *Science* 285, 1505–1510.

Haynes L., Arzey E., Bell C., Buchanan N., Burgess G., Cronan V., *et al.* (2009) Australian surveillance for avian influenza viruses in wild birds between July 2005 and June 2007. *Australian veterinary journal* 87, 266–272.

Hinshaw V.S., Air G.M., Gibbs A.J., Graves L., Prescott B. & Karunakaran D. (1982) Antigenic and genetic characterization of a novel hemagglutinin subtype of influenza A viruses from gulls. *Journal of virology* 42, 865–872.

Hinshaw V.S., Air G.M., Schild G.C. & Newman R.W. (1983) Characterization of a novel haemagglutinin subtype (H13) of influenza A viruses from gulls. *Bulletin of the World Health Organization* 61, 677.

Horimoto T. & Kawaoka Y. (2001) Pandemic Threat Posed by Avian Influenza A Viruses. *Clinical Microbiology Reviews* 14, 129–149.

Hoye B.J., Munster V.J., Nishiura H., Fouchier R.A.., Madsen J. & Klaassen M. (2011) Reconstructing an annual cycle of interaction: natural infection and antibody dynamics to avian influenza along a migratory flyway. *Oikos*.

del Hoyo J. del (1996) *Hoatzin to auks*. Lynx Edicions.

Hurt A.C., Hansbro P.M., Selleck P., Olsen B., Minton C., Hampson A.W., *et al.* (2006) Isolation of avian influenza viruses from two

different transhemispheric migratory shorebird species in Australia. *Archives of virology* 151, 2301–2309.

IOC (2012) IOC World Bird List.

Ito T., Gorman O.T., Kawaoka Y., Bean W.J. & Webster R.G. (1991) Evolutionary analysis of the influenza-A virus M-gene with comparison of the M1 and M2 proteins. *Journal of virology* 65, 5491–5498.

Janout V., Uvízl M., Chmela J., Tůmová B., Stumpa A. & Smékal M. (1979) A study on the role of birds in the spread of infections. *Journal of hygiene, epidemiology, microbiology, and immunology* 23, 457–461.

Järhult J.D., Muradrasoli S., Wahlgren J., Söderström H., Orozovic G., Gunnarsson G., *et al.* (2011) Environmental Levels of the Antiviral Oseltamivir Induce Development of Resistance Mutation H274Y in Influenza A/H1N1 Virus in Mallards. *PLoS ONE* 6, e24742.

Jourdain E., Gunnarsson G., Wahlgren J., Latorre-Margalef N., Bröjer C., Sahlin S., *et al.* (2010) Influenza Virus in a Natural Host, the Mallard: Experimental Infection Data. *PLoS ONE* 5, e8935.

Jourdain E., van Riel D., Munster V.J., Kuiken T., Waldenström J., Olsen B., *et al.* (2011) The Pattern of Influenza Virus Attachment Varies among Wild Bird Species. *PloS one* 6, e24155.

Kawaoka Y., Chambers T.M., Sladen W.L. & Gwebster R. (1988) Is the gene pool of influenza viruses in shorebirds and gulls different from that in wild ducks? *Virology* 163, 247–250.

Kawaoka Y., Gorman O.T., Ito T., Wells K., Donis R.O., Castrucci M.R., *et al.* (1998) Influence of host species on the evolution of the nonstructural (NS) gene of influenza A viruses. *Virus research* 55, 143–156.

Kleijn D., Munster V.J., Ebbinge B.S., Jonkers D.A., Müskens G., Van Randen Y., *et al.* (2010) Dynamics and ecological consequences of avian influenza virus infection in greater white-fronted geese in their winter staging areas. *Proceedings of the Royal Society B: Biological Sciences* 277, 2041–2048.

Koehler A.V., Pearce J.M., Flint P.L., Franson J.C. & Ip H.S. (2008) Genetic evidence of intercontinental movement of avian influenza in a migratory bird: the northern pintail (Anas acuta). *Molecular Ecology* 17, 4754–4762.

Kohls A., Hafez H.M., Harder T., Jansen A., Lierz P., Lüschow D., *et al.* (2011) Avian influenza virus risk assessment in falconry. *Virology journal* 8, 187.

Kou Z., Li Y., Yin Z., Guo S., Wang M., Gao X., *et al.* (2009) The survey of H5N1 flu virus in wild birds in 14 Provinces of China from 2004 to 2007. *PloS one* 4, e6926.

Krauss S., Obert C.A., Franks J., Walker D., Jones K., Seiler P., *et al.* (2007) Influenza in Migratory Birds and Evidence of Limited Intercontinental Virus Exchange. *PLoS Pathogens* 3, e167.

L Perkins L.E. & Swayne D.E. (2001) Pathobiology of A/chicken/Hong Kong/220/97 (H5N1) avian influenza virus in seven gallinaceous species. *Veterinary Pathology Online* 38, 149.

L'vov D.., Yamnikova S.., Gambaryan A.., Fedyakina I.. & Matrosovich M.. (2001) Isolation of influenza viruses from wild birds in the Volga River basin and in the North Caspian Region. *International Congress Series* 1219, 251–258.

L'vov D.K., Andreev V.P., Braude N.A., Shchukina N.V. & Podcherniaeva R.I. (1978) [Isolation of influenza virus with the antigenic formula Hav4 Nav2 and Hav5 Nav2 during epizootic infection among sea gulls in the Astrakhan district in the summer of 1976]. *Voprosy virusologii*, 399–403.

L'vov D.K., Shchelkanov M.I., Deriabin P.G., Fediakina I.T., Burtseva E.I., Prilipov A.G., *et al.* (2006) Isolation of highly pathogenic avian influenza (HPAI) A/5H5N1 strains from wild birds in the epizootic outbreak on the Ubsu-Nur Lake (June 2006) and their incorporation to the Russian Federation State Collection of viruses.

Lam T.T.Y., Ip H.S., Ghedin E., Wentworth D.E., Halpin R.A., Stockwell T.B., *et al.* (2012) Migratory flyway and geographical distance are barriers to the gene flow of influenza virus among North American birds. *Ecology letters*.

Latorre-Margalef N., Gunnarsson G., Munster V.J., Fouchier R.A.., Osterhaus A.D.M.., Elmberg J., *et al.* (2009) Effects of influenza A virus infection on migrating mallard ducks. *Proceedings of the Royal Society B: Biological Sciences* 276, 1029 –1036.

Lebarbenchon C. (2010) Persistence of Highly Pathogenic Avian Influenza Viruses in Natural Ecosystems. *Emerging Infectious Diseases* 16.

Lebarbenchon C., BROWN S.A.M.., Poulin R., Gauthier-Clerc M. & Thomas F. (2008) Evolution of pathogens in a man-made world. *Molecular ecology* 17, 475–484.

Lebarbenchon C., Chang C.M., Van Der Werf S., Aubin J.T., Kayser Y., Ballesteros M., *et al.* (2007) Influenza A virus in birds during spring migration in the Camargue, France. *Journal of wildlife diseases* 43, 789.

Lebarbenchon C., Chang C.M., Gauthier-Clerc M., Thomas F., Renaud F. & Van Der Werf S. (2009) H9N2 avian influenza virus in a Mediterranean gull.

Lebarbenchon C., Chang C.M., Grandhomme V., Dietrich M., Kayser Y., Elguero E., *et al.* (2010) Avian Influenza Circulation in the Camargue (South of France) During the 2006–07 Season. *Avian diseases* 54, 446–449.

Lee K.A. (2006) Linking immune defenses and life history at the levels of the individual and the species. *Integrative and Comparative Biology* 46, 1000–1015.

Lee M.S., Chang P.C., Shien J.H., Cheng M.C. & Shieh H.K. (2001) Identification and subtyping of avian influenza viruses by reverse transcription-PCR. *Journal of virological methods* 97, 13–22.

Leroy E.M., Kumulungui B., Pourrut X., Rouquet P., Hassanin A., Yaba P., *et al.* (2005) Fruit bats as reservoirs of Ebola virus. *Nature* 438, 575–576.

Lin B., Malanoski A.P., Wang Z., Blaney K.M., Long N.C., Meador C.E., *et al.* (2009) Universal detection and identification of avian influenza virus by use of resequencing microarrays. *Journal of clinical microbiology* 47, 988–993.

Lindskog C., Ellström P., Olsen B., Ponten F., Van Riel D., Munster V., *et al.* (In Prep.) H16 Gull Influenza Virus Attaches to Human Upper Respiratory Tract but not to Mallard Digestive Tract.

Lisnizer N., Garcia-Borboroglu P. & Yorio P. (2011) Spatial and temporal variation in population trends of Kelp Gulls in northern Patagonia, Argentina. *Emu* 111, 259–267.

Liu J., Xiao H., Lei F., Zhu Q., Qin K., Zhang X., *et al.* (2005) Highly pathogenic H5N1 influenza virus infection in migratory birds. *Science* 309, 1206–1206.

Mackenzie J.S., Edwards E.C., Holmes R.M., Hinshaw V.S. & others (1984) Isolation of ortho-and paramyxoviruses from wild birds in Western Australia, and the characterization of novel influenza A viruses. *Aust. J. Exp. Biol. Med. Sci* 62, 89–99.

Marchenko V.I., Alekseev A.I., Susloparov I.M., Sharshov K.A., Il'inykh F.A., Zolotykh S.I., *et al.* (2010) [Isolation of influenza A virus from wild birds in western part of Mongolia]. *Zhurnal mikrobiologii, epidemiologii, i immunobiologii*, 18–21.

De Marco M.A., Foni E., Campitelli L., Delogu M., Raffini E., Chiapponi C., *et al.* (2005) Influenza virus circulation in wild aquatic birds in Italy during H5N2 and H7N1 poultry epidemic periods (1998 to 2000). *Avian Pathology* 34, 480_/485.

Munster V.J., Baas C., Lexmond P., Waldenström J., Wallensten A., Fransson T., *et al.* (2007) Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. *PLoS pathogens* 3, e61.

Munster V.J. & Fouchier R.A.M. (2009) Avian influenza virus: of virus and bird ecology. *Vaccine* 27, 6340–6344.

Muzinic J., Feare C.J., Pavlak M., Cvitkovic D. & Tadic M. (2010) The Epidemiological Environment of Avian Influenza H5N1 Outbreaks in Wild Birds in Croatia. *Polish Journal of Environmental Studies* 19, 379–385.

NIAID (2012) National Institute of Allergy and infectious Diseases. Flu (Influenza).

Nicholls J.M., Chan R.W.Y., Russell R.J., Air G.M. & Peiris J.S. (2008) Evolving complexities of influenza virus and its receptors. *Trends in microbiology* 16, 149–157.

Obenauer J.C., Denson J., Mehta P.K., Su X., Mukatira S., Finkelstein D.B., *et al.* (2006) Large-scale sequence analysis of avian influenza isolates. *Science* 311, 1576–1580.

Olsen B., Munster V.J., Wallensten A., Waldenstrom J., Osterhaus A.D.M.. & Fouchier R.A.. (2006) Global patterns of influenza A virus in wild birds. *Science* 312, 384–388.

Orozovic G., Orozovic K., Lennerstrand J. & Olsen B. (2011) Detection of resistance mutations to antivirals oseltamivir and zanamivir in avian influenza a viruses isolated from wild birds. *PLoS One* 6, e16028.

Otsuki K., Takemoto O., Fujimoto R., Kawaoka Y. & Tsubokura M. (1987) Isolation of influenza A viruses from migratory waterfowls in San-in District, Western Japan in winters of 1980-1982. *Zentralblatt für Bakteriologie, Mikrobiologie, und Hygiene. Series A, Medical microbiology, infectious diseases, virology, parasitology* 265, 235–242.

Patz J.A., Daszak P., Tabor G.M., Aguirre A.A., Pearl M., Epstein J., *et al.* (2004) Unhealthy landscapes: policy recommendations on land use change and infectious disease emergence. *Environmental Health Perspectives* 112, 1092.

Pearce J.M., Ramey A.M., Ip H.S. & Gill Jr R.E. (2009) Limited evidence of trans-hemispheric movement of avian influenza viruses among contemporary North American shorebird isolates. *Virus research*.

Pearce J.M., Ramey A.M., Ip H.S. & Gill Jr R.E. (2010) Limited evidence of trans-hemispheric movement of avian influenza viruses among contemporary North American shorebird isolates. *Virus research* 148, 44–50.

Pearce-Duvet J.M.C., Gauthier-Clerc M., Jourdain E. & Boulinier T. (2009) Maternal antibody transfer in yellow-legged gulls. *Emerging infectious diseases* 15, 1147.

Pereda A.J., Uhart M., Perez A.A., Zaccagnini M.E., La Sala L., Decarre J., *et al.* (2008) Avian influenza virus isolated in wild waterfowl in Argentina: evidence of a potentially unique phylogenetic lineage in South America. *Virology* 378, 363–370.

Pourrut X., Kumulungui B., Wittmann T., Moussavou G., Délicat A., Yaba P., *et al.* (2005) The natural history of Ebola virus in Africa. *Microbes and infection* 7, 1005–1014.

Ramey A.M., Pearce J.M., Ely C.R., Sheffield Guy L.M., Irons D.B., Derksen D.V., *et al.* (2010) Transmission and reassortment of avian influenza viruses at the Asian–North American interface. *Virology* 406, 352–359.

Raven S.J. & Coulson J.C. (1997) The distribution and abundance of Larus gulls nesting on buildings in Britain and Ireland. *Bird Study* 44, 13–34.

Reeves A.B., Pearce J.M., Ramey A.M., Meixell B.W. & Runstadler J.A. (2011) Interspecies transmission and limited persistence of low pathogenic avian influenza genomes among Alaska dabbling ducks. *Infection, Genetics and Evolution* 11, 2004–2010.

Röhm C., Horimoto T., Kawaoka Y., Süss J. & Webster R.G. (1995) Do hemagglutinin genes of highly pathogenic avian influenza viruses constitute unique phylogenetic lineages? *Virology* 209, 664–670.

Sandrock C., Kelly T. & others (2007) Clinical review: update of avian influenza A infections in humans. *Crit Care* 11, 209.

Savić V., Labrović A., Zelenika T.A., Balenović M., Šeparović S. & Jurinović L. (2010) Multiple introduction of Asian H5N1 avian influenza virus in Croatia by wild birds during 2005–2006 and isolation of the virus from apparently healthy black-headed gulls (Larus ridibundus). *Vector-Borne and Zoonotic Diseases* 10, 915–920.

Sharshov K. (2010) Avian Influenza (H5N1) Outbreak among Wild Birds, Russia, 2009. *Emerging Infectious Diseases*.

Sivanandan V., Halvorson D.A., Laudert E., Senne D.A. & Kumar M.C. (1991) Isolation of H13N2 influenza A virus from turkeys and surface water. *Avian diseases*, 974–977.

Slepuskin A.N., Pysina T.V., Gonsovsky F.K., Sazonov A.A., Isacenko V.A., Sokolova N.N., *et al.* (1972) Haemagglutination-inhibiting activity to type a influenza viruses in the sera of wild

birds from the far east of the USSR. *Bulletin of the World Health Organization* 47, 527–530.
Spackman E., Swayne D.E., Gilbert M., Joly D.O., Karesh W.B., Suarez D.L., *et al.* (2009) Characterization of low pathogenicity avian influenza viruses isolated from wild birds in Mongolia 2005 through 2007. *Virology Journal* 6, 190.
Stallknecht D.E. & Brown J.D. (2007) Wild birds and the epidemiology of avian influenza. *Journal of Wildlife Diseases* 43, S15–S20.
Staszewski V., Gasparini J., MCCOY K. & others (2007a) Evidence of an interannual effect of maternal immunization on the immune response of juveniles in a long-lived colonial bird. *Journal of Animal Ecology* 76, 1215–1223.
Staszewski V., MCCOY K., Tveraa T. & Boulinier T. (2007b) Interannual dynamics of antibody levels in naturally infected long-lived colonial birds. *Ecology* 88, 3183–3191.
Suarez D.L. & Perdue M.L. (1998) Multiple alignment comparison of the non-structural genes of influenza A viruses. *Virus research* 54, 59–69.
Suzuki Y. (2005) Sialobiology of influenza: molecular mechanism of host range variation of influenza viruses. *Biological and pharmaceutical bulletin* 28, 399–408.
Toennessen R., Germundsson A., Jonassen C.M., Haugen I., Berg K., Barrett R.T., *et al.* (2011) Virological and serological surveillance for type A influenza in the black-legged kittiwake (Rissa tridactyla). *Virology Journal* 8, 21.
Tong S., Li Y., Rivailler P., Conrardy C., Castillo D.A.A., Chen L.M., *et al.* (2012) A distinct lineage of influenza A virus from bats. *Proceedings of the National Academy of Sciences*.
Tsubokura M., Otsuki K., Yamamoto H., Kawaoka Y. & Nerome K. (1981) Isolation of an Hswl Nav4 influenza virus from a tufted duck (Aythya fuligula) in Japan. *Microbiology and immunology* 25, 819–825.
USDA (2012) USDA - APHIS - APHIS Newsroom.
Velarde R., Calvin S.E., Ojkic D., Barker I.K. & Nagy E. (2010) Avian influenza virus H13 circulating in ring-billed gulls (Larus

delawarensis) in southern Ontario, Canada. *Avian Diseases* 54, 411–419.

Wallensten A., Munster V.J., Latorre-Margalef N., Brytting M., Elmberg J., Fouchier R.A.M., *et al.* (2007) Surveillance of Influenza Virus A in Migratory Waterfowl in Northern Europe. *Emerging infectious diseases* 13, 404.

Webster R.G., Bean W.J., Gorman O.T., Chambers T.M. & Kawaoka Y. (1992) Evolution and ecology of influenza A viruses. *Microbiology and Molecular Biology Reviews* 56, 152–179.

Widjaja L., Krauss S.L., Webby R.J., Xie T. & Webster R.G. (2004) Matrix gene of influenza a viruses isolated from wild aquatic birds: ecology and emergence of influenza a viruses. *Journal of virology* 78, 8771.

Wille M., Robertson G.J., Whitney H., Bishop M.A., Runstadler J.A. & Lang A.S. (2011) Extensive Geographic Mosaicism in Avian Influenza Viruses from Gulls in the Northern Hemisphere. *PloS one* 6, e20664.

Winker K. & Gibson D.D. (2010) The Asia-to-America Influx of Avian Influenza Wild Bird Hosts Is Large. *Avian Diseases* 54, 477–482.

Winker K., McCracken K.G., Gibson D.D., Pruett C.L., Meier R., Huettmann F., *et al.* (2007) Movements of birds and avian influenza from Asia into Alaska. *Emerging Infectious Diseases* 13, 547.

Winker K., Spackman E. & Swayne D.E. (2008) Rarity of influenza A virus in spring shorebirds, southern Alaska. *Emerging Infectious Diseases* 14, 1314.

Yamnikova S.S., Gambaryan A.S., Tuzikov A.B., Bovin N.V., Matrosovich M.N., Fedyakina I.T., *et al.* (2003) Differences between HA receptor-binding sites of avian influenza viruses isolated from Laridae and Anatidae. *Avian diseases* 47, 1164–1168.

Zakstel'skaja L.J., Isacenko V.A., Osidze N.G., Timofeeva C.C., Slepuskin A.N. & Sokolova N.N. (1972) Some observations on the circulation of influenzaviruses in domestic and wild birds. *Bulletin of the World Health Organization* 47, 497–501.

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, Hinshaw *et al.*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)

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)

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

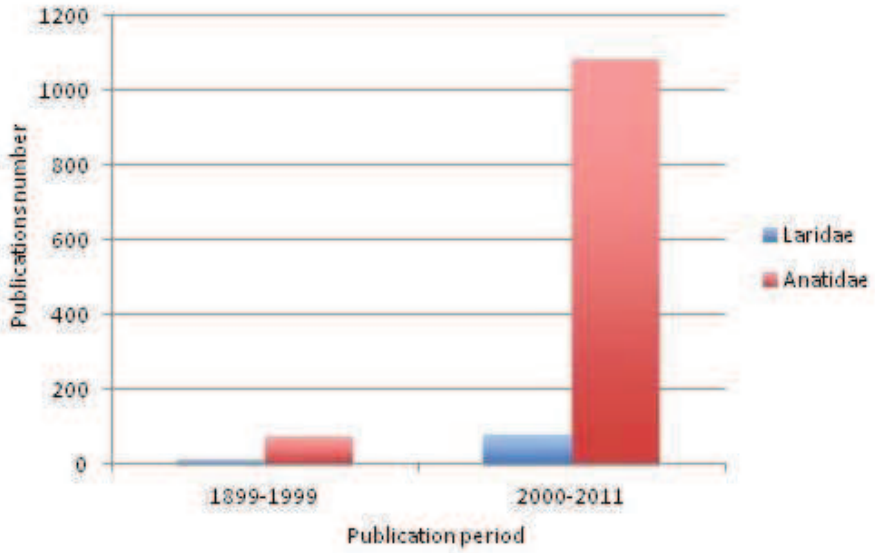
This table presents the different HPAIV subtypes detected naturally or experimentally in different Laridae species.

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)18/09/2012 14:18:00
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	Lvov *et al.* 1978

Figure legends

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

Figures


1200
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Publications number
1899-1999
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Publication period
Laridae
Anatidae

Article 2

Prevalence of Influenza A antibodies in yellow-legged gull (*Larus michahellis*) eggs and adults in southern Tunisia

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Abstract

Wild waterbirds are natural reservoirs for low pathogenic avian influenza A viruses. These viruses can be transmitted to domestic birds and/or humans and subsequently become highly pathogenic. Although the risk of transmission may be influenced by the prevalence of influenza virus in wild birds, little is known about this pathogen's dynamics in natural populations. We investigated influenza A exposure in nature by measuring the prevalence of anti-influenza A antibodies in the eggs and adults of an abundant and anthropophilic waterbird species common around the Mediterranean sea, the yellow-legged gull (*Larus michahellis*). Sampling took place on two colonies located in the gulf of Gabès in southern Tunisia: Sfax and Djerba. Antibodies were detected in the two sites, with a higher prevalence at Sfax (95% of adults and 67 % of eggs) than Djerba (57% of adults and 43% of eggs). Across both colonies, clutches that were laid later in the season, and thus more likely by younger parents, showed lower prevalence. Using patch occupancy modeling applied to egg clutches, we found that it is unnecessary to sample all the eggs in a given nest; nest status (antibody positive or negative) can be reliably estimated from a single egg. Differences in the richness and density of birds between the two sites may explain the observed differences in prevalence. The higher concentration of waterfowl in the Sfax colony could favor the transmission of influenza A viruses between individuals and species. This study highlights the importance of further developing ecological-based approaches to the factors determining the circulation of infectious agents in species like the yellow-legged gull, which exist at the interface between diverse biological communities and human activities.

Running head: *AIV antibodies in the yellow-legged gull*

Keywords: *Avian Influenza, maternal antibodies, patch occupancy modelling, North Africa, waterbirds.*

Introduction

Avian flu is an infectious disease caused by type A influenza viruses. It represents a serious threat to bird and human populations because of its impact on global health and economy (Webster et al. 1992, De Wit and Fouchier 2008). Low pathogenic subtypes of avian influenza A viruses, which are characterized by the full range of protein types in a wide variety of combinations, are commonly found in wild waterbirds. However, they are not evenly distributed across species or locations (Webster et al. 1992, Fouchier et al. 2005, Olsen et al. 2006). In particular, waterfowl and shorebirds, Anseriforms (ducks, geese, swans) and Charadriforms (gulls, terns and waders), are considered to be their natural host reservoirs (Webster et al. 1992, Olsen et al. 2006). Most previous studies and monitoring efforts of AIV in the wild have directly identified the viral agent using molecular detection methods (Wallensten et al. 2007, De Marcoa et al. 2004, Gaidet et al. 2007, Lebarbenchon et al. 2007a, Lebarbenchon et al. 2007b), but other approaches can be useful.

Antibody detection methods have repeatedly shown their efficiency in monitoring natural avian influenza A prevalence (Webster et al. 1992, De Marco et al. 2004, Velarde et al. 2010, Fereidouni et al. 2010, Perez-Ramirez et al. 2010). The presence of detectable anti-influenza A antibody in bird plasma indicates that the individual has been exposed to the virus in the past, but does not require the bird to be currently infected (Brown et al. 2009). Furthermore, the presence of antibodies in egg yolks can reflect the past exposure of mothers to infectious agents without the need to capture adults (Gasparini et al. 2001, Grindstaff et al. 2003, Trampel et al. 2006, Boulinier and Staszewski 2008, Pearce-Duvet et al. 2009). The antibody quantification approach is especially useful when the proportion of individuals shedding virus at any point in time is low, which is common

in the case of avian influenza (e.g., detection of viruses in only 0.5% of more than 300 yellow-legged gull (*Larus michahellis*) droppings sampled; Lebarbenchon et al. 2010). Thus, when used on long-lived birds, the antibody detection approach enables one to detect antibodies that may have persisted following exposure to a virus several years prior, providing a powerful tool for investigating factors affecting bird exposure to AIV in the wild and for identifying high-risk zones. These issues are paramount to understanding the spread and dynamics of AIV in the wild, as well as for prevention purposes.

Spatial variation in bird exposure to AIV is commonly linked to bird migratory behaviour; it is supposed that bird migratory routes and stopover sites constitute particularly important high-risk zones (e.g., Gilbert et al. 2006, Jourdain et al. 2007). Within these sites, high numbers of migrant birds of different origins aggregate in mixed flocks with resident birds, thus increasing the probability of AIV transmission between birds and among species, especially as the viruses can be easily and broadly transmitted via the fecal-oral or the respiratory route (Webster et al. 2006, Munster and Fouchier 2009, Roche et al. 2009). However, even at the scale of a given migratory route (landscape scale), bird exposure to AIV could be hypothesized to vary among local stopover sites due to possible differences in the diversity and turnover rates of their bird assemblages, which may be due to differences in local habitat conditions and attractiveness. Higher exposure to AIV is expected to occur in the most attractive sites for birds with different phenologies (resident, wintering and crossing migrants).

In this paper, we investigated these issues using data on anti-AIV antibodies in the plasma and eggs of yellow-legged gulls (*Larus michahellis*) breeding in the gulf of Gabès, in south-eastern Tunisia. This species nests in large colonies and is a predator and scavenger, both behaviors that could enhance disease transmission. Moreover, because it is a long-lived bird, the yellow-legged gull offers a suitable

model for investigating bird exposure to pathogens through antibody detection. In the gulf of Gabès, this bird species breeds in three distinct sites (Isenmann et al. 2005). One of these sites, namely the Sfax salina, is considered the most important bird area in Tunisia (Fishpool and Evans 2001, Isenmann et al. 2005) and has been listed as a "wetland of international importance" under the RAMSAR convention since 2007 (Ramsar Convention Secretariat 2007). It hosts an exceptional diversity of resident, wintering and spring migrant waterbirds (Chokri et al. 2008). The commercial exploitation of this salina has resulted in stable levels of water and food, as well as a constant protection against disturbance, which make it particularly attractive for waterbirds compared to natural wetlands in the same region. The remaining two breeding sites, namely Kneiss and Djerba islands, correspond to natural wetlands hosting relatively lower diversities of waterbirds.

Because the gulf of Gabès is at a migratory crossroad for palearctic birds moving between two epizootic areas of AIV, sub-Saharan Africa and Western Europe (Olsen et al. 2006, Gaidet et al. 2007, Jourdain et al. 2007), and given its importance as an overwintering area (Van dijk et al. 1986, Isenmann et al. 2005), it may be one of the most important high-risk zones in the Mediterranean; anti-AIV antibody prevalence in yellow-legged gulls breeding in this area is thus expected to be high overall. In particular, anti-AIV prevalence should be highest in the Sfax salina, as it is the most attractive site for waterbirds within this gulf and thus expected to be a hotspot of bird exposure.

We also investigated factors affecting the detection and occurrence of AIV antibodies in yellow-legged gull clutches. In particular, we test if the detection probability of antibodies in a given egg varies with its laying order and if the occurrence probability of antibodies in a given clutch varies with laying date. Antibody detection probability was hypothesised to vary with egg rank because of possible

differences in antibody quantity among eggs of the same clutch, due to earlier suggestions of differential antibody transmission rates from the mother to the eggs reported in other bird species (Groothuis et al. 2006, Hargitai et al. 2006). Moreover, given the fact that the laying date of females is likely related to their ages (older ones laying earlier; Sydeman et al. 1991) and could thus be related to their history of risk of exposure to virus, we predicted a decrease in antibody occurrence probability with laying date. The latter hypotheses were tested by considering eggs from the same nest as sampling replicates of the same subject (i.e., the nest) and by using a capture-recapture-like approach specifically conceived to estimate site occupancy rate by a given species from count replicates while accounting for the effects of various sampling and site parameters on both detection and occurrence probabilities (see Mackenzie et al. 2002, Mackenzie et al. 2003). The use of this approach has recently been expanded to various ecological and epidemiological fields, such as the estimation of parasite prevalence in host populations (e.g. Thompson 2007, Gomez-Diaz et al. 2010) and its application may prove especially useful in disease ecology (McClintock et al. 2009).

Methods

Study species

The yellow-legged gull is a colonial seabird of the family *Laridae*. It is long-lived and shows strong interannual breeding site fidelity (Cramp and Simons 1983). It lays one clutch of 1-3 eggs per season, although females can lay replacement clutches. As in other gull species (Sydeman et al. 1991), individuals that lay earlier in the season are often older/more experienced than individuals that lay later. In the Mediterranean region, the yellow-legged gull is a common and widespread species, where it is locally regarded as a pest and subject to population control measures (Bosch et al. 2000, Vidal et al. 1998, Oro

and Martinez-Abrain 2007). This species is an opportunistic feeder; it forages at sea but also predates upon other birds, eats earthworms, and feeds on refuse dumps (Ramos et al. 2009). As this bird is anthropophilic, often nesting in urban areas and feeding on trash dumps, it may be more likely to exchange pathogens with humans and domestic birds (Bonnedahl et al. 2009). Furthermore, its wide distribution and its feeding behavior make it an important species for monitoring the environment (Abdennadher et al. 2010).

Study area

Our work was conducted during the spring of 2009 in two yellow-legged gull breeding sites situated 100 km apart in the gulf of Gabès, in southeastern Tunisia (Fig. 1). The yellow-legged gull is a common and abundant resident in this area, where it is often seen feeding on open air rubbish dumps and discards of commercial fisheries. The sampled colonies are located on opposite sides of the gulf: Sfax salina to the north (34°42'28"N-10°45'02"E) and Djerba island (33°39'10"N-10°58'59"E) to the south (Fig. 1). Sfax salina extends 15 kilometers south from Sfax city, along the Mediterranean coast, and is bordered on the west by Sfax suburbs. It is formed by more than 200 ponds interspersed with artificial silty dykes that are used as breeding sites by a great diversity of waterbird species. In this salina, the yellow-legged gull breeds in two distinct subcolonies, composed of approximately 100 pairs each and situated 10 km apart. Due to logistical and time constraints, we only sampled the subcolony situated in the northern part of the salina close to Sfax city. The Djerba site consists of a small natural islet (of approximately 50 ha) partly covered by Salicornia tuffs and situated between Djerba island and Zarzis peninsula to the south. At the time of sampling, this colony was constituted of approximately 500 pairs of yellow-legged gulls. Other colonies were established on the neighboring islets, but could not be sampled for practical reasons.

Data collection

Egg and blood sampling

In order to estimate the prevalence of anti-AIV antibodies in the studied colonies, we sampled both adults and entire clutches of a given group of nests. In each study colony, randomly selected nests were marked with a small wooden pole placed nearby. The nests were then checked every 1-2 days and egg(s) were collected. Each egg was marked according to its rank and laying date and then replaced by a dummy egg to prevent females from abandoning their nests. The eggs were brought to the laboratory on the day of collection for processing. Collected eggs were thus 1 to 2 days old and their yolk composition was not yet affected by embryonic development. The yolk of each egg was separated from the albumen, homogenized and frozen at -20°C.

After clutch completion, we trapped as many of the incubating parents as possible by means of noose-carpet traps that we placed on the nests. Each captured bird was marked with a patch of paint on the head to avoid resampling. Upon capture, a 1-ml blood sample was taken from the brachial vein using a sterile syringe. We measured the head, bill, wing and tarsus length (±0.1 mm) using a caliper. We also determined body mass (±20 g) with a spring scale (PESOLA®, Switzerland) before releasing the bird. The blood sample was immediately transferred to heparinized tubes and maintained in a cooler while in the field (4°C). Once in the laboratory, it was centrifuged at 2500 rpm for 15 min. The plasma and blood cells were frozen separately at -20°C until immunological analyses could be performed. The sampled birds were sexed following the protocol described by Griffiths et al. (1998). DNA was extracted from blood using a DNeasy Blood & Tissue Handbook 07/2007 kit and used in PCR with primers 2550 Forward and 2718 Reverse to amplify introns from the CHD-*Z* and CHD-*W* genes (Fridolfsson and Ellegren 1999).

Immunological analyses

Plasma samples were used directly in the immunological assays. However, yolk antibodies first had to be extracted (Mohammed et al. 1986, Gasparini et al. 2001). Egg yolks were thawed and homogenized; a subsample of 800 mg was then taken. The yolk subsample was diluted 1:1 in phosphate-buffered saline (PBS) and the solution was vortexed after adding glass beads. An equal volume of reagent-grade chloroform was added to the mixture. The yolk chloroform mixture was then centrifuged at 16 000 rpm for 15 min and the clear supernatant was used in the immunological assays.

We detected anti-AIV antibodies in gull plasma and yolk using a commercial competitive enzyme-linked immunosorbent assay (ELISA) developed for use in birds (ID Screen® Antibody Influenza A Competition, IDVET, Montpellier, France). The assay is designed to detect antibodies directed against the internal nucleocapsid of the AIV and thus it will pick up all AIV subtypes. Plasma and egg yolk samples were diluted 1:100 and were incubated at 37°C (± 2°C) for one hour. After a washing step, a conjugate solution was added to each well, and incubated 30 min at 21°C (± 5°C). The plates were then washed again; the substrate solution was added to each well and was incubated 10 min at 21°C (± 5°C) in the dark. Finally, a stop solution was added to each well in order to stop the reaction. Optical density (OD) was read at 450 nm using an ELISA plate reader. A subset of samples was tested several times, both within and across plates. Repeatability tests showed that OD measurements were highly repeatable within ($r = 0.913$, $F_{[29,30]} = 22.84$, $P < 0.0001$) and across ($r = 0.85$, $F_{[33,24]} = 9.56$, $P < 0.0001$) plates.

According to the kit instructions, the results were expressed as the percentage competition (PC) between the OD of the sample being tested and the mean OD of a negative control sample (NC), such that $PC=(OD_{specimen}/OD_{NC})x100$. The percentage competition values were

then transformed into percentage inhibition values (PI) using the formula: PI=100-PC. According to kit instructions, plasma samples with a PI greater than or equal to 50 % are considered positive, those with a PI less than or equal to 45% are considered negative and those with a PI between 45% and 50% are considered as doubtful. Doubtful samples were excluded from the data in the statistical analyses. The same threshold of positivity was applied to the yolks, although the PI obtained for the eggs cannot be directly compared (in absolute terms) with those of the plasma as the ELISA was developed for bird plasma. However, we did verify that there was a strong correlation between the values obtained for eggs and that for the plasma of the female that laid these eggs (Hammouda et al. unpublished results).

Data analyses

First, plasma data were used to estimate the overall prevalence of anti-AIV antibodies in the study population (the proportion of positive birds relative to the total number sampled). We then compared antibody prevalence between the two colonies (Sfax *vs* Djerba) and between males and females by means of χ^2 tests. Within breeding pairs for which both adults were sampled, we tested if male and female serological status was related. This was done using a Fisher exact test (appropriate for small sample sizes). All statistical tests were carried out using SAS software (SAS 1998).

We used yolk data to estimate the occurrence of anti-AIV antibodies in the sampled nests. Our sample units were not the individual eggs but rather the nests. Given that imperfect antibody detection in the yolk samples could lead to biased estimates of antibody prevalence in the corresponding nests, we used an estimation approach that takes into account this detectability issue. We used the capture-recapture-like methodology developed by Mackenzie et al. (2002, 2003) and implemented in program PRESENCE (available for download from

http://www.proteus.co.nz/). It takes into account the possible effects of different parameters on both occurrence and detection probabilities by incorporating them as covariates in the estimations. Each nest was considered as a sampling unit and eggs from the same nest were considered as sampling replicates of the same subject. This provided us with a list of detection/non-detection of anti-AIV antibodies from each sampled nest (doubtful eggs were considered as lacking information). The obtained lists (one list per sampled nest) were then used to estimate the prevalence of anti-AIV antibodies in the studied nests and to investigate (1) the effect of laying rank on antibody detection probability in a given egg (p) and (2) the effects of both laying date and colony on antibody occurrence probability in a given nest (ψ).

We employed a two-step process in our analyses. We first selected the best model for detectability by holding occurrence probability constant ($\psi(.)$) and evaluating two models: 1) one that assumed antibody detectability was constant within a nest ($p(.)$) and 2) one that assumed that antibody detectability varied within a nest according to egg rank (p(Rank)). In a second step, we used the best detectability model to estimate antibody occurrence probability. We defined a set of *a priori* models we believed might explain antibody occurrence probability in our nests. The simplest model assumed that antibody occurrence probability was constant throughout the nests ($\psi(.)$). We then developed candidate models accounting for the possible effects of colony (Sfax *vs* Djerba) and laying date. Given that the laying period extended over two weeks, we classified the nests as early or late (early = laid the first week *vs* late = laid the second week). The competing models were ranked according to their Akaike's Information Criterion (AIC), and Akaike weights were applied to estimate the relative support of the models from the data (Burnham and Anderson 2002). In order to account for model selection uncertainty (Burnham and Anderson 2002, Wintle et al. 2003), we used a model averaging

technique for estimating covariate effects (averaged β) and their 95% confidence intervals (Burnham and Anderson 2002). The relationship between a given covariate and antibody occurrence probability was significant if the estimated 95% confidence interval of the corresponding averaged β included zero. We also computed odds ratios so as to interpret the effects of covariates on antibody occurrence.

Results

A total of 64 birds were captured and sampled across our two study colonies: 22 birds from Sfax (16 females and 6 males) and 42 birds from Djerba (20 females and 22 males). Only two birds were of doubtful serostatus and excluded from the analyses (one Sfax male and one Djerba male). Anti-AIV antibody prevalence in plasma was 77% (48 birds out of 62). It was not related to bird sex (females: 81 %; males: 73%; $\chi^2_{[1]} = 0.4830$, $P = 0.4871$). It did, however, differ significantly with location; prevalence was higher at Sfax (95%) than Djerba (68%) ($\chi^2_{[1]} = 5.7676$, $P = 0.0163$). There was no association of antibody status within breeding pairs (Fisher's test: $P = 0.2727$, n = 11).

We sampled a total of 135 nests, corresponding to a total of 333 eggs (45 nests and 127 eggs at Sfax and 90 nests and 206 eggs at Djerba). Clutch size ranged from 1 to 3 eggs, with a modal clutch size of 3 eggs in both colonies (86% at Sfax and 53% at Djerba). Fifteen eggs were classified as having doubtful serostatus and were excluded from the analyses (5 Sfax eggs and 10 Djerba eggs). The total number of nests with at least one positive egg was 75 (32 nests in Sfax and 43 nests in Djerba), which represents a naïve antibody prevalence of 56 % (71% at Sfax and 53% at Djerba).

In our first step of capture-recapture model selection, we found that both detectability models showed a similar fit to the data (ΔAIC less than 2) and a relatively high antibody detection probability; it did not matter if antibody detectability was assumed to be constant or if it varied with egg rank (Table 1). In the constant detectability model, the estimated detection probability of antibodies in a given nest was 0.9015 (95% CI: 0.8467 - 0.9381). When egg rank was included as a covariate, antibody detection probability tended to decrease with egg rank, but the trend was not significant (β±SE = -0.23±0.32; Table2).

The constant detectability model was used for modeling antibody occurrence in nests. Our results gave less support to the constant occurrence model (ψ(.)), as it was the last ranked one (lowest weight) (Table 3). Conversely, the model including both colony and laying date as nest covariates was the most supported model, with an Akaike weight of 72% (Table 3). Using the model averaging technique, we found that the occurrence probability of antibodies in a given nest differed significantly between the two colonies and also according to laying date. The 95% CI associated with the β estimates of both variables did not overlap 0 (Table 4). The occurrence probability of antibodies in a given nest was almost 3 times lower in Djerba compared to Sfax (odds ratio = 0.36) and almost twice as high in early clutches compared to late ones (odds ratio = 1.84). These results suggest a higher circulation of AIV in the Sfax colony compared to the Djerba colony, which is consistent with the results obtained from the bird plasma data. They also support the fact that early-laying females, which are likely to be older on average, may have had a longer time of risk of exposure to the virus than later-laying ones. Overall, the estimated antibody prevalence varied between 35% (late clutches in Djerba) to 79% (early clutches in Sfax) (Table 5).

Discussion

We found a high prevalence of detectable antibodies against AIV in the two yellow-legged gull colonies we sampled, which underlines the potential importance of the yellow-legged gull in the epidemiology of AIV. This also confirms that the monitoring of antibodies against AIV in the eggs can provide an easy and powerful tool for monitoring AIV circulating in wild birds (see Trampel et al. 2006, Pearce-Duvet et al. 2009). The prevalence of anti-AIV antibodies found in the two yellow-legged gull colonies was relatively higher than those commonly reported in other bird studies (e.g., Olsen et al. 2006). The observed difference may be due to differences in the species sampled and specificities of the laboratory diagnostic used (Alexander 2003, De Marco et al. 2003, Sala et al. 2003). As gull species are relatively long lived (e.g., annual survival of adults > 0.8), it is not unexpected that a high proportion of the population shows signs of having been exposed to AIV if anti-AIV antibodies persist over long periods in these species. A recent study on ringed-billed gull (*Larus delawarensis*) in Southern Ontario, Canada, also reported a high seroprevalence among breeding individuals (Velarde et al. 2010).

The yellow-legged gull nests in dense colonies and can feed on open air rubbish dumps. The aggregation of individuals at nesting and feeding sites is likely to lead to high rates of contact between individuals (McCallum et al. 2001), which could facilitate the transmission of AIV between individuals and lead to a high prevalence of viruses in the population (Bin Muzaffar et al. 2006). Further investigations of the aggregative behavior and interactions among birds at their feeding and nesting sites would tell us more about this issue (Munster and Fouchier 2009). Interestingly, our results show that antibody prevalence in the eggs of yellow-legged gulls nesting in the gulf of Gabès was higher than that reported for the same species nesting

in the northern border of the Mediterranean (Pearce-Duvet et al. 2009). However, such a difference could simply be due to possible differences in the competitive ELISA tests used and the threshold used for positive values. Both kits provided highly correlated results in terms of relative amounts of detected antibodies, but the ID Vet ELISA kit used in our study can detect more positive samples (Pearce-Duvet pers. comm.).

Our results also show that antibody prevalence did not differ between sexes. In the yellow-legged gull, males and females incubate eggs, rear offspring and frequent the same feeding areas, and thus likely share the same risk of exposure to AIV. In contrast, antibody prevalence in plasma and also in nests was significantly higher in Sfax than in Djerba. This result gives support to our main prediction and could be interpreted as a possible consequence of the great difference between the two sites in terms of the diversity and density of their avifaunas. Sfax salina is considered as one of the most important bird areas in Africa (Fishpool and Evans 2001). It hosts the most rich and dense community of wintering and nesting waterbirds in Tunisia (Chokri et al. 2008). Over 30,000 birds belonging to 49 migrant species and 8,500 birds from 9 resident and breeding species are known to regularly inhabit this area and to share the same feeding and/or breeding sites every year (Chokri et al. 2008). During our field work, we clearly noticed a greater waterbird density and diversity in Sfax salina compared to Djerba islets. In the latter area, the yellow-legged gull shares the breeding sites with a small colony of Little egrets (*Egretta garzetta*). The higher concentration of nesting and migratory waterbirds in the Sfax salina compared to Djerba islets is likely to result in higher frequencies of contacts, and, hence, to higher transmission rates of AIV among individuals and among species (Olsen et al. 2006, Munster and Fouchier 2009).

The difference in antibody prevalence between Sfax and Djerba's colonies could notably be explained by the variety of AIV

subtypes that are circulating in each site (Munster and Fouchier 2009). The role of migratory waterbirds as main reservoir and carrier for AIV of several subtypes has been well known for a long time (Alexander 2003, De Marco et al. 2003). Sfax salina being an important stopover site lying on the cross-road of many migratory routes of waterbirds, viruses subtypes exchanges between species could be responsible for a higher prevalence of infected birds in Sfax colony than in Djerba's. Furthermore, in Sfax salina, the large populations of waterbirds may be more capable of sustaining a large variety of different AIV subtypes. Conversely, the smaller population sizes near Djerba might limit the maintenance of multiple AIV subtypes and allow only a limited number of AIV subtypes to co-circulate within these populations. Further exploration of the prevalence of antibodies against different sub-types of AIV could thus be revealing.

The capture-recapture approach we used allowed us to ensure that the detection probability of antibodies did not differ among eggs from the same clutch, although there was a tendency for decreasing detectability with increasing egg rank. This result would suggest that the sampling of only one egg per nest, of whatever rank, is efficient to provide unbiased results regarding the prevalence of antibodies in the nest population. Furthermore, the approach we used allowed us to indirectly assess the relevance of female age as a predictor of the occurrence probability of anti-AIV antibodies in its nest. Our results support our prediction and would suggest that in the two studied colonies, the older is the female (i.e., the earlier the laying date), the higher is the occurrence probability of anti-AIV antibodies in her nest. Older females are likely to have been exposed to the infection for a longer time than the younger ones.

Mounting and maintaining an immune response is assumed to be energetically and nutritionally costly (Sheldon and Verhulst 1996). Consequently, to boost their immune system, birds have to reallocate

some of their resources from other demanding functions, such as growth, development, molting and reproduction (Ilmonen et al. 2000). The immune system may be adversely affected when such reallocation of energy or nutritional conditions are not ideal. In this context, AIV prevalence may be affected by different factors such as food supply, the age and the sex structure in a colony and individual immunocompetence (Weber and Stilianakis 2007) and it should be possible to investigate such processes by monitoring the incidence of exposure in the young birds of the population.

In conclusion, our study suggests that the yellow-legged gull has the potential to play a major role in the epidemiology of AIV. It also suggests that even at the relatively small geographic scale of the studied area, there can be important variation in the level of exposure of individuals. We hypothesize that this spatial variation is related to differences in avifaunal density and diversity between the two studied breeding sites. Further investigations of the ecology of the yellow-legged gull and its interactions with sympatric waterbird species on series of comparable sites are needed to test our predictions.

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References

Abdennadher, A, Ramírez, F, Romdhane, MS, Ruiz, X, et al. Biomonitoring of coastal areas in Tunisia: Stable isotope and trace element analysis in the Yellow-legged Gull. Mar Pollut Bull 2010; 60: 440-447.

Alexander, DJ. Report on avian influenza in the eastern hemisphere during 1997-2002. Avian Dis 2003; 47: 792-797.

Bin Muzaffar, S, Ydenberg, RC, Jones, IL. Avian Influenza: An Ecological and Evolutionary Perspective for Waterbird Scientists. Waterbirds 2006; 29: 243-257

Bonnedahl, J, Drobni, M, Gauthier-Clerc, M, Hernandez, J, et al. Dissemination of Escherichia coli with CTX-M Type ESBL between Humans and Yellow-Legged Gulls in the South of France. PLoS ONE 2009; 4: e5958

Bosch, M, Oro, D, Cantos, FJ, Zabala, M. Short-term effects of culling on the ecology and population dynamics of the Yellow-legged Gull. J Appl Ecol 2000; 37: 369-385.

Boulinier, T, Staszewski, V. Maternal transfer of antibodies: raising immuno-ecology issues. Trends Ecol Evol 2008; 23: 282-290.

Brown, JD, Stallknecht, DE, Berghaus, RD, Luttrell, MP, et al. Evaluation of a commercial blocking enzyme-linked immunosorbent assay to detect Avian Influenza Virus antibodies in multiple experimentally infected avian species. Clin Vaccine Immunol 2009; 16: 824-829

Burnham, KP, Anderson, DR. Model selection and multimodel inference: a practical information-theoretic approach. Springer Verg, Berlin, Germany 2002.

Chokri, MA, Sadoul, N, Medhioub, K, Béchet, A. Analyse comparative de la richesse avifaunistique du salin de Sfax dans

le contexte tunisien et méditerranéen. Rev Ecol Terre Vie 2008; 63:4.

Cramp, S, Simmons, KEL. Handbook of the birds of Europe, the Middle East and the North Africa. Oxford: Oxford University Press 1983.

De Marco, MA, Fonic, E, Campitelli, L, Raffini, E, et al. Circulation of influenza viruses in wild waterfowl wintering in Italy during the 1993–1999 period: evidence of virus shedding and sero-conservation in wild ducks. Avian Dis 2003; 47: 861-866.

De Marco, MA, Campitelli, L, Foni, E, Raffini, E, et al. Influenza surveillance in birds in Italian wetlands (1992–1998): is there a host restricted circulation of influenza viruses in sympatric ducks and coots? Vet Microbiol 2004; 98: 197-208.

De Wit, E, Fouchier, RAM. Emerging influenza. J Clin Virol 2008; 41: 1-6

Fereidouni, SR, Grund, C, Häuslaigner, R, Lange, E, et al. Dynamics of Specific Antibody Responses Induced in Mallards After Infection by or Immunization with Low Pathogenicity Avian Influenza Viruses. Avian Dis 2010; 54: 79-85.

Fishpool, LDC, Evans MI (Eds). Important bird areas in Africa and associated islands: Priority for conservation. 2001; Newbury and Cambridge, UK: Pisces Publications and Birdlife international (Birdlife Conservation Series No. 11).

Fouchier, RAM, Munster, V, Wallensten, A, Bestebroer, TM, et al. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol 2005; 79: 2814-2822.

Fridolfsson, AK, Ellegren H. A simple and universal method for molecular sexing of non-ratite birds. J Avian Biol 1999; 30:116-121.

Gaidet, N, Dodman, T, Caron, A, Balança, G, et al. Avian Influenza Viruses in Water Birds, Africa. Emerg Infect Dis. 2007; 13: 626-629.

Gasparini, J, McCoy, KD, Haussy, C, Tveraa, T, et al. Induced maternal response to the Lyme disease spirochaete Borrelia burgdorferi sensu lato in a colonial seabird, the kittiwake Rissa tridactyla. P Roy Soc Lond B Bio 2001; 268: 647-650.

Gilbert, M, Xiao, X, Domenech, J, Lubroth, J, et al. Anatidae Migration in the Western Palearctic and Spread of Highly Pathogenic Avian Influenza H5N1 Virus. Emerg Infect Dis 2006; 13: 1650-1656.

Gómez-Díaz, E, Doherty, PF, Duneau, D, McCoy, KD. Cryptic vector divergence masks vector-specific patterns of infection: an example from the marine cycle of Lyme borreliosis. Evolutionary Applications 2010; 3: 391-401.

Griffiths, R, Double, MC, Orr K, Dawson, RJG. A DNA test to sex most birds. Mol Ecol 1998; 7: 1071-1075.

Grindstaff, JL, Brodie, ED III, Ketterson, ED. Immune function across generations: integrating mechanism and evolutionary process in maternal antibody transmission. P Roy Soc Lond B Bio 2003; 270: 2309-2319.

Groothuis, TGG, Eising, CM, Blount, JD, Surai, P, et al. Multiple pathways of maternal effects in black-headed gull eggs: constraint and adaptive compensatory adjustment. J Evolution Biol 2006; 19: 1304-1313.

Hargitai, R, Prechl, J, Torok, J. Maternal immunoglobulin concentration in Collared Flycatcher (Ficedula albicollis) eggs in relation to parental quality and laying order. Funct Ecol 2006; 20: 829-838.

Ilmonen, P, Taarna, T, Hasselquist, D. Experimentally activated immune defence in female pied flycatchers results in reduced breeding success. P Roy Soc Lond B Bio 2000; 267: 665-670.

Isenmann, P, Thierry, G, Hili, A, Azafzaf, H, et al. (Eds). Oiseaux de Tunisie. Société d'Études Ornithologiques de France, Paris, France 2005.

Jourdain, E, Gauthier-Clerc, M, Bicout, DJ, Sabatier, P. Bird migration routes and pathogen dispersion risk in western Mediterranean wetlands. Emerg Infect Dis 2007; 13: 365-372

Lebarbenchon, C, Chang, C-M, Vander Werf, S, Aubin, J-T, et al. Influenza A Virus in Birds during Spring Migration in the Camargue, France. J Wildlife Dis 2007a; 43: 789-793

Lebarbenchon, C, Vander Werf, S, Thomas, F, Aubin, J-T, et al. Absence of detection of highly pathogenic H5N1 in migratory waterfowl in southern France in 2005-2006. Infect Genet Evol 2007b; 7: 604-608.

Lebarbenchon, C, Chang CM, Grandhomme, V, Dietrich, M, et al. Avian Influenza circulation in the Camargue (south of France) during the 2006-07 season. Avian Dis 2010; 54: 446-449.

Mackenzie, DI, Nichols, JD, Lachman, GB, Droege, S, et al. Estimating site occupancy rates when detection probabilities are less than one. Ecology **2**002; 83: 2248-2255.

Mackenzie, DI, Nichols, JD, Hines, JE, Knutson, MG, et al. Estimating site occupancy, colonisation, and local extinction when a species is detected imperfectly. Ecology 2003; 84: 2200-2207.

McCallum, H, Barlow, N, Hone, J. How should pathogen transmission be modelled? Trends Ecol Evol 2001; 16: 295-300.

McClintock, BT, Nichols, JD, Bailey, LL, MacKenzie, DI, et al. Seeking a second opinion: uncertainty in disease ecology. Ecol Lett 2009; 13: 659-674.

Mohammed, HO, Yamamoto, R, Carpenter, TE, Ortmayer, HB. Comparison of egg yolk and serum for the detection of Mycoplasma gallisepticum and M. synoviae antibodies by enzyme-linked immunosorbent assays. Avian Dis 1986; 30: 398-408.

Munster, VJ, Fouchier, RAM. Avian influenza virus: of virus and bird ecology. Vaccine 2009; 27: 6340-6344.

Olsen, B, Munster, VJ, Wallensten, A, Waldenstrom, J, et al. Global patterns of influenza A virus in wild birds. Science 2006; 312: 384-388.

Oro, D, Martinez-Abrain, A. Deconstructing myths on large gulls and their impact on threatened sympatric waterbirds. Anim Conserv 2007; 10: 117-126.

Pearce-Duvet, J, Gauthier-Clerc, M, Jourdain, E, Boulinier, T. Maternal antibody transfer in Yellow-legged gulls. Emerg Infect Dis 2009; 15: 1148-1149.

Perez-Ramirez, E, Rodriguez, V, Sommer, D, Blanco, JM, et al. Serologic Testing for Avian Influenza Viruses in Wild Birds: Comparison of Two Commercial Competition Enzyme-Linked Immunosorbent Assays. Avian Dis 2010; 54: 729-733.

Ramos, R, Ramirez, F, Sanpera, C, Jover, L, et al. Diet of Yellow-legged Gull (Larus michahellis) chicks along the Spanish Western Mediterranean coast: the relevance of refuse dumps. J Ornithol 2009; 150: 265-272

Roche, R, Lebarbenchon, C, Gauthier-Clerc, M, Chang, C-M, et al. Water-borne transmission drives avian influenza dynamics in wild birds: The case of the 2005–2006 epidemics in the Camargue area. Infect Genet Evol 2009; 9: 800-805

Sala, G, Cordioli, P, Moreno-Martin, A, Tollis, M, et al. ELISA Test for the Detection of Influenza H7 Antibodies in Avian Sera. Avian Dis 2003; 47:1057-1059.

SAS Statistical Institute (Ed). SAS/STAT user's guide, version 8. SAS Statistical Institute, Cary, NC. USA 1998.

Sheldon, BC, Verhulst, S. Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. Trends Ecol Evol 1996; 11: 317-321.

Sydeman, WJ, Penniman, JF, Penniman, TM, Pyle, P, et al. Breeding performance in the Western gull – Effects of parental age, timing of breeding and year in relation to food availability. J Anim Ecol 1991; 60: 135-149.

Thompson, KJ. Use of site occupancy models to estimate prevalence of Myxobolus cerebralis infection in Trout. Aquat Anim Health 2007; 19: 8-13.

Trampel, DW, Zhou, EM, Yoon, KJ, Koehler, KJ. Detection of antibodies in serum and egg yolk following infection of chickens with an H6N2 avian influenza virus. J Vet Diagn Invest 2006; 18: 437-442.

Van Dijk, AJ, Van Dijk, K, Dijksen, LJ, Van Spanje, TM, et al. (Eds). Wintering Waders and Waterfowl in the Gulf of Gabès, Tunisia, January–March 1984. Working Group on International Waderbird and Wetland Research (WIWO), Zeist, The Netherlands 1986.

Velarde, R, Calvin, SE, Ojkic, D, Barker, IK et al. Avian Influenza Virus H13 circulating in Ring-billed Gulls (Larus delawarensis) in Southern Ontario, Canada. Avian Dis 2010; 54: 411-419.

Vidal, E, Médail, F, Tatoni, T. Is the Yellow-legged Gull a superabundant species in the Mediterranean? Impact on fauna and flora, conservation measures and research priorities. Biodivers Conserv 1998; 7: 1013-1026.

Wallensten, A, Munster VJ, Latorre-Margalef N, Brytting M, et al. Surveillance of Influenza A Virus in Migratory Waterfowl in Northern Europe. Emerg Infect Dis**.** 2007; 13: 404-411.

Weber, TP, Stilianakis, NI. Ecologic Immunology of Avian Influenza (H5N1) in Migratory Birds. Emerg Infect Dis 2007; 13: 1139-1143.

Webster, RG, Bean, WJ, Gorman, OT, Chambers, TM et al. Evolution and ecology of influenza A viruses. Microbiol Rev 1992; 56:152-79.

Webster, RG, Peiris, M, Chen, H, Guan, Y. H5N1 outbreaks and enzootic influenza. Emerg Infect Dis 2006; 12: 3-8.

Wintle, BA, McCarthy, MA, Volinsky, CT, Kavanagh, RP. The use of Bayesian model averaging to better represent uncertainty in ecological models. Conserv Biol 2003; 17: 1579-1590

Table 1. Summary of the numbers of collected samples and the obtained results.

		Sfax colony	Djerba colony	Total
Blood samples	Number of sampled birds	22	42	64
	Males	6	22	28
	Females	16	20	36
	Range of PI (%)	29-95	00-98	00-98
	Males	45-95	00-97	00-97
	Females	29-95	08-98	08-98
	Number of positive birds	20	28	48
	Males	5	14	19
	Females	15	14	29
	Number of doubtful birds	1	1	2
	Males	1	1	2
	Females	0	0	0
Egg samples	Number of sampled eggs	127	206	333
	Number of sampled nests	45	90	135
	Range of PI (%)	47-100	32-98	32-100
	Number of positive eggs	84	87	171
	Number of doubtful eggs	5	10	15
	Number of positive nests	32	43	75
	Number of doubtful nests	0	2	2

Table 2. Summary of AIC model selection for antibody occurrence probability in the studied nests.

Model	K	AIC	ΔAIC	W
ψ(Colony,Laying date),p(.)	4	294.20	0.00	0.7213
ψ(Colony),p(.)	3	296.64	2.44	0.2129
ψ(Laying date),p(.)	3	300.06	5.86	0.0385
ψ(.),p(.)	2	300.75	6.55	0.0273

Table 3. Parameter estimates for variables explaining variation in antibody occurrence probability in the studied nests.

		Averaged β		**Odds ratio**	
Covariate	**Relative weight**	**Estimate**	**95% CI**	**Estimate**	**95% CI**
Colony (Djerba = 1 *vs* Sfax = 0)	0.9342	-1.01	[-1.76 ; -0.26]	0.36	[0.17 ; 0.77]
Laying date (Early = 1 *vs* Late = 0)	0.7598	0.61	[0.03 ; 1.19]	1.84	[1.03 ; 3.28]

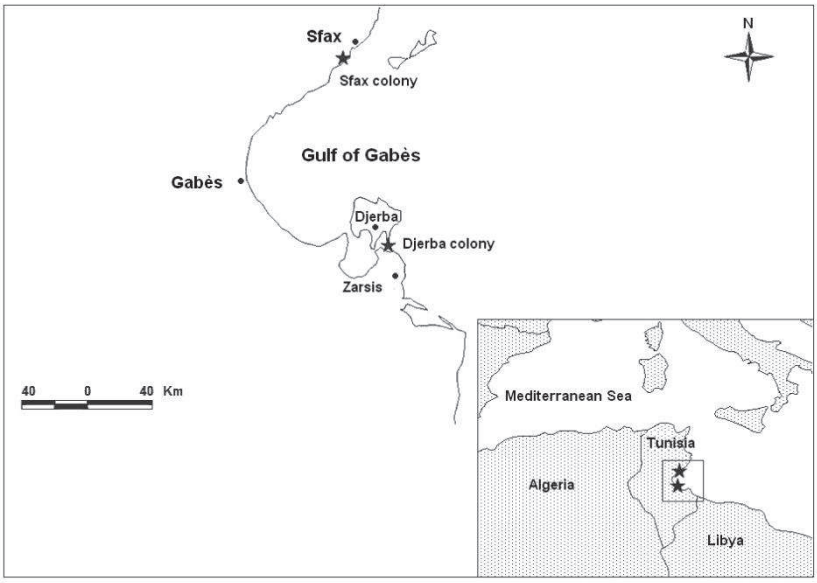
Table 4. Estimated antibody occurrence probabilities and 95% confidence intervals in early and late nests in the two studied colonies. The estimates were obtained under model ψ(Colony,Laying date),p(.).

	Djerba colony		Sfax colony	
	ψ estimate	**95% CI**	**ψ estimate**	**95% CI**
Early laying date	0.5515	[0.4304 ; 0.6668]	0.7898	[0.6314 ; 0.8918]
Late laying date	0.3534	[0.2158 ; 0.5204]	0.6254	[0.4429 ; 0.7781]

Figure legend

Figure Map of the gulf of Gabès showing the location of the two studied colonies.

Figure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Libya

Article 3

Counting eggs before they hatch: Yolk antibodies reveal avian influenza prevalence patterns in Mediterranea

Jessica Pearce-Duvet, Audrey Arnal, Slaheddine Selmi, Abdessalem Hammouda, Jacob Gonzalez-Solis, Wided Alioua, Samraoui Boudjéma, Romain Garnier, Michel Gauthier-Clerc and Thierry Boulinier

In preparation

Abstract

The monitoring of emerging diseases is important in the characterization of host-pathogen dynamics in natural wildlife populations, but it is not always easy to accomplish. Avian influenza viruses (AIVs) are an example of emerging infectious pathogens of concern that need continual surveillance. Maternal antibody transfer may be useful in monitoring AIVs, as well other emergent diseases. Using Mediterranean yellow-legged gulls as our study system, we determined if eggs can serve as proxies for adults in estimating pathogen prevalence and reveal information about avian influenza ecology. In the spring of 2009, we first sampled blood and eggs from adult females in two colonies in Tunisia. Second, we collected eggs from an additional eight yellow-legged gull colonies along the Mediterranean coast (France, Spain, and Algeria) to examine prevalence patterns. The levels of anti-AIV antibodies found in a female's plasma correlated with those found in the yolks of her eggs; the relationship allowed us to establish a separate ELISA positivity threshold for the egg yolks (70%). Antibody prevalence was heterogenous across colonies, suggesting AIV exposure varies according to location. Egg volume was not correlated with antibody status, suggesting female quality and/or age does not explain this pattern. Nor was there much spatial autocorrelation of status within colonies (i.e. positive or negative eggs were not clustered together). In contrast, prevalence was higher in both small and large colonies. Taken together, these results suggest that AIV exposure is linked to factors outside the colony and linked to colony size; foraging behavior, such as trash dump feeding, should be examined further. We propose that egg sampling may be incorporated into surveillance efforts as an easy and less expensive means of a) revealing pathogen "hot spots" worthy of further characterization b) divulging valuable information about host-pathogen ecology.

Keywords: Avian influenza, maternal antibody transfer, serology, surveillance, yellow-legged gull

Introduction

Over the past fifty years, the number of plant and animals disease emerging in natural populations has increased Daszak *et al.* 2000; Jones *et al.* 2008. This increase is not simply due to differences in reporting patterns over time Jones *et al.* 2008 but more likely represents a shift in ecological conditions Woolhouse 2002, resulting in a pathogen either increasing in incidence in its host species or infecting novel hosts Woolhouse *et al.* 2001; Woolhouse *et al.* 2005. Many diseases classified as emerging come from pathogens with long antecedents in natural reservoirs. While the monitoring of emerging diseases is important in the characterization of host-pathogen dynamics in natural wildlife populations Grenfell *et al.* 1995; Hudson *et al.* 2002, it is not always easily accomplished.

Avian influenza is an example of an emerging infectious disease of concern. AIV first emerged in domestic poultry in Asia in 1996 Xu *et al.* 1999 and has since shown the ability to jump from its poultry hosts to wild birds as well as to wild and domestic mammals, including humans, causing significant ensuing mortality Olsen *et al.* 2006; Gauthier-Clerc *et al.* 2007. Wild birds may contribute to the emergence of highly pathogenic strains because they are the hosts of a variety of low pathogenic (LP) strains Alexander 2000; Alexander 2007; Fouchier *et al.* 2007 that they transfer to domestic poultry Boyce *et al.* 2008, in whom the strains transform to high pathogenic (HP) subtypes, likely because of dense living conditions Gauthier-Clerc *et al.* 2007. Indeed, all known subtypes of influenza A have been found in birds and most appear to circulate asymptomatically Olsen *et al.* 2006. Anseriformes (ducks, geese, and swans) and Charadriiformes (gulls, terns, and waders) have been identified as the main reservoirs of LP strains and their viral subtypes appear to be genetically distinct Kawaoka *et al.* 1988; Stallknecht *et al.* 1988; Stallknecht *et al.* 2007; however, natural

transmission cycles in these groups remain to be explored Caron *et al.* 2009.

Monitoring efforts are a crucial part of revealing the ecological epidemiology of avian influenza and studies continue to call for improved surveillance e.g., Hoye *et al.* 2010; Knight-Jones *et al.* 2010; Krauss *et al.* 2010; Lee *et al.* 2010. Currently, surveillance efforts most often involve sampling via cloacal and respiratory tract swabbing and then testing for virus presence using RT-PCR. While this approach yields quality information about actual infection and immediate risk, it is nevertheless costly and time-consuming for the limited epidemiological information acquired Caron *et al.* 2009; for instance, intensive sampling efforts may reveal little to no viral prevalence e.g., Haynes *et al.* 2009; Pannwitz *et al.* 2009; Lebarbenchon *et al.* 2010; Ip *et al.* 2012. This result is not surprising given that animals may not be actively infected for very long. Because the antibody response remains even after infection clears, serological surveys are as a useful complement to approaches that emphasize virus detection and are increasingly being used Haynes *et al.* 2009; Marché *et al.* 2010; Han *et al.* 2012; Redig *et al.* 2012. Furthermore, samples are most often acquired from adult birds, which is not a trivial process. Wild birds are difficult to capture and susceptible to significant stress as a result Arnold *et al.* 2008.

We propose that maternal effects and, specifically, maternal antibody transfer may provide a useful tool in avian influenza monitoring efforts. Maternal effects are an adaptive response in which mothers can selectively tailor the embryonic environment so as to enhance growth and immunological protection in their offspring relative to perceived risk Mousseau *et al.* 1998; Grindstaff *et al.* 2003; Boulinier *et al.* 2008. In bird species, adult females that have been exposed to pathogens will mount an immune response and the antibodies produced as a consequence will be transferred to their young, in the yolk of the

eggs they produce, a pattern that has been observed in avian host-pathogen systems in nature e.g., Gasparini *et al.* 2002; Gibbs *et al.* 2005; Gasparini *et al.* 2006; Hammouda *et al.* 2011 as well as in domestic species Beck *et al.* 2003; Trampel *et al.* 2006; Gharaibeh *et al.* 2008; Rutten *et al.* 2012; Sa e Silva *et al.* 2012. By using eggs as a proxy for adult exposure, prevalence patterns within numerous populations and/or species could be ascertained rapidly and at reduced cost, and locations with high levels of exposure could then be targeted for more in-depth sampling Rutten *et al.* 2012. Given that environmental transmission has recently been recognized as playing a role in epidemics Bradley *et al.* 2008; Rohani *et al.* 2009, egg sampling could also help identify pathogen hot spots, in which ecological conditions, whether behavioral or abiotic, promote pathogen transmission.

We used yellow-legged gulls (*Larus michahellis*) as a model for studying the feasibility of characterizing avian influenza exposure in natural populations using egg sampling. This gull species is infected with at least two unique LP subtypes that have not been found in any other bird species Fouchier *et al.* 2007 and do not readily infect ducks (following experimental infection), even though ducks sustainably host a range of strains Hinshaw *et al.* 1985; Fouchier *et al.* 2005. This pattern suggests co-evolution between host and pathogen Fouchier *et al.* 2005 and hosts would demonstrate corresponding immunological responses Pearce-Duvet 2009; Hammouda *et al.* 2011; Hammouda *et al.* 2012. Lab studies suggest LP strains may cause minor clinical effects Jourdain *et al.* 2010, and the latter may translate into the detrimental, costly effects observed when hosts are in their natural environment van Gils *et al.* 2007; Latorre-Margalef *et al.* 2009. Thus infection may incur a real ecological cost that organisms may mediate via an adaptive immunological response.

Low pathogenic viruses in yellow-legged gulls are an ideal system not only because they should provide an excellent source of observational data but also because they potentially involve human epidemiological issues. *L. michahellis* is an extremely abundant species whose historical numbers have increased dramatically in response to the anthropogenic modifications of the environment, especially the presence of trash dumps Bosch *et al.* 1994; Duhem *et al.* 2007; Duhem *et al.* 2008. Additionally, because of their association with humans, their colonies are often located in close proximity to urban areas, leading to their classification as a "nuisance" bird Belant 1997. They are opportunistic omnivores and will eat other bird species Oro *et al.* 1994, both alive and dead, increasing the probability of pathogen infection. Overall, the yellow-legged gull represents a pathogen reservoir of significant concern, by being a numerical abundant species at high risk of infection and highly affiliated with human populations, yet little to nothing is known of its transmission dynamics Lebarbenchon 2008; Roche *et al.* 2009; Lebarbenchon *et al.* 2010.

Using the gull-avian influenza study system, we wished to determine the utility of egg sampling to disease surveillance programs as well as gather some initial data on avian influenza ecology in yellow-legged gulls. First, we determined whether there was a strong and reliable correlation between antibody levels in maternal sera and their egg yolks. Second, we characterized spatial patterns of influenza exposure using eggs gathered from across the Mediterranean coast. We looked at both broad-scale patterns, by asking if antibody prevalence was heterogeneous across colonies and examining the relationship between colony prevalence, egg volume, and size, and local patterns, by determining if egg serostatus was spatially correlated within a subsample of colonies.

Methods

Data Collection

We conducted two complementary sampling efforts in the spring of 2009. First, we concurrently collected eggs and blood samples from gulls in the context of study aimed at clarifying evolutionary ecology of yellow-legged gulls see Hammouda *et al.* 2011; Hammouda *et al.* 2012 for more details. These data were used to determine the correlation between adult serum and egg yolk antibody levels. Sampling took place in the gulf of Gabès, south-eastern Tunisia, specifically in the Sfax salina and the small islets between Djerba island and the Zarzis peninsula (Table 1). Randomly selected nests from these colonies were marked with stakes and checked every 1-2 days for egg collection. Multiple eggs were collected per nest to verify that eggs from the same nest had correlated antibody levels Hammouda *et al.* 2011. The eggs we collected were replaced by dummies to prevent nest abandonment and immediately returned to the laboratory for processing (see below). Adults were trapped once their clutches were completed. We captured as many of the incubating parents as possible using noose-carpet traps placed on the nests. A 1-ml blood sample was taken from the brachial vein of each captive adult using a sterile syringe, and the bird's head was marked with paint to avoid resampling. The blood sample was immediately transferred to a heparinized tube and maintained in a cooler at 4°C while in the field.

Second, we sampled eggs from a wide diversity of yellow-legged gull colonies along the Mediterranean coast to characterize spatial patterns of influenza exposure. In addition to the two colonies in Tunisia, eight colonies were located in France, three colonies in Spain, and one colony in Algeria (Table 1). Whenever possible, we determined the approximate number of breeding pairs per colony (rounded to the nearest 500) by examining the literature and contacting researchers or

government officials who study the associated yellow-legged gull populations (Table 2). In three of the French colonies (Riou Islands, Villeneuve-Lès-Maguelones, and Corrège), nest spatial locations were determined using GPS in order to test for spatial autocorrelation within colonies. The Riou Islands consisted of two separate subcolonies, Plane and Riou, each located on a different islet.

Eggs were collected by sampling along transects, with the number of transects and their length depending on the spatial configuration of the colony. The observers walking the transects collected one egg from any nests they perceived; antibody levels in a nest can be reliable predicted from a single egg Hammouda *et al.* 2011. In smaller colonies, transects traversed the whole colony and a majority of nests were sampled. For larger colonies, transects were performed within a randomly chosen subsection of the colony. At least 50 eggs were collected from each site with the exception of Medes and Sfax, where sample size was limited by small colony sizes and sampling constraints. The freshness of the eggs was determined in the field using a float test; we preferentially collected the freshest egg in a nest to control as much as possible for differences in laying date. We limited the time spent on the colony so as to not overly disrupt the incubation process. However, at many of these sampling locations, eggs were collected immediately prior to governmental sterilization efforts aimed at reducing yellow-legged gull population size.

Laboratory Analyses

Upon our return from the field, blood samples were centrifuged at 2500 rpm for 15 min, generating plasma and blood cells. For the eggs, we noted length and width and then separated out the yolk from the albumen. Egg volume, a potential correlate of parental quality

and/or environmental conditions, was calculated Hoyt 1979. Plasma, blood cells, and yolks were kept frozen at -20°C until further analyses could be performed.

We sexed birds molecularly Griffiths *et al.* 1998. DNA was extracted from the blood cells using a DNeasy Blood & Tissue kit (Qiagen Inc., Valencia, CA) and used in PCR with primers 2550 Forward and 2718 Reverse to amplify introns from the CHD-Z and CHD-W genes, located on the avian sex chromosomes Fridolfsson *et al.* 1999. PCR fragments were then separated on an electrophoresis agarose gel. In this method, a single band of DNA on the gel indicated that a bird was a male, while two bands were present for females.

Plasma samples were used directly in our immunological analyses. In contrast, antibodies were extracted from frozen egg yolks using an adaptation of a standard protocol Mohammed *et al.* 1986; Gasparini *et al.* 2001. Yolks were first thawed and homogenized. They were then diluted 1:1 in phosphate-buffered saline and the solution was shaken in a mill until it formed a homogenous emulsion. Equal volumes of the emulsion and chloroform were vortexed together and the resulting mixture was centrifuged at 6,000 g for 15 minutes. The resulting supernatant was sequestered and frozen at -20°C until its use in the immunological assay.

The presence of antibodies against avian influenza type A was determined using a competitive enzyme-linked immunosorbent assay (ELISA) test using a kit that detected antibodies against any avian influenza A subtype (IDVet, Montpellier, France); the influenza A nucleoprotein (NP) is fixed in the wells and will form a complex with antibodies present in the samples. The test was performed according to kit instructions. The samples were diluted in the buffer provided in the kit (dilution 1/10) and 100 µl were transferred to the ELISA plate wells. The plates were incubated for 1 hour at 37°C. They were then washed 5

times using the kit's rinsing solution (dilution 1/20). The furnished anti-NP peroxydase conjugate was diluted in a second buffer and 50 µl was added to each well. The plates were covered with aluminium foil and incubated for 30 minutes at 21°C. They were then washed 3 times and, in low-light conditions, 50 µl of the TMB substrate was added to the wells. The plates were then incubated for 10 minutes at 21°C in the dark. Fifty µl of the stop solution (0.5 M H_2SO_4) was added to the wells and the optical density (OD) of the samples was read at a wavelength of 450 nm. Intra- and interplate repeatability of sample OD were high (intraplate: r=0.93; interplate: r=0.85) Lessells *et al.* 1987. The percentage of competition was calculated as follows: $OD_{sample}/OD_{negative\ control}$ x 100. The kit that we used considers that a sample is positive for avian influenza when its competition value is 45% or less. However, as this methodology had been developed using plasma and not yolk extracts, it was necessary to determine if the same positivity threshold was applicable for egg samples. This was done by examining the correlation between antibody levels in adult females and their corresponding eggs.

Statistical Analyses

All statistical analyses were performed in R 2.15.0 R Development Core Team 2012.

Female-Egg Antibody Correlation

Using the results of the molecular sexing of adult gulls, we were to identify and associate the results for female gulls with their eggs. A linear regression was performed with female plasma competition values as the predictor and egg yolk competition values as the response. The data were first transformed using the natural log. When multiple eggs

were available for a given female, the mean competition value for the nest was used.

Broad and Local Scale Prevalence Patterns

The significance of different factors in the following analyses was determined using model comparison employing backwards term elimination starting from a full model. The prevalence of anti-AIV antibodies across different colonies was compared using a generalized linear model with a binomial distribution. The response variable was contained the combined number of antibody positive and negative eggs. Due to sampling issues, egg characteristics were not available for some colonies. Consequently, a subset of the data was used to examine the influence of egg volume. In this subset, the relationship of colony and egg volume (predictor variables) and the likelihood of being antibody positive (response variable) was determined using binomial logistic regression. To determine if autocorrelation in egg volume within colonies could affect the results, a generalized linear mixed model was also performed, incorporating colony as a random effect as opposed to a fixed factor. The difference in mean egg volume among colonies was tested using a linear model with colony as the predictor and egg volume as the response.

The relationship between colony size, colony mean egg volume, and egg antibody prevalence was examined using a generalized linear model with quasibinomial error. The number of antibody positive and negative eggs was the combined response variable and colony size (the number of breeding pairs) was the predictor. We also initially included colony mean egg volume after finding that it was not correlated with colony size ($F=3.96$, $df=1,6$, $p=0.09$). Because of non-linearity in the

relationship between prevalence and colony size, colony size was included as a quadratic term in the model.

Estimates of spatial autocorrelation within colonies were performed using the spdep package Bivand 2012. Using Join count tests and a matrix of weights based on nearest neighbor distances, we sought to determine whether eggs found closer together were more likely to have the same antibody status (0 or 1). This analysis was performed separately for VLM, Corrège, and each of the Riou Islets (Plane and Riou).

Results

Correlation between female serum and egg yolk antibody levels

The levels of anti-AIV antibodies found in a female's plasma correlated with those found in her eggs as indicated by the significant relationship between female and egg percentage competition (n=37 pairs; $F=36.1$; $df=1,35$; $p<0.001$) (Fig. 1a). Using the equation describing this relationship (log egg competition = 0.78 * log female competition + 1.26), the seropositivity threshold of 45% described for plasma approximates to a back-transformed positivity threshold of 70% for egg yolk. The frequency distributions of female and egg yolk competition values further support these thresholds for both plasma (Fig. 1b) and egg yolk (Fig. 1c); although somewhat irregular, they show evidence of a bimodal frequency distribution within positive and negative serostatus categories. If we consider eggs with competition values of 70% or less to be positive, there are nonetheless five eggs for which the mother is seropositive but the egg yolk is antibody-negative. In contrast, there are no false positives (mother negative but egg

positive). This result signifies that we will have a tendency to underestimate prevalence by 13%.

As a consequence of this finding, we considered that the eggs from our broader sampling of the Mediterranean were antibody-positive for AIV when they had a competition value of 70% or less.

Spatial patterns of influenza exposure revealed by egg yolks

Broad-scale patterns

Antibody prevalence was not homogenous across the Mediterranean coast (Figure 2); it ranged from 31-74% (Table 1). Interestingly, all colonies demonstrated some exposure to avian influenza. Colonies contained significantly different proportions of antibody-positive eggs (retention of colony id: $p<0.001$) (Table 1). Colony importance was confirmed in the analyses that included egg volume. In the best-fit model predicting an egg's antibody status, only colony was retained ($p<0.001$); an egg's volume did not make it more or less likely to be antibody positive ($p=0.98$), even though mean egg volume was found to differ among colonies (linear model: $F=8.5$; $df=10, 782$; $p<0.001$). The generalized linear mixed model showed limited support for autocorrelation of egg volumes within colonies (correlation=0.015), supporting the use of the simpler generalized linear models.

The proportion of antibody positive eggs was non-linearly related to colony size (retention of quadratic term: $p<0.001$). Prevalence was higher at both small and large colony sizes (Figure 3). Colony mean egg volume did not significantly contribute to explaining model variance and was thus dropped ($p=0.65$).

Within-colony patterns

There was no spatial structuring of antibody status in the majority of the colonies examined ($p>0.05$ for Corrège, Villeneuve, and the Riou islet). On Plane, however, antibody-negative nests were more likely to be located near other antibody-negative nests (standard deviate = 1.96, $p=0.02$) although antibody-positive nests were freely scattered throughout the colony ($p=0.2$).

Discussion

The primary goal of this study was to determine if egg sampling could be a useful technique in broad-scale disease surveillance, thus allowing researchers a rapid and easy way to identify regions of interest and eliminating the need for the more challenging and costly sampling of adults. We found that there was indeed a strong correlation between maternal sera and egg yolks. We were able to use this relationship to estimate and examine AIV antibody prevalence in yellow-legged gull colonies all along the Mediterranean coast. Based on our results, it seems likely that exposure to AIV is influenced less by processes such as colony demography and interactions at the nest level and more by factors contributing to colony size.

The strong relationship that we found between antibody values in maternal sera and egg yolks (Figure 1a), as well as the serostatus frequency distributions (Figure 1b,c), support the utility of this approach in surveillance. It demonstrates that eggs can indeed serve as proxies for the adults that laid them, as has previously suggested Pearce-Duvet 2009. More importantly, the specific nature of the relationship allows us to establish a new, different threshold for seropositivity. Since commercial competitive ELISAs are developed so

as to be used with blood, not egg yolk, it was necessary to determine if the same level of seropositivity would apply. Instead, we found that the kit's threshold of 40% to be inappropriately low and thus likely to severely underestimate prevalence. Using the regression equation, we calculated that a threshold of 70% was more accurate. This finding emphasizes the need to either develop ELISAs that have been validated using egg yolk samples or establish the threshold individually for a given host-pathogen system, if this technique is to enter more general use.

The proxy assumption is not completely accurate. Using the new threshold, five eggs coming from seropositive mothers were classified as seronegative. This result may reflect variability in a female's ability or choice to transfer antibodies to her eggs Hammouda *et al.* 2012. However, there were no false positives, thus the primary risk is that prevalence will still be slightly underestimated. Given that the intention is that this approach would occur in tandem with others and that overall prevalence is relatively high, it does not seem that this level of underestimation should be of major concern.

We found that all of the colonies we sampled showed evidence of past or present avian influenza exposure. The fact that they also differed widely in prevalence, ranging from 31% in the Ebro Delta of Spain to 74% in the French colony of Carteau, suggests two things. First, yellow-legged gulls, in general, are frequently exposed to AIVs in nature. This result concurs with previously published findings on gulls from Canada Velarde *et al.* 2010, France Pearce-Duvet 2009, Norway Toennessen *et al.* 2011, Tunisia Hammouda *et al.* 2011, and the eastern coast of the U.S. Graves 1992; this also suggests that our technique provides biologically reasonable results. Second, the significant differences between sites suggest that the risk of AIV exposure, either the likelihood of encounter or transmission, is not homogenous. Although we did not collect detailed data on environmental conditions

or individual birds that allow us to address the reason for these differences, our preliminary data nonetheless permit some speculation.

Bird demography does not appear to play a decisive role in determining prevalence. We might predict that likelihood of exposure could be linked to age. In birds, including marine birds, older and more experienced females tend to lay larger eggs see Amundsen *et al.* 1996; Christians 2002 and older gulls are found nesting at higher quality sites Pugesek *et al.* 1983. Different colonies had significantly different mean egg volumes, suggesting that their age structures and perhaps their quality were also likely to be different. However, egg antibody status was not correlated with volume: larger eggs were not more or less likely to contain anti-AIV antibodies than smaller eggs. Furthermore, a colony's mean egg volume was not correlated with its prevalence. This suggests either that exposure is age-independent, indeed chicks have been found to be infected Velarde *et al.* 2010, or that the age-linked pattern of exposure gets washed out because antibodies are retained over time. The longevity of anti-AIV antibodies is a topic that remains to be explored.

In contrast, colony size was a significant predictor of colony prevalence. Both smaller and larger, but not intermediate, colonies contained a greater percentage of birds that had been exposed to avian influenza. In large colonies, it could be that a density-dependent effect is involved. Gulls nest in large, dense colonies. Low pathenogenic viruses in birds are shed in the feces of infected individuals and are transmitted to susceptible hosts upon ingestion Olsen *et al.* 2006, via drinking or filtering of contaminated water Hinshaw *et al.* 1979; Brown *et al.* 2009, or perhaps both Roche *et al.* 2009. Larger colony sizes could result in a greater accumulation of feces and virus, higher encounter rates, and thus greater disease risk Müller *et al.* 2004; Fouchier *et al.* 2007. Density-dependence could equally be at play in small-sized colonies. For instance, one of the smallest colonies,

Carteau, had the highest prevalence value. It has been found that the small colonies have extremely high densities, perhaps because suitable nesting substrate is limited on the small islands where they are found Duhem *et al.* 2007.

The fact that some colonies achieve large sizes can reflect their access to urban trash dumps Duhem *et al.* 2007; Duhem *et al.* 2008. This foraging behavior may present an even greater disease risk because it further concentrates gulls in a food-rich area while facilitating fecal-oral transmission because they are defecating where they are eating. Although we do not have data available for all of our colonies, it is known that gulls from some of the larger colonies forage in trash dumps Frioul, Riou: Duhem *et al.* 2003; Duhem *et al.* 2005; Ebro, Medes: Ramos *et al.* 2009; Ramos *et al.* 2010. The fact that we found limited evidence for spatial clustering of antibody status within colonies tends to support this hypothesis; it suggests that exposure is taking place outside of the colony, perhaps as a consequence of trash foraging or other as yet unidentified factors.

We recognize that this study only represents an initial effort in understanding AIV dynamics in nature, and future studies should clarify additional facets of this system that are relevant to surveillance. For instance, we detected general AIV antibodies by using an immunological approach that targets the nucleoprotein common to all subtypes. As a consequence, we don't know the identity of subtypes to which the gulls were exposed nor if concurrent infection with several subtypes could have taken place. It could be that we are detecting gull exposure to the two taxon-specific subtypes, characterized by H13 and H16 Fouchier *et al.* 2005; Fouchier *et al.* 2007. However, they may also have been infected with other subtypes and as well as being cross-infected. It should be recalled that the first recorded isolation of an influenza virus in the wild was found in terns (*Sterna hirundo*) Becker 1966, a sister taxonomic group (family Sternidae) to the gulls (family

Laridae) van Tuinen *et al.* 2004. The HP subtype, H5N3, resulted in serious outbreak in South Africa Becker 1966; Alexander 2000. The unpredictable dynamics of avian influenza have taught us that we should not underestimate its potential for emergence and re-emergence Landolt *et al.* 2007; Neumann *et al.* 2009.

Based on the results of this study, we recommend that egg sampling be incorporated into the suite of techniques currently employed for avian influenza surveillance. Indeed, its utility in domestic, farm settings has already been suggested Beck *et al.* 2003; Trampel *et al.* 2006; Gharaibeh *et al.* 2008; Rutten *et al.* 2012; Sa e Silva *et al.* 2012. The ease of sampling eggs contrasts sharply with that of capturing adults. As demonstrated here, the antibodies present in egg yolk can reveal the locations of interest, allowing researchers to narrow in on pathogen "hot spots", areas that may present the greatest concern either because of their high prevalence levels or their proximity to human urban environments. Once such locations are identified, a more precise pathogen characterization of the pathogen (virus presence and type) and the factors that contribute to infection risk as well as disease emergence can take place. We consider this approach could be advantageous not only in the surveillance of avian influenza but in monitoring other pathogens as well. Further work could and should examine its tractability in other systems.

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References

Alexander, D. J. 2000. A review of avian influenza in different bird species. Veterinary Microbiology **74**:3-13.

Alexander, D. J. 2007. An overview of the epidemiology of avian influenza. Vaccine **25**:5637-5644.

Amundsen, T., L. Svein-Hakon, and T. Tveraa. 1996. Effects of Egg Size and Parental Quality on Early Nestling Growth: An Experiment with the Antarctic Petrel. Journal of Animal Ecology **65**:545-555.

Arnold, J. M., S. A. Oswald, C. C. Voigt, R. Palme, A. Braasch, C. Bauch, and P. H. Becker. 2008. Taking the stress out of blood collection: comparison of field blood-sampling techniques for analysis of baseline corticosterone. Journal of Avian Biology **39**:588-592.

Beck, J. R., D. E. Swayne, S. Davison, S. Casavant, and C. Gutierrez. 2003. Validation of egg yolk antibody testing as a method to determine influenza status in white leghorn hens. Avian Diseases **47**:1196-1199.

Becker, W. B. 1966. The isolation and classification of Tern virus: Influenza Virus A/Tern/South Africa/1961. Journal of Hygiene **64**:309-320.

Belant, J. L. 1997. Gulls in urban environments: landscape-level management to reduce conflict. Landscape and Urban Planning **38**:245-258.

Bivand, R. 2012. spdep: spatial dependence: weighting schemes, statistics and models.

Bosch, M., D. Oro, and X. Ruiz. 1994. Dependence of yellow-legged gulls (*Larus cachinnans*) on food from human activity in two western Mediterranean colonies. Avocetta **18**:135-139.

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

Boyce, W. M., C. Sandrock, C. Kreuder-Johnson, T. Kelly, and C. Cardona. 2008. Avian influenza in wild birds: a moving target. Comparative Immunology, Microbiology and Infectious Diseases:in press.

Bradley, C. A., S. E. J. Gibbs, and S. Altizer. 2008. Urban land use predicts West Nile virus exposure in songbirds. Ecological Applications **18**:1083-1092.

Brown, J. D., G. Goekjian, R. Poulson, S. Valeika, and D. E. Stallknecht. 2009. Avian influenza virus in water: infectivity is dependent on pH, salinity and temperature. Veterinary Microbiology **136**:20-26.

Caron, A., N. Gaidet, M. de Garine-Wichatitsky, S. Morand, and E. Z. Cameron. 2009. Evolutionary biology, community ecology and avian influenza research. Infection, Genetics and Evolution **9**:298-303.

Christians, J. K. 2002. Avian egg size: variation within species and inflexibility within individuals. Biological Reviews **77**:1-26.

Daszak, P., A. A. Cunningham, and A. D. Hyatt. 2000. Emerging infectious diseases of wildlife: threats to biodiversity and human health. Science **287**:443-449.

Duhem, C., P. Roche, E. Vidal, and T. Tatoni. 2007. Distribution of breeding sites and food contrains size and density of yellow-legged gull colonies. Ecoscience **14**:535-543.

Duhem, C., P. Roche, E. Vidal, and T. Tatoni. 2008. Effects of anthropogenic food resources on yellow-legged gull colony size on Mediterranean islands. Population Ecology **50**:91-100.

Duhem, C., E. Vidal, J. Legrand, and T. Tatoni. 2003. Opportunistic feeding responses of the yellow-legged gull *Larus michahellis* to accessibility of refuse dumps. Bird Study **50**:61-67.

Duhem, C., E. Vidal, P. Roche, and J. Legrand. 2005. How is the diet of yellow-legged gull chicks influenced by parents' accessibility to landfills? Waterbirds **28**:46-52.

Fouchier, R. A. M., V. J. Munster, J. Keawcharoen, A. D. M. E. Osterhaus, and T. Kuiken. 2007. Virology of avian influenza in relation to wild birds. Journal of Wildlife Diseases **43**:S7-S14.

Fouchier, R. A. M., V. J. Munster, A. Wallensten, T. M. Bestebroer, S. Herfst, D. Smith, G. F. Rimmelzwaan, B. Olsen, and A. D. M. E. Osterhaus. 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from Black-headed Gulls. Journal of Virology **79**:2814-2822.

Fridolfsson, A. K., and H. Ellegren. 1999. A simple and universal method for molecular sexing of non-ratite birds. Journal of Avian Biology **30**:116-121.

Gasparini, J., K. D. McCoy, C. Haussy, T. Tveraa, and T. Boulinier. 2001. Induced maternal response to the Lyme disease spirochaete *Borrelia burgdorferi sensu lato* in a colonial

seabird, the kittiwake *Rissa tridactyla*. Proceedings of the Royal Society of London B **268**:647-650.

Gasparini, J., K. D. McCoy, V. Staszewski, C. Haussy, and T. Boulinier. 2006. Dynamics of anti-*Borrelia* antibodies in Black-legged Kittiwake (*Rissa tridactyla*) chicks suggest a maternal educational effect. Canadian Journal of Zoology **84**:623-627.

Gasparini, J., K. D. McCoy, T. Tveraa, and T. Boulinier. 2002. Related concentrations of specific immunoglobins against the Lyme disease agent *Borrelia burgdorferi* sensu lato in eggs, young, and adults of the kittiwake (*Rissa tridactyla*). Ecology Letters **5**:519-524.

Gauthier-Clerc, M., C. Lebarbenchon, and F. Thomas. 2007. Recent expansion of highly pathogenic avian influenza H5N1: a critical review. Ibis **149**:202-214.

Gharaibeh, S., K. Mahmoud, and M. Al-Natour. 2008. Field evaluation of maternal antibody transfer to a group of pathogens in meat-type chickens. Poultry Science **87**:1550-1555.

Gibbs, S. E. J., D. M. Hoffman, L. M. Stark, N. L. Marlenee, B. J. Blitvich, B. J. Beaty, and D. E. Stallknecht. 2005. Persistence of antibodies to West Nile virus in naturally infected rock pigeons (*Columba livia*). Clinical and Diagnostic Laboratory Immunology **12**:665-667.

Graves, I. L. 1992. Influenza viruses in birds of the Atlantic Flyway. Avian Diseases **36**:1-10.

Grenfell, B. T., and A. P. Dobson. 1995. Ecology of infectious diseases in natural populations. Cambridge University Press, Cambridge, U.K.

Griffiths, R., M. C. Double, K. Orr, and R. J. G. Dawson. 1998. A DNA test to sex most birds. Molecular Ecology **7**:1071-1075.

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

Hammouda, A., J. M. C. Pearce-Duvet, M. A. Chokri, A. Arnal, M. Gauthier-Clerc, T. Boulinier, and S. Selmi. 2011. Prevalence of influenza A antibodies in yellow-legged gull (*Larus michahellis*) eggs and adults in southern Tunisia. Vector-Borne and Zoonotic Diseases **11**:1583-1590.

Hammouda, A., S. Selmi, J. Pearce-Duvet, M. A. Chokri, A. Arnal, M. Gauthier-Clerc, and T. Boulinier. 2012. Maternal antibody transmission in relation to mother fluctuating asymmetry in a long-lived colonial seabird: the yellow-legged gull *Larus michahellis*. PLoS ONE **7**:e34966.

Han, Y., G. Hou, W. Jiang, C. Han, S. Liu, J. Chen, J. Li, P. Zhang, B. Huang, Y. Liu, and J. Chen. 2012. A survey of avian influenza in tree sparrows in China in 2011. PLoS ONE **7**:e33092.

Haynes, L., E. Arzey, C. Bell, N. Buchanan, G. Burgess, V. Cronan, C. Dickason, H. Field, S. Gibbs, P. M. Hansbro, T. Hollingsworth, A. C. Hurt, P. Kirkland, H. McCracken, J. O'Connor, J. Tracey, J. Wallner, S. Warner, R. Woods, and C. Bunn. 2009. Australian surveillance for avian influenza viruses in wild birds between July 2005 and June 2007. Australian Veterinary Journal **87**:266-272.

Hinshaw, V. S., R. G. Webster, and B. Turner. 1979. Water-borne transmission of influenza A viruses? Intervirology **11**:66-68.

Hinshaw, V. S., J. M. Wood, R. G. Webster, R. Deibel, and B. Turner. 1985. Circulation of influenza viruses and paramyxoviruses in waterfowl originating from two different areas of North America. Bulletin of the World Health Organization **63**:711-719.

Hoye, B. J., V. J. Munster, H. Nishiura, M. Klaassen, and R. A. M. Fouchier. 2010. Surveillance of wild birds for avian influenza virus. Emerging Infectious Diseases **16**:1827-1834.

Hoyt, D. F. 1979. Practical methods of estimating volume and fresh weight of bird eggs. The Auk **96**:73-77.

Hudson, P. J., B. T. Rizzoli, B. T. Grenfell, H. Heesterbeek, and A. P. Dobson. 2002. The ecology of wildlife diseases. Oxford University Press, Oxford, U.K.

Ip, H. S., R. J. Dusek, and D. M. Heisey. 2012. The effect of swab sample choice on the detection of avian influenza in apparently healthy wild ducks. Avian Diseases **56**:114-119.

Jones, K. E., N. G. Patel, M. A. Levy, A. Storeygard, D. Balk, J. L. Gittleman, and P. Daszak. 2008. Global trends in emerging infectious diseases. Nature **451**:990-993.

Jourdain, E., G. Gunnarsson, J. Wahlgren, N. Latorre-Margalef, C. Bröjer, S. Sahlin, L. Svensson, J. Waldenström, A. Lundkvist, and B. Olsen. 2010. Influenza virus in a natural host, the mallard: experimental infection data. PLoS ONE **5**:e8935.

Kawaoka, Y., T. M. Chambers, W. L. Sladen, and R. Gwebster. 1988. Is the gene pool of influenza viruses in shorebirds and gulls different from that in wild ducks? Virology **163**:247-250.

Knight-Jones, T. J. D., R. Hauser, D. Matthes, and K. D. C. Stärk. 2010. Evaluation of effectiveness and efficiency of wild bird surveillance for avian influenza. Veterinary Research **41**:50-66.

Krauss, S., and R. G. Webster. 2010. Avian influenza virus surveillance and wild birds: past and present. Avian Diseases **54**:394-398.

Landolt, G. A., and C. W. Olsen. 2007. Up to new tricks: a review of cross-species transmission of influenza A viruses. Animal Health Research Reviews **8**:1-21.

Latorre-Margalef, N., G. Gunnarsson, V. J. Munster, R. A. M. Fouchier, A. D. M. E. Osterhaus, J. Elmberg, B. Olsen, A. Wallensten, P. D. Haemig, T. Fransson, L. Brudin, and J. Waldenström. 2009. Effects of influenza A virus infection on migrating mallard ducks. Proceedings of the Royal Society B: Biological Sciences **276**:1029-1036.

Lebarbenchon, C. 2008. Maladie infectieuses et écosystèmes: écologie des virus influenza aviaires en Camargue. Université Montpellier II, Montpellier.

Lebarbenchon, C., C.-M. Chang, V. Grandhomme, M. Dietrich, Y. Kayser, E. Elguero, F. Renaud, F. Thomas, S. van der Werf, and M. Gauthier-Clerc. 2010. Avian influenza circulation in the Camargue (south of France) during the 2006-07 season. Avian Diseases **54**:446-449.

Lee, D.-H., H.-J. Lee, Y.-J. Lee, H.-M. Kang, O.-M. Jeong, M.-C. Kim, J.-S. Kwon, J.-H. Kwon, C.-B. Kim, J.-B. Lee, S.-Y. Park, I.-S. Choi, and C.-S. Song. 2010. DNA barcoding techniques for avian influenza virus surveillance in migratory bird habitats. Journal of Wildlife Diseases **46**:649-654.

Lessells, C. M., and P. T. Boag. 1987. Unrepeatable repeatabilities: a common mistake. The Auk **104**:116-121.

Marché, S., and T. van den Berg. 2010. Evaluation of different strategies for the use of ELISA tests as first screening tools for serologic surveillance of low pathogenic avian influenza in the Belgian poultry sector. Avian Diseases **54**:627-631.

Mohammed, H. O., R. Yamamoto, T. E. Carpenter, and H. B. Ortmayer. 1986. Comparison of egg yolk and serum for the detection of *Mycoplasma gallisepticum* and *M. synoviae* antibodies by enzyme-linked immunosorbent assay. Avian Diseases **30**:398-408.

Mousseau, T. A., and C. W. Fox. 1998. Maternal Effects as Adaptations. Oxford University Press, New York.

Müller, W., T. G. G. Groothuis, C. Dijkstra, H. Siitari, and R. V. Alatalo. 2004. Maternal antibody transmission and breeding densities in the Black-headed Gull *Larus ridibundus*. Functional Ecology **18**:719-724.

Neumann, G., T. Noda, and Y. Kawaoka. 2009. Emergence and pandemic potential of swine-origin H1N1 influenza virus. Nature **459**:931-939.

Olsen, B., V. J. Munster, A. Wallensten, J. Waldenström, A. D. M. E. Osterhaus, and R. A. M. Fouchier. 2006. Global patterns of influenza A virus in wild birds. Science **312**:384-388.

Oro, D., and A. Martinez-Vilalta. 1994. Factors affecting kleptoparasitism and predation rates upon a colony of Audouin's gull (*Larus audouinii*) by yellow-legged gulls (*Larus cachinnans*) in Spain. Colonial Waterbirds **17**:35-41.

Pannwitz, G., C. Wolf, and T. Harder. 2009. Active surveillance for avian influenza virus infection in wild birds by analysis of avian fecal samples from the environment. Journal of Wildlife Diseases **45**:512-518.

Pearce-Duvet, J. M. C. 2009. Maternal antibody transfer in yellow-legged gulls. Emerging Infectious Diseases **15**:1147-1149.

Pugesek, B. H., and K. L. Diem. 1983. A Multivariate Study of the Relationship of Parental Age to Reproductive Success in California Gulls. Ecology **64**:829-839.

Ramos, R., M. Cerdá-Cuéllar, F. Ramírez, L. Jover, and X. Ruiz. 2010. Influence of refuse sites on the prevalence of *Campylobacter* spp. and *Salmonella* serovars in seagulls. Applied and Environmental Microbiology **76**:3052-3056.

Ramos, R., F. Ramírez, C. Sanpera, L. Jover, and X. Ruiz. 2009. Diet of yellow-legged gull (*Larus michahellis*) chicks along the Spanish Western Mediterranean coast: the relevance of refuse dumps. Journal of Ornithology **150**:265-272.

Redig, P. T., and S. M. Goyal. 2012. Serologic evidence of exposure of raptors to influenza A virus. Avian Diseases **56**:411-413.

Roche, B., C. Lebarbenchon, M. Gauthier-Clerc, C.-M. Chang, F. Thomas, F. Renaud, S. van der Werf, and J.-F. Guégan. 2009. Water-borne transmission drives avian influenza dynamics in wild birds: the case of the 2005–2006 epidemics in the Camargue area. Infection, Genetics and Evolution **9**:800-805.

Rohani, P., R. Breban, D. E. Stallknecht, and J. M. Drake. 2009. Environmental transmission of low pathogenicity avian influenza viruses and its implications for pathogen invasion.

Proceedings of the National Academy of Sciences **106**:10365-10369.

Rutten, N., J. L. Gonzales, A. R. W. Elbers, and A. G. J. Velthuis. 2012. Cost analysis of various low pathogenic avian influenza surveillance systems in the Dutch egg layer sector. PLoS ONE **7**:e33930.

Sa e Silva, M., and D. E. Swayne. 2012. Serum and egg yolk antibody detection in chickens infected with low pathogenicity avian influenza virus. Avian Diseases **in press**.

Stallknecht, D. E., and J. D. Brown. 2007. Wild birds and the epidemiology of avian influenza. Journal of Wildlife Diseases **43**:S15-S20.

Stallknecht, D. E., and S. M. Shane. 1988. Host range of avian influenza in free-living birds. Veterinary Research Communications **12**:125-141.

Team, R. D. C. 2012. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Toennessen, R., A. Germundsson, C. Jonassen, I. Haugen, K. Berg, R. Barrett, and E. Rimstad. 2011. Virological and serological surveillance for type A influenza in the black-legged kittiwake (*Rissa tridactyla*). Virology Journal **8**:1-8.

Trampel, D. W., E. Zhou, K. Yoon, and K. J. Koehler. 2006. Detection of antibodies in serum and egg yolk following infection of chickens with an H6N2 avian influenza virus. Journal of Veterinary Diagnostic Investigation **18**:437-442.

van Gils, J. A., V. J. Munster, R. Radersma, D. Liefhebber, R. A. M. Fouchier, and M. Klaasen. 2007. Hampered foraging and migratory performance in swans infected with low-pathogenic avian influenza A virus. PLoS ONE **2**:e184.

van Tuinen, M., D. Waterhouse, and G. J. Dyke. 2004. Avian molecular systematics on the rebound: a fresh look at modern shorebird phylogenetic relationships. Journal of Avian Biology **35**:191-194.

Velarde, R., S. E. Calvin, D. Ojkic, I. K. Barker, and E. Nagy. 2010. Avian influenza virus H13 circulating in ring-billed gulls (*Larus delawarensis*) in Southern Ontario, Canada. Avian Diseases **54**:411-419.

Woolhouse, M. E. J. 2002. Population biology of emerging and re-emerging pathogens. Trends in Microbiology **10**:s3-s7.

Woolhouse, M. E. J., D. T. Haydon, and R. Antia. 2005. Emerging pathogens: the epidemiology and evolution of species jumps. Trends in Ecology & Evolution **20**:238-244.

Woolhouse, M. E. J., L. H. Taylor, and D. T. Haydon. 2001. Population biology of multihost pathogens. Science **292**:1109-1112.

Xu, X., K. Subbarao, N. J. Cox, and Y. Guo. 1999. Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. Virology **261**:15-19.

Table 1. Prevalence of anti-AIV antibodies in yellow-legged gull eggs sampled from across Mediterranean coast. Colonies are ordered counter clock-wise, starting in southeastern France and ending in southeastern Tunisia, and their longitudinal and latitudinal coordinates are provided. Note that Plane and Riou are islets within the complex of islands called Riou. GDR stands for Grau du Roi and VLM stands for Villeneuve-lès-Maguelone. *Total Eggs* are the number of eggs that were collected from the colony, *Ab+ Eggs* the number whose yolks were found to contain anti-AIV antibodies, and *Prevalence* is *Ab+ Eggs*/*Total Eggs*.

Country	Colony	Location	Total Eggs	Ab+ Eggs	Prevalence
France	Riou (overall)		127	65	51%
	Plane (islet)	43°11'14"N, 5°23'10"E	60	30	50%
	Riou (islet)	43°10'35"N, 5°23'08"E	65	65	54%
	Frioul	43°16'27"N, 5°18'15"E	79	55	70%
	Carteau	43°22'40"N, 4°51'27"E	102	75	74%
	Besson	43°29'15"N, 4°27'47"E	79	47	59%
	GDR	43°30'52"N, 4°08'36"E	50	33	66%
	VLM	42°02'50"N, 3°13'21"E	70	31	44%
	Gruissan	43°06'36"N, 3°06'25"E	99	54	55%
	Corrège	42°51'15"N, 3°01'21"E	70	24	34%
	Medes	42°02'50"N, 3°13'21"E	38	16	42%
Spain	Ebro Delta	40°34'17"N, 0°39'37"E	55	17	31%
	Isla Grosa	37°43'40"N, 0°42'27"E	66	26	39%
Algeria	Chetaïbi	37°05'79"N, 7°17'53"E	55	26	47%
Tunisia	Sfax	34°42'28"N, 10°45'02"E	45	28	62%
	Djerba	33°39'10"N, 10°58'59"E	89	36	40%

Table 2. Available data on the number of breeding pairs (colony size) at our sampling locations as well as their source. VLM stands for Villeneuve-lès-Maguelone.

Colony	Size	Information Source
Riou (islet)	6000	Duhem 2004
Plane (islet)	2000	Duhem 2004
Frioul	8000	P. Mayet pers. comm.
Corrège	4000	P. Mayet pers. comm.
Gruissan	350	P. Mayet pers. comm.
VLM	400	P. Mayet pers. comm.
Carteau	400	P. Mayet pers. comm.
Medes Islands	6500	Ramos et al. 2009
Ebro Delta	6000	Ramos et al. 2009
Isla Grosa	1400	D. Oro pers. comm.
Sfax	100	Hammouda pers. comm.
Djerba	2500	Hammouda pers. comm.

Figure legend

Figure 1: a) Linear regression between ELISA percentage competition values for female plasma (x-axis) and corresponding egg yolk (y-axis); values have been log-transformed b) Histogram of ELISA percentage competition values for female plasma b) Histogram of ELISA percentage competition values for egg yolk

Figure 2: Antibody prevalence patterns estimated from eggs in a) France; b) Spain; c) North Africa. The locations of the colonies sampled are indicated by black dots. The pie charts represent the number of eggs found seropositive (in black) and seronegative (in white) for anti-AIV antibodies; details are provided in Table 1.

Figure 3: Non-linear regression between colony size and egg antibody prevalence

Figure 1

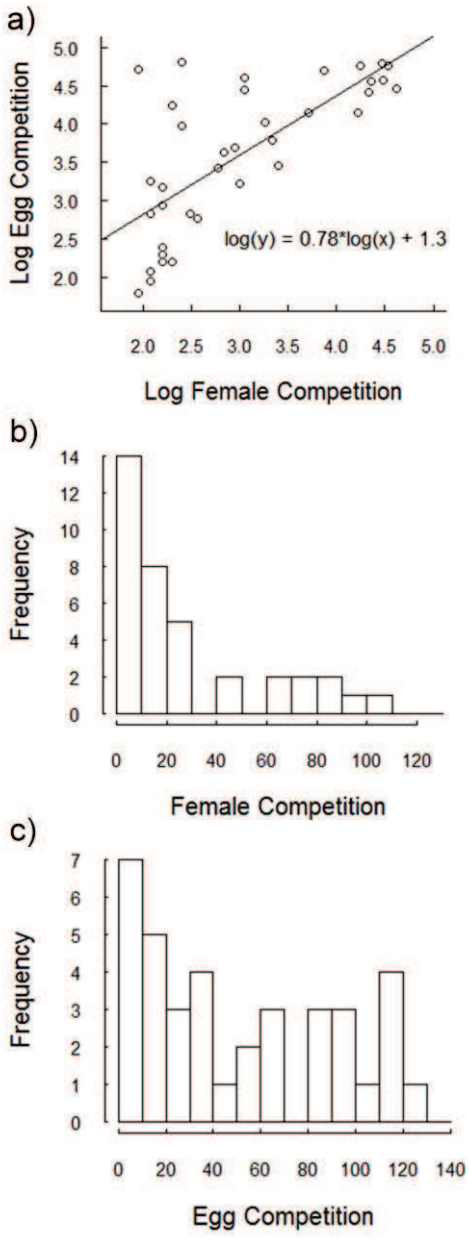

a)
Log Egg Competition
5.0
4.5
4.0
3.5
3.0
2.5
2.0
log(y) = 0.78*log(x) + 1.3
2.0 2.5 3.0 3.5 4.0 4.5 5.0
Log Female Competition
b)
Frequency
14
12
10
8
6
4
2
0
0 20 40 60 80 100 120
Female Competition
c)
Frequency
7
6
5
4
3
2
1
0
0 20 40 60 80 100 120 140
Egg Competition

Figure 2

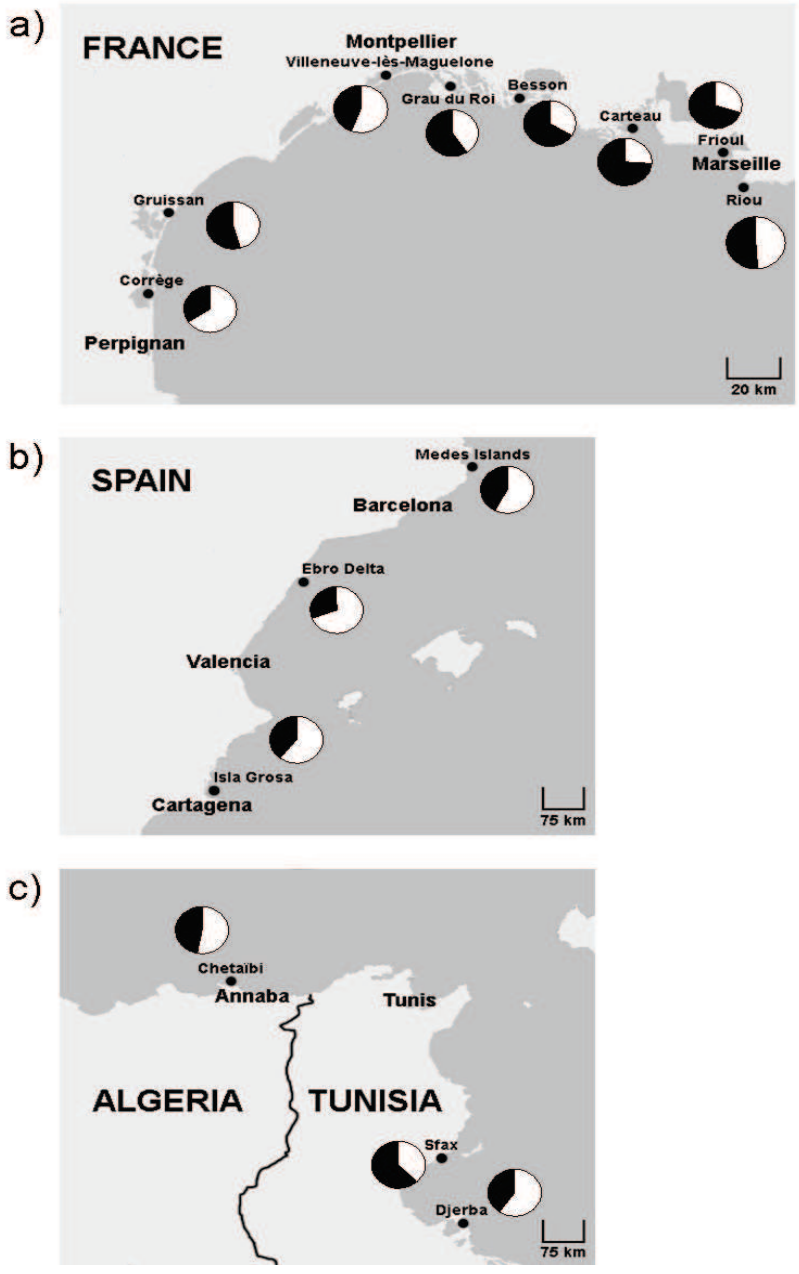
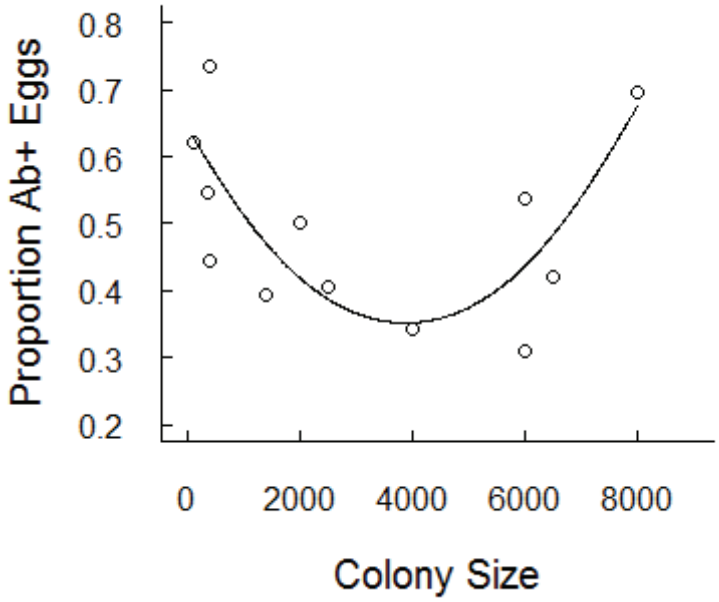

a)
FRANCE
Montpellier
Villeneuve-lès-Maguelone
Grau du Roi
Besson
Carteau
Frioul
Marseille
Riou
Gruissan
Corrège
Perpignan
20 km
b)
SPAIN
Medes Islands
Barcelona
Ebro Delta
Valencia
Isla Grosa
Cartagena
75 km
c)
Chetaïbi
Annaba
Tunis
ALGERIA
TUNISIA
Sfax
Djerba
75 km

Figure 3


Proportion Ab+ Eggs
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0
2000
4000
6000
8000
Colony Size

Article 4

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

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Abstract

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.

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.

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.

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 Anker *et al.* 2002. However, many anthropogenic environmental disturbances have contributed to pathogen emergence Lebarbenchon *et al.* 2008. 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 Patz *et al.* 2004; Lebarbenchon *et al.* 2008. 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 Daszak *et al.* 2000; Gortazar *et al.* 2007; Jones *et al.* 2008. 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 Chen *et al.* 2005; De Wit *et al.* 2008. These viruses have complex epidemiological dynamics Webster *et al.* 1992, characterized by the ability to jump from wild birds to domestic hosts as well as to wild and domestic mammals, including humans Olsen *et al.* 2006; Gauthier-Clerc *et al.* 2007.

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 Webster *et al.* 1992. 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 Webster *et al.* 1992; Alexander 2000. 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) Ito *et al.* 2001; Lebarbenchon *et al.* 2010. To date, H5 and H7 are the only subtypes that have been able to evolve from low to high pathogenicity Alexander 2000; Fouchier *et al.* 2007. However, a wide variety of subtypes circulate in wild birds Webster *et al.* 1992; Alexander 2000. 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 Zhou *et al.* 1999.

For various reasons, including the fact that Anatidae species represent the main natural host reservoir for AIVs Stallknecht *et al.* 2007b, given the global distribution of the taxon Cramp *et al.* 1983, most studies have focused on AIV circulation in Anatidae and given far less attention to Laridae Arnal *et al.* in prep.. However, studies that have investigated AIV dynamics in Laridae have shown that several species are regularly infected by LPAIVs Becker 1966; Wille *et al.* 2011 and may be healthy carriers of HPAIVs Olsen *et al.* 2006; Muzinic *et al.* 2010; Savic *et al.* 2010. Moreover, specific subtypes such as H13 and H16 are essentially maintained in gull populations Yamnikova *et al.* 2003; Fouchier *et al.* 2005; Olsen *et al.* 2006, although they are also occasionally detected in other avian taxa, including the mallard (*Anas platyrhynchos)* Munster *et al.* 2007, the domestic turkey (*Meleagris gallopavo)* Sivanandan *et al.* 1991, or the American white pelican (*Pelecanus erythrorhynchos*) Lebarbenchon *et al.* 2010.

Some Laridae species, such as the yellow-legged gull (*Larus michahellis*), are colonial seabirds that nest in large and dense colonies Oro *et al.* 2007, creating good opportunities for virus spread through aerial and feacal transmission Markwell *et al.* 1982; Webster *et al.* 1992. 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 Brown *et al.* 2009; Costa *et al.* 2011. Yellow-legged gulls are also predators for ducks Tamisier *et al.* 1999, and it has been shown that Laridae species can be infected with HPAI H5N1 through the consumption of contaminated meat Brown *et al.* 2008. Finally, they have colonized some urban areas and can exploit resources resulting from human activities Duhem *et al.* 2008; Lisnizer *et al.* 2011, 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 Munster *et al.* 2007; Germundsson *et al.* 2010. 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 Lebarbenchon *et al.* 2010.

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 Kuno 2001. 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 Kuno 2001. 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 Staszewski *et al.* 2007a. Moreover, the long lifespan of Laridae Cramp *et al.* 1983 could favor the development of persistent acquired immune responses Lee 2006.

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 Gasparini *et al.* 2002; Hammouda *et al.* 2012; Pearce-Duvet *et al.* in prep. and therefore reflects a female's prior exposure to infectious agents Pearce-Duvet *et al.* 2009; Hammouda *et al.* 2011; Pearce-Duvet *et al.* in prep..

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 Pearce-Duvet *et al.* 2009; Hammouda *et al.* 2011, 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 Krauss *et al.* 2004; Lebarbenchon *et al.* 2007; Wallensten *et al.* 2007; Hanson *et*

al. 2008. Knowing that yellow-legged gulls breed in dense colonies Sol *et al.* 1995, 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 Fouchier *et al.* 2005; Olsen *et al.* 2006; Fouchier *et al.* 2007, 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 Muzinic *et al.* 2010; Savic *et al.* 2010.

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 Hammouda *et al.* 2011.

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 Gasparini *et al.* 2001, 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 Kuiken *et al.* 1998; WHO manual on animal influenza diagnosis and surveillance 2002. 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 Burnham *et al.* 2002. 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 Vicente *et al.* 2007 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 $q^* = 0.05$ Benjamini *et al.* 1995. 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 Garcia 2003.

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 Pearce-Duvet *et al.* 2009; Hammouda *et al.* 2011; Pearce-Duvet *et al.* in prep.. They are also consistent with serological results observed in adults of black-legged kittiwakes (*Rissa tridactyla*) living in Norway Toennessen *et al.* 2011. 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 Keeling *et al.* 2002 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 Staszewski *et al.* 2007a, 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 McCallum *et al.* 2001; Bin Muzaffar *et al.* 2006. During the winter, when temperature conditions are favourable to the persistence of AIVs in the environment Brown *et al.* 2009, 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 Garnier *et al.* 2012.

Prevalence levels are not homogeneous among colonies. This result confirms the variation in prevalence previously detected in two colonies in Tunisia Hammouda *et al.* 2011. 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 Stallknecht *et al.* 2007b, are only marginally present in Algeria Cramp *et al.* 1983; Samraoui *et al.* 2008; Samraoui *et al.* 2011, as opposed to certain areas of France (Camargue; Lebarbenchon *et al.* 2010) or Spain (Ebro Delta, Mateo *et al.* 1998). 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 Muzinic *et al.* 2010; Savic *et al.* 2010.

Knowing that H13 and H16 AIV subtypes are common in gull populations Fouchier *et al.* 2005; Olsen *et al.* 2006; Fouchier *et al.* 2007, 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 Obenauer *et al.* 2006; Toennessen *et al.* 2011. 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 Velarde *et al.* 2010, where 92% and 80% of the adult had antibodies against H13 AIVs in 2000 and 2004 Velarde *et al.* 2010.

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 Toennessen *et al.* 2011. These methods can detect both Immunoglobulin type M (IgM) and Immunoglobulin type Y (IgY, which is a functional equivalent of the mammalian IgG West *et al.* 2004). In birds, the acquired immune response is mainly mediated by IgY, which is the only immunoglobulin that can be transferred into the egg yolk West *et al.* 2004. 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 Toennessen *et al.* 2011. 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 Toennessen *et al.* 2011. 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 Toennessen *et al.* 2011. Seropositivity for both H13 and H16 antigens may be explained by a possible co-exposure to different AIV subtypes Sharp *et al.* 1997 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 Chen *et al.* 2006; Van Poucke *et al.* 2010. 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.

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References

1. Anker M, Schaaf D: **WHO Report on global surveillance of epidemic-prone infectious diseases. WHO/CDS/CSR/ISR/2000.1.** *World Health Organisation, Geneva, Switzerland* 2002.

2. Lebarbenchon C, Brown SP, Poulin R, Gauthier-Clerc M, Thomas F: **Evolution of pathogens in a man-made world.** *Molecular Ecology* 2008, **17:**475-484.

3. Patz JA, Daszak P, Tabor GM, Aguirre AA, Pearl M, Epstein J, Wolfe ND, Kilpatrick AM, Foufopoulos J, Molyneux D, et al: **Unhealthy landscapes: Policy recommendations on land use change and infectious disease emergence.** *Environmental Health Perspectives* 2004, **112:**1092-1098.

4. Daszak P, Cunningham AA, Hyatt AD: **Wildlife ecology - Emerging infectious diseases of wildlife - Threats to biodiversity and human health.** *Science* 2000, **287:**443-449.

5. Gortazar C, Ferroglio E, Hoefle U, Froelich K, Vicente J: **Diseases shared between wildlife and livestock: a European perspective.** *European Journal of Wildlife Research* 2007, **53:**241-256.

6. Jones KE, Patel NG, Levy MA, Storeygard A, Balk D, Gittleman JL, Daszak P: **Global trends in emerging infectious diseases.** *Nature* 2008, **451:**990-U994.

7. Chen H, Smith GJD, Zhang SY, Qin K, Wang J, Li KS, Webster RG, Peiris JSM, Guan Y: **H5N1 virus outbreak in migratory waterfowl.** *Nature* 2005, **436:**191-192.

8. De Wit E, Fouchier RAM: **Emerging influenza.** *Journal Of Clinical Virology* 2008, **41:**1-6.

9. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y: **Evolution And Ecology Of Influenza-A Viruses.** *Microbiological Reviews* 1992, **56:**152-179.

10. Gauthier-Clerc M, Lebarbenchon C, Thomas F: **Recent expansion of highly pathogenic avian influenza H5N1: a critical review.** *Ibis* 2007, **149:**202-214.

11. Olsen B, Munster VJ, Wallensten A, Waldenstrom J, Osterhaus A, Fouchier RAM: **Global patterns of influenza A virus in wild birds.** *Science* 2006, **312:**384-388.

12. Alexander DJ: **A review of avian influenza in different bird species.** *Veterinary Microbiology* 2000, **74:**3-13.

13. Lebarbenchon C, Chang C-M, Grandhomme V, Dietrich M, Kayser Y, Elguero E, Renaud F, Thomas F, van der Werf S, Gauthier-Clerc M: **Avian Influenza Circulation in the Camargue (South of France) During the 2006-07 Season.** *Avian Diseases* 2010, **54:**446-449.

14. Ito T, Goto H, Yamamoto E, Tanaka H, Takeuchi M, Kuwayama M, Kawaoka Y, Otsuki K: **Generation of a highly pathogenic avian influenza A virus from an avirulent field isolate by passaging in chickens.** *Journal Of Virology* 2001, **75:**4439-4443.

15. Fouchier RAM, Munster VJ, Keawcharoen J, Osterhaus ADME, Kuiken T: **Virology of avian influenza in relation to wild birds.** *Journal Of Wildlife Diseases* 2007, **43:**S7-S14.

16. Zhou NN, Senne DA, Landgraf JS, Swenson SL, Erickson G, Rossow K, Liu L, Yoon KJ, Krauss S, Webster RG: **Genetic reassortment of avian, swine, and human influenza A viruses in American pigs.** *Journal Of Virology* 1999, **73:**8851-8856.

17. Stallknecht DE, Brown DJ: **Wild birds and the epidemiology of avian influenza.** *Journal of Wildlife Diseases* 2007b, **43:**15-20.

18. Cramp S, Simmons KEL, Brooks DJ, Collar NJ, Dunn E, Gillmor R, Hollom PAD, Hudson R, Nicholson EM, Ogilvie MA, et al: *Handbook of the Birds of Europe, the Middle East and North Africa: The Birds of the Western Palearctic, .* Oxford; 1983.

19. Arnal A, Vittecoq M, Jourdain E, Pearce J, Gauthier-Clerc M, Boulinier T: **Laridae: a neglected reservoir that could play a major role in avian influenza virus dynamics.** in prep.

20. Becker WB: **The isolation and classification of Tern virus: Influenza Virus A/Tern/South Africa/1961.** *Journal of Hygiene-Cambridge* 1966, **64:**309-&.

21. Wille M, Robertson GJ, Whitney H, Bishop MA, Runstadler JA, Lang AS: **Extensive Geographic Mosaicism in Avian Influenza Viruses from Gulls in the Northern Hemisphere.** *PLoS One* 2011, **6**.

22. Muzinic J, Feare CJ, Pavlak M, Cvitkovic D, Tadic M: **The Epidemiological Environment of Avian Influenza H5N1 Outbreaks in Wild Birds in Croatia.** *Polish Journal of Environmental Studies* 2010, **19:**379-385.

23. Savic V, Labrovic A, Zelenika TA, Balenovic M, Separovic S, Jurinovic L: **Multiple Introduction of Asian H5N1 Avian Influenza Virus in Croatia by Wild Birds During 2005-2006 and Isolation of the Virus from Apparently Healthy Black-Headed Gulls (Larus ridibundus).** *Vector-Borne and Zoonotic Diseases* 2010, **10:**915-920.

24. Fouchier RAM, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, Rimmelzwaan GF, Olsen B, Osterhaus A: **Characterization of a novel influenza a virus hemagglutinin subtype (H16) obtained from black-headed gulls.** *Journal Of Virology* 2005, **79:**2814-2822.

25. Yamnikova SS, Gambaryan AS, Tuzikov AB, Bovin NV, Matrosovich MN, Fedyakina IT, Grinev AA, Blinov VM, Lvov DK, Suarez DL, Swayne DE: **Differences between HA receptor-binding sites of avian influenza viruses isolated from Laridae and Anatidae.** *Avian Diseases* 2003, **47:**1164-1168.

26. Munster VJ, Baas C, Lexmond P, Waldenstrom J, Wallensten A, Fransson T, Rimmelzwaan GF, Beyer WEP, Schutten M, Olsen B, et al: **Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds.** *Plos Pathogens* 2007, **3:**630-638.

27. Sivanandan V, Halvorson DA, Laudert E, Senne DA, Kumar MC: **Isolation of H13N2 influenza-A virus from turkeys and surface-water.** *Avian Diseases* 1991, **35:**974-977.

28. Lebarbenchon C, Sreevatsan S, Ramakrishnan MA, Poulson R, Goekjian V, Di Matteo JJ, Wilcox B, Stallknecht DE: **Influenza A Viruses in American White Pelican (*Pelecanus erythrorhynchos*).** *Journal Of Wildlife Diseases* 2010, **46:**1284-1289.

29. Oro D, Martinez-Abrain A: **Deconstructing myths on large gulls and their impact on threatened sympatric waterbirds.** *Animal Conservation* 2007, **10:**117-126.

30. Markwell DD, Shortridge KF: **POSSIBLE WATERBORNE TRANSMISSION AND MAINTENANCE OF INFLUENZA-VIRUSES IN DOMESTIC DUCKS.** *Applied And Environmental Microbiology* 1982, **43:**110-116.

31. Brown JD, Goekjian G, Poulson R, Valeika S, Stallknecht DE: **Avian influenza virus in water: Infectivity is dependent on pH, salinity and temperature.** *Veterinary Microbiology* 2009, **136:**20-26.

32. Costa TP, Brown JD, Howerth EW, Stallknecht DE: **Variation in viral shedding patterns between different wild bird species infected experimentally with low-pathogenicity avian influenza viruses that originated from wild birds.** *Avian Pathology* 2011, **40:**119-124.

33. Tamisier A, Dehorter O, Jay M: *Camargue, canards et foulques : Fonctionnement et devenir d'un prestigieux quartier d'hiver.* Broché; 1999.

34. Brown JD, Stallknecht DE, Swayne DE: **Experimental infections of herring gulls (*Larus argentatus*) with H5N1 highly pathogenic avian influenza viruses by intranasal inoculation of virus and ingestion of virus-infected chicken meat.** *Avian Pathology* 2008, **37:**393-397.

35. Duhem C, Roche P, Vidal E, Tatoni T: **Effects of anthropogenic food resources on yellow-legged gull colony size on Mediterranean islands.** *Population Ecology* 2008, **50:**91-100.

36. Lisnizer N, Garcia-Borboroglu P, Yorio P: **Spatial and temporal variation in population trends of Kelp Gulls in northern Patagonia, Argentina.** *Emu* 2011, **111:**259-267.

37. Germundsson A, Madslien KI, Hjortaas MJ, Handeland K, Jonassen CM: **Prevalence and subtypes of Influenza A Viruses in Wild Waterfowl in Norway 2006-2007.** *Acta Veterinaria Scandinavica* 2010, **52**.

38. Kuno G: **Persistence of arboviruses and antiviral antibodies in vertebrate hosts: its occurrence and impacts.** *Reviews in Medical Virology* 2001, **11:**165-190.

39. Staszewski V, McCoy KD, Tveraa T, Boulinier T: **Interannual dynamics of antibody levels in naturally infected long-lived colonial birds.** *Ecology* 2007a, **88:**3183-3191.

40. Lee KA: **Linking immune defenses and life history at the levels of the individual and the species.** *Integrative and Comparative Biology* 2006, **46:**1000-1015.

41. Gasparini J, McCoy KD, Tveraa T, Boulinier T: **Related concentrations of specific immunoglobulins against the Lyme disease agent Borrelia burgdorferi sensu lato in eggs, young and adults of the kittiwake (Rissa tridactyla).** *Ecology Letters* 2002, **5:**519-524.

42. Hammouda A, Selmi S, Pearce-Duvet J, Chokri MA, Arnal A, Gauthier-Clerc M, Boulinier T: **Maternal Antibody Transmission in Relation to Mother Fluctuating Asymmetry in a Long-Lived Colonial Seabird: The Yellow-Legged Gull *Larus michahellis*.** *PLoS One* 2012, **7**.

43. Pearce-Duvet JMC, Arnal A, Selmi S, Hammouda A, Gonzalez-Solis J, Alioua W, Garnier R, Mayet P, Gauthier-Clerc M, Boulinier T: **Counting eggs before they hatch: yolk antibodies reveal avian Influenza prevalence patterns.** in prep.

44. Hammouda A, Pearce-Duvet J, Chokri M, Arnal A, Gauthier-Clerc M, Boulinier T, Selmi S: **Prevalence of Influenza A antibodies in yellow-legged gull (*Larus michahellis*) eggs and adults in southern Tunisia.** *Vector-Borne and Zoonotic Diseases* 2011.

45. Pearce-Duvet JMC, Gauthier-Clerc M, Jourdain E, Boulinier T: **Maternal antibody transfer in yellow-legged gulls.** *Emerging Infectious Diseases* 2009, **15:**1147-1149.

46. Hanson BA, Luttrell MP, Goekjian VH, Niles L, Swayne DE, Senne DA, Stallknecht DE: **Is the occurrence of avian influenza virus in Charadriiformes species and location dependent?** *Journal Of Wildlife Diseases* 2008, **44:**351-361.

47. Krauss S, Walker D, Pryor SP, Niles L, Li CH, Hinshaw VS, Webster RG: **Influenza A viruses of migrating wild aquatic birds in North America.** *Vector-Borne and Zoonotic Diseases* 2004, **4:**177-189.

48. Lebarbenchon C, Chang C-M, van der Werf S, Aubin J-T, Kayser Y, Ballesteros M, Renaud F, Thomas F, Gauthier-Clerc M: **Influenza A virus in birds during spring migration in the Camargue, France.** *Journal Of Wildlife Diseases* 2007, **43:**789-793.

49. Wallensten A, Munster VJ, Latorre-Margalef N, Brytting M, Elmberg J, Fouchier RAM, Fransson T, Haemig PD, Karlsson M, Lundkvist A, et al: **Surveillance of influenza A virus in migratory waterfowl in northern Europe.** *Emerging Infectious Diseases* 2007, **13:**404-411.

50. Sol D, Arcos JM, Senar JC: **The influence of refuse tips on the winter distribution of Yellow-legged gulls *Larus cachinnans*.** *Bird Study* 1995, **42:**216-221.

51. Gasparini J, McCoy KD, Haussy C, Tveraa T, Boulinier T: **Induced maternal response to the Lyme disease spirochaete *Borrelia burgdorferi* sensu lato in a colonial seabird, the kittiwake *Rissa tridactyla*.** *Proceedings Of The Royal Society Of London Series B-Biological Sciences* 2001, **268:**647-650.

52. WHO manual on animal influenza diagnosis and surveillance: **Available from** http://whqlibdoc.who.int/hq/2002/WHO_CDS_CSR_NCS_2002.5.pdf**.** In *[cited 2012 June 9]*; 2002.

53. Kuiken T, Leighton FA, Wobeser G, Danesik KL, Riva J, Heckert RA: **An epidemic of Newcastle disease in double-crested cormorants from Saskatchewan.** *Journal Of Wildlife Diseases* 1998, **34:**457-471.

54. Burnham KP, Anderson DR: *Model Selection and Multi-Model Inference: A Practical Information-Theoretic Approach.* 2nd edn: Springer; 2002.

55. Vicente J, Hofle U, Garrido JM, Fernandez-de-Maria IG, Acevedo P, Juste R, Barral M, Gortazar C: **Risk factors associated with the prevalence of tuberculosis-like lesions in fenced wild boar and red deer in south central Spain.** *Veterinary Research* 2007, **38:**451-464.

56. Benjamini Y, Hochberg Y: **Controlling the false discovery rate a practical and powerful approach to multiple testing.** *Journal of the Royal Statistical Society Series B-Methodological* 1995, **57:**289-300.

57. Garcia LV: **Controlling the false discovery rate in ecological research.** *Trends In Ecology & Evolution* 2003, **18:**553-554.

58. Toennessen R, Germundsson A, Jonassen CM, Haugen I, Berg K, Barrett RT, Rimstad E: **Virological and serological surveillance for type A influenza in the black-legged kittiwake (*Rissa tridactyla*).** *Virology Journal* 2011, **8**.

59. Keeling MJ, Rohani P: **Estimating spatial coupling in epidemiological systems: a mechanistic approach.** *Ecology Letters* 2002, **5:**20-29.

60. McCallum H, Barlow N, Hone J: **How should pathogen transmission be modelled?** *Trends In Ecology & Evolution* 2001, **16:**295-300.

61. Bin Muzaffar S, Ydenberg RC, Jones IL: **Avian influenza: An ecological and evolutionary perspective for waterbird scientists.** *Waterbirds* 2006, **29:**243-257.

62. Garnier R, Ramos R, Staszewski V, Militao T, Lobato E, Gonzalez-Solis J, Boulinier T: **Maternal antibody persistence: a neglected life-history trait with implications from albatross**

conservation to comparative immunology. *Proceedings Of The Royal Society B-Biological Sciences* 2012, **279:**2033-2041.

63. Samraoui B, Samraoui F: **An ornithological survey of Algerian wetlands: Important Bird Areas, Ramsar sites and threatened species.** *Wildfowl* 2008, **58:** 71–96.

64. Samraoui F, Alfarhan AH, Al-Rasheid KAS, Samraoui B: **Aa appraisal of the status and distribution of waterbirds of Algeria: indicators of global changes?** *Ardeola* 2011, **58:**137-163.

65. Mateo R, Gamez A, Guitart R: **Organochlorine residues in hunted wild mallards in the Ebro Delta, Spain.** *Bulletin of Environmental Contamination and Toxicology* 1998, **60:**134-141.

66. Obenauer JC, Denson J, Mehta PK, Su XP, Mukatira S, Finkelstein DB, Xu XQ, Wang JH, Ma J, Fan YP, et al: **Large-scale sequence analysis of avian influenza isolates.** *Science* 2006, **311:**1576-1580.

67. Velarde R, Calvin SE, Ojkic D, Barker IK, Nagy E: **Avian Influenza Virus H13 Circulating in Ring-Billed Gulls (*Larus delawarensis*) in Southern Ontario, Canada.** *Avian Diseases* 2010, **54:**411-419.

68. West AP, Herr AB, Bjorkman PJ: **The chicken yolk sac IgY receptor, a functional equivalent of the mammalian MHC-related Fc receptor, is a phospholipase A(2) receptor homolog.** *Immunity* 2004, **20:**601-610.

69. Sharp GB, Kawaoka Y, Jones DJ, Bean WJ, Pryor SP, Hinshaw V, Webster RG: **Coinfection of wild ducks by influenza a viruses: Distribution patterns and biological significance.** *Journal Of Virology* 1997, **71:**6128-6135.

70. Chen R, Holmes EC: **Avian influenza virus exhibits rapid evolutionary dynamics.** *Molecular Biology and Evolution* 2006, **23:**2336-2341.

71. Van Poucke SGM, Nicholls JM, Nauwynck HJ, Van Reeth K: **Replication of avian, human and swine influenza viruses in porcine respiratory explants and association with sialic acid distribution.** *Virology Journal* 2010, **7**.

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	2009	127	42 (33%)	10
	(43°10'39"N-5°23'04"E)	2010	8	4 (50%)	0
	Frioul	2009	79	38 (48%)	9
	(43°16'27"N-5°18'15"E)	2010	51	17 (33%)	3
	Carteau	2009	102	57 (56%)	6
	(43°22'40"N-4°51'27"E)	2010	56	25 (45%)	5
	Besson	2009	79	34 (43%)	2
	(43°29'15"N-4°27'47"E)	2010	59	35 (59%)	5
	Villeneuve	2009	70	20 (29%)	6
	(42°02'50"N-3°13'21"E)	2010	64	34 (53%)	3
	Gruissan	2009	99	33 (33%)	12
	(43°06'36"N-3°06'25"E)	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	2009	38	11 (29%)	2
	(42°02'50"N-3°13'21"E)	2010	49	27 (55%)	0
	Ebro delta	2009	55	12 (22%)	0
	(40°34'17"N-0°39'37"E)	2010	29	15 (52%)	1
	Isla Grosa	2009	66	13 (20%)	6
	(37°43'40"N-0°42'27"O)	2010	50	16 (32%)	1
Algeria	Chetaïbi	2009	55	17 (31%)	4
	(37°05'79"N, 7°17'53"E)	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	2009	40 (127 ; 31%)	9 (22.5%)	0
	(43°10'39"N-5°23'04"E)	2010	4 (8 ; 50%)	2 (50%)	1 (25%)
	Frioul	2009	37 (79 ; 47%)	11 (30%)	0
	(43°16'27"N-5°18'15"E)	2010	17 (51 ; 33%)	2 (12%)	1 (6%)
	Carteau	2009	55 (102 ; 54%)	8 (15%)	0
	(43°22'40"N-4°51'27"E)	2010	25 (56 ; 45%)	8 (32%)	2 (8%)
	Besson	2009	34 (79 ; 43%)	12 (35%)	0
	(43°29'15"N-4°27'47"E)	2010	35 (59 ; 59%)	12 (34%)	2 (6%)
	Villeneuve	2009	20 (70 ; 29%)	10 (50%)	0
	(42°02'50"N-3°13'21"E)	2010	34 (64 ; 53%)	22 (65%)	3 (9%)
	Gruissan	2009	32 (99 ; 32%)	5 (16%)	1 (3%)
	(43°06'36"N-3°06'25"E)	2010	49 (91 ; 54%)	21 (43%)	4 (8%)
	Corrège	2009	14 (70 ; 20%)	12 (86%)	1 (7%)
	(42°51'15"N-3°01'21"E)	2010	27 (54 ; 50%)	7 (26%)	0
Spain	Medes	2009	10 (38 ; 26%)	5 (50%)	1 (10%)
	(42°02'50"N-3°13'21"E)	2010	27 (49 ; 55%)	6 (22%)	2 (7%)
	Ebro delta	2009	12 (55 ; 22%)	3 (25%)	0
	(40°34'17"N-0°39'37"E)	2010	15 (29 ; 52%)	9 (60%)	2 (13%)
	Isla Grosa	2009	13 (66 ; 20%)	7(54%)	4 (31%)
	(37°43'40"N-0°42'27"O)	2010	16 (50 ; 32%)	4 (25%)	4 (25%)
Algeria	Chetaïbi	2009	17 (55 ; 31%)	6 (35%)	1 (6%)
	(37°05'79"N, 7°17'53"E)	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

Figure legends

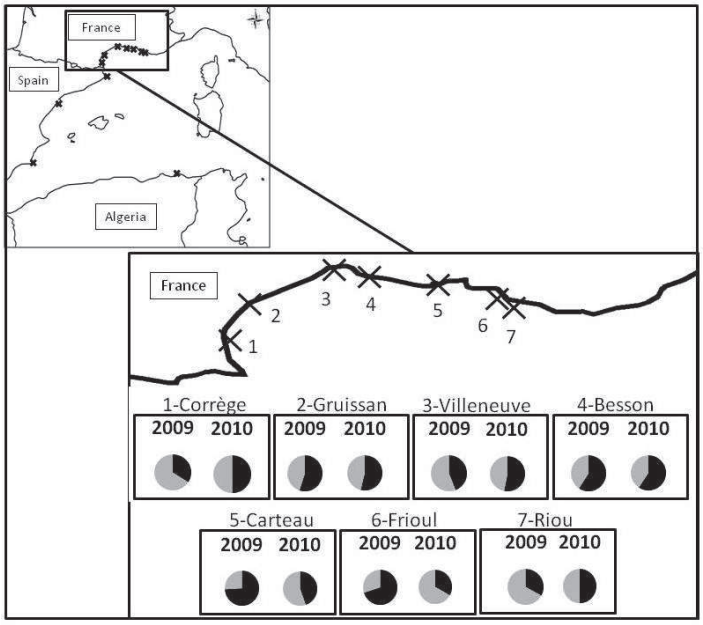
Figure 1: **Prevalence patterns of antibodies against AIVs estimated from eggs in a) France; b) Spain; c) Algeria.**

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.

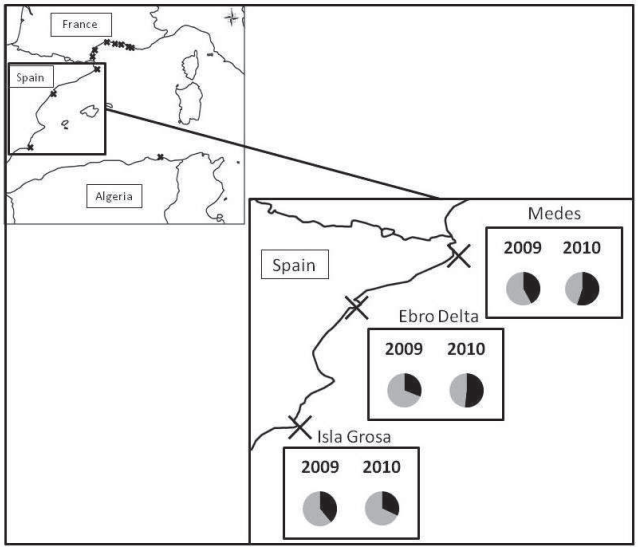
Figure 2: HI titer observed for AIV-subtypes a) H13 and b) H16.

Figure 1

a)


France
Spain
Algeria
France
1
2
3
4
5
6
7
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

b)


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

c)

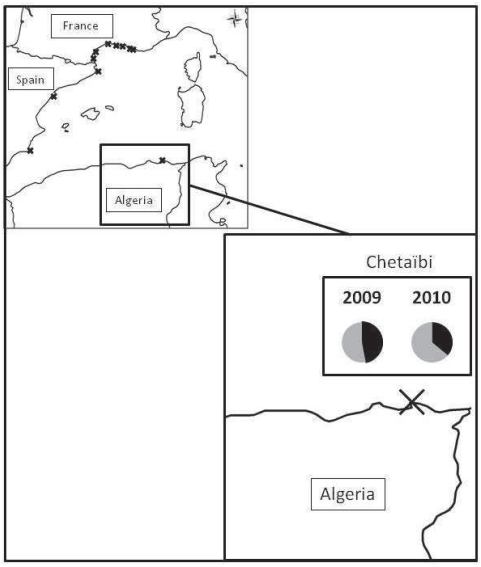
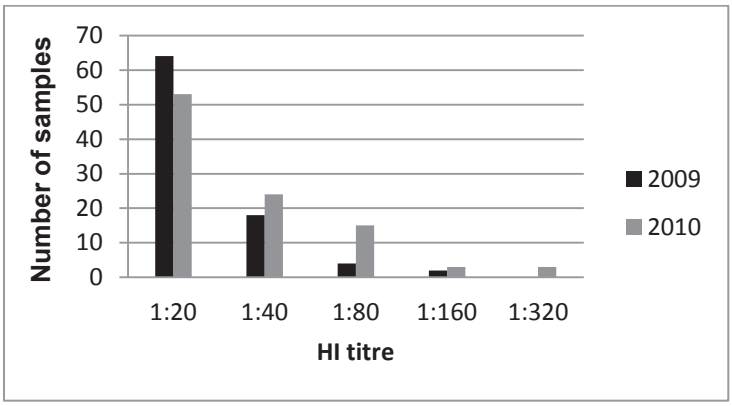
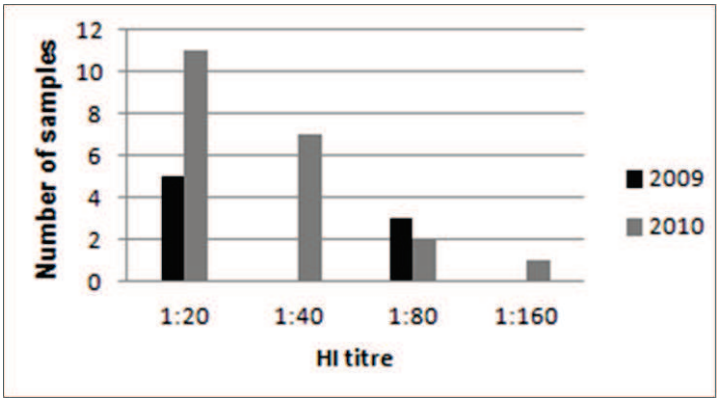

France
Spain
Algeria
Chetaïbi
2009 2010
Algeria

Figure 2

a)


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

b)


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

Article 5

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

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Abstract
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.

Running title
Meaban virus in gulls and seabird ticks in Spain

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

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.

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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.

References

1. Gould EA, Higgs S. Impact of climate change and other factors on emerging arbovirus diseases. Transactions of the Royal Society of Tropical Medicine and Hygiene. 2009 Feb;103(2):109-21.
2. Gubler DJ. The global resurgence of arboviral diseases. Transactions of the Royal Society of Tropical Medicine and Hygiene. 1996 Sep-Oct;90(5):449-51.
3. Porterfield JS. Basis of arbovirus classification. Medical biology. 1975 1975;53(5):400-5.
4. Chastel C, Main AJ, Guiguen C, Lelay G, Quillien MC, Monnat JY, et al. The isolation on Meaban virus, a new *Flavivirus* from the seabird tick *Ornithodoros* (*Alectorobius*) *maritimus* in France. Archives of Virology. 1985a;83(3-4):129-40.
5. Hubalek Z, Wegner E, Halouzka J, Tryjanowski P, Jerzak L, Sikutova S, et al. Serologic survey of potential vertebrate hosts for West Nile virus in Poland. Viral Immunology. 2008 Jun;21(2):247-53.
6. Calistri P, Giovannini A, Hubalek Z, Ionescu A, Monaco F, Savini G, et al. Epidemiology of West Nile in Europe and in the Mediterranean basin. Open Virol J. 2010;4:29-37.
7. EpiSouth. West Nile virus circulation in the EpiSouth countries and neighbouring areas (Hungary and Russia). Update 6th of October 2010; www.episouth.org/download.php?&id=12182.
8. Calzolari M, Bonilauri P, Bellini R, Albieri A, Defilippo F, Maioli G, et al. Evidence of simultaneous circulation of West Nile and Usutu viruses in mosquitoes sampled in Emilia-Romagna region (Italy) in 2009. PLoS One. 2010 Dec;5(12):e14324.
9. Busquets N, Alba A, Allepuz A, Aranda C, Nunez JI. Usutu virus sequences in Culex pipiens (Diptera : *Culicidae*), Spain. Emerging Infectious Diseases. 2008 May;14(5):861-3.
10. Cavrini F, Gaibani P, Longo G, Pierro AM, Rossini G, Bonilauri P, et al. Usutu virus infection in a patient who underwent orthotropic liver transplantation, Italy, August-September 2009. European communicable disease bulletin. 2009 Dec;14(50).

11. Dobler G. Zoonotic tick-borne flaviviruses. Veterinary Microbiology. 2010 Jan 27;140(3-4):221-8.
12. Chastel C, Guiguen C, Lelay G, Monnat JY, Hardy E, Kerdraon G, et al. Arbovirus serosurvey among seabirds and landbirds in Brittany. Bulletin De La Societe De Pathologie Exotique. 1985b;78(5):594-605.
13. Chastel C, Guiguen C, Lelay G, Monnat JY, Quillien MC, Beaucournu JC. Are the Arboviruses from seabirds colonies in Brittany able to infect man. Revue d'épidemiologie et de sante publique. 1983;31(4):445-57.
14. Mansfield KL, Johnson N, Phipps LP, Stephenson JR, Fooks AR, Solomon T. Tick-borne encephalitis virus - a review of an emerging zoonosis. Journal of General Virology. 2009 Aug;90:1781-94.
15. Sol D, Arcos JM, Senar JC. The influence of refuse tips on the winter distribution of Yellow-legged gulls *Larus cachinnans*. Bird Study. 1995 Nov;42:216-21.
16. Oro D, Martinez-Abrain A. Deconstructing myths on large gulls and their impact on threatened sympatric waterbirds. Animal Conservation. 2007 Feb;10(1):117-26.
17. Baaloudj A, Samraoui F, Laouar A, Benoughidene M, Hasni D, Bouchahdane I, et al. Dispersal of yellow-legged gulls *Larus michahellis* ringed in Algeria: a preliminary analysis. Ardeola. 2012;59(1):137-44.
18. Malkinson M, Banet C, Weisman Y, Pokamunski S, King R, Drouet MT, et al. Introduction of West Nile virus in the Middle East by migrating white storks. Emerging Infectious Diseases. 2002 Apr;8(4):392-7.
19. Jourdain E, Toussaint Y, Leblond A, Bicout DJ, Sabatier P, Gauthier-Clerc M. Bird species potentially involved in introduction, amplification, and spread of West Nile virus in a Mediterranean wetland, the Camargue (Southern france). Vector-Borne and Zoonotic Diseases. 2007 Spr;7(1):15-33.
20. Hammouda A, Pearce-Duvet J, Chokri M, Arnal A, Gauthier-Clerc M, Boulinier T, et al. Prevalence of Influenza A antibodies in yellow-legged gull (*Larus michahellis*) eggs and

adults in southern Tunisia. Vector-Borne and Zoonotic Diseases 2011.
21. Pearce-Duvet JMC, Gauthier-Clerc M, Jourdain E, Boulinier T. Maternal antibody transfer in yellow-legged gulls. Emerging Infectious Diseases. 2009 Jul;15(7):1147-9.
22. Kuno G. Persistence of arboviruses and antiviral antibodies in vertebrate hosts: its occurrence and impacts. Reviews in Medical Virology. 2001 May-Jun;11(3):165-90.
23. Gasparini J, McCoy KD, Tveraa T, Boulinier T. Related concentrations of specific immunoglobulins against the Lyme disease agent Borrelia burgdorferi sensu lato in eggs, young and adults of the kittiwake (Rissa tridactyla). Ecology Letters. 2002 Jul;5(4):519-24.
24. Gibbs SEJ, Hoffman DM, Stark LM, Marlenee NL, Blitvich BJ, Beaty BJ, et al. Persistence of antibodies to West Nile virus in naturally infected rock pigeons (*Columba livia*). Clinical and Diagnostic Laboratory Immunology. 2005 May;12(5):665-7.
25. Mansfield KL, Horton DL, Johnson N, Li L, Barrett ADT, Smith DJ, et al. Flavivirus-induced antibody cross-reactivity. Journal of General Virology. 2011 Dec;92:2821-9.
26. Gasparini J, McCoy KD, Haussy C, Tveraa T, Boulinier T. Induced maternal response to the Lyme disease spirochaete *Borrelia burgdorferi* sensu lato in a colonial seabird, the kittiwake *Rissa tridactyla*. Proceedings Of The Royal Society Of London Series B-Biological Sciences. 2001;268(1467):647-50.
27. Garnier R, Ramos R, Staszewski V, Militao T, Lobato E, Gonzalez-Solis J, et al. Maternal antibody persistence: a neglected life-history trait with implications from albatross conservation to comparative immunology. Proceedings Of The Royal Society B-Biological Sciences. 2012 May 22;279(1735):2033-41.
28. Dauphin G, Zientara S. West Nile virus: Recent trends in diagnosis and vaccine development. Vaccine. 2007 Jul 26;25(30):5563-76.

29. Oliphant T, Diamond MS. The molecular basis of antibody-mediated neutralization of West Nile virus. Expert Opinion on Biological Therapy. 2007 Jun;7(6):885-92.
30. Figuerola J, Jimenez-Clavero MA, Rojo G, Gomez-Tejedor C, Soriguer R. Prevalence of West Nile virus neutralizing antibodies in colonial aquatic birds in southern Spain. Avian Pathology. 2007b Jun;36(3):209-12.
31. Sanchez-Seco MP, Rosario D, Domingo C, Hernandez L, Valdes K, Guzman MG, et al. Generic RT-nested-PCR for detection of flaviviruses using degenerated primers and internal control followed by sequencing for specific identification. Journal Of Virological Methods. 2005 Jun;126(1-2):101-9.
32. Grard G, Moureau G, Charrel RN, Lemasson J-J, Gonzalez J-P, Gallian P, et al. Genetic characterization of tick-borne flaviviruses: New insights into evolution, pathogenetic determinants and taxonomy. Virology. 2007 Apr 25;361(1):80-92.
33. Bofill D, Domingo C, Cardenosa N, Zaragoza J, de Ory F, Minguell S, et al. Human West Nile virus infection, Catalonia, Spain. Emerging Infectious Diseases. 2006 Jul;12(7):1163-4.
34. EstradaPena A, Bosch M, Pedrocchi V. First record of *Ornithodoros (Alectorobius) maritimus* Vermeil & Marguet (Acari: Argasidae) in Spain. Acarologia. 1996 Apr;37(1):19-21.
35. Oro D. Living in a ghetto within a local population: An empirical example of an ideal despotic distribution. Ecology. 2008 Mar;89(3):838-46.
36. Cramp S, Simmons KEL, Brooks DJ, Collar NJ, Dunn E, Gillmor R, et al. Handbook of the Birds of Europe, the Middle East and North Africa: The Birds of the Western Palearctic, . Oxford; 1983.
37. McCoy KD, Boulinier T, Tirard C. Comparative host-parasite population structures: disentangling prospecting and dispersal in the black-legged kittiwake *Rissa tridactyla*. Molecular Ecology. 2005 Aug;14(9):2825-38.

38. Gomez-Diaz E, Morris-Pocock JA, Gonzalez-Solis J, McCoy KD. Trans-oceanic host dispersal explains high seabird tick diversity on Cape Verde islands. Biology letters. 2012.
39. Nuttall PA, Kelly TC, Carey D, Moss SR, Harrap KA. Mixed Infections with Tick-Borne Viruses in a Seabird Colony in Eire. Archives of Virology. 1984 1984;79(1-2):35-44.
40. Gomez-Diaz E, Doherty PF, Jr., Duneau D, McCoy KD. Cryptic vector divergence masks vector-specific patterns of infection: an example from the marine cycle of Lyme borreliosis. Evolutionary Applications. 2010 Jul;3(4):391-401.

Table.1. Prevalence of antibodies directed against the flavivirus envelop protein found in yellow-legged gulls for all colonies sampled

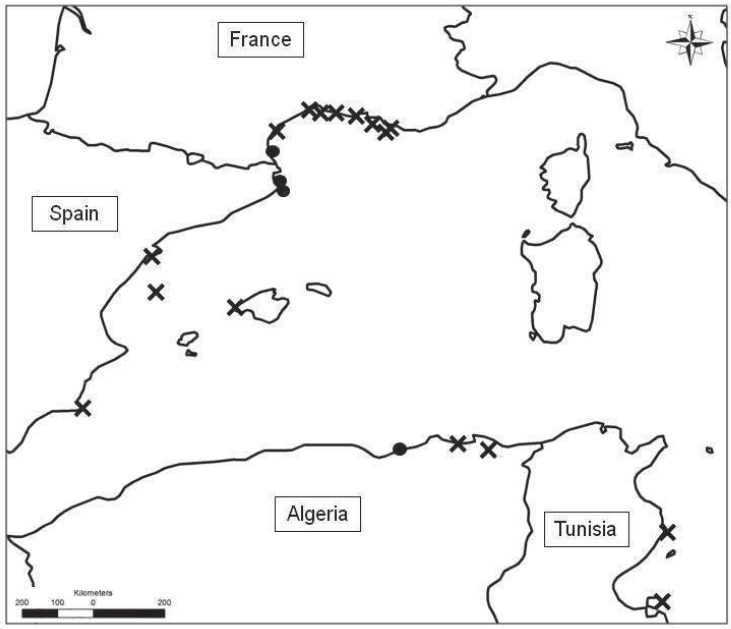
Country	Colony	Year	Sample size	Number of positive samples	Number of doubtful samples
France	1-Plane	2010	20	0	
	2-Riou	2009	32	0	
		2010	8	0	
	3-Frioul	2009	32	0	
		2010	32	0	
	4-Carteau	2009	32	0	
		2010	32	0	
		2011	30	0	
	5-Besson	2009	32	0	
		2010	32	0	
	6-Grau-du- roi	2009	32	0	
	7-Villeneuve	2009	32	0	
		2010	32	0	
	8-Gruissan	2009	32	0	
		2010	32	0	
	9-Corrège	2009	32	1	1
		2010	32	1	
Spain	10-Escala	2010	6	4	
	10-Medes	2009	38	14	6
		2010	49	24	1
		2011	49	33	3
	11-Ebro delta	2009	32	0	
		2010	29	0	
		2011	30	0	
	12-Columbretes	2010	50	0	
	13-Dragonera	2010	39	0	
	14-Isla Grosa	2009	32	0	
		2010	32	0	
		2011	30	0	
Algéria	15-Jijel	2010	31	1	
	16-Skikda	2010	17	0	
	17-Chetaïbi	2009	32	0	
		2010	32	0	
Tunisia	18-Djerba	2009	32	0	
	19-Sfax	2009	32	0	1

Table.2. Results of virus neutralization tests to identify if the antibodies were specific to West Nile, Usutu, Tick-borne encephalitis, and Meaban viruses

Country	Colony	Year	West Nile virus sample size (positive)	Usutu virus sample size (positive)	Tick-borne encephalitis sample size(positive)	Meaban virus sample size (positive)
France	Corrège	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.

Figure



Article 6

Recent circulation of West Nile and other flaviviruses

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Abstract

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.

Running title
Circulation of flaviviruses in Southern France

Keywords
West Nile virus, Flavivirus, Serology, Birds

Text

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.

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Disclosure Statement

No competing financial interests exist.

References

Balança G, Gaidet N, Savini G, Vollot B, et al. Low West Nile virus circulation in wild birds in an area of recurring outbreaks in Southern France. Vector Borne Zoonotic Dis 2009;9:737-741

Becker N, Jöst H, Ziegler U, Martin Eiden M, et al. Epizootic emergence of Usutu virus in wild and captive birds in Germany. PLoS ONE 2012;7:e32604. DOI:10.1371/journal.pone.0032604

European Centre for Disease Prevention and Control (ECDC). West Nile fever maps [cited July 03, 2012]. Available from: http://ecdc.europa.eu/en/healthtopics/west_nile_fever/West-Nile-fever-maps/Pages/index.aspx

Figuerola J, Jiménez-Clavero MA, Rojo G, Gómez-Tejedor C, et al. Prevalence of West Nile virus neutralizing antibodies in colonial aquatic birds in southern Spain. Avian Pathol 2007; 36:209-212.

Jourdain E, Schuffenecker I, Korimbocus J, Reynard S, et al. West Nile virus in wild resident birds, Southern France, 2004. Vector Borne Zoonotic Dis 2007;7:448-452.

Jourdain E, Gauthier-Clerc M, Sabatier P, Grège O, et al. Magpies as hosts for West Nile virus, Southern France. Emerg Infect Dis 2008;14:158-160.

Savini G, Monaco F, Terregino C, Di Gennaro A et al. Usutu virus in ITALY: An emergence or a silent infection? Vet Microbiol 2011;151:264–274.

Svensson L. Identification guide to European passerines. British Trust for Ornithology 1992.267-268.

Vazquez A, Sanchez-Seco MP, Palacios G, Molero F, et al. Novel flaviviruses detected in different species of mosquitoes in Spain. Vector Borne Zoonotic Dis 2012;12:223-229.

Weissenbock H, Hubálek Z, Bakonyi T, Nowotny N. Zoonotic mosquito-borne flaviviruses: Worldwide presence of agents with proven pathogenicity and potential candidates of future emerging diseases. Vet Microbiol 2010;140:271-280.

Tables

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

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

Article 7

Maternal antibody transmission in relation to mother fluctuating asymmetry in a long-lived colonial seabird: yellow-legged gull *Larus michahellis*

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Abstract

Female birds transfer antibodies to their offspring via the egg yolk, thus possibly providing passive immunity against infectious diseases to which hatchlings may be exposed, and affecting their fitness. It is nonetheless unclear whether the amount of maternal antibodies transmitted into egg yolks varies with female quality and egg laying order. In this paper, we investigated the transfer of maternal antibodies against type A influenza viruses (anti-AIV antibodies) by a long-lived colonial seabird, the yellow-legged gull (*Larus michahellis*), in relation to fluctuating asymmetry in females, i.e. the random deviation from perfect symmetry in bilaterally symmetric morphological and anatomical traits. In particular, we tested whether females of greater asymmetry transmitted fewer antibodies to their eggs, and whether within-clutch variation in yolk antibodies varied according to the maternal level of fluctuating asymmetry. We found that asymmetric females were in worse physical condition, produced fewer antibodies, and transmitted lower amounts of antibodies to their eggs. We also found that, within a given clutch, yolk antibody level decreased with egg laying order, but this laying order effect was more pronounced in clutches laid by the more asymmetric females. Overall, our results support the hypothesis that maternal quality interacts with egg laying order in determining the amount of maternal antibodies transmitted to the yolks. They also highlight the usefulness of fluctuating asymmetry as a sensitive indicator of female quality and immunocompetence in birds.

Running title: *Fluctuating asymmetry and maternal antibodies*

Key-words: *female quality, fluctuating asymmetry, laying order, maternal antibodies*

Introduction

Most organisms face a dangerous world, in which parasitic species outnumber host species [1], and vertebrates have evolutionarily responded to this threat by developing a complex immune system in which antibodies provide tailored protection against the particular pathogens encountered. In vertebrates, antibodies are known to be maternally transferred, thus conferring passive immune protection against parasites faced by the offspring after birth or hatching and potentially impacting offspring growth and survival [2, 3, 4]. In birds, as the production of immunoglobulins and their transfer are energetically costly [5, 6, 2, 7], the degree of immunity provided by a mother to her offspring should be strongly affected by her quality.

Female quality may affect maternal antibody transfer in two ways: through the overall capacity to produce and deposit antibodies and their distribution within the clutch. Overall, higher quality females should be more capable of synthesizing immunoglobulins and depositing them in egg yolks than lower quality ones [2, 7, 8]. At the level of the clutch, patterns become more complicated and have received far less attention. The quantity of maternal antibodies deposited is known to vary among eggs according to egg laying order, but the directionality of this relationship depends on the reproductive strategy adopted [9, 10, 11].

In altricial birds, two opposite reproductive strategies evolved in response to asynchronous egg hatching in circumstances of unpredictable food availability [see 12]. In many species, females may seek to improve whole brood survival by increasing their investment in the last-laid egg, thus reducing the effect of hatching asynchrony on nestling competition and improving the survival probability of the youngest hatchling [13, 14]. However, species more commonly demonstrate adaptive brood reduction. In this system, females may

improve the survival probability of the first nestlings by allocating more resources to the first-laid eggs, which are likely to have the highest reproductive value, and thus sacrifice the ones that hatch last [15, 16]. In both systems, the ability of females to maintain a differential antibody transmission to eggs according to their laying order, and thus the extent of the laying order effect, would reflect their quality. In the case of a brood reduction strategy, two opposite trends could be predicted. First, lower quality females may be less able to control antibody deposition into egg yolks in accordance with laying order, resulting in a reduced laying order effect. Alternatively, because lower quality females could have a lesser amount of antibodies to deposit in their eggs, they may transmit most of this amount to the first eggs, at the cost of the last ones. This would result in a more pronounced laying order effect.

Female quality is often estimated by determining the level of fluctuating asymmetry (FA), i.e. the random deviation from perfect symmetry in bilaterally symmetric morphological traits [17, 18]. FA reflects deficiency in the early-life developmental processes, i.e. developmental instability, due to stressful conditions such as food limitation, parasitism and other challenges [19, 20, 21, 22]. It is generally negatively correlated with fitness-related traits [23] and is increasingly viewed as a reliable morphological indicator of individual quality [23, 24, 25]. In general, more asymmetric birds have lower survival and breeding success than symmetric ones. Using this line of reasoning, FA could be used as an indicator of female immunocompetence and one could expect females with greater asymmetry to produce fewer antibodies and to transmit lower amounts of antibodies to their eggs than do the more symmetric ones.

We investigated the extent to which maternal quality, as estimated through fluctuating asymmetry, contributes to within-clutch variation in yolk antibodies using avian influenza in the yellow-legged

gull (*Larus michahellis*) as a model. The yellow-legged gull is a suitable model for such an investigation as it is a long-lived species, demonstrates asynchronous egg hatching [26, 27], and appears to adopt the brood reduction strategy [28].

Methods

Ethics statement

This study complies with the current Tunisian laws regarding ethics and animal use for scientific purposes. It was approved by the "Forest Department" in the Tunisian Ministry of Agriculture, which is the relevant authority in charge of wildlife use and conservation in Tunisia, through the permit number 518-28/02/2009.

In the Mediterranean region, the yellow-legged gull is an abundant species, and is subject to population control measures [29, 30, 31], which makes it amenable to adult capture and egg sampling. Upon capture, birds were kept in a safe position as is standard practice in bird ringing studies. Blood samples were taken from the brachial vein using a sterile syringe and the puncturing site was accurately disinfected. All individuals were released as soon as possible. After being released, the sampled birds behaved normally and resumed their normal breeding activities.

Study species and area

The yellow-legged gull is a socially monogamous and semicolonial bird of the family *Laridae*. It is long-lived and shows strong interannual breeding site fidelity [32]. It typically lays one clutch

per season, with a modal clutch size of 3 eggs, although replacement clutches are possible. Eggs are laid at 1-3 day intervals and hatching is typically asynchronous. Egg volume decreases along the laying sequence, with markedly smaller last-laid eggs [26, 27]. Chicks are semiprecocial and remain around the nest for the first few days of life, after which they become highly mobile. In Tunisia, the yellow-legged gull is a common and abundant resident bird that nests on the numerous small islands along the coast, as well as in some coastal wetlands [33]. Our work was conducted during the spring of 2009 in two breeding colonies situated in the gulf of Gabès, in south-eastern Tunisia: Sfax salina (34°42'28"N-10°45'02"E) and the small islets between Djerba island and the Zarzis peninsula (33°39'10"N-10°58'59"E). The yellow-legged gull in this area is often seen feeding on open air rubbish dumps and discards of commercial fisheries.

Data collection

Egg and blood sampling and morphological measurements

Randomly selected nests were marked with small wooden stakes. The nests were checked every 1-2 days and any eggs were collected. Each egg was marked according to its rank and laying date and then replaced by a dummy gull egg to prevent females from abandoning their nests. The eggs were brought to the laboratory on the day of collection for processing. Collected eggs were thus 1 to 2 days old and their yolk composition was not yet affected by embryonic development. The yolk of each collected egg was separated from the albumen, homogenized, and frozen at -20°C. Only the results from nests containing three eggs were considered in this study.

Following clutch completion, we trapped as many of the incubating parents as possible by means of noose-carpet traps we placed

on the nests. Each captured bird was marked with a patch of paint on the head to avoid resampling. Upon capture, birds were weighed (± 20 g) with a spring scale (PESOLA®, Switzerland). Four bilateral morphological traits were then measured with a digital caliper (±0.01 mm) on both the left (l) and right (r) sides: (1) Nalospi length (N), defined as the distance from the tip of the bill to the nostril, (2) lower mandible length (B), defined as the distance from the tip of the lower mandible to the corner of the mouth, (3) tarsus length (T), defined as the tarso-metatarsus length, and (4) middle toe length (P), defined as the distance from the first scale to the base of the nail of the middle toe. All measurements were carried out by the same observer (A. Hammouda). In order to assess measurement repeatability, each measurement was performed 3 times on each side.

Before releasing the bird, a 1-ml blood sample was taken from the brachial vein using a sterile syringe. The blood sample was immediately transferred to a heparinized tube and maintained in a cooler at 4°C while in the field. Once in the laboratory, a subsample of blood (50-70µl) was placed in microcapillary tubes, and centrifuged at 11 500 r.p.m. for 10 min. The haematocrit value was defined as the proportion of the microcapillaries that were occupied by red blood cells. Haematocrit value was regarded as an index of the health status of the bird [34, 35]. Whole blood was centrifuged at 2500 rpm for 15 min. The plasma and blood cells were frozen separately at -20°C until immunological analyses could be performed.

Sex determination

The sampled birds were sexed following the molecular method [36]. DNA was extracted from the blood using a DNeasy Blood & Tissue Handbook 07/2007 kit and used in PCR with primers 2550

Forward and 2718 Reverse to amplify introns from the CHD-*Z* and CHD-*W* genes, located on the avian sex chromosomes [37]. PCR fragments were then separated on an electrophoresis agarose gel. In this method, a single band of DNA on the gel indicated that a bird was a male, while two bands were present for females.

Immunological analyses

Anti-AIV antibodies in plasma and yolk samples were measured using a commercial competitive enzyme-linked immunosorbent assay (ELISA) developed for use in birds (ID Screen® Antibody Influenza A Competition, ID VET, Montpellier, France). The assay is designed to detect antibodies directed against the internal AIV nucleocapsid and thus it will detect all AIV subtypes. Plasma samples were used directly in the immunological assays. However, yolk antibodies were first extracted [38, 39]. Egg yolks were thawed and homogenized. A subsample of 800 mg of yolk was then diluted 1:1 in phosphate-buffered saline solution (PBS) to which a few glass beads were added. The solution was shaken in a mill until a homogenous emulsion was obtained and an equal volume of reagent-grade chloroform was added to the mixture. The yolk-chloroform blend was then centrifuged at 16 000 rpm for 15 min and the clear supernatant was used in the immunological assays.

Plasma and yolk supernatant samples were diluted 1:100 and incubated at 37°C for one hour. After a washing step, a peroxidase-marked conjugate was added to each well and the samples were incubated for 30 min at 21°C. The plates were then washed again, a substrate solution was added to each well, and the samples were incubated for 10 min at 21°C in the dark. Finally, a stop solution was added to each well in order to stop the reaction. Optical density (OD)

was read at 450 nm using a spectrophotometer. A subset of samples were repeated, both within and across plates and we found that OD measurements were highly repeatable (within: $r = 0.913$, $F_{[29,30]} = 22.84$, $P < 0.0001$; across: $r = 0.85$, $F_{[33,24]} = 9.56$, $P < 0.0001$).

According to the kit instructions, the results were expressed as the percentage competition (PC) between the OD of the sample being tested and the mean OD of a negative control sample (NC), such that $PC = (OD_{specimen} / OD_{NC}) \times 100$. The percentage competition values were then transformed into percentage inhibition values (PI) using the formula: PI = 100 - PC. The PI was used as measure of anti-AIV antibody concentration.

Data analyses

Fluctuating asymmetry and body condition

For each measured bilateral trait (B, N, T and P), we calculated the signed difference between the left and right sides ($l - r$), the absolute difference between left and right ($|l - r|$), and the average size [$(l + r) / 2$]. To evaluate the possibility of anti-symmetry (i.e. a tendency away from bilateral symmetry), we checked for departures from normality of the distribution of the signed differences ($l - r$) using the Shapiro-Wilk test. To test for any directional asymmetry (i.e. biased to one side), a Student's *t*-test was used to determine whether the mean of signed differences between left and right sides ($l - r$) was significantly different from zero [40]. If no directional asymmetry is present and the distribution of signed differences is normal, then the variation in these differences represents classical fluctuating asymmetry [40]. For all the four traits (B, N, T and P), we calculated a size-corrected index of fluctuating asymmetry: $FA_i = [(|l - r|) / ((l + r) / 2)]$. We then calculated a composite fluctuating asymmetry index FA for

each female, by summing the FA_i values across the four traits: $FA = FA_B + FA_N + FA_T + FA_P$.

In order to obtain one composite measure of female body condition, a Principal Components Analysis was carried out on the following parameters: hematocrit value (%), clutch volume (cm^3), average egg volume (cm^3), and the residuals of the regression of body mass (g) on tarsus length (cm) as a measure of size-corrected female mass. Only factors whose eigenvalues exceeded 1 were retained (see results). Egg volume was calculated using the following formula: egg volume (cm^3) = 0.000476 x length (mm) x width^2 (mm) [41]. Larger eggs and clutches are supposed to be laid by females with better body conditions [42].

Body condition, fluctuating asymmetry and patterns of maternal antibody transfer

The relationship between female body condition index (BCI), as a response variable, and fluctuating asymmetry score (FA), as an explanatory variable, was assessed by means of simple linear regression. We also used separate linear regressions to investigate the relevance of female BCI and FA as possible predictors of female anti-AIV antibody levels. In the latter regressions, female anti-AIV antibody level was arcsin-transformed to ensure the normal distribution of model residuals. Furthermore, in order to check if females with higher antibody levels transmitted higher amounts of antibodies to their eggs, we calculated the average antibody level (average PI) for each clutch and then regressed these average egg antibody levels (arcsin-transformed) on the plasma antibody levels of the corresponding mothers (arcsin-transformed). We also regressed the average egg antibody levels on the fluctuating asymmetry scores of the

corresponding mothers to verify whether more symmetric females transmitted more antibodies to their eggs. As we were also interested in testing whether yolk antibody level varied within clutches according to egg laying order, we conducted a repeated-measure ANOVA on yolk antibody level as a function of nest identity and egg laying order (categorical variable with three classes: eggs 1, 2 and 3). A Duncan post-hoc test was conducted to identify significant differences. Moreover, in order to determine if the extent of intra-clutch variation in yolk antibody level varied according to the level of fluctuating asymmetry in the corresponding mothers, we calculated the coefficient of variation of yolk antibody levels (intra-clutch CV) for each clutch, and then tested for the significance of female fluctuating asymmetry score as a predictor of intra-clutch CV (log-transformed) by means of simple linear regression. Finally, we calculated the difference in antibody level between the first-laid and the last-laid egg in each clutch, and we checked whether this difference (log(x+10)-transformed) was related to maternal FA by means of linear regression. All statistical analyses and tests were carried out using SAS software [43]. All means are reported in the text are ±1SE.

Results

We were able to obtain complete data (blood and yolk antibody levels, morphological measurements, hematocrit values, and clutch and egg volumes) on a total of 18 anti-AIV antibody positive females. Anti-AIV antibody level (%) varied between 75 and 97 (mean = 90.96 ± 1.21) in plasma samples and between 34 and 100 (mean = 80.58 ± 2.03) in egg yolk samples. Female hematocrit (%) ranged from 39 to 59, with an average of 46 ± 1. Clutch and egg volumes (cm^3) ranged respectively from 223 to 306 (mean = 256.22 ± 4.74) and from 74 to 102 (mean =

85.41 ± 1.58). Female weight (g) ranged from 936 to 1100, with an average of 1005.17±11.28.

Female fluctuating asymmetry, body condition and plasma anti-AIV antibody level

Descriptive statistics of signed ($l - r$) values for each of the four measured morphological traits are shown in Table 1. No trait had an average ($l - r$) that significantly departed from zero ($P > 0.05$ for all comparisons, Table 1). Furthermore, for all traits, the signed ($l - r$) values showed a normal distribution ($P > 0.05$ for all traits, Table 1). Overall, these results would suggest that in all traits the observed asymmetry was fluctuating and that no problem of directional asymmetry or antisymmetry occurred in the data. The composite fluctuating asymmetry index (FA), obtained by summing the calculated size-corrected fluctuating asymmetry values across the four traits, ranged from 0.016 to 0.087, with a mean value of 0.046 ± 0.005.

In order to obtain a single estimate of female body condition, the Principal Component Analysis conducted on the four original variables (i.e., the hematocrit value, clutch volume, average egg volume and size-corrected weight) allowed us to summarise these variables into one factor accounting for 66% of the original variance. This factor had an eigenvalue of 2.62, while the eigenvalue of the second factor did not exceed 1 (0.97). The first factor derived from this PCA provided one composite index of body condition (BCI) that is positively correlated with female hematocrit value ($r = 0.502$, $P = 0.033$), clutch volume ($r = 0.928$, $P < 0.0001$), average egg volume, ($r = 0.928$, $P < 0.0001$) and size-corrected body weight ($r = 0.802$, $P < 0.0001$). Females with the highest BCI values thus had the highest hematocrit values, the largest egg- and clutch sizes, and greatest size-corrected mass.

The results of the regression analyses show that female BCI was negatively associated with FA ($r^2 = 0.28$, $\beta \pm SE = -23.44 \pm 9.36$, $F_{1,16} = 6.27$, $P = 0.0235$, Fig.1-A). We also found that anti-AIV antibody levels in female plasma were negatively related to FA ($r^2 = 0.26$, $\beta \pm SE = -2.50 \pm 1.05$, $F_{1,16} = 5.63$, $P = 0.0306$, Fig.1-B), but were not correlated with BCI ($r^2 = 0.06$, $\beta \pm SE = 0.03 \pm 0.03$, $F_{1,16} = 0.95$, $P = 0.3449$). These results would suggest that females demonstrating greater asymmetry are also characterized by poorer body conditions and lower plasma levels of anti-AIV antibodies than the more symmetric ones.

Egg antibody level in relation to female FA and laying order

A positive relationship between the average level of anti-AIV antibodies in egg yolks and the level of anti-AIV antibodies in the plasma of the corresponding mother was found ($r^2 = 0.26$, $\beta \pm SE = 1.17 \pm 0.49$, $F_{1,16} = 5.80$, $P = 0.0285$, Fig. 2). Clutches laid by females with higher levels of anti-AIV antibodies in their plasma received more antibodies than those laid by females with lower levels of anti-AIV antibodies. Furthermore, we found that the average level of anti-AIV antibodies in egg yolks was negatively related to maternal FA ($r^2 = 0.36$, $\beta \pm SE = -379.77 \pm 124.68$, $F_{1,16} = 9.28$, $P = 0.0077$, Fig. 1-C), yet no significant correlation was found between female FA and the residuals of the regression of average egg antibody level on female antibody level (n = 18, $r = -0.40$, $P = 0.10$). Taken together, these results suggest that the eggs of more asymmetric females contain fewer antibodies because they are less competent at producing antibodies.

Within a given clutch, yolk anti-AIV antibody level varied significantly according to egg laying order (Table 2). The level of anti-AIV antibodies in the yolk decreased with the order in which an egg was laid (average values (%): 83 ± 3 for egg 1, 80 ± 4 for egg 2 and 78

± 4 for egg 3). However, only the difference between the first and the third egg was significant (post-hoc Duncan, $P < 0.05$). These results would suggest that, within a given clutch, the amount of antibodies received from the mother decreased gradually from the first-laid to the last-laid egg. Finally, we found that the extent of within-clutch variation in yolk anti-AIV antibodies (intra-clutch CV) was positively correlated with mother FA ($r^2 = 0.26$, $\beta \pm SE = 38.83 \pm 15.98$, $F_{1,16} = 2.43$, $P = 0.027$, Fig. 1-D), but the positive relationship between the difference in antibody level between the first and the last egg and mother FA was marginally significant ($r^2 = 0.17$, $\beta \pm SE = 10.39 \pm 5.72$, $F_{1,16} = 3.30$, $P = 0.0881$). Overall, the latter results would suggest that the laying order effect was more pronounced in the clutches laid by the more asymmetric females.

Discussion

The aim of this study was to investigate the interaction between female quality, as estimated by fluctuating asymmetry, and egg laying order with regards to maternal antibody transfer using the yellow-legged gull model. Little attention has previously been paid to the link between a female's morphological asymmetry and her capacity to produce and transmit antibodies into the eggs. Overall, our results support the hypothesis that clutches laid by asymmetric females received fewer antibodies and exhibited a more pronounced laying order effect than those laid by the more symmetric ones.

Both female body condition and plasma antibody level were found to be negatively correlated with the degree of fluctuating asymmetry. As fluctuating asymmetry is generally associated with a reduced ability to satisfy nutritional needs and face environmental challenges [44, 45, 18, 24], the negative relationship we found between

fluctuating asymmetry and body condition was expected. Furthermore, because individuals facing stressful conditions may be unable to mount an efficient immune response [46], the negative relationship found between female fluctuating asymmetry and plasma antibody level was also expected. The latter result stresses once more that higher amounts of plasma immunoglobulins often characterize females of higher phenotypic quality [6]. Surprisingly, no significant correlation was found between female body condition and plasma antibody level. This result seems to be in contradiction with the findings of some previous studies showing that humoral immucompetence is strongly related to female body condition during egg production [e.g., 47]. This result may signify that fluctuating asymmetry provides a better predictor of female quality and thus plasma antibody levels because it takes into account the longer term developmental history of the females studied; in contrast, the simple measure of body condition may instead reflect a shorter term snapshot of current breeding conditions. Diminished body condition and limited humoral immunocompetence should both be viewed as consequences of developmental instability due to stressful conditions during the early life stages.

Our results show that the level of antibodies in the eggs was positively correlated with the level of antibodies in their mother's plasma. This finding is consistent with the general trend that females with higher levels of antibodies in their plasma transmit more antibodies to their eggs [48, 7], whether passively or actively. Moreover, our results show that eggs laid by asymmetric females contained fewer antibodies than those laid by the more symmetric ones. Under the hypothesis that antibody transfer is a passive mechanism, such a pattern could be a direct consequence of differences among females in their capacity to produce antibodies. Alternatively, it could result from differences in antibody transmission capacity, i.e. asymmetric females transmitted fewer antibodies than predicted by the

levels in their plasma. Our results support the former rather than the latter argument: when female antibody level was controlled for, the correlation between egg antibody level and female fluctuating asymmetry disappeared. Our results thus suggest that eggs laid by asymmetric females contained fewer antibodies than those laid by more symmetric females because of maternal differences in antibody production.

These findings highlight the fact that the stressful developmental conditions that females suffer during their early life stages can not only negatively affect their phenotype, but also those of their offspring. Asymmetric females may face a greater selective disadvantage compared to more symmetric ones because, in addition to their own diminished body conditions and immunocompetence, their offspring also receive lower immune protection and are, consequently, exposed to higher mortality risks. Indeed, maternal antibodies are known to provide the young with a protection against pathogens during critical life stages [49, 50]. Low passive immune protection is generally associated with high chick mortality in birds [51, 9, 52].

Our results also show a strong laying order effect for yolk antibodies within clutches. The amount of antibodies in the yolk gradually decreased with egg laying order. This result is consistent with previous findings from related gull species [e.g., 10, 11]. It also supports the hypothesis that the yellow-legged gull adopts the brood reduction strategy [28]. By allocating less immunity to the last-laid egg, females may be enhancing the survival of earlier hatched, more reproductively valuable nestlings while lowering the survival prospects of the nestling with the lowest reproductive value (the last nestling) [15, 16]. More interestingly, we found that the degree to which yolk antibodies decrease with laying order within the clutch increases with maternal fluctuating asymmetry. Differences in antibody transmission seem to be more pronounced in the more asymmetric females compared

to the symmetric ones. This result supports the hypothesis that because asymmetric females have fewer antibodies to deposit in their eggs, the majority of their antibodies may be transferred into the first eggs to the detriment of the last ones. The brood reduction strategy seems thus to be more acutely manifest in clutches laid by the more asymmetric females. Further investigations of how egg laying order and female fluctuating asymmetry relate to chick immunocompetence, growth, and survival in other species and other reproductive systems would tell us more about this issue. In particular, an experimental approach that includes a controlled exposure of females to an antigen would help confirm the causal nature of the reported relationships.

In conclusion, the results of our work show the great complexity of factors affecting the transmission of maternal antibodies to eggs in birds. In particular, they stress the need to consider female developmental history when dealing with the ecology and evolution of immunity transfer. They also highlight the usefulness of fluctuating asymmetry as a measure of individual quality and immunocompetence.

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References

1. Dobson A, Lafferty KD, Kuris AM, Hechinger R F, Jetz W (2008) Homage to Linnaeus: How many parasites? How many hosts? Proc Natl Acad Sci USA 105: 11482-11489.
2. Grindstaff JL, Brodie EDIII, Ketterson ED (2003) Immune function across generations: integrating mechanism and evolutionary process in maternal antibody transmission. P Roy Soc Lond B Bio 270: 2309-2319.
3. Boulinier T, Staszewski V (2008) Maternal transfer of antibodies: raising immuno-ecology issues. Trends Ecol Evol 23: 282-290.
4. Hasselquist D, Nilsson JÅ (2009) Maternal transfer of antibodies in vertebrates: Trans-generational effects on offspring immunity. Phil. Trans. R. Soc. B 364: 51-60.
5. Klasing KC (1998) Nutritional modulation of resistance to infectious diseases. Poult. Sci. 77: 1119-1125.
6. Saino N, Martinelli R, Møller A P (2001) Immunoglobulin plasma concentration in relation to egg laying and mate ornamentation of female barn swallows (*Hirundo rustica*). J Evol Biol14: 95-109.
7. Grindstaff JL, Demas GE, Ketterson ED (2005) Diet quality affects egg size and number but does not reduce maternal antibody transmission in Japanese quail *Coturnix japonica*. J. Anim. Ecol. 74: 1051-1058.
8. Hargitai R, Prechl J, Török J (2006) Maternal immunoglobulin concentration in collared flycatcher (*Ficedula albicollis*) eggs in relation to parental quality and laying order. Funct Ecol 20: 829-838.
9. Buechler K, Fitze PS, Gottstein B, Jacot A, Richner H (2002) Parasite-induced maternal response in a natural bird population. J. Anim. Ecol. 71: 247-252.
10. Blount JD, Surai PF, Nager RG, Houston DC, Møller A P, et al. (2002) Carotenoids and egg quality in the lesser black-backed gull *Larus fuscus*: a supplemental feeding study of maternal effects. Proc. R. Soc. B 269: 29-36.

11. Müller W, Groothuis TGG, Dijkstra C, Siitarni H, Alatalo RV (2004) Maternal antibody transmissionand breeding densities in the Black-Headed Gull *Larus ridibundus*. Funct. Ecol. 18: 719-724.
12. Slagsvold T, Sandvik J, Rofstad G, Lorentsen O, Husby M (1984). On the adaptive value of intraclutch egg-size variation in birds. Auk 101: 687-697.
13. Schwabl H (1993) Yolk is a source of maternal testosterone for developing birds. Proc. Natl Acad. Sci. USA 90: 11446-11450.
14. Lipar JL, Ketterson ED, Nolan VJr (1999) Intra-clutch variation in testosterone content of red-winged blackbird eggs. Auk 116: 231-235.
15. Schwabl H, Mock DW, Gieg JA (1997) A hormonal mechanism for parental favouritism. Nature 386: 231.
16. Gil D, Graves J, Hazon N, Wells A (1999) Male attractiveness and differential testosterone investment in zebra finch eggs. Science 286: 126-128.
17. Watson PJ, Thornhill R (1994) Fluctuating asymmetry and sexual selection. Trends Ecol Evol 9: 21-25.
18. Møller AP, Swaddle JP (1997) Developmental Stability & Evolution. Oxford University Press, Oxford.
19. Parsons PA (1990). Fluctuating asymmetry: an epigenic measure of stress. Biol. Rev. 65: 131-154.
20. Fair JM, Hassen ES, Ricckelefs RE (1999) Growth, developmental stability and immune response in juvenvenile Japanese quail (*Coturnix coturnix japonica*). Proc. R. Soc. Lond. B. 266: 1735-1742.
21. Brown CR, Brown MB (2002) Ectoparasites cause increased bilateral asymmetry of naturally selected traits in a colonial bird. J. Evol. Biol 15: 1067-1075
22. Amat JA, Aguilera E, Visser GH (2007) Energetic and developmental costs of mounting an immune response in greenfinches (*Carduelis chloris*). Ecol Res 22: 282-287.
23. Møller AP (1999) Condition-dependent asymmetry is fluctuating asymmetry. J. Evol. Biol.12: 450-459.
24. Polak M (2003) Developmental Instability: Causes and Consequences. Oxford University Press, Oxford.

25. Mateos C, Alarcos S, Carranza J, Sánchez-Prieto CB, Valencia, J (2008) Fluctuating asymmetry of red deer antlers negatively relates to individual condition and proximity to prime age. Anim. Behav 75: 1629-1640.
26. Cramp S, (1998) The Birds of the Western Paleartic on CD Rom. *Oxford University Press, Oxford.*
27. Rubolini D, Romano M, Martinelli R, Saino N (2006) Effects of elevated yolk testosterone levels on survival, growth and immunity of male and female yellow-legged gull chicks. Behav. Ecol. Sociobiol 59: 344-352.
28. Saino N, Bertacche V, Bonisoli-Alquati1 A, Romano1 M, Rubolini D (2008) Phenotypic Correlates of Yolk and Plasma Carotenoid Concentration in Yellow-Legged Gull Chicks. Physiol. Biochem. Zool 81: 211-225
29. Bosch M, Oro D, Cantos FJ, Zabala M (2000) Short-term effects of culling on the ecology and population dynamics of the Yellow-legged Gull. J Appl Ecol 37: 369-385.
30. Vidal E, Médail F, Tatoni T (1998) Is the Yellow-legged Gull a superabundant species in the Mediterranean? Impact on fauna and flora, conservation measures and research priorities. Biodivers Conserv 7: 1013-1026.
31. Oro D, Martinez-Abrain A (2007) Deconstructing myths on large gulls and their impact on threatened sympatric waterbirds. Anim Conserv 10: 117-126.
32. Cramp, S. & Simmons KEL (Eds) (1983). Handbook of the Birds of Europe, the Middle East and North Africa: The Birds of the Western Palearctic, Volume. III. Oxford University Press, Oxford.
33. Isenmann P, Gaultier T, Hili A, Azafzaf H, Dlensi H, et al. (2005) (Eds) Oiseaux de Tunisie. Société d'Études Ornithologiques de France, Paris, France.
34. Potti J, Moreno J, Merino S, Frias O, Rodriguez R (1999) Environmental and genetic variation in the haematocrit of fledgling pied flycatchers *Ficedula hypoleuca*. Oecologia 120: 1-8.
35. Hoi-Leitner M, Romero-Pujante M, Hoi H, Pavlova A (2001) Food availability and immune capacity in serin (Serinus serinus) nestlings. Behav Ecol Sociobiol 49: 333-339.

36. Griffiths R, Double MC, Orr K, Dawson RJG (1998) A DNA test to sex most birds. Mol Ecol 7: 1071-1075.
37. Fridolfsson A K, Ellegren H (1999) A simple and universal method for molecular sexing of non-ratite birds. J Avian Biol 30: 116-121.
38. Mohammed HO, Yamamoto R, Carpenter TE, Ortmayer HB (1986) Comparison of egg yolk and serum for the detection of *Mycoplasma gallisepticum* and *M. synoviae* antibodies by enzyme-linked immunosorbent assays. Avian Dis 30: 398-408.
39. Gasparini J, McCoy KD, Haussy C, Tveraa T, Boulinier T (2001) Induced maternal response to the Lyme disease spirochaete *Borrelia burgdorferi sensu lato* in a colonial seabird, the kittiwake Rissa tridactyla. P Roy Soc Lond B 268: 647-650.
40. Palmer AR (1994) Fluctuating asymmetry analyses: A primer. In: Developmental stability: its Origins and Evolutionary Implications (ed. T. A. Markow), pp. 335-364. Kluwer Academic Publishers, Dordrecht.
41. Harris MP (1964) Aspects of the breeding biology of the gulls *Larus argentatus, L. fuscus and L. marinus.* Ibis 106: 432-456.
42. Bolton M, Monaghan P, Houston DC (1993) Proximate determination of clutch size in lesscr black-backed gulls: the roles of food supply and body condition. Can. J. Zool 71: 273-279
43. SAS Statistical Institute (1998) SAS/STAT user's guide, version 8. SAS Statistical Institute, Cary, N.C.
44. Balmford A, Jones IL, Thomas ALR (1993) On avian asymmetry: evidence of natural selection for symmetrical tails and wings in birds. Proc. R. Soc. Lond. B. 252: 245-251.
45. Thomas ALR (1993) The aerodynamic costs of asymmetry in the wings and tails of birds: asymmetric birds can't fly round tight corners. Proc. R. Soc. Lond. B 254: 181-189.
46. Read AF, Allen JA (2000) The economics of immunity. Science 290: 1104-1105
47. Pihlaja M, Siitari H, Alatalo RV (2006) Maternal antibodies in a wild altricial bird: effects on offspring immunity, growth and survival. J. Anim. Ecol. 75: 1154-1164.
48. Gasparini J, McCoy KD, Tveraa T, Boulinier T (2002) Related concentrations of specific immunoglobulins against the Lyme

disease agent Borrelia burgdorferi sensu lato in eggs, young and adults of the kittiwake (*Rissa tridactyla*). Ecol. Lett 5: 519-524.
49. Klasing KC, Leshchinsky TV (1999) Function, costs and benefits of the immune system during development and growth. In *Proceedings of the 22nd International Ornithology Congress Durban* (ed. N. J. Adams & R. H. Slotow), pp. 2817-2835. Johannesburg: Bird Life, South Africa.
50. Grindstaff JL (2008) Maternal antibodies reduce costs of an immune response during development. J. Exp. Biol 211: 654-660.
51. Heeb P, Werner I, Kölliker M, Richner H (1998) Benefits of induced host responses against an ectoparasite. Proc. R. Soc. B 265: 51-56.
52. Sahin O, Luo ND, Huang SX, Zhang QJ (2003) Effect of Campylobacter-specific maternal antibodies on *Campylobacter jejuni* colonization in young chickens. Appl Environ Microbiol 69: 5372-5379

Table. 1. Statistical properties of signed differences between the right and left sides of female yellow-legged gulls for the four measured morphological traits and results of tests of normality and mean difference with zero. N = sample size.

Trait	N	Mean±SE	Skewness	Kurtosis	Student t-test for « mean = 0 »		Shapiro-Wilk normality test	
					t	*P*	*W*	*P*
Lower mandible	18	0.09±0.18	-0.29	-0.65	0.50	0.6218	0.97	0.7045
Nalospi	18	0.13±0.07	-1.02	3.02	1.80	0.0899	0.91	0.0648
Tarsus	18	-0.11±0.22	-0.34	1.10	-0.50	0.6243	0.95	0.4818
Middle toe	18	-0.58±0.30	1.12	2.99	-1.96	0.0661	0.92	0.1135

Table 2. Results of repeated-measure ANOVA on the yolk level of anti-AIV antibodies as a function of egg laying order. Model $R^2 = 0.89$, $F_{19,34} = 14.87$, $P < 0.0001$.

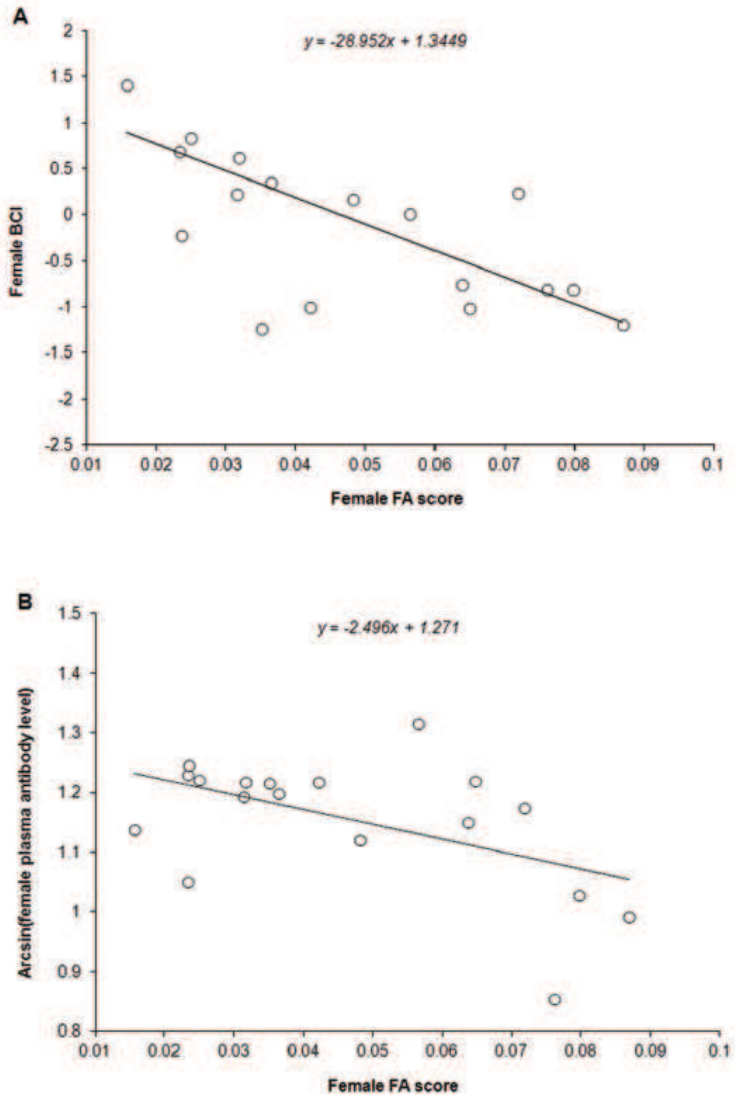
Effect	DF	Type III SS	F	P
Nest identity	17	10268.155	16.23	< 0.0001
Laying order	2	242.267	3.26	0.0509

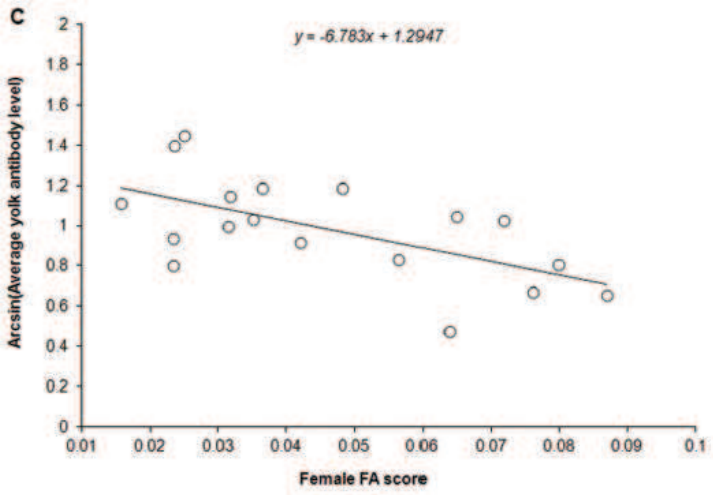
Figure legends

Figure 1. Relationship between female fluctuating asymmetry and (A) female body condition index, (B) anti-AIV antibody level in female plasma, (C) average yolk anti-AIV antibody level, and (D) intra-clutch CV of yolk anti-AIV antibody levels.

Figure 2. Mean egg antibody level as a function of plasma antibody level across all females sampled.

Figure 1.


A
y = -28.952x + 1.3449
Female BCI
Female FA score
B
y = -2.496x + 1.271
Arcsin(female plasma antibody level)
Female FA score


C
y = -6.783x + 1.2947
Arcsin(Average yolk antibody level)
Female FA score

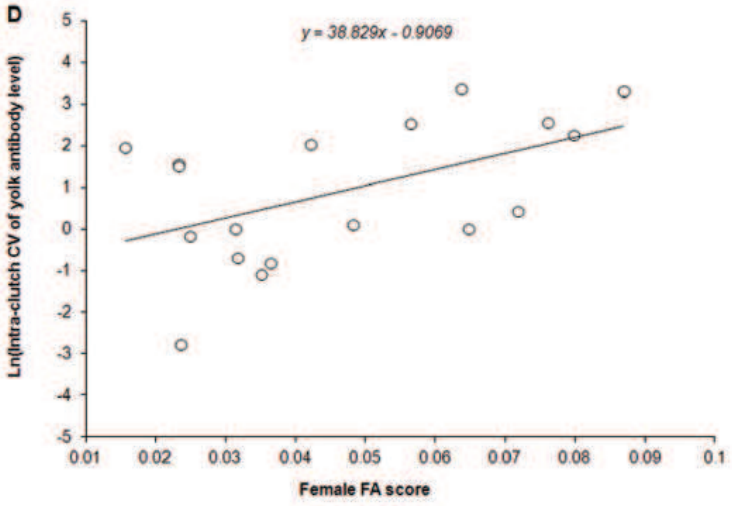
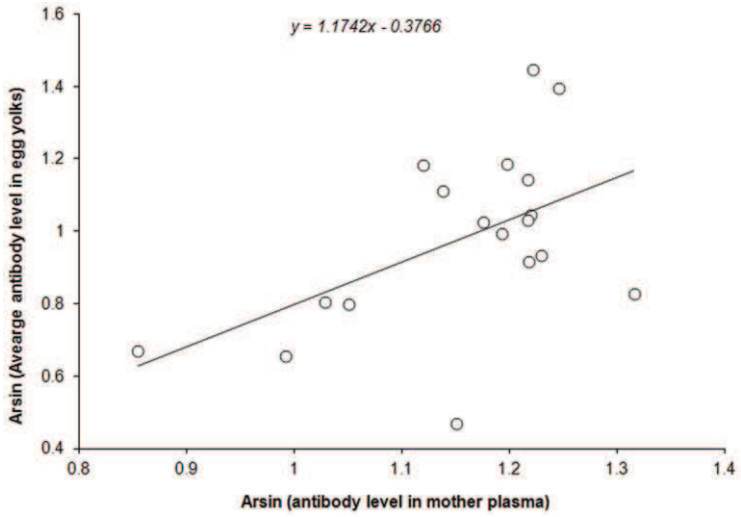

D
y = 38.829x - 0.9069
Ln(Intra-clutch CV of yolk antibody level)
Female FA score

Figure 2.


y = 1.1742x - 0.3766
Arsin (Avearge antibody level in egg yolks)
Arsin (antibody level in mother plasma)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.8
0.9
1
1.1
1.2
1.3
1.4

Partie III

Annexes

Annexe 1

Circulation des virus Influenza A: *apports de la modélisation*

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Résumé

Les modèles mathématiques qui permettent de produire une description simplifiée de la réalité apparaissent comme des outils importants de l'épidémiologie moderne. En effet, en épidémiologie, le problème majeur est de chercher à comprendre comment les pathogènes se transmettent d'un individu à l'autre afin de prédire les épidémies, leur ampleur dans le temps et l'espace. Les modèles ont ainsi le potentiel à la fois de servir de fil conducteur aux praticiens en santé publique en comparant différentes stratégies de gestion de la maladie, mais aussi d'améliorer notre compréhension de la transmission du pathogène.

Le but de cet article est de se concentrer sur quelques exemples de modèles pour souligner leur utilité pratique dans la compréhension de la circulation des virus Influenza. Nous nous intéresserons tout d'abord à des modèles épidémiologiques simples permettant de comprendre la démarche à adopter pour formaliser un modèle. Puis nous verrons comment utiliser ces modèles simples pour comprendre des processus épidémiques à une échelle plus globale. Enfin, nous discuterons en quoi la modélisation peut permettre de répondre à des questions aussi larges et variées que : dans quelles mesures un agent pathogène peut déclencher une épidémie ou encore quels sont les effets des mesures de santé publique sur l'évolution des virus grippaux.

Mots clefs : modélisation, éco-épidémiologie évolutive, virus Influenza.

Abstract

The mathematical models used to produce a simplified description of reality appear as important modern epidemiology tools. Indeed, a goal in epidemiology is to understand how pathogens are transmitted between individuals. This would predict epidemics and their extent in time and space. The models can provide a guide to public health practitioners, through comparison of different strategies in disease management. Also it improves our understanding of pathogen transmission.

Here, I focused on some of these models to emphasize their practical value in understanding the influenza viruses' circulation. First, I present a simple epidemiological model that provides an understandable approach to follow in order to build a model. Second, I show how to use such simple models to include the epidemic process in a more global scale. Finally, I explain how modeling can help investigate the extent to which a pathogen can trigger an epidemic or the effects of public health measures on evolving influenza viruses.

Keywords: modelling, evolutionary eco-epidemiology, Influenza viruses.

Introduction

Les maladies infectieuses, provoquées par la transmission de micro-organismes (virus, bactéries), constituent une des causes majeures de mortalité chez l'homme avec environ 15 millions de décès pas an dans le monde Fauci *et al.* 2005. Au cours des deux derniers siècles, les progrès enregistrés en matière d'hygiène, le développement de nombreux vaccins (contre la variole, le choléra, la rage, le tétanos…) ainsi que la découverte de la pénicilline en 1929 ont fait penser, au milieu des années 1970, que la bataille contre les micro-organismes allait être gagnée. C'était sans compter sur les nombreux facteurs écologiques et évolutifs impliqués dans l'émergence, la réémergence ou la persistance des maladies infectieuses dans le monde. En particulier, leur capacité d'adaptation permet l'apparition continue de nouvelles souches ou variants échappant au système immunitaire de leur hôte ou encore l'acquisition de propriétés de résistance aux traitements. Cette faculté d'évolution constitue une des principales causes d'émergence ou de réémergence de ces agents pathogènes.

Dans les populations humaines (ensemble des individus qui occupe simultanément un même environnement), il apparaît que la diversité spécifique en agents pathogènes et parasitaires n'est pas distribuée de manière aléatoire Guernier *et al.* 2004. Elle montre une distribution géographique assez proche de celle que l'on connaît pour de nombreux groupes d'animaux et de végétaux en biogéographie Blondel 1995 : les pathogènes sont concentrés dans les zones intertropicales et en particulier sur le continent africain. Cette diversité maximale s'explique sans doute par la richesse exceptionnelle en espèces animales, représentant autant de réservoirs et vecteurs de microorganismes potentiellement pathogènes pour l'homme.

Mais des changements écologiques survenus dans de nombreux endroits de la planète ont provoqué le déplacement géographique d'agents pathogènes, qui se retrouvent aujourd'hui au sein de populations démunies de toutes formes de résistances. Par exemple, le virus du Nil Occidental, a été introduit en 1999 aux Etats-Unis, où il a induit une épidémie majeure chez les chevaux, les humains et les oiseaux. Le déplacement des agents pathogènes est de plus favorosié par le développement des transports qui permet une diffusion extrêmement rapide et efficace des micro-organismes à travers le monde Colizza *et al.* 2007.

L'ensemble de ces facteurs contribue au maintien des agents causatifs de maladies infectieuses et peut être même à leur diversification. Mais d'autres facteurs aggravants, directement connectés à la distribution géographique et aux mouvements des agents pathogènes, peuvent favoriser la diffusion d'une épidémie. En effet, la taille de la population mondiale augmente continuellement, induisant l'accroissement du nombre d'individus sensibles, ce qui peut potentiellement accroitre l'importance et la durée des épidémies. L'étude de Jones *et coll.* Jones *et al.* 2008 montre d'ailleurs que parmi les quelques facteurs explicatifs d'émergences d'agents pathogènes utilisés dans leur analyse, la densité humaine est incontestablement celui qui présente le pouvoir explicatif le plus important.

Dans la plupart des cas, ces émergences sont associées à la capacité des pathogènes à passer de populations d'animaux domestiques et/ou sauvages aux populations humaines Cleaveland *et al.* 2001. On parle alors de zoonose émergente. Cette dynamique de transmission dépend en partie des mécanismes de défense de l'hôte Thomas *et al.*

2007 mais aussi des caractéristiques de l'agent pathogène. Or il est souvent difficile de connaitre la transmissibilité, le pouvoir pathogène ou encore le potentiel pandémique d'un agent émergent. Il peut alors être utile de comparer différentes situations théoriquement plausibles. De plus, devant les problèmes de santé publique globale associés à la circulation d'agents infectieux, il est important de développer des outils performants permettant à la fois de prédire une éventuelle menace de pandémie et d'évaluer l'impact des différentes politiques de santé publique sur la propagation de maladies infectieuses. Ces outils doivent être suffisamment souples pour être adaptés à un large éventail de situations. Ils doivent notamment pouvoir représenter divers types de caractéristiques démographiques des populations ou permettre de tester différents types d'interventions (vaccination, antiviraux, confinement des individus malades, interventions de masse…). Dans ce contexte, outre l'acquisition de données sur le terrain et en laboratoire, le développement et l'utilisation de modèles mathématiques peut se révéler très utile Hethcote 2000.

Les virus influenza, agents de la grippe, apparaissent comme de bons candidats pour illustrer l'utilité des modèles mathématiques. En effet, ils sont à la fois responsables d'épidémies annuelles mais aussi sont régulièrement cités comme potentiels agents de pandémies. L'origine des virus pandémiques humains proviendrait de recombinaisons à partir de virus Influenza A circulant naturellement dans les populations d'oiseaux sauvages et domestiques. De plus, le virus de la grippe possède une capacité à évoluer rapidement pour contourner la réponse immunitaire de l'hôte, comme l'illustre la nécessité de réactualiser le vaccin contre ce virus à chaque nouvelle épidémie annuelle (OMS WHO. 2009). Différents facteurs, et en particulier des facteurs sociaux, écologiques et évolutifs sont responsables de ces dynamiques complexes d'émergences de nouvelles

souches pandémiques et de persistance de souches en constante modification. L'évolution rapide des virus grippaux contribue à la complexité de leurs dynamiques épidémiologiques, d'autant plus qu'il est difficile de séparer écologie et évolution d'un point de vue temporel. Le but de cet article n'est pas de proposer une présentation exhaustive des modèles mathématiques utilisés en épidémiologie, mais plutôt de se concentrer sur certains de ces modèles pour mettre en avant leur utilité pratique dans la compréhension de la circulation des virus Influenza.

Qu'est ce qu'un modèle mathématique ?

Le recours aux modèles mathématiques est assez ancien en épidémiologie Kermack *et al.* 1927; Anderson *et al.* 1978 mais depuis quelques années, on observe une augmentation très importante du nombre de publications utilisant des modèles mathématiques (Fig. 1). Ce phénomène est en partie associé au fait que l'on comprend de mieux en mieux l'utilité du langage mathématique pour produire une description nécessairement **simplifiée** mais **utile** de la réalité. Il est irréalisable de construire un modèle parfait puisqu'il restera toujours par définition une simplification de la réalité, mais l'inclusion d'un plus ou moins grand réalisme permet d'aborder des questions générales, ou au contraire plus précises. Il est important de comprendre que la complexité mathématique n'est pas un critère suffisant pour juger de la pertinence d'un modèle Hethcote 2000. Or en général, pour bien faire coller un modèle à la réalité, on a tendance à augmenter le nombre de paramètres pour capturer le maximum d'aspects qui semblent importants dans le système. Par exemple dans les modèles épidémiologiques, il peut paraître important d'intégrer de nombreux paramètres en rapport avec la transmission et le contrôle de la maladie. Mais cette complexification rend difficile la compréhension générale du comportement du modèle et pose le problème de la qualité des données

utilisées pour fixer les paramètres. Dans de nombreux cas, il est important de commencer avec un modèle parcimonieux, compréhensible, et surtout de s'assurer qu'il est adapté à la fois aux questions posées et aux données épidémiologiques dont on dispose. Nous verrons plus loin que certaines études essayent de construire des modèles fortement réalistes Colizza *et al.* 2006; Colizza *et al.* 2007.

Un modèle simple d'épidémie ?

En épidémiologie, le problème majeur est de chercher à **comprendre** comment les pathogènes se transmettent d'un individu à l'autre afin d'être capable de **décrire** les épidémies, leur ampleur dans le temps et l'espace. On comprend donc l'importance d'utiliser des modèles mathématiques, à la fois pour servir de fil conducteur aux praticiens en santé publique en comparant les performances attendues de différentes stratégies de gestion de la maladie, mais aussi pour améliorer notre compréhension de la transmission du pathogène.

Nous nous intéresserons dans ce chapitre à un des modèles compartimentaux les plus simples, le modèle SIR (Fig. 2). Ce modèle décrit l'état de la population d'hôtes en la compartimentant en groupes d'individus différenciés en fonction de leur statut vis-à-vis de l'agent pathogène : sensibles (S), infectieux (I) et guéris (R, « recovered » en anglais). Les individus sensibles deviennent infectés (contaminé par le pathogène, mais ne pouvant pas le transmettre) et infectieux (contaminé par le pathogène et pouvant le transmettre) au taux λ communément appelé force d'infection. Ces individus peuvent présenter une mortalité accrue, traduisant l'effet généralement négatif de la présence du pathogène. Le taux α traduit la virulence du pathogène soit la surmortalité des individus infectés. Enfin, les individus infectés

guérissent au taux γ. Ces différents compartiments sont donc reliés entre eux par des flux reflétant soit la biologie de l'hôte (taux de mortalité, taux de guérison), soit celle du pathogène (force d'infection, virulence) Anderson *et al.* 1991; Hethcote 2000; Keeling *et al.* 2008.

L'élément crucial de ce modèle en compartiment est indubitablement le processus de contagion (λ) : comment un individu sensible devient-il infecté ? Comme ce processus de contagion rend compte du passage du pathogène d'un individu infectieux à un individu sensible, on comprend que le nombre de nouveaux malades dépend non seulement du nombre d'individus sensibles, mais également du nombre d'individus déjà infectieux présents dans la population Keeling *et al.* 2008. La force d'infection est donc proportionnelle à la fois à la capacité des individus à transmettre l'infection (β, taux de transmission) et soit à la densité d'individus infectieux I dans la population (transmission densité dépendante, $\lambda = \beta I$) ou la fréquence des individus infectieux I/N dans la population (transmission fréquence dépendante, $\beta = \lambda\ I/N$) Cette écriture de la force de l'infection implique que tout individu exposé est immédiatement infectieux. Prendre en compte un temps de latence entre l'exposition et le stade infectieux va donc directement jouer sur la force de l'infection en modifiant la densité et la fréquence des individus sensibles. En particulier, dans le cadre des virus Influenza A, on peut tenir compte d'une période de latence (1 à 2 jours), après l'infection, durant laquelle les individus exposés ne sont pas encore infectieux Potter 2001. Dans ce cas, il faut ajouter un compartiment au modèle contenant les individus en état de latence (fig. 3). Après cette période de latence, les individus présentent les symptômes grippaux et sont donc considérés comme infectieux (environ 3 à 4 jours après infection) Ferguson *et al.* 2005. Enfin après cet intervalle d'infection, les individus guérissent et acquièrent une immunité à vie au variant infectant et aux variants antigéniques proches.

Mais cette immunité n'est en réalité que temporaire, compte tenu de l'importante diversité génétique du virus grippal et de son évolution génétique rapide au cours du temps. On peut donc modéliser ce processus de perte d'immunité en imposant aux individus guéris (R) de perdre leur résistance acquise et de repasser dans le compartiment des individus sensibles (S) après quelques années Dushoff *et al.* 2004.

A l'aide d'un tel modèle simple, la première question qu'un acteur en santé publique peut se poser est de savoir si l'épidémie d'une maladie donnée peut apparaitre et se propager dans une population naïve (i.e. qui n'a jamais connu de maladie). Répondre à cette question peut se faire aisément en calculant le nombre reproducteur de base R_0 du pathogène. Il représente le nombre moyen d'infections secondaires dues à l'introduction d'un individu infecté dans une population entièrement sensible Keeling *et al.* 2008. C'est un paramètre clef en épidémiologie puisque sa valeur renseigne sur la possibilité d'une épidémie. En effet, pour qu'une épidémie survienne, il faut qu'un individu infecté transmette son infection au minimum à un autre individu ($R_0>1$), sinon le pathogène est éliminé de la population ($R_0<1$). La valeur du R_0 de la grippe est variable selon la souche. Par exemple, elle est comprise entre 1.25 et 1.38 pour le virus pandémique A/H1N1 Tuite *et al.* 2010 ou encore entre 1.4 et 2.8 pour le virus A/H1N1 de la grippe espagnole de 1918 Bootsma *et al.* 2007. Ces valeurs restent relativement faible lorsqu'on les comparent aux R_0 de la coqueluche ou de la rougeole compris entre 16 et 18 Anderson *et al.* 1982, mais cela ne signifie pas que la grippe doit être négligée par les acteurs en santé publique. En effet, ce paramètre reste informatif et ne décrit ni la virulence de la maladie ou la dynamique épidémique exacte de la grippe.

Comprendre la dynamique des épidémies de grippe nécessite de prendre en compte les variations de la population exposée au virus, tant dans le temps que dans l'espace. Ceci nécessite de décrire la démographie de la population qui va influencer la disponibilité des hôtes sensibles pour le virus. Ainsi, à la fin de l'épidémie, la majorité des hôtes a développé une immunité et le redémarrage de l'épidémie va dépendre à la fois (i) de la reproduction qui produit de nouveaux individus accessibles au parasite et (ii) de la perte d'immunité des individus guéris. Bien que nous n'ayons pas traité de cela ici, les modèles S(E)IR permettent de modéliser les caractéristiques démographiques de l'hôte et d'en étudier les conséquences épidémiologiques. En revanche, la prise en compte des dynamiques spatiales nécessite de faire appel à d'autres types de modèles dont un exemple fait l'objet de la section suivante.

Modélisation de processus épidémiques à l'échelle mondiale

Une des questions fondamentales dans l'étude de la dynamique des virus grippaux concerne les conditions spatio-temporelles favorisant la diffusion d'une épidémie. Pour répondre à cette question, nous allons à présent nous intéresser à une des grandes familles de modèles mathématiques : les graphes Bergé 1983. Un graphe est la donnée de deux objets : une liste de nœuds et une liste de liens. En épidémiologie, un nœud schématise un hôte ou une population d'hôtes. Un lien conceptualise le chemin entre deux nœuds : c'est le chemin d'infection que peuvent emprunter par exemple les virus Influenza. Donc d'après le schéma (Fig. 4), à partir du site *a* du graphe, le virus ne peut atteindre directement que les hôtes qui y sont reliés par un lien. Autrement dit, l'évolution de l'état des sites *b* et *d* entre deux pas de temps ne dépend que de l'état initial (infecté ou non) du site *a*. L'hypothèse qui est faite dans cette approche est que l'agent infectieux se propage d'un hôte vers

un petit nombre d'autres hôtes. C'est ce que l'on appelle une propagation « par contact ». Ce cadre est donc bien adapté à l'épidémiologie humaine où chaque individu a effectivement des contacts avec un petit nombre d'individus auxquels il peut potentiellement transmettre la maladie.

L'utilisation des graphes en épidémiologie n'est pas restreinte à l'échelle de l'individu : elle peut s'étendre aux populations humaines. Il est ainsi possible de modéliser la diffusion mondiale d'une épidémie. Pour cela, il est utile de distinguer deux échelles à laquelle la maladie se diffuse. La première échelle permet de comprendre la dynamique locale du virus, pour laquelle un modèle de type SEIR (vu précédemment) est classiquement utilisé Anderson *et al.* 1991. La deuxième échelle concerne la diffusion mondiale entre villes via le transport aérien. En effet, à l'heure actuelle, l'expansion du transport aérien permet de relier les pays du monde entre eux. Il n'est donc pas surprenant de voir de plus en plus d'études s'intéressant à l'impact de la structure du réseau aérien mondial sur la diffusion planétaire de maladies telles que le virus de la grippe A/H5N1 Hufnagel *et al.* 2004; Colizza *et al.* 2006; Colizza *et al.* 2007.

Les données concernant le réseau aérien mondial ont été obtenues auprès de l'Association Internationale du Transport Aérien (AITA). Cet organisme regroupe 3880 aéroports qui font office de nœuds dans le modèle, et de 18 810 connexions aériennes qui représentent les liens entre les aéroports. Ces données ont permis à Fraser *et coll.* Fraser *et al.* 2009 d'analyser la diffusion de la nouvelle souche Influenza pandémique A/H1N1 à partir du Mexique. Sur une échelle plus large, Colizza *et coll.* Colizza *et al.* 2007 ont étudié à l'aide

de simulations comment une maladie se propage au niveau mondial via le réseau aérien. La question primordiale qu'ils se sont posé est de déterminer quelles sont les zones et les périodes les plus à risque de voir débuter une épidémie. Comme les zones les plus touchées par le H5N1 ont été l'Asie WHO. 2009 et l'Europe de l'est Cox *et al.* 2000, il était cohérent de choisir le point de départ de l'épidémie à l'intérieur de ces régions (Hanoï et Bucarest). De même pour considérer les débuts de l'épidémie, les mois d'avril et octobre ont été retenus.

L'étude de Colizza *et coll.* Colizza *et al.* 2007 confirme qu'il existe des conditions spatiales et temporelles qui semblent très défavorables à la diffusion d'une épidémie grippale. Ceci permet d'identifier les zones et les périodes qui sont les plus à risque pour le déclenchement d'une épidémie et d'y concentrer les efforts de surveillance. Elle permet également de mettre en évidence l'utilité d'associer des modèles simples, de type SEIR, à la notion de graphe, qui permet de formaliser différents chemins d'infection. Ces modèles sont cependant plus difficiles à analyser que les modèles non spatiaux ou non structurés. Mais ils trouvent tout leur intérêt dès que les contacts au sein d'une population sont hétérogènes.

Emergence d'une nouvelle épidémie humaine

Après avoir étudié les conditions spatio-temporelles favorables à la diffusion d'une épidémie, il semble intéressant de se demander dans quelle mesure un agent pathogène peut déclencher une épidémie chez l'Homme. En effet, certains agents pathogènes, tels que le sous-type H5N1 de la grippe aviaire, provoquent des infections sporadiques chez l'Homme mais semblent peu capables de s'établir dans la population humaine (impasse épidémiologique), tandis que d'autres réussissent à

surmonter cette barrière inter-espèces (par exemple, le virus responsable du Syndrome Respiratoire Aigu Sévère SRAS). Arinaminpathy *et coll.* Arinaminpathy *et al.* 2009 étudient l'épidémiologie associée à l'établissement d'un pathogène dans un nouvel hôte. Comme discuté par Antia *et coll.* Antia *et al.* 2003, même un pathogène avec une faible transmission inter-humaine, c'est à dire capable seulement de cas sporadiques, peut acquérir des adaptations pour devenir capable de se transmettre à l'Homme. D'autres adaptations peuvent se produire en réponse à des pressions de sélection exercées par de nouveaux environnements. Les changements dans les contacts interhumains et les facteurs environnementaux peuvent avoir le même effet en améliorant la transmission du pathogène. Par exemple, le sous-type H5N1 de la grippe aviaire a causé plus de 400 cas de décès chez l'Homme (World Health Organization 2009), principalement suite à des contact étroits et prolongés avec des volailles infectées Beigel *et al.* 2005. Bien qu'il ait montré pas ou peu de transmission inter-humaine, la possibilité de son adaptation ultérieure à l'homme ne peut être exclue. Arinaminpathy *et coll*. Arinaminpathy *et al.* 2009 se sont donc demandé dans quelle mesure un agent pathogène qui provoque des centaines de cas mais ne se transmet pas entre humains serait effectivement incapable de s'adapter à la transmission chez l'Homme. Plus précisément, combien de cas humains 'manqués' devraient se produire pour conclure qu'une émergence peut être considérée comme improbable ? Pour répondre à cette question, ils utilisent un modèle mathématique simple pour étudier les marqueurs potentiels de l'adaptation.

Pour cela, ils s'intéressent à la probabilité d'émergence, en considérant chaque événement de cas sporadiques comme une tentative d'émergence. Cette probabilité dépend fortement du R_0 du pathogène : si le R_0 d'un pathogène est élevé, lors d'une infection sporadique, un individu infecté a une probabilité plus forte de transmettre la maladie au sein d'une population et donc de provoquer une épidémie. Cette

augmentation du R_0 nécessite une adaptation du pathogène à son hôte. L'évolution du virus est ici modélisée par une augmentation graduelle d'un R_0 peu élevé à un R_0 élevé. A partir de ce modèle, ils montrent qu'indépendamment du nombre de cas consécutifs où le pathogène a échoué dans son émergence, il n'est jamais possible de conclure que cette probabilité d'émergence est nulle (Fig. 5). De plus, la probabilité d'émergence est diminuée de 98% après les 100 premiers cas d'émergence ratée. Autrement dit, après 100 cas sans émergence, il y a alors une très faible probabilité d'émergence. Au-delà de ce point, le nombre de cas d'échec d'émergence par le pathogène ne fournit que peu d'informations sur l'adaptabilité de l'agent pathogène. Dans l'exemple de l'influenza H5N1, ce compte a été obtenu dès le 8 Juin 2005 WHO. 2009 . Quantitativement, selon ce modèle, des cas sporadiques humains postérieurs à cette date ne nous apprennent pas beaucoup plus d'information sur comment cette souche de virus pourrait s'adapter à l'homme.

Ce type de modèle mathématique peut fournir des renseignements précieux sur la mise en place de potentiels scénarii d'émergence. Mis entre les mains des acteurs concernés, ces modèles mathématiques peuvent jouer un rôle important dans notre boîte à outils pour la préparation et l'établissement de politiques en matière de santé publique.

Modélisation de l'évolution d'un pathogène : influence d'une protection hétérogène de la population

Après avoir fourni les outils nécessaires à la mise en place d'une politique de santé publique, il faut anticiper et comprendre l'impact de ces politiques sur les populations humaines. Or jusqu'ici, nous avons considéré des populations homogènes, dans la mesure où tous les individus ont les mêmes caractéristiques démographiques et épidémiologiques. Il s'agit d'une hypothèse simplificatrice puisque les

populations humaines sont hétérogènes. Cette hétérogénéité peut être déterminée génétiquement (les différents génotypes sont plus ou moins résistants aux pathogènes), liée à l'histoire des individus (certains peuvent avoir été infectés auparavant et n'ont pas le même statut immunologique), ou encore résulter de mesures de santé publique (comme la vaccination).

Pour comprendre au mieux l'impact d'une politique de santé publique et dans le but de l'améliorer, il serait donc intéressant d'étudier les effets épidémiologiques de cette hétérogénéité de la population d'hôte induite par une mesure de santé publique comme la vaccination. En effet, une couverture vaccinale n'étant jamais totale, on distingue dans la population les individus vaccinés et non vaccinés (appelés naïfs). De plus, en supposant que la vaccination n'est pas totalement efficace, un individu vacciné pourrait être infecté (avec une fréquence moindre qu'un individu naïf), transmettre la maladie voire en mourir.

Gandon *et coll.* Gandon *et al.* 2001 ont montré qu'un vaccin imparfait peut aboutir à la sélection de pathogènes plus virulents. Le but de ce type de modèles est de définir la valeur de la virulence qui maximise la transmission du pathogène (soit son R_0). La virulence (au sens théorique du terme) est la mortalité induite par la présence d'un pathogène autrement dit la surmortalité des hôtes infectés. Certains vaccins peuvent limiter la croissance du pathogène dans l'hôte diminuant ainsi les risques de mortalité suite à l'infection (vaccin anti-croissance) Gandon *et al.* 2007. Des pathogènes plus virulents peuvent alors être sélectionnés et les hôtes naïfs de la population peuvent subir une mortalité accrue (en considérant que les hôtes vaccinés sont potentiellement protégés). Ainsi, même si l'utilisation d'un vaccin anti-croissance peut limiter à court terme la mortalité induite par la maladie,

à long terme (en prenant en compte que le pathogène évolue) la mortalité totale induite par le pathogène augmente du fait d'une vaccination imparfaite.

Le modèle présenté ici n'a pas été paramétré pour des virus Influenza mais illustre l'utilité de prendre en compte ce type de modèle dans les politiques de santé publique. En effet, cet exemple nous montre que la vaccination peut avoir d'importants effets sur l'évolution des pathogènes : le bien de l'individu (être soigné et guérir) peut alors entrer en conflit avec le bien de la population (éviter l'évolution du pathogène, et une mortalité accrue).

Conclusion

Cet article permet d'illustrer par quelques **questions biologiques** l'intérêt des modèles épidémiologiques dans la compréhension de la dynamique des virus de la grippe. Cet article n'est cependant pas limité aux virus grippaux est peut être étendu à d'autres micro-parasites. Nous sommes donc partie d'un modèle des plus simples (SEIR) qui nous a permis de comprendre la démarche suivie par le modélisateur : toujours être guidé par une question biologique centrale qui doit guider le choix et la mise en place d'un modèle aussi simple que nécessaire pour répondre à cette question. La qualité et la quantité croissante des données épidémiologiques disponibles va aussi permettre le développement de modèles de plus en plus complexes pour répondre à des questions biologiques de plus en plus précises Ferguson *et al.* 2003. Ainsi le modèle SEIR introduit au début de ce mémoire peut être complexifié à l'infini suivant la question posée. Cette complexification se traduit, par exemple, par l'utilisation de modèles individus-centrés qui permettent de tenir compte de la variabilité

individuelle ou encore par la prise en compte de différentes formes d'hétérogénéité. Cependant, cet article n'est pas exhaustif et les exemples présentés ici ne sont qu'un échantillon réduit de l'ensemble des modèles épidémiologiques existants et de leurs utilités.

Les modèles épidémiologiques sont donc des outils particulièrement intéressants à utiliser pour comprendre au mieux la circulation des virus Influenza mais également d'autres agents pathogènes, prédire les épidémies ou encore servir de fil conducteur pour les acteurs en santé publique Moghadas *et al.* 2009. Il ne faut donc pas percevoir les modèles comme des mathématiques pures, mais essayer de voir au travers des formules la question biologique sous-jacente.

Remerciements

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Bibliographie

1 Fauci, A.S.*, et al.* (2005) Emerging infectious diseases: a 10-year perspective from the National Institute of Allergy and Infectious Diseases. *Emerging Infectious Diseases* 11, 519-525

2 Guernier, V.*, et al.* (2004) Ecology drives the worldwide distribution of human diseases. *Plos Biology* 2, 740-746

3 Blondel, J. (1995) *Biogéographie: Approche écologique et évolutive*.

4 Colizza, V.*, et al.* (2007) Modeling the worldwide spread of pandemic influenza: Baseline case and containment interventions. *Plos Medicine* 4, 95-110

5 Jones, K.E.*, et al.* (2008) Global trends in emerging infectious diseases. *Nature* 451, 990-U994

6 Cleaveland, S.*, et al.* (2001) Diseases of humans and their domestic mammals: pathogen characteristics, host range and the risk of emergence. *Philosophical Transactions Of The Royal Society Of London Series B-Biological Sciences* 356, 991-999

7 Thomas, F.*, et al.* (2007) *Ecologie et évolution des systèmes parasités*.

8 Hethcote, H.W. (2000) The mathematics of infectious diseases. *Siam Review* 42, 599-653

9 WHO. (2009) World Health Organization. Cumulative number of confirmed human cases of avian influenza A/(H5N1) reported to WHO. See http://www.who.int/csr/disease/avian_influenza/country/en/ (Retrieved 7 June 2009)

10 Kermack, W.O. and McKendrick, A.G. (1927) Contributions to the mathematical theory of epidemics. *Proceedings of the Royal Society A.* 115, 700-721

11 Anderson, R.M. and May, R.M. (1978) Regulation and Stability of Host-Parasite Population Interactions .1. Regulatory Processes. *Journal of Animal Ecology* 47, 219-247

12 Colizza, V.*, et al.* (2006) The role of the airline transportation network in the prediction and predictability of global epidemics. *Proceedings Of The National Academy Of Sciences Of The United States Of America* 103, 2015-2020

13 Keeling, M.J. and Rohani, P. (2008) *Modeling Infectious Diseases in humans and animals*. Press, Princeton University

14 Anderson, R.M. and May, R.M. (1991) *Infectious Diseases of Humans. Dynamics and Control*.

15 Potter, C.W. (2001) A history of influenza. *Journal Of Applied Microbiology* 91, 572-579

16 Ferguson, N.M.*, et al.* (2005) Strategies for containing an emerging influenza pandemic in Southeast Asia. *Nature* 437, 209-214

17 Dushoff, J.*, et al.* (2004) Dynamical resonance can account for seasonality of influenza epidemics. *Proceedings Of The National Academy Of Sciences Of The United States Of America* 101, 16915-16916

18 Tuite, A.R.*, et al.* (2010) Estimated epidemiologic parameters and morbidity associated with pandemic H1N1 influenza. *Canadian Medical Association Journal* 182, 128-133

19 Bootsma, M.C.J. and Ferguson, N.M. (2007) The effect of public health measures on the 1918 influenza pandemic in US cities. *Proceedings Of The National Academy Of Sciences Of The United States Of America* 104, 7588-7593

20 Anderson, R.M. and May, R.M. (1982) Directly Transmitted Infectious-Diseases - Control By Vaccination. *Science* 215, 1053-1060

21 Bergé, C. (1983) *Graphes*. Gauthier-Villars-Bordas

22 Hufnagel, L.*, et al.* (2004) Forecast and control of epidemics in a globalized world. *Proceedings Of The National Academy Of Sciences Of The United States Of America* 101, 15124-15129

23 Fraser, C.*, et al.* (2009) Pandemic Potential of a Strain of Influenza A (H1N1): Early Findings. *Science* 324, 1557-1561

24 Cox, N.J. and Subbarao, K. (2000) Global epidemiology of influenza: Past and present. *Annual Review Of Medicine* 51, 407-421

25 Arinaminpathy, N. and McLean, A.R. (2009) Evolution and emergence of novel human infections. *Proceedings Of The Royal Society B-Biological Sciences* 276, 3937-3943

26 Antia, R.*, et al.* (2003) The role of evolution in the emergence of infectious diseases. *Nature* 426, 658-661

27 Beigel, H.*, et al.* (2005) Current concepts - Avian influenza A (H5N1) infection in humans. *New England Journal Of Medicine* 353, 1374-1385

28 Gandon, S.*, et al.* (2001) Imperfect vaccines and the evolution of pathogen virulence. *Nature* 414, 751-756

29 Gandon, S. and Day, T. (2007) The evolutionary epidemiology of vaccination. *Journal Of The Royal Society Interface* 4, 803-817

30 Ferguson, N.M.*, et al.* (2003) Planning for smallpox outbreaks. *Nature* 425, 681-685

31 Moghadas, S.M.*, et al.* (2009) Managing public health crises: the role of models in pandemic preparedness. *Influenza And Other Respiratory Viruses* 3, 75-79

Légendes des Figures

Fig. 1 Modifié de Keeling *et coll.* (2008) Keeling *et al.* 2008. Une indication de l'importance croissante de l'utilisation des modèles mathématiques dans la littérature épidémiologique. Les barres blanches représentent le nombre approximatif de publications dans l'ensemble de la littérature scientifique utilisant les modèles de maladies infectieuses (données obtenues via ISI Web of Science). En gris et noir, le nombre de ces publications trouvées, respectivement, dans *Nature* et *Science* donnant une indication du fort impact de ces travaux.

Fig. 2 Modèle SIR. Les individus hôtes sont compartimentés selon leur état infectieux en individus sains (S), infectieux (I) et guéris (R). Les flèches entre les compartiments représentent les flux d'individus selon le taux indiqué au-dessus des flèches. Le taux λ représente le processus de contagion, α traduit la virulence du pathogène soit la surmortalité des individus infectés, enfin γ décrit le taux de guérison.

Fig. 3 Modèle SEIR. Les individus hôtes sont compartimentés selon leur état infectieux en individus sains (S), exposé (E), infectieux (I) et guéris (R). Les flèches entre les compartiments représentent les flux d'individus selon le taux indiqué au-dessus des flèches. Le taux $\beta I = \lambda$

représente le processus de contagion (β, taux de transmission), α traduit la virulence du pathogène soit la surmortalité des individus infectés, enfin γ décrit le taux de guérison.

Fig. 4 Graphe. Les ronds représentent les nœuds, annotés de *a* à *d.* Les droites représentent les liens entre nœuds. En épidémiologie, ils conceptualisent le chemin d'infection que peut emprunter un agent pathogène.

Fig. 5 Modifié de Arinaminpathy *et coll.* (2009) Arinaminpathy *et al.* 2009. Probabilité d'émergence d'un pathogène en fonction du nombre d'introduction ne produisant pas d'émergences.

Fig.1.

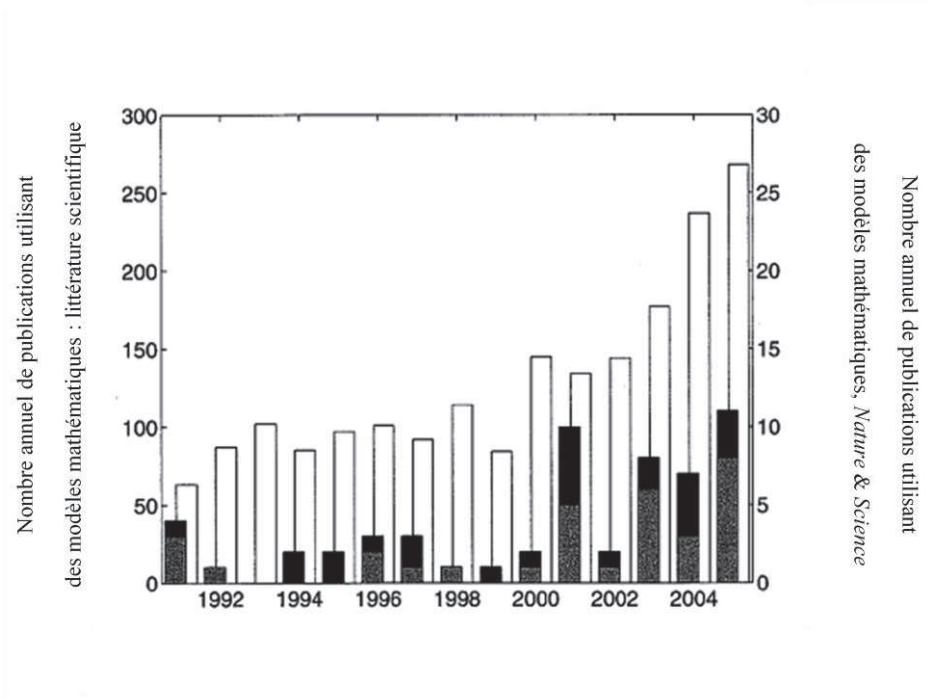

Nombre annuel de publications utilisant
des modèles mathématiques : littérature scientifique
300
250
200
150
100
50
0
30
25
20
15
10
5
0
1992
1994
1996
1998
2000
2002
2004
Nombre annuel de publications utilisant
des modèles mathématiques, *Nature & Science*

Fig.2.

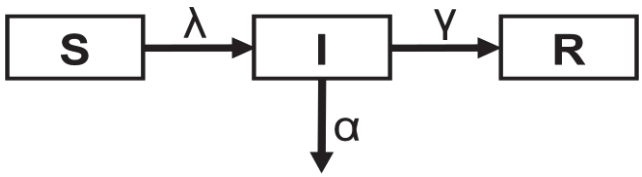

S
λ
I
γ
R
α

Fig.3.

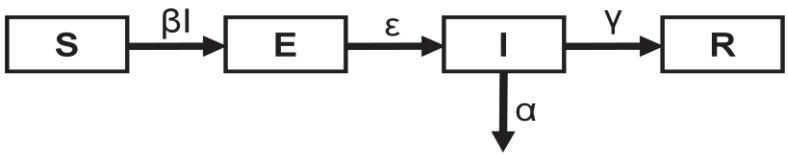


Fig.4.

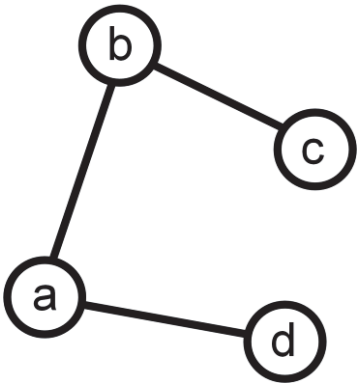
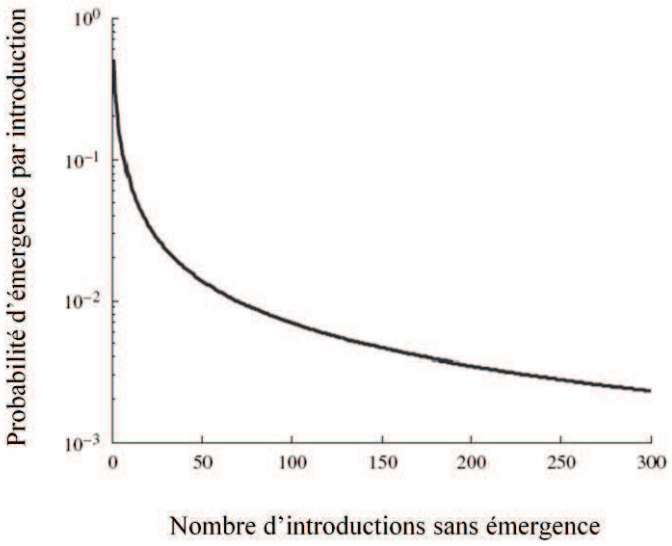


Fig.5.


Probabilité d'émergence par introduction
10^0
10^{-1}
10^{-2}
10^{-3}
0
50
100
150
200
250
300
Nombre d'introductions sans émergence

Annexe 2

Poster in international WDA conference

Lyon, France, July 2012

Flavivirus antibodies in yellow-legged gull (*Larus michahellis*) eggs in the western mediterranean basin.

Audrey Arnal, Elsa Jourdain, Jessica Pearce-Duvet, Sylvie Lecollinet, Marion Vittecoq, Jacob González-Solís, Hammouda Abdesslem, Samraoui Boudjéma, Romain Garnier, Slaheddine Selmi, Michel Gauthier-Clerc et Thierry Boulinier

Flavivirus antibodies in yellow-legged gull (*Larus michahellis*) eggs in the western Mediterranean basin

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In recent years, an unexpected number of flaviviral zoonoses have emerged worldwide and wild birds represent the most important reservoir

Objectives

Investigating wild bird exposure to flavivirus at a large spatial scale

Screening of the egg yolks of yellow-legged gulls (*Larus michahellis*) sampled from different colonies of the western Mediterranean for detecting the presence of flavivirus-specific antibodies (Ab)

Materials and Methods

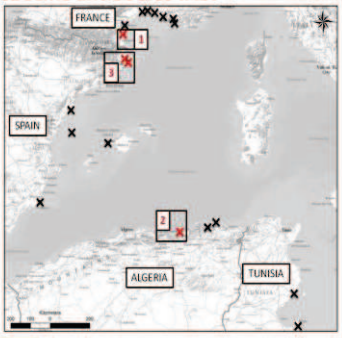
Breeding season from 2009 to 2011
19 colonies; 1 098 eggs collected (8 to 50 eggs per colony)

↓

Competitive ELISA to detect Ab against Flaviviruses
West Nile Virus (WN) envelop protein (ID-Vet)

↓

ELISA positive eggs screened for neutralizing Ab specific for WN, Usutu (USU), Tick-borne encephalitis (TBE) viruses



Results

1 and 2: three eggs ELISA-positive (Jijel in Algeria and Corrège in France)
3: High number of ELISA-positive eggs in Medes Island, Spain
37%, 49% and 67% for 2009, 2010 and 2011 (n > 38 each year)
In addition, 4/6 eggs ELISA-positive from the village of l'Escala (11 km from Medes Island)

No detection of neutralizing antibodies specific for WN, USU and TBE viruses
Soft ticks sampled in Medes → Detection of virus that share 95% similarity with Meaban
(See the poster of Elena Gómez-Díaz *et al.*)

Conclusion and perspectives

Gulls are exposed to flaviviruses but differences in antibody prevalence among colonies: Flavivirus was only detected on Medes Island and the nearby village of L'Escala (Spain) despite a large sampling effort conducted in 19 breeding colonies in 4 countries.
Our results suggest that gulls may be locally important in flavivirus circulation.
Maternal antibody detection : powerful way to detect the circulation of other pathogens

Abstract: The emergence of zoonotic diseases is directly linked to the noise generated by humans on the natural environment to a greater or lesser extent. Of all the emerging zoonoses in humans, the majority comes from wild animals. The study of the role of wildlife in the circulation of pathogens is crucial, especially when wildlife/human interfaces are prominent. The aim of this thesis is to understand, using large scale eco-epidemiological approaches, the circulation of pathogens in a close to human population of wild bird, the yellow-legged gull (Larus michahellis). The first chapter presents the different methods used in the monitoring of pathogens and their limitations when they are conducted in natural populations. This chapter further highlights, through a study of avian influenza viruses, that the quantification of maternal antibodies in eggs is an effective tool. The second chapter expands the spatial scale of the study to highlight the finer eco-epidemiological factors influencing the transmission of avian influenza viruses within and between populations of Western Mediterranean yellow-legged gull. Finally, the last chapter is based on the comparison of exposition patterns obtained for vectorial transmission mode pathogens: flaviviruses. This thesis highlights patterns of exposure for certain pathogens (avian influenza virus and flaviviruses) and enables the understanding of the eco-epidemiological factors potentially involved in their circulations. The results allow to consider future areas of research needed to more accurately assess the dispersion of these viruses over the Mediterranean.

Keywords: Eco-epidemiology, infectious diseases, avian influenza virus, flaviviruses, Meaban virus, Laridae, quantification of maternal antibodies, western Mediterranean

Résumé : L'émergence des zoonoses est à relier directement avec les perturbations générées par l'Homme sur son environnement naturel à plus ou moins grande échelle. Sur l'ensemble des zoonoses émergentes chez l'Homme, la majorité provient d'animaux sauvages. L'étude du rôle de la faune sauvage dans la circulation des agents pathogènes est donc cruciale en particulier quand les interfaces faune sauvage/Homme sont fortes. L'objectif de cette thèse a été de comprendre par des approches éco-épidémiologiques à large échelle, la circulation d'agents pathogènes dans les populations d'un oiseau sauvage en contact étroit avec l'Homme, le goéland leucophée (*Larus michahellis*). Le premier chapitre présente les différentes méthodes utilisées dans la surveillance d'agents pathogènes ainsi que leurs limites lorsqu'elles sont menées en populations naturelles. Ce chapitre met en évidence au travers d'une étude portant sur les virus influenza aviaires, que la quantification des anticorps maternels dans les œufs est un outil efficace. Le second chapitre consiste à élargir l'échelle spatiale de l'étude afin de mettre en évidence plus finement les facteurs éco-épidémiologiques influençant la transmission des virus influenza aviaires dans et entre les populations de goéland leucophée de l'ouest méditerranéen. Enfin, le dernier chapitre repose sur la comparaison des patrons d'expositions obtenus pour des agents pathogènes au mode de transmission vectoriel : les flavivirus. Cette thèse permet de mettre en évidence les patrons d'exposition de certains agents pathogènes (virus influenza aviaire et flavivirus) et d'appréhender les facteurs éco-épidémiologiques potentiellement impliqués dans leurs circulations. Les résultats permettent d'envisager de futurs axes de recherches, nécessaires pour évaluer plus précisément la dispersion de ces virus en Méditerranée.

Mots clés: Eco-épidémiologie, maladies infectieuses, virus influenza aviaire, flavivirus, virus Meaban, Laridés, quantification d'anticorps maternels, ouest méditerranéen