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## Protozoa and molds: deciphering the molecular mechanisms that hijack host's cell machinery

Veronica Risco-Castillo

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# Protozoa and molds: deciphering the molecular mechanisms that hijack host's cell machinery

Habilitation à diriger des recherches  
de l'Université Paris-Saclay

présentée et soutenue à Maisons-Alfort, le 12 décembre 2022,  
par

**Veronica RISCO CASTILLO**

## Composition du jury

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<b>Andrew HEMPHILL</b> PhD, Principal Investigator, Institute of Parasitology, Faculties of Veterinary Medicine and Medicine, University of Berne	Rapporteur
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<b>Coralie MARTIN</b> PhD, PR, UMR 7245 MCAM, MNHN/CNRS	Examinatrice
<b>Ladislav SIMO</b> PhD, HDR, DR, UMR BIPAR, ANSES/INRAe/EnvA	Examineur
<b>Olivier SILVIE</b> MD PhD, HDR, DR, CIMI-Paris, Inserm U1135	Examineur

**Titre :** titre (en français) **Protozoaires et moisissures : décryptage des mécanismes moléculaires qui détournent la machinerie cellulaire de l'hôte**

**Mots clés :** *Neospora*, *Plasmodium*, *Aspergillus*, hôte, expression génique et immunité

**Résumé :** La coévolution des agents pathogènes et de leurs hôtes cibles a permis l'établissement d'interactions étroites et complexes entre eux. Des protozoaires, jusqu'aux moisissures, les interactions mises en place pendant la colonisation de l'hôte permettent l'évasion du parasite voire le contrôle de l'hôte pour le détournement de ses ressources, pouvant entraîner son épuisement et sa mort programmée au bénéfice du parasite.

Tout au long de mon parcours scientifique, j'ai eu la chance de pouvoir faire partie de plusieurs équipes de recherche intéressées, comme moi, à comprendre la capacité des parasites à manipuler la biologie de la cellule hôte et les signaux de danger qu'elle déclenche. Ainsi, lors de mes études de master, doctorat et de mon poste postdoctoral, j'ai été amenée à étudier, dans un premier temps, des protozoaires intracellulaires tels que *Neospora caninum* et *Plasmodium* sp.. Ensuite, lors de mon recrutement comme maître de conférences à l'EnvA, j'ai voulu appliquer les connaissances acquises dans la compréhension de l'interaction cellulaire entre la moisissure *Aspergillus fumigatus* et les cellules responsables de la première ligne de défense pulmonaire.

Alors que *Neospora* est responsable des graves pertes économiques associées à des avortements chez les bovins, l'infection par *Plasmodium* continue d'avoir des conséquences dévastatrices sur la santé et les moyens de subsistance des populations à travers le monde. Le caractère intracellulaire de ces deux parasites est un exemple de leur capacité de camouflage face aux mécanismes de défense de l'hôte au niveau cellulaire et immunitaire.

Dans la première partie de ce document, je montre l'importance de la régulation de l'expression des protéines parasitaires de surface et de celles produites par des vésicules sécrétoires lors de l'invasion du parasite et de sa transformation à l'intérieur de la cellule hôte, telles que les protéines SAG1 et BSR4 chez *Neospora*, ou encore PLP1 et RON4 chez *Plasmodium*.

La caractérisation et la manipulation des gènes exprimant ses protéines ont permis le développement de stratégies visant le diagnostic précoce et/ou la prévention de l'infection.

Alors que la manipulation de la cellule hôte est un mécanisme attendu chez les protozoaires intracellulaires, cette capacité est peu évoquée chez d'autres eucaryotes tels que la moisissure *Aspergillus fumigatus*. Ce champignon opportuniste est capable de provoquer différentes formes de mycoses chez l'Homme et chez les animaux. Ainsi, les aspergilloses peuvent être responsables des troubles respiratoires handicapants voire mortelles, et des graves pertes économiques dans le secteur avicole.

Dans la deuxième partie de ce document, j'explique comment l'issue de l'infection aspergillaire dépend des mécanismes précoces de la réponse innée activés chez deux espèces d'oiseaux par des motifs moléculaires associés à la surface d'*A. fumigatus*. La stimulation de la réponse pro-inflammatoire, mais aussi la production de certaines immunoglobulines semble essentielle pour assurer la colonisation du parenchyme respiratoire au détriment de sa fonction chez l'oiseau atteint, et ce avec plus ou moins d'efficacité selon l'espèce aviaire affectée.

Enfin, dans l'équipe de recherche que je viens d'intégrer au sein de l'UMR-BIPAR (ANSES, EnvA, INRAE), je vais continuer avec cette approche co-évolutive, mais cette fois-ci en explorant l'interaction plus complexe entre un protozoaire (*Babesia*) et son vecteur (tique Ixodidae). Je profiterai des outils et des techniques de pointe développés par mon équipe pour analyser leurs interactions cellulaires et proposer des moyens de contrôle et de prévention des piroplasmoses, un fléau pour la santé animale et un problème de santé publique en augmentation au niveau mondial.

**Title :** Titre (en anglais) **Protozoa and moulds : deciphering the molecular mechanisms that hijack host's cell machinery**

**Keywords :** *Neospora*, *Plasmodium*, *Aspergillus*, host, protein regulation and immunity

**Abstract :** The co-evolution of pathogens and their target hosts has led to the establishment of close and complex interactions between them. From protozoa to moulds, the interactions set up during host colonisation allow the parasite to escape or even to control the host to divert its resources, which can lead to cell exhaustion and programmed cell death for the benefit of the parasite.

Throughout my scientific career, I have been lucky enough to be part of several research teams interested, as I was, in understanding the ability of parasites to manipulate the biology of their host cell and the danger signals it triggers. Thus, during my master's and doctoral studies, and my postdoctoral position, I studied intracellular protozoa such as *Neospora caninum* and *Plasmodium* sp. After my recruitment as associate professor at the EnvA, I wanted to apply the knowledge acquired in the understanding of the cellular interaction between the mould *Aspergillus fumigatus* and the cells responsible for the first line of lung defence.

While *Neospora* is responsible for the severe economic losses associated with abortion in cattle, *Plasmodium* infection continues to have devastating consequences for the health and livelihoods of people around the world. The intracellular nature of these two parasites is an example of their ability to camouflage themselves from host defence mechanisms at the cellular and immune level.

In the first part of this paper, I show the importance of regulating the expression of parasitic surface proteins and those produced by secretory vesicles during the invasion and transformation within the host cell, such as SAG1 and BSR4 in *Neospora*, or PLP1 and RON4 in *Plasmodium*.

The characterisation and manipulation of the genes expressing these proteins has allowed the development of strategies for the early diagnosis and/or prevention of infection.

While host cell manipulation is an expected mechanism in intracellular protists, this ability is not well known in other eukaryotes such as the mould *Aspergillus fumigatus*. This opportunistic fungus is capable of causing various forms of mycosis in humans and animals. Thus, aspergillosis can be responsible for disabling and even fatal respiratory disorders and serious economic losses in the poultry sector.

In the second part of this paper, I explain how the outcome of aspergillosis infection depends on early innate response mechanisms activated in two avian hosts by molecular patterns associated with the surface of *A. fumigatus*. The stimulation of the pro-inflammatory response, but also the production of certain immunoglobulins appears to be essential to ensure colonisation of the respiratory parenchyma to the detriment of its function in the affected birds, with varying degrees of success depending on the affected avian species.

Finally, in the research team that I have just joined within the UMR-BIPAR (ANSES, EnvA, INRAe), I will continue with this co-evolutionary approach, but this time by exploring the more complex interaction between a protozoan (*Babesia*) and its vector (Ixodidae tick). I will take advantage of the cutting-edge tools and techniques developed by my team to analyse their cellular interactions and propose means of controlling and preventing piroplasmiasis, a scourge for animal health and a growing public health concern worldwide.

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# 1. Curriculum vitae

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## 1.1 Personal information

**Veronica RISCO CASTILLO**

Birth Date: 15/01/1975 (Lima, Peru)

Nationality: Peruvian - French

Married (one enfant)

## 1.2 Professional information

Maître de Conférences hors classe (associate professor)

CNECA section n° 7

Discipline: Parasitology, mycology, parasite and fungal diseases

Institution : École Nationale Vétérinaire d'Alfort (EnvA)

Laboratory of affiliation: UMR BIPAR (ANSES, INRAe, EnvA) – MiTick team

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ORCID account: <https://orcid.org/0000-0002-1586-6793>

IdRef identification: 232659451

## 1.3 Professional experience

- |                        |   |
|------------------------|---|
| <b>2004 to 2008</b>    | <b>PhD candidate.</b> University Complutense of Madrid, Spain<br>Research group SALUVET   |
| <b>2009 to 2011</b>    | <b>Postdoctoral researcher</b> , UMR S945 INSERM - Pierre and Marie Curie University<br>Winner of a "Poste Vert" postdoctoral position<br>Topic: Development of particle-based vaccines by transcutaneous and intradermal routes of vaccination in a new humanized mouse model: an example of <i>Plasmodium falciparum</i> antigens.<br>Supervisor: Dominique Mazier  |
| <b>2012 to 2013</b>    | <b>Postdoctoral researcher</b> , UMR S945 INSERM - Pierre and Marie Curie University<br>Topic: Malaria Liver Infection: molecular mechanisms of host cell invasion by <i>Plasmodium</i> sporozoites<br>Supervisor: Olivier Silvie   |
| <b>2014</b>            | <b>Lecturer (Maitre de conférences stagiaire)</b> in Parasitology, mycology, parasite and fungal diseases, Ecole nationale vétérinaire d'Alfort (EnvA)<br>Member of EA7380 DYNAMYC (UPEC/ EnvA/ANSES) from 2014 to 2021.  |
| <b>2015 to present</b> | <b>Assistant Professor (Maître de Conférences)</b> in Parasitology, mycology, parasite and fungal disease, Ecole nationale vétérinaire d'Alfort (EnvA)<br>Member of the Department of Biological and Pharmaceutical Sciences (DSBP)<br>Veterinary collaborator at the Wildlife Hospital of Alfort (ChuvFS)<br>Member of UMR BIPAR (ANSES/EnvA/INRAe), research team MiTick (Microorganismes & Tiques) since 2022. |

#### 1.4 Academic education

##### **1998 - Doctor on Veterinary Medicine**

Institution: University San Marcos - Faculty of Veterinary Medicine, Lima-Peru

Exercise thesis: Detection of viral charge and antibodies against Bovine viral diarrhoea virus in an alpacas (*Vicugna pacos*) herd during the reproductive season. Honours degree (*cum laude*)

Supervisor: Hermelinda Rivera Geronimo

##### **2004 - Academic degree recognition on Veterinary medicine**

Institution: University Complutense of Madrid, Spain

##### **2004 - MsSc degree in Infectious Diseases**

Institution: Veterinary Faculty, Complutense University of Madrid, Spain

Thesis: Mechanisms of tachyzoite-to-bradyzoite transformation in *Neospora caninum*. Honours degree (*cum laude*)

Supervisor: DVM PhD Aurora Fernandez Garcia

##### **2008 - PhD in Animal Sciences**

Institution: Complutense University of Madrid, Spain

Thesis: Identification and Characterization of bradyzoite-specific antigens of *Neospora caninum*. Honours degree (*cum laude*)

Supervisor: DVM PhD Luis Miguel Ortega Mora

##### **Since 2020 – Resident of the European Veterinary Parasitology College (EVPC)**

#### 1.5 Complementary training

- 2010** Authorisation for animal experimentation level 1. Pierre and Marie Curie University. Paris, France
- 2011** Extension of the authorisation in rodent surgery in the context of animal experimentation. Pierre and Marie Curie University. Paris, France.
- 2017** Teaching training for lecturers (4 weeks). AgroParistech Paris, France.
- 2019** Training in medical mycology (4 weeks). Pasteur Institute. Paris, France.
- 2021** Training in veterinary entomology (1 week). National College of Toulouse (ENVT). Toulouse, France.

#### 1.6 Membership to Commissions/Workgroups/Societies

- Since 2012** Member of the World Association for the Advancement of Veterinary Parasitology (WAAVP)
- Since 2014** Member of the International Society for Human and Animal Mycology (ISHAM)
- Since 2014** Member of the Association for the development of veterinary parasitology of Alfort (ADPVA)
- Since 2014** Member of the French Society of Parasitology (SFP), and of the French Society of Medical Mycology (SFMM)

#### 1.7 Prizes and distinctions

- 1998** Prize for the best research work "Association of classical swine fever virus and perinatal mortality in technical farms" (III National Congress of Veterinary Sciences, Lambayeque-Peru).
- 2002-2003** Pre-doctoral grant. Complutense University of Madrid.
- 2003-2006** Pre-doctoral fellowship (MAE-AECI). Department of Animal Health, Faculty of

- Veterinary Medicine, Complutense University of Madrid.
- 2007** Accesit Madrid Prize 2007, for the best patent: "Usefulness of *N. caninum* NcSAG4 gene in the diagnosis and prevention of Neosporosis" (Madrid City Council, Spain).
- 2009** Grant to participate in the 4th EuPathDB Workshop (Center for Tropical and Emerging Global Diseases, University of Georgia, Athens, GA, USA, funded by the National Institute of Health, USA).
- 2022** Recognition as the most successful veterinarian in its 25<sup>th</sup> anniversary (Veterinary Faculty, San Marcos University)

## 1.8 Research activity

After my veterinary thesis I worked a couple of years as veterinary practitioner in Peru, but soon I realised that scientific research was a field I wanted and needed to explore. Some years later, my research activity could be structured into successive stages linked to the evolution of my professional career. From my PhD training at the Complutense University of Madrid, following my postdoctoral work in the UMRS 945 at the Pierre and Marie Curie University (Paris VI), to finally join the DYNAMYC host team (EnvA, UPEC).

My master and PhD studies, under the supervision of Drs. Aurora Fernandez Garcia and Luis Miguel Ortega Mora, focused in the development of tools for the diagnosis and prevention of bovine neosporosis. It relied on the identification of proteins that are specifically expressed at different stages of the parasite, their role during the development of the parasite and its interaction with the host cell. All along my PhD research, I was able to participate in four Spanish and three European research projects, and my results led to the filing of three patents.

After my PhD, the possibility to work with new molecular and cellular tools brought me to Paris to work with Prof. Dominique Mazier team. The challenge was to develop vaccine candidates based on the model of "retrovirus like particles" (VLPs) that were able to stimulate an immune response against antigens expressed during the sporozoite stage of *Plasmodium*, the form transmitted by the mosquito. Later, we developed a second project under the supervision of Dr. Olivier Silvie regarding the study of host-parasite relationships during the invasion of the sporozoite into the liver cell. Thanks to these two research topics, I participated in three ANR research projects.

My experience in the study of host-parasite interactions at the cellular level was one of the reasons of my recruitment at EnvA. I devoted my research project to the understanding of the early interaction of the mould *Aspergillus fumigatus* and the lung environment in birds in the research group Dynamyc, under the direction of Prof. Jacques Guillot. In addition to the results that elucidate the importance of an early activation of the pro-inflammatory response during lung colonisation, our model allowed the collaboration with the joint research unit BIPAR (ANSES, INRAe, EnvA). Finally, this collaboration also opened the door to the opportunity to continue with the analysis of the interaction between the parasite and its host, this time using *Babesia* sp. as a model.

In all cases, my main research objective has always been to understand the underlying questions about the ability of the parasite to subvert the host machinery. For this, it has been always important to establish strong collaborations with other research groups, and to participate to multidisciplinary teams in order to be able to understand the interactions among gene regulation of parasite's machinery for cell invasion, host response at cellular and immune response level, and comparative analysis between *in vitro* and *in vivo* models.

### 1.8.1 **Obtained funding**

a. As coordinator

- 2017** Host-pathogen interaction in invasive aspergillosis (IA): characterisation of the

- local host immune response  
 Financing source: EnvA's Scientific Council  
 Amount: 9 938 euros (project financing and master 2 stipend)
- 2020** Pathophysiology of aspergillosis in turkeys: role of the respiratory microbiota and particularities of the local immune response  
 Financing source: EnvA's Scientific Council  
 Amount: 5 000 euros (master 2 stipend)
- 2020-2022** Genetic diversity and susceptibility to acaricides in *Sarcoptes* mites infecting vicuñas and alpacas in Peru  
 Amount: financing of travel expenses for sampling and co-ordination meetings (France-Peru or vice versa, twice a year), 10 000 euros approximately
- b. As collaborator
- 2005-2007** Evaluation of the health impact of new management systems in the Peruvian vicuña population.  
 Financing source: Complutense University of Madrid  
 Amount: 18 000 euros  
 Scientific collaborator: San Marcos University (Peru)
- 2017** Study of pathogens in Animal Health: installation of a confocal microscope at Alfort's site (scientific coordinator: Dr. Grégory Karadjian, UMR BIPAR, ANSES, EA DYNAMYC)  
 Financing source: DIM-1Health investments  
 Amount: 232 323 euros
- 2017** Study of pathogens in Animal and Human Health: mass spectrometry platform at Alfort's site (scientific coordinator: Prof. Henri-Jean Boulouis, EnvA, UMR-BIPAR)  
 Financing source: DIM-1Health investments  
 Amount: 238 113 euros
- 2020** Fungal/bacterial interactions: *Trichosporon asahii*, a partner for exacerbated virulence of *Chlamydia gallinacea*?  
 Financing source: Paris-Est University (amorçage program)  
 Amount: 13 000 euros (reagents and master 2 stipend)
- 2022** Prevalence survey of heartworm or worm skin disease caused by *Dirofilaria immitis* and *Dirofilaria repens* in military dogs in mainland France (DIROMYL survey)(scientific coordinator: Dr Stéphanie Watier-Grillot)  
 Financing source: Centre for Epidemiology and Public Health of the Armed Forces  
 Amount: 10 000 euros (reagents and diagnostic techniques)

### 1.8.2 Research supervision

- a. Veterinary thesis supervision as director
2023. GROSBOIS Amélia. Study of the prevalence of dirofilariasis in military working dogs in mainland France (2021-2022).
2023. SRUN Camille. Detection and molecular characterisation of cryptosporidium sp. and giardia sp. in European hedgehogs (*erinaceus europaeus*) arriving at CEDAF.
2021. HILKA SARAH. Haemosporidia of raptors and corvids in care centres: epidemiological survey 2018-2019 at the wildlife reception centre of the Alfort Veterinary School (CEDAF).
2020. EHKIRCH CAMILLE. Hemosporidia infestation in avian wildlife: a retrospective survey of the Cévennes rescue centre between 2011 and 2018
2020. JACQUESSON THIBAUT. Haemosporidia in raptors and corvids: epidemiological study in birds treated at the Garrigues Cévennes Wildlife Hospital in Ganges from 2011 to 2017
2019. DENAES Chloé. Epidemiological study of dermatophytosis in European hedgehogs (*Erinaceus europaeus*) treated at the Alfort Wildlife Care Centre (CEDAF) in 2016
2019. DEFIVES Chloé. Coccidiosis in llamas (*Lama glama*) and alpacas (*Vicugna paos*) reared in France: epidemiological and analytical survey of infestations by *Eimeria* spp.

2018. LOISEAUX Juliette. Evaluation of the presence of gastrointestinal nematodes and scabies agents in small camelids in France and therapeutic trial on scabies in alpacas from southern Peru.
- b. Veterinary thesis supervision as examiner
- 2022 PAQUET Chloé. Methods and applications of ethology in wildlife care centres - a first approach to the assessment of welfare in rock pigeons (*Columba livia*) at CHUV-FS
- 2022 De MIJOLLA Eloïse. Preventive medicine beyond vaccination. Description of the chuva preventive medicine service between October 2019 and October 2021.
- 2022 MAUGER Maud. The health of the hive: the interests of connected hives for beekeepers and veterinarians.
2021. LISE Solène. Identification of corynebacteria of the diphtheriae complex in wildlife.
2021. CAUBET Betty. Study of the activity of essential oils on bacterial strains.
- 2020 LOUIS EDWIN. Information sheets for dog owners: proposal for a standardised methodology and application to the fields of behaviour and vaccine and parasite prevention in puppies and kittens.
2018. LAURENT Gautier. Creation of a new clinical file to improve the care of European wildlife animals received at the Centra d'Accueil de la Faune Sauvage (CEDAF), as well as the recording and statistical processing of data for the year 2017.
2017. CHEMOUNI Raphael. Bovine besnoitiosis in France: retrospective survey on the epidemiological situation, the means of surveillance and control recommended by the GDS of metropolitan France (CONFIDENTIAL until 31/12/2023).
- c. Master 1 supervision
- 2016 ZAPATA Laura. Development of a protocol for isolation and characterization by flow cytometry of immune cells, in a turkey model of aspergillosis infection. UPMC
- 2016 STYM-POPPER Grégoire. Evaluation of avian thrombocyte recruitment during *Aspergillus fumigatus* infection. UPMC.
- 2016 HALIFA Ruben. Development of an *in vitro* culture method and infection of avian thrombocytes by *Aspergillus fumigatus*. UPMC.
- 2017 FILLEUL Caroline. *In vitro* study of the role of avian thrombocytes against the fungus *Aspergillus fumigatus*. UPMC
- 2018 BIGEARD Pierre. *Aspergillus* and phagocytosis: *in vitro* study of the phagocytic activity of avian thrombocytes and heterophils. Sorbonne Universities
- d. Master 2 supervision
- 2012 SON Olivia. Production of transgenic parasites in *Plasmodium yoelii* for the study of sporozoite entry into hepatocytes. UPMC / UMRS 945.
- 2017 ZAPATA Laura. Host-pathogen interaction during acute aspergillosis: characterization and comparison of the local immune response in two avian models. Sorbonne Universities / EA7380 DYNAMYC.
- 2018 VAHSEN Tobias. Characterization of the inflammatory response to acute avian aspergillosis. Université de Paris-Saclay / EA7380 DYNAMYC.
- 2021 PICAVET Léa. Cellular and molecular interaction between a biofilm of *Aspergillus fumigatus* and avian bronchial epithelial cells. University of Paris-Saclay / EA7380 DYNAMYC.
- 2022 HOGAN Patrick. *Trichosporon asahii*, an exacerbated virulence partner of *Chlamydia gallinacea*?. University of Poitiers / EA7380 DYNAMYC.
- e. PhD thesis supervision
- 2022 Caina NING (1<sup>st</sup> year). Cholinomimetics-Mediated Activities of Peptidergic Neurons Innervating the Salivary Glands of the Tick *Ixodes ricinus*. Ecole Doctorale ABIES, Univ. Paris-Saclay, AgroParisTech, INRAe. (20% of co-supervision).

2022 Apolline MAITRE (1<sup>st</sup> year). Anti-microbiota vaccines for the control of tick-borne pathogens affecting livestock in Corsica. Ecole Doctorale ABIES, Univ. Paris-Saclay, AgroParisTech, INRAe. (20% of co-supervision).

### 1.8.3 Scientific production

#### a. Articles published

**37 publications in indexed international journals**  
**7 publications in non-indexed journals**  
**13 publications as first or second author**  
**3 Publications as last author**  
**Citations: 1331 (628 since 2017); h index: 21 (15 since 2017) (according Google Scholar)**

#### a.1 Publications in indexed scientific journals

1. Čmoková, A.; Kolařík, M.; Guillot, J.; **Risco-Castillo, V.**; Cabañes, F.J.; Nenoff, P.; Uhrlaß, S.; Dobiáš, R.; Mallátová, N.; Yaguchi, T.; Kano, R.; Kuklová, I.; Lysková, P.; Mencl, K.; Hamal, P.; Peano, A.; Hubka, V. 2022. Host-driven subspeciation in the hedgehog fungus, *Trichophyton erinacei*, an emerging cause of human dermatophytosis. *Persoonia - Molecular Phylogeny and Evolution of Fungi*, Volume 48, June 2022, pp. 203-218(16). <https://doi.org/10.3767/persoonia.2022.48.06>
2. Thorel M, Mateos-Hernandez L, Mulot B, Azzouni MN, Hodžić A, Gaillot H, Ruel Y, Desoubeaux G, Delaye JB, Obregon D, Wu-Chuang A, de la Fuente J, Bermúdez-Humarán LG, **Risco-Castillo V**, Leclerc A, Cabezas-Cruz A. 2022. Assessment of the Safety and Efficacy of an Oral Probiotic-Based Vaccine Against *Aspergillus* Infection in Captive-Bred Humboldt Penguins (*Spheniscus humboldti*). *Front Immunol*.13:897223. doi: 10.3389/fimmu.2022.897223. eCollection 2022.
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  32. Serrano-Martínez E; Collantes-Fernández E; Chàvez-Velàsquez A; Rodríguez-Bertos A; Casas-As-tos E; **Risco-Castillo V**; Rosadio-Alcantara R; Ortega-Mora L M. 2007. Evaluation of *Neospora caninum* and *Toxoplasma gondii* infections in alpaca (*Vicugna pacos*) and llama (*Lama glama*) aborted fetuses from Peru. *Veterinary parasitology* 2007;150(1-2):39-45.
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bradyzoite-specific gene from *Neospora caninum*. International journal for parasitology 2007;37(8-9):887-96.

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37. Von Blumröder D; Schares G; Norton R; Williams D J L; Esteban-Redondo I; Wright S; Björkman C; Frössling J; **Risco-Castillo V**; Fernández-García A; Ortega-Mora L M; Sager H; Hemphill A; van Maanen C; Wouda W; Conraths F J. 2004. Comparison and standardisation of serological methods for the diagnosis of *Neospora caninum* infection in bovines. Veterinary parasitology 2004;120(1-2):11-22.

#### a.2 Publications in non-indexed journals

1. Delsart M., Dufour B., Arné P., Risco-Castillo V., Bernigaud C., Le Roux D., Guillot J., Rose N. 2019. Evaluation des performances d'une grille de notation des lésions cutanées induites par la gale sarcoptique chez le porc. Approche comparée de la sensibilité et de la spécificité des méthodes de notation visuelle des lésions et d'un test elisa. Épidémiol. et santé anim. 76, 127-140.
2. Veronica Risco-Castillo, Olivia Son, Jean-François Franetich, Eric Rubinstein, Dominique Mazier et Olivier Silvie. 2013. *Plasmodium* sporozoite entry pathways during malaria liver infection. Biologie Aujourd'hui, 207 (4), 219-229. <https://doi.org/10.1051/jbio/2013021>
3. G. Alvarez-Garcia; A. Aguado Martinez; V. Risco-Castillo; E. Collantes-Fernandez. Mortalidad Perinatal en ovejas (I).Produccion Animal. 2007; 22(228), 4-13.
4. E. Collantes-Fernandez; A. Aguado Martinez; V. Risco-Castillo; G. Alvarez-Garcia. Mortalidad Perinatal en ovejas (II).Produccion Animal. 2007; 22(229), 4-15.
5. V. Risco-Castillo; A. Aguado Martinez; G. Alvarez-Garcia; E. Collantes-Fernandez. Mortalidad Perinatal en ovejas (III).Produccion Animal. 2007; 22(230), 52-63.
6. Raul Rosadio; Veronica Risco. Variations on the weight of Alpacas on an intensive production system. Revista de Investigaciones Veterinarias del Perú. 1999; 10-1.
7. Veronica Risco; Hermelinda Rivera; Nieves Sandoval; Danilo Pezo; Wilber Garcia; Raul Rosadio. Detection of BVD antibodies and virus in alpacas at reproductive age. Revista de Investigaciones Pecuarias. 1998; 2.

#### b. Participation to national and international events

- c. **Participation to 7 local and 26 international events**
- d. **15 oral communications (4 as invited speaker)**
- e. **18 poster presentations**

#### b.1 Oral presentations as invited speaker

1. **Risco-Castillo, V.** Ringworm in wild European hedgehogs: clinical and therapeutic approach in a French wildlife rehabilitation centre. Onygenales International Workshop, 10-11 September 2020, Prague, Czech Republic.
2. **Risco-Castillo V**, Amadoudji Zin M, Yahia N, Joulin-Giet A, Morosan S, Moris A, Mazier M, Combadière B, Bellier B. Development of particle-based vaccines by cutaneous route of vaccination in murine

models: example of *Plasmodium* antigens. Colloque de l'Agence National de la Recherche (ANR), Programme Emergence –Bio. Paris, France. November 2012.

## b.2 Participation to international scientific events

### **Oral communications**

1. Hilka, S., Le Barzic, C., Arne, P., **Risco Castillo, V.** Haemosporidian infection in urban and peri-urban wild birds admitted to a French Wildlife rehabilitation Center between 2018 and 2019. 15<sup>th</sup> International Congress in Parasitology (ICOPA), 21-26 August 2022, Copenhagen, Denmark.
2. C. Le Barzic; C. Denaes; P. Arne; C. Angebault; F. Botterel; J. Guillot, **V. Risco-Castillo.** *Trichophyton erinacei* in European hedgehogs admitted to wildlife rehabilitation centers: results of a prospective epidemiological survey in Ile-de-France, France. Congress of the International Society for Human and Animal Mycology (ISHAM), 28-31 May 2018, Amsterdam, Netherlands.
3. E. Melloul, L. Roisin, P.L. Woerthe, **V. Risco**, E. Dannaoui, J. Guillot, J-W. Decousser, F. Botterel. Assessment of clinical, animal and environmental strains of *Aspergillus fumigatus* and *Stenotrophomonas maltophilia* in an *in vitro* mixed biofilm model. 8th Trends in Medical Mycology (TIMM), 6-9 October 2017, Belgrade, Serbia.
4. Guillot J, **Risco V**, Chermette R, Arné P. Aspergillosis: a real threat for poultry industry? Joint symposium of the Japanese Society of Veterinary Science, ISHAM-Veterinary Mycology and Medical Phylogeny working groups, 8 septembre 2016, Fujisawa, Japon
5. **Risco Castillo, Veronica**; Ortega, L.M. Toxoplasmosis and Neosporosis in South American Camelids: Actual situation. Alvarez-García, G.; XIV Latin American Congress of Buiatrics. Lima, Peru, 2009.
6. SALUVET group. VIII Madrid on Science. "Parasites on marine mammal from the antartics. Madrid, Spain. 12-15 April 2007.
7. **V. Risco-Castillo**, A. Fernàndez-García, A. Zaballos, G. Àlvarez-García, L.M. Ortega-Mora. Molecular characterization of BSR4, a novel bradyzoite-specific gene from *Neospora caninum*. European Union international congress COST–action 857, 3rd annual workshop. Dresden-Germany. May 2006.
8. **V. Risco-Castillo**, A. Fernàndez-García, A. Zaballos, G. Àlvarez-García, L.M. Ortega-Mora. Characterization of NcSAG4 expression in *Neospora caninum* bradyzoites. European Union international congress COST–action 857, 2nd annual workshop. Beatenberg-Switzerland. May 2005.
9. E. Serrano-Martínez, A. Chàvez-Velàzquez, E. Collantes-Fernàndez, G. Àlvarez-García, E. Casas-Astos, R. Rosadio-Alcàntara, **V. Risco-Castillo**, L.M. Ortega-Mora. Neosporosis and Toxoplasmosis in South American Camelids from Peru. European Union international congress COST ACTION 854 Annual Meeting. Warsaw-Poland. 2005.

### **Poster presentations**

1. P. Bigeard, C. Filleul, T. Vahsen, L. Zapata, F. Botterel, J. Guillot, P. Arné, **V. Risco-Castillo.** *In vitro* phagocytosis of *Aspergillus fumigatus* conidia by chicken thrombocytes and heterophils. 9th Trends in Medical Mycology (TIMM), 11-14 October 2019, Nice, France.
2. Vahsen, T.; Zapata, L.; Guabiraba, R.; Melloul, E.; Guibert, M-J.; Botterel, F.; Guillot, J.; Arné, P.; **Risco-Castillo, V.** Cellular and molecular insights on pro-inflammatory immune response regulation during acute Aspergillosis in chicken and turkey poults. Congress of the International Society for Human and Animal Mycology (ISHAM), 28-31 May 2018, Amsterdam, Netherlands.
3. Guillot J, **Risco V**, Chermette R. Epidemiology of animal dermatophytoses. Developments in clinical and fundamental aspects of dermatophytes, ISHAM workshop, 29-30 octobre 2016, Utrecht, Pays-Bas.
4. **Risco-Castillo V**, Manzoni G, Briquet S, Topçu S, Silvie O. Targeted gene deletion of perforin-like protein 1/spect2 in *Plasmodium yoelii*. 9th Annual BioMalPar I EVIMalaR Conference, Biology and Pathology of the Malaria Parasite. EMBL Heidelberg, Germany. May 2013.
5. **Risco-Castillo, V.**, Son, O., Silvie, O. Novel Transgenic *Plasmodium yoelii* parasites to study sporozoite invasion. Annual Molecular Parasitology Meeting. WoodsHole, Etats Unis. Aout, 2012.
6. Aguado-Martinez, A. Fernandez-Garcia, G. Àlvarez-García, S. Rodriguez-Marcos, **V. Risco-Castillo, V.** Marugan-Hernandez & L. M. Ortega-Mora. Stage-specific NcSAG1 and NcSAG4 genes as molecular

- markers of acute and chronic *Neospora caninum* infection in a mouse model. COST-action 857, 4th annual conference (Apicomplexa in the postgenomic era). Kolymbari-Greece. May 2008.
7. Aguado-Martínez, G. Álvarez-García, A. Fernández-García, **V. Risco-Castillo**, I. Arnaiz-Seco, X. Rebordosa-Trigueros, V. Navarro-Lozano, L.M. Ortega-Mora. Usefulness of the recombinant proteins rNcSAG4 and rNcGRA7-based ELISA tests to distinguish primo-infection, recrudescence and chronic bovine neosporosis. WAAVP congress, The 21st International Conference of the World Association for the Advancement of veterinary Parasitology. Ghent, Belgium. May 2007.
  8. López-Pérez IC, Collantes-Fernández E, **Risco-Castillo V**, Pérez-Pérez V, Pereira-Bueno J. and Ortega-Mora LM; Pathological and immunological findings on placenta after infection with *Neospora caninum* in a pregnant mouse model; WAAVP congress, The 21st International Conference of the World Association for the Advancement of veterinary Parasitology. Ghent, Belgium. May 2007.
  9. A. Aguado-Martínez, G. Alvarez García, A. Fernández García, **V. Risco-Castillo**, V. Marugan-Hernandez, L.M. Ortega Mora. Protective immunity induced by recombinant antigens rNcSAG4 and rNcGRA7 against chronic and congenital neosporosis in an experimental murine model. WAAVP congress, The 21st International Conference of the World Association for the Advancement of veterinary Parasitology. Gante, Belgium. May 2007.
  10. A. Fernández-García, G. Álvarez-García, A. Aguado-Martínez, **V. Risco-Castillo**, J.A. Castillo, L.M. Ortega-Mora; Development and validation of an indirect ELISA for the diagnosis of bovine besnoitiosis. AVEDILA Congress. Bilbao, Spain. 2007.
  11. A. Aguado-Martínez, G. Álvarez-García, A. Fernández-García, **V. Risco-Castillo**, I. Arnaiz-Seco, X. Rebordosa-Trigueros, V. Navarro-Lozano, L.M. Ortega-Mora. Usefulness of two recombinant ELISAs using recombinant proteins rNcGRA7 y rNcSAG4 for the discrimination between primo-infection, recrudescence and chronic infection of *Neospora caninum* on cattle. AVEDILA Congress. Bilbao, Spain. 2007.
  12. Schares G, von Blumröder D, Norton R, Williams DJ, Esteban-Redondo I, Wright S, Björkman C, Frössling J, **Risco-Castillo V**, Fernández-García A, Ortega-Mora LM, Sager H, Hemphill A, van Maanen C, Wouda W, Conraths FJ. Comparison of commercial and in-house Elisas for the diagnosis of bovine *Neospora caninum* infection after epidemic and endemic abortion. European Union international congress COST-action 854, final conference. Liege-Belgium. Sept 2006.
  13. Fernández-García, A., **Risco-Castillo, V.**, Zaballos, A., Álvarez-García, G., Ortega-Mora, L.M. Molecular cloning, expression and characterization of the *Neospora caninum* SAG4 gene. European Union international congress COST-action 857, 1st annual workshop. Lisbon-Portugal. May 2004.
  14. López-Pérez, **V. Risco-Castillo**, E. Collantes-Fernández, L.M. Ortega-Mora. Effect of infection with *Neospora caninum* in different stages of gestation in balb/c mice. European Union international congress COST-action 854, joint meeting of working groups 2 and 3. Derio-Spain. Jun 2004.
  15. E. Serrano-Martínez, A. Chàvez-Velàsquez, G. Álvarez-García, E. Collantes-Fernández, E. Casas-Astos, **V. Risco-Castillo** and L.M. Ortega-Mora. *Neospora caninum* infection in alpacas (*Vicugna pacos*) and llamas (*Lama glama*). European Union international congress COST-action 854, 1st annual workshop. Liverpool-UK. Sep 2004.
  16. D. Von Blumröder, G. Schares, Williams, R. Norton, E.A. Innes, I. Esteban, J. Mattson, C. Björkman, **V. Risco Castillo**, A. Fernández García, L.M. Ortega Mora, A. Hemphill, H. Sager, C. Van Maanen, W. Wouda, B. Fontijn, F. Conraths. A multi-centred study to compare serological tests. Cost Action 854 (Protozoal reproduction losses in farm ruminants). Rheinsberg, Germany. 2003.
  17. J. Pereira-Bueno, A. Quintanilla-Gozaolo, V. Pérez-Pérez, G. Álvarez-García, **V. Risco-Castillo**, E. Collantes-Fernández, L.M. Ortega-Mora. Evaluation of ovine abortion associated with *Toxoplasma gondii* in Spain by different diagnostic techniques. COST 854 joint meeting of working groups 3 (diagnosis) and 4 (epidemiology, risk assessments, economics and control). Rheinsberg-Germany. May 2003.

### b.3 Participation to to local/national scientific events

1. Laroucau, K., **Risco-Castillo**, V. Fungal/bacterial interactions: *Trichosporon asahii*, a partner for exacerbated virulence of *Chlamydia gallinacea*?. JS COMUE 2020 projects, Paris-Est University. 28<sup>th</sup> june 2022.
2. **V. Risco-Castillo**. Bovine neosporosis and its reproductive risks. National breeding control laboratory – CST 13rd Nov 2018.

3. **V. Risco-Castillo.** Species-dependent susceptibility to avian aspergillosis: new insights on host immunity-pathogen interactions. Virtual conference series, Royal Veterinary College. London, UK., 25th Jul 2017
  4. **V. Risco-Castillo.** The local pulmonary immune response to infection with the mould *A. fumigatus* - a comparative study in two bird species Journées de recherché EnvA. 27<sup>th</sup> June 2017
  5. Marugàn-Hernàndez, Regidor-Cerrillo J, Fernandez-Garcia A, Alvarez-Garcia G, Aguado-Martinez A, **Risco-Castillo V**, Gomez-Bautista M, Ortega-Mora LM. Proteomic analysis in cyst-developing protozoa with interest on animal health. Proteomics congress. Barcelona, España. 2008.
  6. Fernández-García A., Morales A., Pereda J. M., Aguado-Martínez A., Marcén J. M., Rojo-Montejo, S., **Risco-Castillo V.**, Àlvarez-García G., Ortega-Mora L. Description of besnoitiosis outbreak in Central Spain. Anembe meeting, Salamanca-Spain. May 2008.
  7. **V. Risco-Castillo;** Arnaiz Seco, Ignacio; García-Peña, Francisco Javier; Rosadio Alcàntara, Raúl Héctor; Castillo Doloriert, Hugo; Wheeler, Jane; Hoces, Domingo; Ortega Mora, Luis Miguel. Evaluation of health impact of new rearing systems on vicugna population of Peru. Research in Agriculture for the Development Congress. Barcelona, Spain. Dec 2007.
- f. Patents
- European patent DIRV-19-0079, Use of *E. coli* (probiotic strains) to modulate  $\alpha$ -Gal immunity and provide protection against infectious diseases in animals.
  - Patent EP1710309B1 and Prix Accesit Madrid 2007, best patent: “Use Of Gene NcSAG4 For the diagnosis and prevention of neosporosis and as a marker for analysis of the pathogenesis” (Spain, 2008).
  - Patent P200601262, Use of the gene NcBSR4 for the diagnosis and prevention of neosporosis and as a marker for analysis of the pathogenesis.
  - Patent P201001593, Use of the gene NcSRS9 for the diagnosis and prevention of neosporosis and as a marker for analysis of the pathogenesis.

#### **1.8.4 Other publication activities**

- a. Editorial and scientific advisory board activities
  - Reviewer for:
    - o Parasitology International since 2012 (Elsevier)
    - o American Journal of Veterinary Research since 2014 (AVMA)
    - o Acta Tropica since 2017 (Elsevier)
    - o Journal of Global Antimicrobial Resistance since 2018 (Elsevier)
    - o Frontiers in Veterinary Science since 2018 (Frontiers)
    - o Veterinary Record Case Report since 2018 (BMJ)
    - o Journal of Medical Mycology since 2018 (Elsevier)
    - o Frontiers in Health Production since 2019 (Frontiers)
    - o Journal of Fungi since 2021 (MDPI).
  - Guest editor of the special issue “Fungal Infections in Animals” at the Journal of Fungi journal (to be published at the beginning of 2023)

#### **1.9 Teaching activity**

After my recruitment in September 2014, I participated as trainee lecturer for the Unit of veterinary Parasitology, Mycology and Dermatology, at the Department of Biological and Pharmaceutical Sciences (DSBP). This unit was formed by 4 teacher-researchers (1 full professor + 3 associate professor), a contractual teaching assistant and two hospital practitioners. Since my arrival, I have been involved in theoretical and practical teaching.

After the pedagogical reform of 2015, my teaching activity takes place in a transversal way by participating in what is called “units of competence” (UC). They include fundamental subjects such as veterinary parasitology, but also microbiology, immunology and pharmacology, as well as UCs which target health management in terms of individual or herd medicine level, depending on the animal

species. My teaching activities also include close interaction with the Department of Animal Production and Public Health (DPASP) through my participation in clinical and diagnostic activities at the Centre Hospitalier Universitaire Vétérinaire de Faune Sauvage (Chuv-FS). My participation was purely clinical before 2015, but subsequently evolved in line with my teaching responsibilities, perfectly articulated with my teaching and research activities.

Before dermatology was attached to the Department of Equine and Carnivore Breeding and Pathology (DEPEC) in 2020, all of the personnel assigned to these activities were in our teaching unit. In addition to the 5 EC (4 parasitologists and a dermatologist), there were 4 to 5 consultants for clinical activities, 2 to 3 technical staff for laboratory activities and a secretary who were all under the responsibility of the head of the unit. Since then, its activities are carried out with 4 EC parasitologists-mycologists and 1 technical assistant (research training).

Also, I have been participating for several years as a lecturer in the Master of Science program offered by Sorbonne University (Master 1 and Master 2, Molecular and Cellular Biology programme), with 6 hours of lectures in total. These courses are followed each year by an average of fifteen students per master. My participation is unpaid.

#### **1.9.1 Pre-graduated teaching activity**

- |                        |   |
|------------------------|---|
| <b>2004 to 2008</b>    | <b>Veterinary Faculty, Complutense University of Madrid</b> (Spain). Second and fourth year of veterinary studies. Responsible of 20 hours of practical work in veterinary parasitology (second year), and 20 hours of practical work in parasite diseases (fourth year). |
| <b>2014 to Present</b> | <b>Veterinary College of Alfort</b> (EnvA). From first to fifth year of veterinary studies. Participation of theoretical and practical courses in parasitology and parasite diseases, equivalent to 180 hours of teaching per year.                                       |

#### **1.9.2 Post-graduated teaching activity**

- |                        |   |
|------------------------|---|
| <b>2009 to 2013</b>    | <b>Pierre and Marie Curie University</b> . Master degree program (first year). 3-hour lecture and 6 hours of practical course in the framework of a post-graduated formation in Parasitology/Fundamental and medical mycology.  |
| <b>2014 to Present</b> | <b>Sorbonne University</b> . MsSc studies (Molecular and Cellular Biology programme) Participation as lecturer in veterinary parasitology and veterinary mycology, aspergillosis and candidiasis in animals (6 hours per year). |
| <b>2021 and 2022</b>   | San Marcos University, Peru. Participation as lecturer at a MsSc program in animal health organised by the de veterinary faculty (6 hours).   |

#### **1.9.3 Other teaching activities**

- |                        |  |
|------------------------|--|
| <b>2014 to Present</b> | <b>EnvA's Wildlife Hospital</b> (Chuv-FS). Clinic and teaching activity, at least one on-call duty per month. This activity also includes the diagnosis of parasite diseases in birds and mammals arriving to the Hospital, parasites isolation and identification and the establishment of treatment protocols. |
|------------------------|--|

### **1.10 Other activities**

#### **1.10.1 PhD Jury member**

- Jury member for four PhD theses in veterinary science (Complutense University of Madrid, Spain)  
2011: Virginia Marugan Hernandez (jury). Vaccines against bovine neosporosis: use of transgenic strains and identification of new vaccine targets  
2012: Silvia Rojo Montejo (examiner). Development of vaccines against bovine neosporosis using inactivated and attenuated "*Neospora caninum*" isolates  
2013: Elena Jimenez Ruiz (examiner). Vaccines against neosporosis: new adjuvants and vaccine candidates and improvement of experimental mouse models.

2015: Paula Garcia (examiner). Strategies for the improvement of serological diagnosis of bovine besnoitiosis

- Member of the thesis monitoring committee (tutor) of Pierre Bigeard, at the doctoral school Complexity of Life of Sorbonne University (PhD to be defended at the beginning of 2023).

#### **1.10.2 Publication of teaching material**

- In 2006, I collaborated in the conception and production of the chapter about sarcoptic mange for an Atlas in veterinary parasitology (digital version in [www.3tres3.com](http://www.3tres3.com)), targeting veterinary students and young veterinarians.
- In 2007, I participated in the writing of three publications regarding perinatal mortality in lambs, targeting sheep farmers.

#### **1.10.3 Diagnostic activities**

As a young researcher, one of my responsibilities at the laboratory of parasitology of Complutense University was the serological diagnosis of bovine neosporosis with indirect immunofluorescence (IFI) and ELISA techniques. On average, 20 to 30 samples were processed per week.

Within the framework of the Parasitology-Mycolology Laboratory attached to the EnvA Biopole, I provide scientific support in the field of clinical biology, covering the performance of analyses using various parasitological and mycological techniques, as well as the interpretation of results. The laboratory receives an average of 2000 analysis per year.

Thanks to my participation at the Chuv-FS it has been possible to establish a surveillance programme for dermatophytosis in hedgehogs. The Parasitology-Mycolology Laboratory carries out the diagnosis and the results have enabled us to characterise the epidemiological and zoonotic risk of dermatophytosis in the Paris region and its surroundings.

#### **1.10.4 Participation to the institutional life**

I participate by being part of several commission/councils engaged to the amelioration of students' life, to the balance between educational and scientific burden of educators, and to the improvement of the communication of all EnvA actors .

- Since 2018 : elected deputy member of EnvA's teaching council.
- Since 2020: member of the EnvA's working group in professional equality
- Since 2021 : elected member of EnvA's scientific council
- Since 2021: elected deputy member of EnvA's technical council
- Since 2022: elected deputy member of EnvA's Council for Education and Student Life
- Since 2022: elected deputy member of the National Commission for Teacher-Researchers under the Ministry of Agriculture (CNECA, section 7)

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## 2. Preface

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*“In a sense, the hosts select for the best parasites and  
the parasites select for the best hosts”  
Claude Combes (The Art of Being a Parasite, 2001)*

Claude Combes said once that the selective pressures exerted inside a parasite-host system force the parasite to adapt its life cycle through a series of metamorphoses in order to ensure its survival, and this happens while the host triggers highly elaborated defence mechanisms. During the preparation of my PhD, I had the opportunity to observe *in vitro* how the protozoan parasite *Neospora caninum* regulates its gene expression in order to stay in its host cell in a resting stage, and how it avoids intrinsic host cell's defence mechanisms such as apoptosis. The mystery behind of how parasites are able to hijack their host cells in a relative homeostasis has since then appealed to me.

Parasitology is an enticing discipline that involves animal and Public Health as well as the environment, most of the time in a complex manner. Novel research strategies used by the scientific community allow us now to explore parasite diseases in animals and humans using a One Health approach. During my postdoctoral positions, I had the opportunity to work with clinicians in a multidisciplinary team interested in human malaria, with whom I investigated the fine interaction between another protozoan, *Plasmodium* sp., and its host cell. I learned to master innovative cellular and molecular tools to understand host-parasite interaction during host invasion and I realized how basic science provides the foundation of knowledge for the applied science that follows.

After spending more than ten years to basic research, I realized that my real passion lays in the possibility to share and transfer my outcomes to others. Therefore, I decided to orientate my professional activity towards teaching and research at the Veterinary College of Alfort (EnvA). This opportunity boosted my curiosity to develop both applied and epidemiological research in new fields, but at the same time, I could make use of my knowledge regarding the technical approach to study early pathogen-interaction, this time in a mould responsible of human and avian aspergillosis. The objective of this document is to offer a wide sight of my scientific background in order to obtain the accreditation to supervise research. It compiles my research activities but it is not necessarily exhaustive since its purpose is to clearly define how my professional career has evolved throughout my specialisation as veterinary parasitologist in host-parasite interaction.

This dissertation will start with a brief introduction about the importance of the encounter and the ways of interaction between the host and its parasite to ensure parasitism, it will continue with a compilation of my main results structured in three chapters, naming my research in *Neospora caninum*, then in *Plasmodium* and finally in *Aspergillus fumigatus*. The illustration of the results from those three axis will come from original articles published during my scientific activity. The document will finish with a project I will soon start in *Babesia* sp. as a new member of the MiTick team of the UMR BIPAR.

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### 3. The Parasite and its Host: an endless arms race

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Parasite–host interactions are ubiquitous in ecological communities, and coevolution among species -a permanent confrontation between genetic variation and selective pressures- engaged in these interactions may be a potent driver of evolutionary change. Parasites rely on vertebrate or invertebrate organisms acting as hosts or vector hosts to complete their life cycle. Parasite multiplication in the host leads to the development of various systemic or localised pathological processes in certain target organs depending on the specialisation of the parasite, causing significant alterations in tissue homeostasis (Zambrano-Villa et al., 2002; Henriquez, 2008).

Despite constant species coevolution and speciation, both host and parasite genetic diversities are considered indispensable and would need to be renewed to avoid parasite's total adaptation and to resist the defence of one against the other. Virulence is another feature to be included when studying coevolution and parasitism. Even if a weakened parasite virulence has for long being interpreted as a successful parasite adaptation to its host, we know that selective pressures operating either on the host population or on the parasite population will decrease or increase parasite virulence, respectively. For instance, an increased parasite virulence could be observed due to a reduced host availability or susceptibility.

Many parasites, such as protozoans, have developed adaptive mechanisms to ensure their survival and transmission, completing their life cycle with little or no apparent clinical manifestations (Dubey and Lindsay, 1996) and maintaining a balance between multiplication and host survival. In these cases, certain mechanisms associated with the parasite, the host cell, or both are involved, and their mission is to evade the immune response and/or control the exacerbation of the immune response, by means of a parasite-host cell interaction (Zambrano-Villa et al., 2002). In both situations, the conditioning of an advantageous microenvironment is crucial for the multiplication and differentiation of the parasite, and this will allow the persistence of the infection.

Once colonized, the parasite can profit from different ways to divert host's defence. Studies have shown that their adaptive mechanisms are dependent on their location in the host (intracellular or extracellular), the stage of development and the immunological characteristics of the microenvironment, all in order to evade the immune response developed by the host (Frenkel, 2000; Zambrano-Villa et al., 2002). While some parasites are able to infect virtually any nucleated cell (Barragan and Sibley, 2003), other can transform from highly active stages to intracellular dormant forms. Several apicomplexan protozoa are also able to block the development of the host immune response by camouflaging with signalling molecules similar to those produced by the host (Aliberti and Bafica, 2005). Also, antigenic variability between parasite stages would allow the display of variants of an antigen previously recognised by the host immune system (Deitsch et al., 1997; Gupta, 2005).

Regarding the development of an immune response against the parasite, it seems possible that some parasites produce the precursors necessary to control its intracellular proliferation, preventing the occurrence of a disproportionate pro-inflammatory response leading to the destruction of host tissue (Nishikawa et al., 2001; Bannenberg et al., 2004). Intracellular parasites would also be able to efficiently establish an anti-apoptotic state both in the host cell and in adjacent uninfected cells, thus modulating the host immune response and preventing its elimination by macrophages (Lüder and Gross, 2005). Finally, protozoan parasites would be able to manipulate host's Th2-type immune response, facilitating their multiplication (Gazzinelli et al., 1996; Butcher and Denkers, 2002; Kim and Weiss, 2004).

The study of molecular host–parasite interactions is essential to understand parasitic infection and adaptation within the host system. As well, prevention and treatment of infectious diseases require a clear understanding of the molecular crosstalk between parasites and their hosts. A recent study proposed a computational analysis of protein-to-protein interactions between the host and a panel of 15 parasites that may define a global pathogenic hallmark (Figure 1). Their results show that specific lifestyle requirements of

the intracellular parasites revealed two exclusive biological processes related to exploitative mechanisms to acquire nutrients from the cytosol of host cells. Also, they obtained a reactome pathway common to intracellular parasites that belongs to the human innate immune defence by acting as pattern-recognition receptors. This approach will help further studies targeting predicted putative proteins for their analysis *in vitro* and *in vivo*.

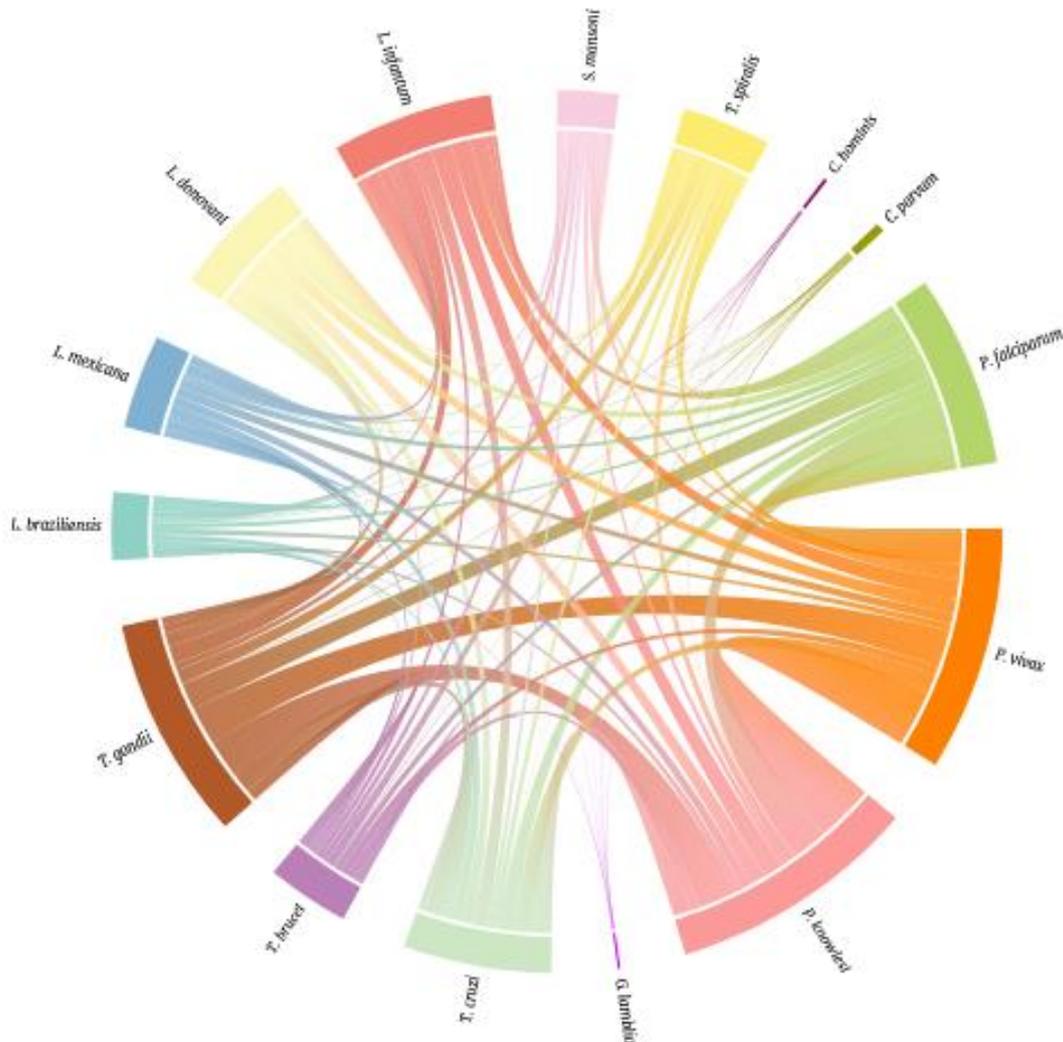


Figure 1. **Predicted interactomes for 15 parasites** (<http://orthohpi.jensenlab.org>)

The epigenetics of host–pathogen interactions is another emerging way to study how parasites have evolved sophisticated strategies to manipulate host gene transcription and protein expression. The complex host–parasite relationships lead to extensive changes in host transcriptome regulation. These changes can have drastic effects on host cell phenotypes, including stress and inflammatory responses. Parasites have developed several strategies to target different epigenetic pathways, such as those associated to the NF-κB pathway, a pivotal mediator of lymphocyte recruitment and of pro-inflammatory response activation. This interference is reached through either NF-κB regulators, with NF-κB signalling by nuclear translocation or by phosphorylation, then creating the parasite epigenator stimuli and fine-tuning of the transcriptional NF-κB programme. Other strategies are the inactivation of the P53 pathway, which regulates cell proliferation and apoptosis (Haller et al., 2010; Bougdour et al., 2013), and the phosphorylation of host cell kinases and chemokines (Cheeseman and Weitzman, 2015).

The physiological and immunological state of the host can be influenced by current infections and infection

history. In that sense, previous infections can affect host susceptibility to another parasite, as well as the impact of such infection. In natural conditions, animals are infected by a succession of different parasites throughout the course of their lives, with probably frequent concomitant infections. In one study, *Babesia microti*-infected BALB/c mice seemed immune to a subsequent *Plasmodium chabaudi* challenge; this conferred protection would rely mainly upon the function of macrophages (Efstratiou et al., 2020). A similar study also confirmed cross-immunity protection during acute *Babesia* coinfection (Zafar et al., 2022).

Finally, it is worth to remind that parasitic infections remain a major cause of morbidity and mortality worldwide; they kill millions annually and inflict debilitating injuries on additional millions. The understanding of host-parasite interactions at the cell, tissue and organism levels will help to developing effective vaccines/therapeutic targets against parasitic diseases and to identifying parasite-derived immune modulatory molecules to control hyper-inflammatory diseases.

## 4. Immunogenicity of bradyzoite-specific proteins of *N. caninum* and usefulness as vaccine candidates

*Neospora caninum* is an apicomplexan protozoan responsible for the disease called neosporosis. This parasite was first described in 1984 in Norway, following the observation by Bjerkås *et al.* of a fatal encephalopathy in dogs, characterised by encephalomyelitis and polymyositis apparently caused by an unidentified cyst-forming protozoan similar to *Toxoplasma gondii* (Bjerkås and Presthus, 1989). Subsequently, in 1988, Dubey *et al.* observed the same parasite in dogs with identical symptomatology and proposed the description of the new genus *Neospora* and the species *N. caninum* (Dubey *et al.*, 1988). In 1989, *N. caninum* was described for the first time as a causative agent of bovine abortion (Thilsted and Dubey, 1989) and subsequent studies demonstrated that this parasite is one of the main pathogens associated with this pathology worldwide (Dubey, 2003).

The life cycle of *N. caninum* involves three distinctive stages, a sporozoite (latent stage within oocysts by which intermediate hosts become infected after ingestion), the fast-replicating tachyzoite (disseminated throughout host tissues), and a slow-dividing bradyzoite (which remains latent in tissue cysts until reactivation) (Antony and Williamson, 2001; Buxton *et al.*, 2002). Since its description, the scientific community is working intensively to understand the biology and epidemiology of *N. caninum*, in an effort to develop diagnostic tools and establish control measures for neosporosis (Dubey, 1999; Moore *et al.*, 2005). One of the main features of bovine neosporosis is the risk of congenital symptomatic or asymptomatic infection, which perpetuates prevalence in the herd (figure 2). Therefore, the development of diagnostic tools to distinguish the different types of infection has become imperative.

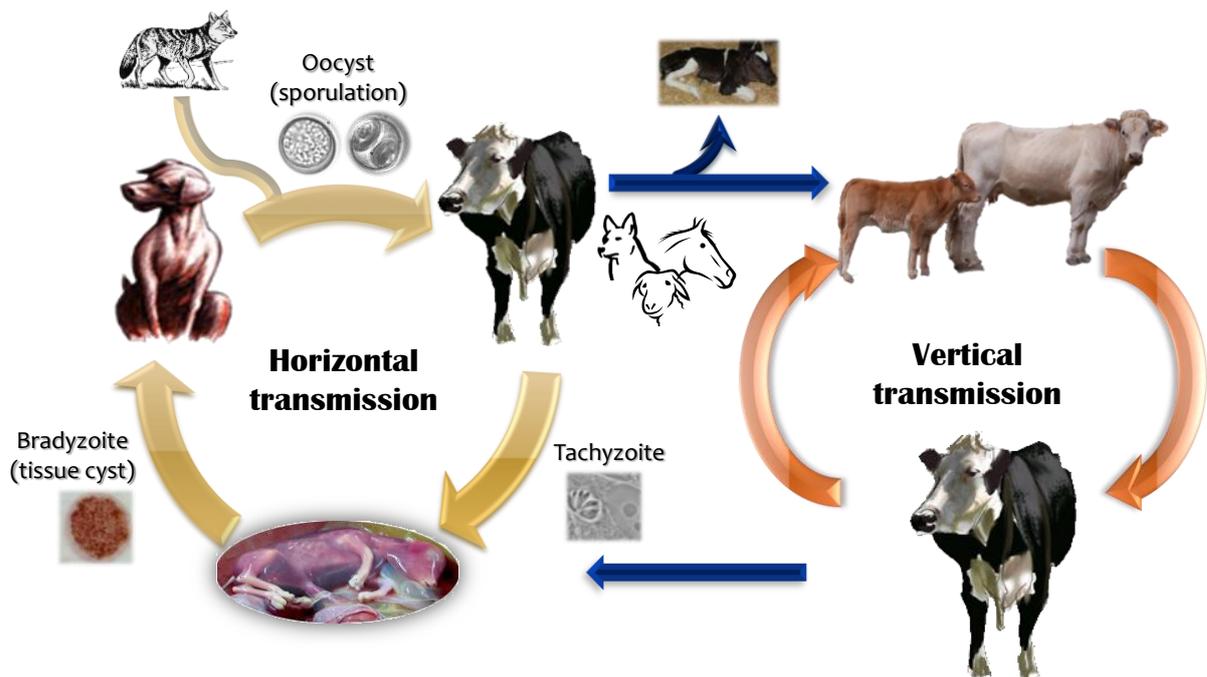


Figure 2. **Life cycle of *Neospora caninum*.** Symptomatic expression of bovine neosporosis relies on pregnancy stage during infection. Asymptomatic heifers are able to transmit the parasite to their progeny (vertical transmission) and ensure the presence of the parasite in the herd without being detected.

From 2002 to 2008, I was granted by the Spanish Ministry of Foreign Affairs and the Spanish Agency of International Cooperation (MAE-AECI). It allowed me to perform my master and PhD studies at the laboratory directed by the Professor Luis Miguel Ortega Mora. My main objective was to characterise target proteins for the design of a recombinant vaccine and to develop stage-specific diagnostic tools to control bovine neosporosis. The first step was to standardise an *in vitro* system to obtain tachyzoite or bradyzoite-enriched populations (Risco-Castillo *et al.*, 2004). We established a bradyzoite culture using the Nc-Liverpool strain in

MARC-145 epithelial-like cells, combined to the treatment with the nitric-oxide donor sodium nitroprusside (SNP). We concluded that the transformation of the parasite is a progressive, individual process achieved at distinct times within a single vacuole. We also observed that SNP treatment would stimulate bradyzoite in a dose-dependant manner.

Parasite conversion might be due to actions on the parasite and/or host cell mitochondrial electron transport in order to switch on appropriate signaling pathways and enhancing bradyzoite conversion (Figure 3 and 4). In *T. gondii*, nitric oxide exerts its effect on enzymes of the respiratory chain containing iron-sulfur centers, mimicking IFN- $\gamma$ -driven immune pressure over cystogenesis (Sokol-Borrelli et al., 2020). Our results also suggest the role of early innate pro-inflammatory response in the conversion and encystation of *Neospora* bradyzoites, but substantial work to investigating any difference in gene expression during the tachyzoite to bradyzoite conversion process is still needed, in order to reliable produce bradyzoites *in vitro* and *in vivo*.

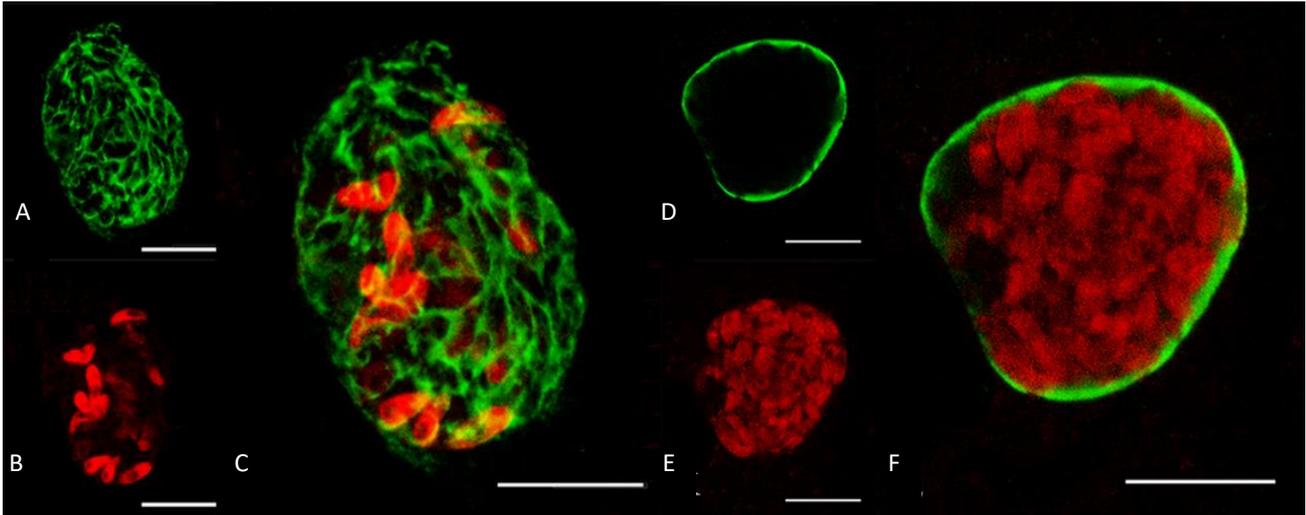


Figure 3. **Double-label immunofluorescence in Nc-Liv-infected MARC-145 cells stressed with 70  $\mu$ M SNP and fixed at day 7 after stress.** Vacuoles expressing maturing cyst-wall protein CC2 are green (A,C) and bradyzoite-specific BAG1-positive parasites are red (B,C). Depending on the maturation stage, some vacuoles react with CC2 on the lumen, with recognition of some BAG1-positive parasites (C), whereas vacuoles reacting with CC2 at their periphery express BAG1 on all zoites (F). Bar scale = 10  $\mu$ M

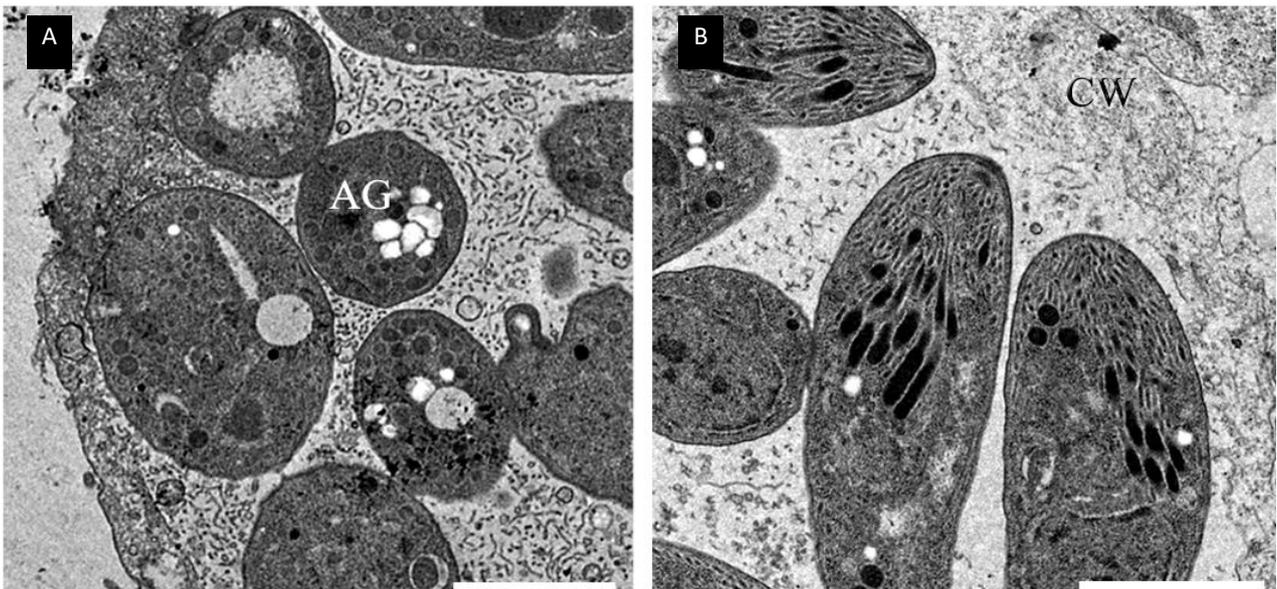


Figure 4. **Transmitted-electron microscopy (TEM) in Nc-Liv-infected MARC-145 cells.** In (A), After 5 days of SNP treatment, large clumps of electron-dense material accumulate in the matrix, whereas some parasites begin to show electron-lucent vacuoles interpreted as amylopectin granules (AG), characteristic of bradyzoites. In (B), a developing tissue cyst is shown. Note electron-dense granular and filamentous material accumulated peripherally, forming a thick

layer bounded to the parasitophorous membrane, which indicates cyst wall formation (CW). Bar scale = 2  $\mu$ M (G,H).

*Neospora caninum* is closely related to *Toxoplasma gondii*, a protozoan responsible of abortion and congenital transmission in ewes and humans. After observing similar morphological and developmental features between both apicomplexan parasites, I confirmed the cross-reactivity of a bradyzoite-specific monoclonal antibody originally produced with a *T. gondii* antigen. This arouse the hypothesis that *Neospora* transformation into bradyzoite triggers the expression of ortholog genes. I used bradyzoite-specific TgSAG4 (surface antigen 4) gene and TgBSR4 (bradyzoite surface-related 4) locus, which code for surface antigens, in order to identify the *N. caninum* orthologs (Fernández-García et al., 2006; Risco-Castillo et al., 2007). Using degenerate primers and sequencing, we identified the genes coding for the surface antigens NcSAG4 and NcBSR4, and we produced their recombinant proteins by using the prokaryotic expression vector system pRSET. We also determined the time of expression *in vitro*. We observed that NcSAG4 is early expressed during the transformation into bradyzoite, while NcBSR4 was expressed later on (Figure 5).

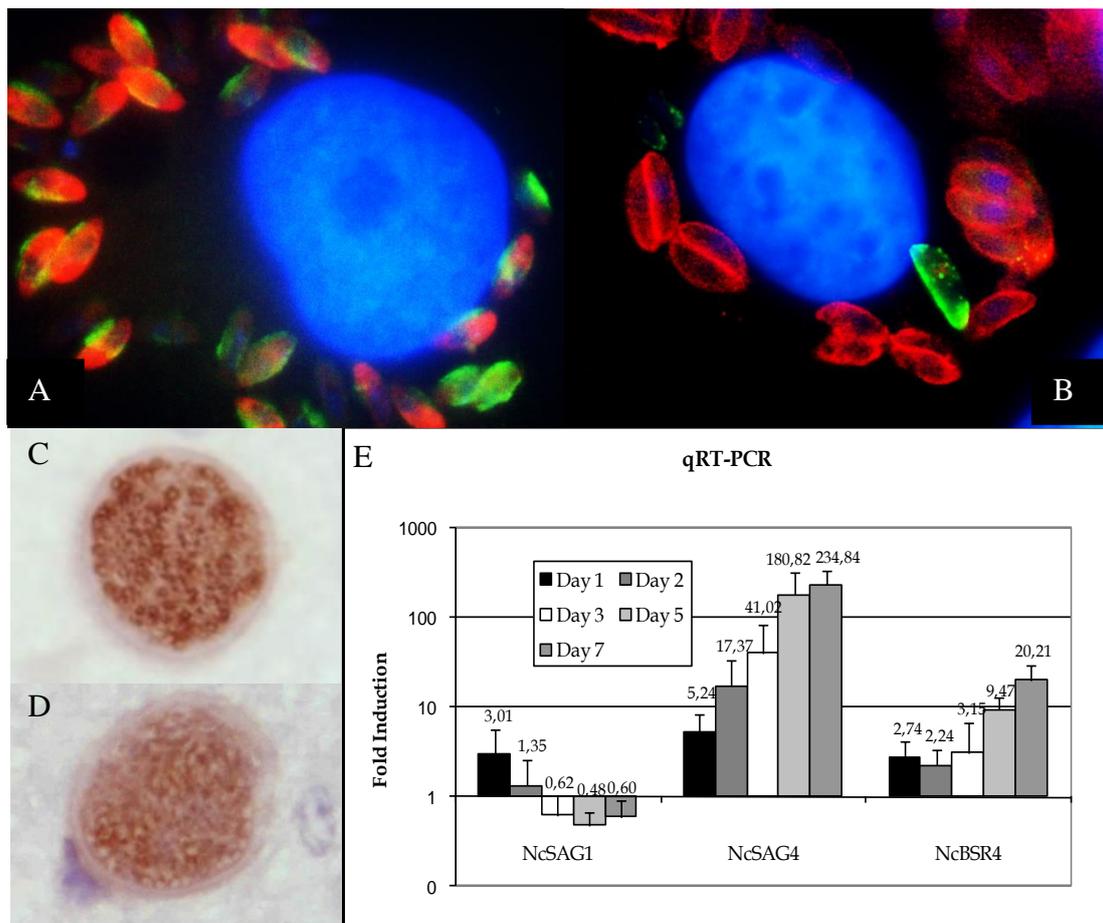


Figure 5. **Expression of NcSAG4 and NcBSR4.** (A,B) Double immunofluorescence staining of *Neospora caninum* bradyzoites at day 7 after stress revealing bradyzoite-specific NcSAG4 and NcBSR4 expression. Organisms labelled against BAG1 (A) or SAG1 (B) are red while those labelled in green are expressing either SAG4 (A) or BSR4 (B). In (A), both proteins are co-localising the parasite surface, but note in (B) that parasites expressing BSR4 do not express SAG1. (C-D) brain-cyst from a congenitally infected calf labelled by immunohistochemistry with rabbit polyclonal antibodies against NcSAG4 and NcBSR4. (E) Transcription rates were determined by real-time RT-PCR and calculated as fold induction when compared with control tachyzoites.

Thanks to a collaboration with the Institute of Parasitology (University of Bern), I had the opportunity to improve my skills on cell culture, immunolabeling techniques and electron microscopy (scanning and transmission EM), in the framework of a short-term internship (one month) granted by the European Union (COST Action 854). The results obtained during this internship completed the experiments needed to confirm the localization of NcSAG4 and NcBSR4 adhesins in the cell membrane of either early- or late-formed bradyzoites (figure 6).

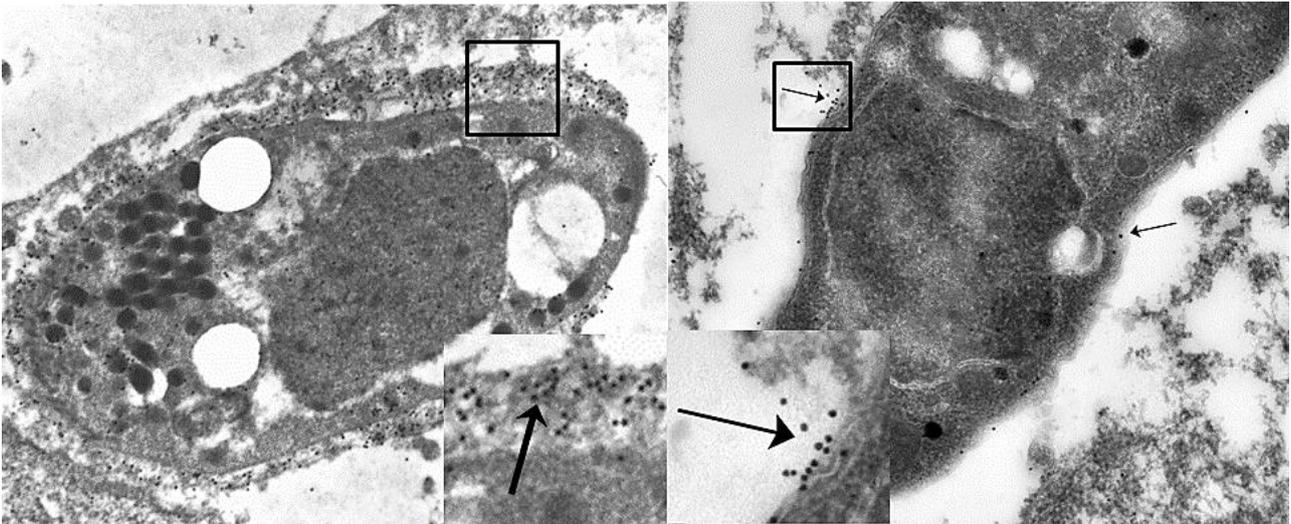


Figure 6. **Immunogold TEM staining of 7-day cultured *N. caninum* bradyzoites.** Anti-NcSAG4 (A) and anti-NcBSR4 (B) mouse and rabbit polyclonal antibodies, respectively. Note the localization of gold particles on the surface membrane of the parasite.

We confirmed the immunogenicity of recombinant NcSAG4 and NcBSR4 by using pre-colostrual sera from infected calves. The potential usefulness of these two SAG-related sequences (SRS) adhesins, for diagnosis and prevention of neosporosis was registered with patents P200302869; PCT/32004/000529 and P200601262. The usefulness of these proteins was confirmed with the design of a serological avidity test using a large sera panel from experimentally infected cows belonging to different groups (primo-infection, recrudescence and chronic infection). The detection of NcSAG4 six weeks after infection, in congenitally infected calves and in experimentally infected mice confirmed that this antigen can be exploited as a good marker of chronic infection (Aguado-Martínez et al., 2009a, 2009b).

Despite the high immunogenicity of NcSAG4, its use as a recombinant vaccine to fight against transplacental transmission of *Neospora caninum* seemed not to stimulate Th1 immune response, nor immunoglobulin G production, but it was able to reduce clinical neosporosis even if transplacental transmission and perinatal mortality remained high (Aguado-Martínez et al., 2009a). During my PhD, I was able to participate to many other studies regarding *N. caninum* infection model and stage-specific proteins were published (von Blumröder et al., 2004; López-Pérez et al., 2006; Regidor-Cerrillo et al., 2008; Marugán-Hernández et al., 2010). These collaborations allowed me to develop multiple skills in a broad range of laboratory techniques, from molecular biology to immunolabeling, including cell culture and experimental *in vivo* models.

The fact that the only two bradyzoite-specific SRS adhesins described for *Neospora caninum* were those characterised by our research team, made me realize the urgent need to further explore the mechanisms involved in parasite transformation and immune response evasion, by first identifying surface proteins associated to early interaction with the host cell during bradyzoite conversion. The cloning and further characterization of stage-regulated genes is critical for uncovering crucial events in *N. caninum* pathogenesis. *Neospora* participates actively during the adhesion to and invasion of its target cell. The mechanisms employed by the parasite would be partially mediated by the expression of surface molecules displayed sequentially and in a stage-specific fashion. However, it is unclear whether SRS proteins expressed during the bradyzoite stage are involved in the mechanisms of host-parasite interaction and, therefore, in the evasion of the host immune system, as hypothesized for *T. gondii*.

In that sense, I applied the genome walking method using the NcBSR4 gene as a framework to elucidate the presence of other bradyzoite up-regulated genes flanking NcBSR4 (Risco-Castillo et al., 2011). Our results introduced preliminary data regarding the *N. caninum* SRS9 locus, which displays significant homology to the *T. gondii* SRS9 locus (Jung et al., 2004). Our efforts led us to locate 3 genes (putative Ubiquinol-cytochrome C reductase, NcBSR4 and NcSRS9) and 1 putative pseudogene (SRS5) on the same locus based on those

observed through *Neospora* genome annotation made by the Wellcome Trust Sanger Institute (data available on <http://www.sanger.ac.uk/sequencing/Neospora/caninum/>) (figure 7).

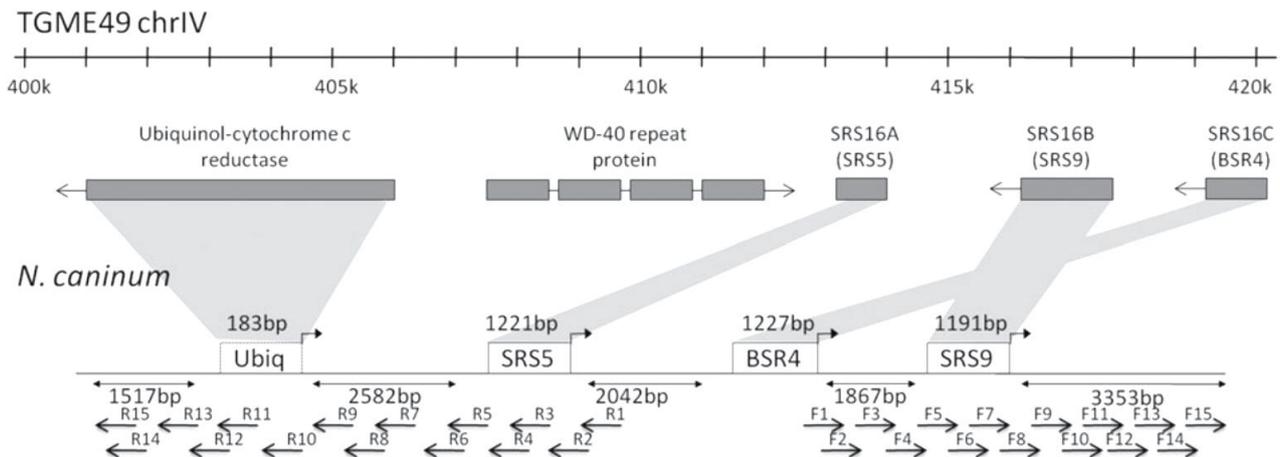


Figure 7. **Synteny representation between *Neospora caninum* and *Toxoplasma gondii*.** Putative genes are depicted in grey (*T. gondii*) or white (*N. caninum*) boxes. Double arrowed lines indicate the size (bp) of intergenic regions, and the orientation of the *N. caninum* genes is given by arrows in the upper right side of the boxes. Single arrowed lines with numbers indicate primer location during genome walking, and either forward (F1-F15) or reverse (R1-R15) was used with a complementary adapter, according to manufacturer's recommendations. The distribution of *T. gondii* genes was generated with ToxoDB v.6.0 (<http://toxodb.org/toxo/>).

The Ubiquinol-cytochrome C reductase is an enzyme and a component of the cytochrome bc complex associated with the mitochondrial system of electron transport and ATP synthesis. This enzyme had been previously identified in *T. gondii* (Morales-Sainz et al., 2008), and orthologues of other components of this complex in *N. caninum* have been also described (Kang et al. 2008). Several major anti-parasitic drugs, such as atovaquone and endochin-like quinolones, act through inhibition of the mitochondrial electron transport chain at the coenzyme Q-cytochrome c oxidoreductase complex (Complex III). The apicomplexan-specific Complex III subunit in *T. gondii* is important for parasite proliferation and mitochondrial oxygen consumption. In *N. caninum*, this enzyme could be also related to this metabolic pathways, and our findings could lead to further studies in order to characterise the Complex III as a target for the potential development of treatments to prevent drug resistance (McFadden et al., 2000)

At the same locus, we identified the NcSRS9 (surface-related sequence 9) gene, orthologous to *T. gondii* TgSRS9, and we further characterised it. The identification of a putative N-terminal signal peptide and a GPI anchor at the C-terminus in the protein encoded by NcSRS9 correlated to that expected for a protein tethered to the cytoplasmic membrane and exposed to the surface of the parasite (Knoll and Boothroyd, 1998; Jung et al., 2004). This feature is similar to the previously described NcSAG4 and NcBSR4 bradyzoite-specific surface proteins (Fernández-García et al., 2006; Risco-Castillo et al., 2007).

The presence of 12 cysteine residues in the NcSRS9 antigen has also been observed in other SRS antigens of *T. gondii* and *N. caninum* (Howe et al. 1998; Risco-Castillo et al. 2007). Their conservation may indicate that they are involved in the intramolecular disulfide bonding of SRS antigens and therefore suggests that they would show a similar folding pattern (Howe and Sibley, 1999; Risco-Castillo et al., 2007). This result further suggests that they may participate in the adhesion of the parasite to some host cell receptors and therefore in an interaction with the host immune response (Henriquez, 2008).

The moderate to low immunogenicity against infected cattle of rNcSRS9 could be explained by a possible late bradyzoite upregulated expression during chronic infection reducing antigenic exposure to the host immune response. However, Western blot and immunohistochemical assays on bradyzoites produced *in vitro* confirmed NcSRS9 membrane-associated expression in bradyzoites from mature tissue cysts.

The transcription factors described in apicomplexan parasites might specifically regulate the expression of developmental stage-specific target genes and shape gene expression in an early or late fashion (Yang and Parmley, 1997; Balaji et al., 2005). Even if the bradyzoite-specific expression of SRS antigens would avoid the induction of a potentially protective immune response, it is important to recall that SRS antigens expressed in bradyzoites are the first to be exposed to the intestinal epithelium of the definitive host during oral infection. In *T. gondii*, it has been observed that a lack of SRS9 expression does not affect parasite infectivity in mice but does compromise its ability to persist in the brain (Kim and Boothroyd, 2005). Furthermore, TgSRS9 seems to play an important role in reactivation of *T. gondii* in intestinal tissues from chronically infected mice (Kim et al., 2007). In that sense, further investigation into an immunological role of NcSRS9 other than the reinforcement of immune response evasion is required to unveil its role in the mechanisms by which *Neospora* is able to evade the host immune response and undergo persistent infection.

During my PhD, further projects included the possibility of studying other Apicomplexan parasites that cause emerging diseases in cattle, such as *Besnoitia besnoiti* (Fernández-García et al., 2009b, 2009a, 2013). I also performed epidemiological studies in neosporosis and toxoplasmosis in South American camelids (Serrano-Martínez et al., 2007; Risco-Castillo et al., 2014b), but I do not intend to delve in this subject since it did not take part of my main research objectives.

Finally, my involvement in *Neospora caninum* bradyzoite characterisation included the development and registration of patents for the use of the genes NcSAG4 (EP1710309B1), NcBSR4 (P200601262) and NcSRS9 (P201001593) for the diagnosis and prevention of neosporosis, and as markers for analysis of the pathogenesis. We also obtained the Accesit Madri+d 2007 prize for the best patent (NcSAG4).

## 5. *Plasmodium* sporozoite proteins: role on cell traversal parasitophorous vacuole formation in hepatocytes

At the end of my PhD studies, my interest in the mechanisms that allow the internalization of parasites into the host cell and its manipulation led me to obtain a postdoctoral contract "Poste Vert" from the French National Institute for Health and Medical Research (INSERM). In January 2012, I joined the team of Dr. Dominique Mazier at the research unit 945 (INSERM, UPMC), located at the Medicine Faculty facilities joining the Pitié-Salpêtrière Hospital. At that time, she was also responsible of the Parasitology and Mycology Hospital department, and she was the scientific reference for the study of the hepatic phase of malaria in France.

Always interested in host cell and parasite interactions, I addressed my work towards the study of the molecular mechanisms involved in the liver phase of malaria. More precisely, I developed a project on the study of host-parasite relationships during sporozoite invasion in the liver cell (MaLI project – Agence Nationale de la Recherche, ANR). For this purpose, I used reverse genetic approaches in *P. yoelii* to study the role of several parasite genes, and I carried out transgenic labelling of proteins of rhoptries, secretory vesicles involved in invasion.

Malaria is one of the oldest documented human diseases, yet it is one of the most prevalent human infectious diseases even today. According to the latest estimates from WHO, there were 241 million new malaria cases and an estimated 627 000 deaths due to malaria in 2021 (World Health Organization, 2021). Malaria is caused by protozoan parasites of the genus *Plasmodium*. *Plasmodium falciparum* is the most virulent form infecting human beings (Sultan, 1999). *Plasmodium* has a complex life cycle alternating between a mosquito vector and a vertebrate host. (Figure 8).

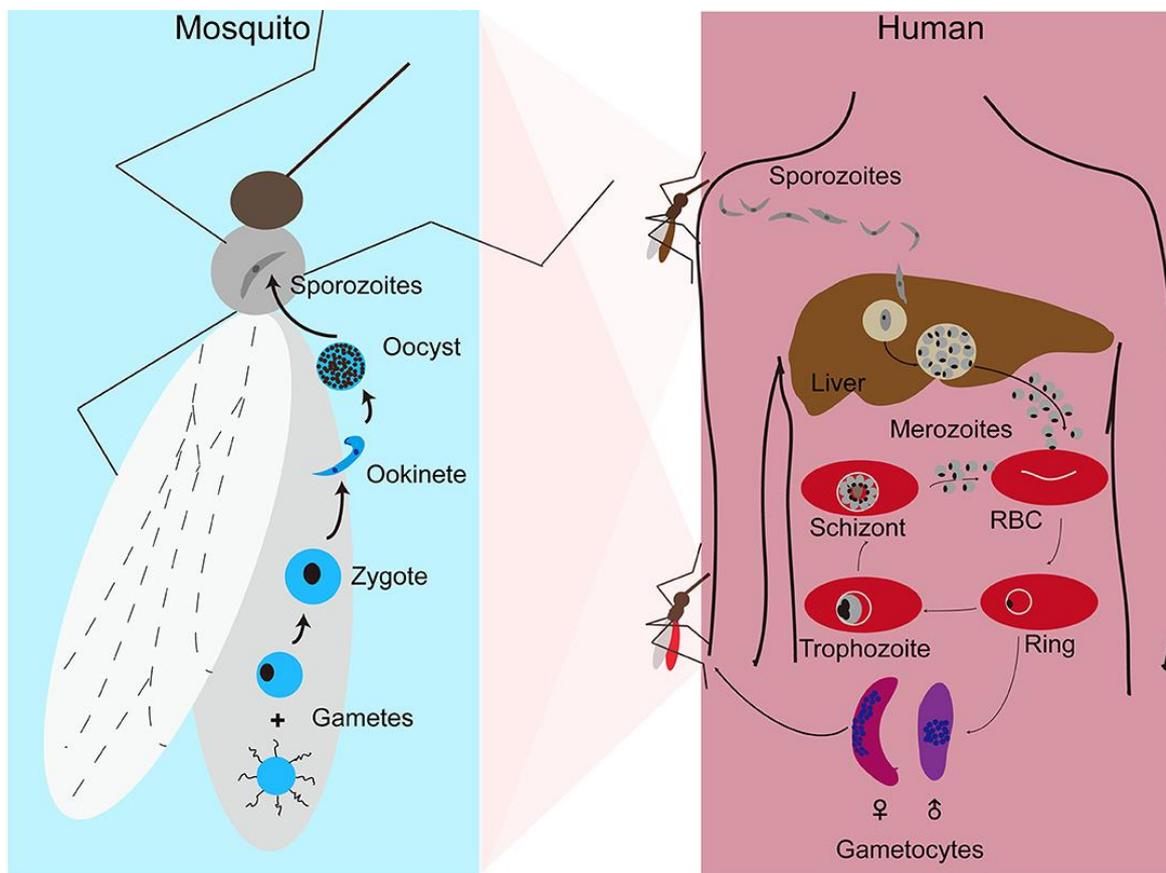


Figure 8. **Life cycle of malaria parasite.** Transmission of the parasite occurs through a bite by a female *Anopheles* mosquito, which injects the parasite into the skin of the host. The parasite grows and multiplies first in the liver cells and

then in the red blood cells until another mosquito comes for a blood meal and ingest the parasite, completing its cycle (adapted from Su et al., 2020).

Right after mosquito biting, the motile sporozoites rapidly travel to the liver and invade hepatocytes, where they differentiate into exoerythrocytic forms and pathogenic merozoites inside a membrane-bound compartment, the parasitophorous vacuole. Sporozoite progression through the host tissues following transmission by the mosquito relies on active gliding motility and the capacity of the parasite to migrate through cells (Ménard et al., 2013). During cell traversal, sporozoites breach the host cell membrane and glide through the traversed cell cytoplasm (Mota and Rodriguez, 2001). Among the parasite factors involved in cell traversal, pore-forming proteins such as Perforin-Like Protein 1 (PLP1, also called SPECT2) would mediate perforation of the host cell plasma membrane to facilitate parasite entry into the traversed cell (Ishino et al., 2005), but the mechanisms of this host cell membrane rupturing have not been elucidated.

In order to investigate the molecular mechanisms of cell traversal and productive invasion during sporozoite infection, we produced Plp1-knockout sporozoites using the Gene Out Marker Out (GOMO) strategy developed in our laboratory (Manzoni et al., 2014). Our goal was to integrate recent advances in experimental genetics protocols to allow concomitant replacement of a gene of interest by a fluorescent cassette, and removal of the drug-selectable marker, thus facilitating both downstream phenotypic analysis and further genetic modifications. We used the HSP70 promoter to obtain parasites expressing GFP constitutively at all stages of parasite development, and the hDHFR-yFCU fusion gene, for positive-negative selection, coupled to a second fluorescent cassette, encoding the red fluorescent protein mCherry. With this strategy, parasites that have excised the drug-selectable marker become GFP<sup>+</sup> mCherry<sup>-</sup>, and can be easily distinguished from GFP<sup>+</sup> mCherry<sup>+</sup> parasites still harbouring the hDHFR-yFCU marker. We then targeted the dispensable *p230* gene as control, and the *plp1* gene (figure 9).

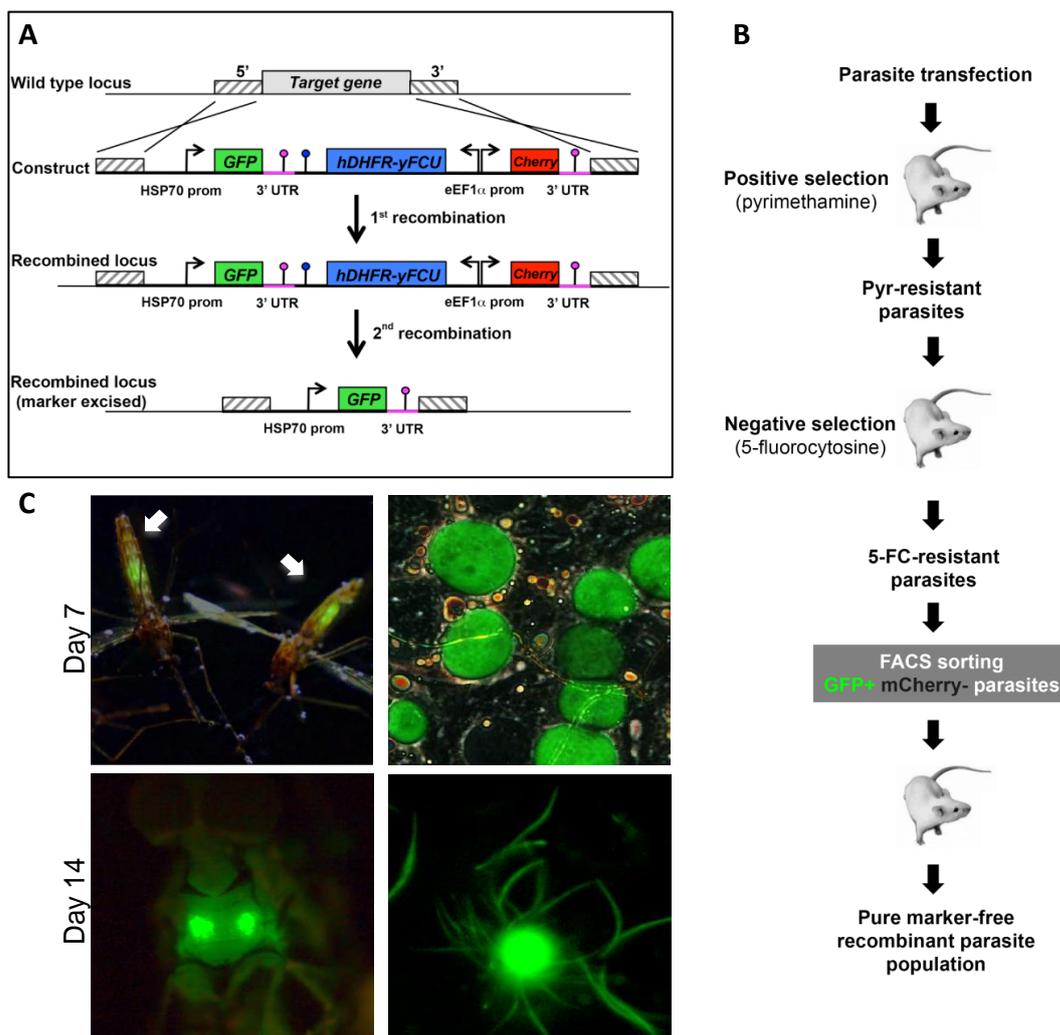


Figure 9. **Production of  $\Delta plp1$ -gfp *Plasmodium yoelii* parasites.** (A) *P. yoelii* 17XNL and *P. berghei* ANKA WT parasites were transfected with Pyplp1 targeting constructs, using standard transfection methods. (B) GFP-expressing parasite mutants were isolated by flow cytometry from infected mouse red blood cells. Correct construct integration was confirmed by analytical PCR using specific primer combinations. (C) Recovery of  $\Delta plp1$ -gfp sporozoites from *Anopheles stephensi* mosquitoes was performed 14 days after blood meal with mouse blood containing parasite mutants.

Tetraspanin CD81, a putative receptor for hepatitis C virus, is required on hepatocytes for human *Plasmodium falciparum* and rodent *Plasmodium yoelii* sporozoite infectivity (Silvie et al., 2003). We exploited this feature in a *P. yoelii* model to compare active invasion of HepG2/CD81 cells to traversal of HepG2 cells. We confirmed that cell traversal precedes productive invasion, and that *Plasmodium* sporozoites actively invade cells inside transient vacuoles during cell traversal, which are distinct from parasitophorus vacuoles. Without PLP1, parasites failed to egress from transient vacuoles and they were eliminated after fusion with the host cell lysosomes (figure 10).

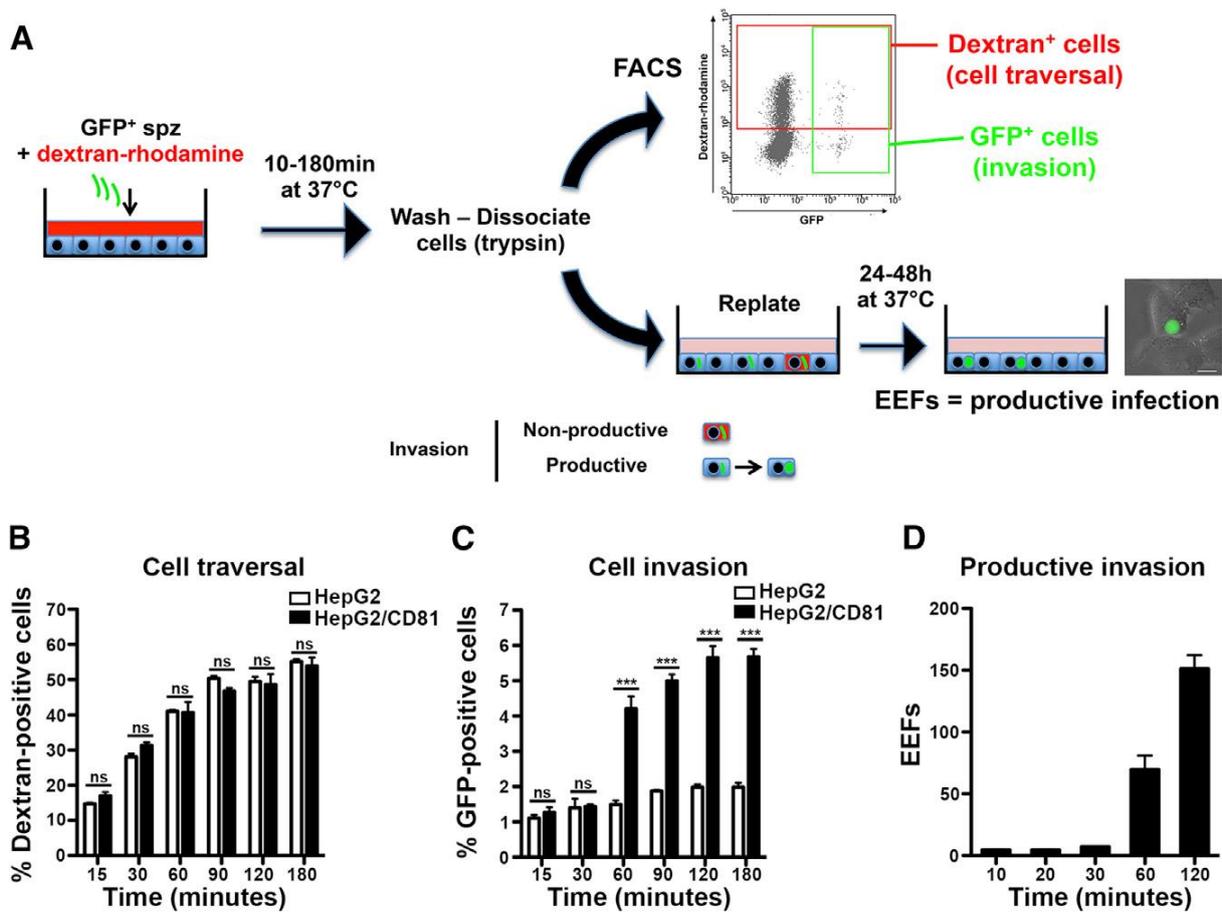
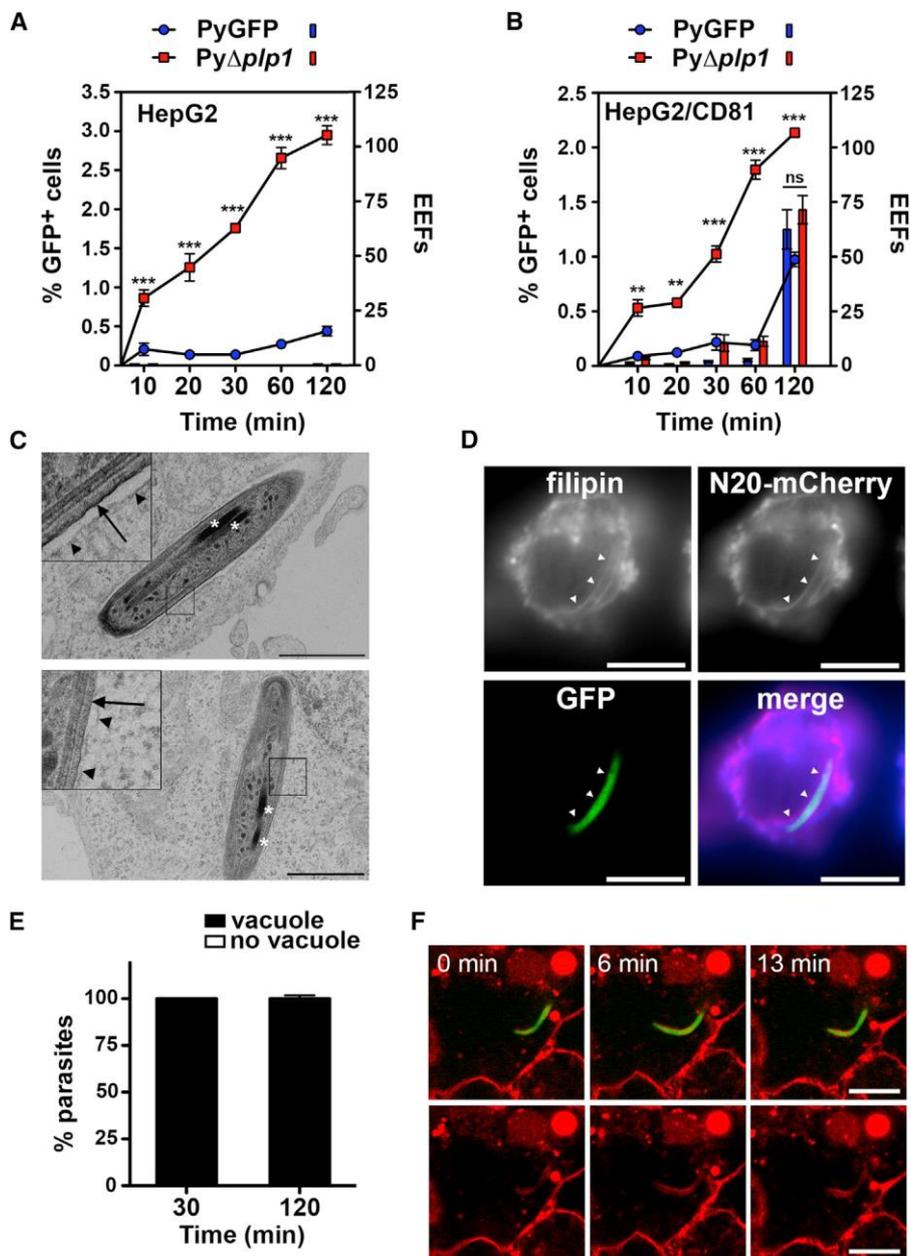


Figure 10. **Kinetics of *P. yoelii* Cell Traversal and Cell Invasion.** (A) Invasion/infection assay. Cell cultures were incubated for 10–180 min with GFP-expressing sporozoites in the presence of rhodamine-labeled dextran, trypsinized, and either directly analysed by FACS, to determine the percentage of traversed (dextran-positive) and invaded (GFP-positive) cells, or replated and further incubated for 24–48 hr, to determine the number of EEF-infected cells by fluorescence microscopy (productive infection). (B and C) HepG2 and HepG2/CD81 cells ( $5 \times 10^4$ ) were incubated at 37° C with PyGFP sporozoites ( $3 \times 10^4$ ) in the presence of rhodamine-conjugated dextran, and analyzed by FACS to determine the percentage of traversed (dextran-positive) cells (B) and invaded (GFP-positive) cells (C). Results are expressed as the mean percentage ( $\pm$ SD) of triplicate wells. Statistical significance was assessed test. \*\*\* $p < 0.001$ . (D) HepG2/CD81 cell cultures were incubated with PyGFP sporozoites for 10–120 min, dissociated and replated, and cultured for an additional 24 hr to determine the number of EEFs by fluorescence microscopy.

Our data revealed that *Plasmodium* sporozoites can actively invade cells inside two different vacuoles, and either use PLP1 and pH sensing to egress from transient non replicative vacuoles (figure 11), or remodel the PV membrane to escape degradation by the host cell lysosomal machinery (Risco-Castillo et al., 2015). Transient vacuoles feature conserved host cell membrane components (e.g. basigin, F-actin) but without PLP1

vacuole and parasites were eliminated. We hypothesized that the host cell lysosomal machinery may be responsible for the elimination of PyDplp1 nonreplicative vacuoles. To test this hypothesis, we used the acidic organelle probe LysoTracker red and antibodies against the lysosomal associated membrane protein 1 (LAMP1), and we confirmed that most of intracellular PyDplp1 parasites in HepG2 cells were labeled by LysoTracker red in HepG2 cells, whereas in HepG2/CD81 both LysoTracker-positive and LysoTracker-negative parasites could be found.



**Figure 11. *PyΔplp1* sporozoites accumulate inside nonreplicative vacuoles.** (A and B) HepG2 (A) or HepG2/CD81 (B) cells ( $5 \times 10^4$ ) were incubated with PyGFP or PyDplp1 sporozoites ( $3 \times 10^4$ ) for 10–120 min, trypsinized, and either directly analyzed by FACS to quantify invaded (GFP-positive) cells (lines) or replated and cultured for an additional 24 hr before quantification of EEFs by fluorescence microscopy (bars). Shown are the mean values ( $\pm$ SD) of triplicate wells. Statistical significance was assessed using two-way ANOVA followed by Bonferroni test. \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; ns, non significant. (C) Electron micrographs of PyDplp1 sporozoites in HepG2 cell. The insets show at higher magnification the parasite plasma membrane (arrow) and the vacuole membrane (arrowheads). The parasite rhoptries are indicated with asterisks. Scale bars, 1  $\mu$ m. (D) HepG2/N20-mCherry cells (red) were incubated with PyDplp1 sporozoites (green) for 30 min, fixed, and labeled with filipin (blue). Scale bars, 10  $\mu$ m. (E) HepG2/N20-mCherry cells were incubated with PyDplp1 sporozoites for 30 or 120 min before fixation and labeling with filipin, and the presence or absence of a vacuole was determined by fluorescence microscopy. (F) Time-lapse confocal microscopy of a PyDplp1 sporozoite (green) in a HepG2/N20-mCherry cell (cell membrane labeled in red).

In *T. gondii*, low pH promotes membrane binding and cytolytic activity of PLP1 (Roiko et al., 2014). We hypothesized that *Plasmodium* PLP1 activity might also be regulated by the pH, and that acidification of the vacuole upon fusion with lysosomes would activate PLP1 and parasite egress from transient vacuoles. To test this hypothesis, we incubated PyGFP sporozoites with host cells pretreated with bafilomycin A1, a selective inhibitor of vacuolar type H<sup>+</sup>-ATPases that blocks lysosomal acidification (Yoshimori et al., 1991). Remarkably, pretreatment of cells with bafilomycin A1 suppressed sporozoite cell traversal, and we observed an increase in the number of PyGFP-invaded cells.

In summary, malaria sporozoites can invade cells transiently, either during cell traversal, or by establishing a resident parasitophorous vacuole, where they further develop into exoerythrocytic forms. Transmigrating sporozoites do not necessarily breach the host cell membrane at the time of invasion, but enter cells inside transient vacuoles, from which they subsequently egress using PLP1 and pH sensing. Apicomplexan zoites productively invade host cells through a moving junction, a structure composed in part by rhoptry proteins secreted by the parasite (Besteiro et al., 2011). The moving junction anchors the invading parasite to the host cell and serves as a molecular sieve that selectively excludes host proteins from the membrane of the nascent vacuole, resulting in protection from the host cell lysosomes (Mordue et al., 1999).

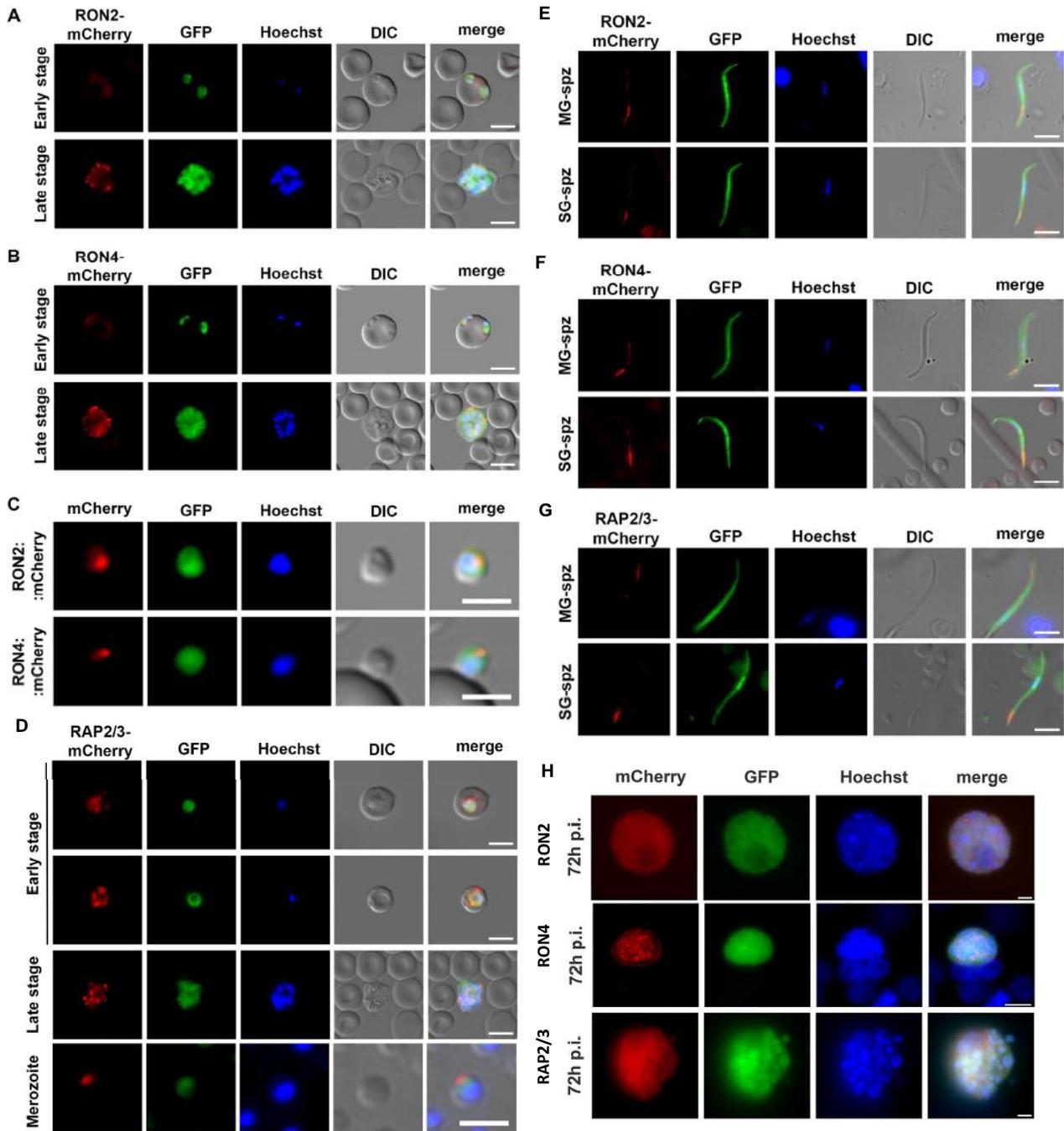
CD81 acts at an early stage of infection, possibly at the entry step, but the mechanisms involved are still unknown. We confirmed that *Plasmodium* sporozoites migrate through cells before establishing a replicative parasitophorous vacuole inside hepatocytes. They also actively enter transient nonreplicative vacuoles during cell traversal, and use the perforin-like protein PLP1 to egress and escape degradation by the host cell lysosomes. Thus, we decided to investigate the role of CD81 during sporozoite entry, we generated transgenic *P. yoelii* parasites expressing fluorescent versions of three known rhoptry proteins, RON2, RON4 and RAP2/3 (Risco-Castillo et al., 2014a).

*Toxoplasma* yields a complex of RON proteins that is exported to the host plasma membrane during cell invasion. *Plasmodium* sporozoites also express AMA-1, RON2 and RON4 proteins (Le Roch et al., 2003; Silvie et al., 2004; Srinivasan et al., 2004; Tufet-Bayona et al., 2009; Giovannini et al., 2011). RON2 can be inserted into the host cell membrane and can interact *in trans* with the protein AMA-1, which is secreted from the micronemes onto the parasite surface (Besteiro et al., 2009; Tonkin et al., 2011; Tyler et al., 2011). Direct interaction of RON2 with AMA1 is conserved in *Plasmodium* and has been proposed to build the moving junction during merozoite invasion (Narum et al., 2008; Cao et al., 2009; Richards et al., 2010; Srinivasan et al., 2011; Hossain et al., 2012; Vulliez-Le Normand et al., 2012).

A recent conditional mutagenesis approach in *P. berghei* has shown that RON4 plays a critical role during hepatocyte infection (Giovannini et al., 2011). Another protein, the Rhoptry Associated Protein 2/3 (RAP2/3), is present in the rhoptries of *P. berghei* merozoites and sporozoites (Tufet-Bayona et al., 2009). RAP2/3 is part of a low-molecular-weight protein complex made of three rhoptry bulb proteins in *P. falciparum*, RAP1, RAP2 and RAP3 (Baldi et al., 2000, 2002). Whilst *P. falciparum* possesses two proteins, RAP2 and RAP3, encoded by adjacent genes organised in a head-to-tail fashion, rodent malaria parasites have only one single copy syntenic *rap2/3* gene. Like RON2 and RON4 (Tufet-Bayona et al., 2009; Giovannini et al., 2011), RAP2/3 is refractory to gene deletion by standard approaches in *P. berghei*, suggesting that it fulfils essential yet unknown functions during asexual blood stage development.

Our strategy was to produce *P. yoelii* parasites expressing the mcherry-tagged rhoptry neck proteins RON2, RON4 and the rhoptry protein RAP2/3 constitutively, by using single crossover recombination at the endogenous genes (figure 12). During the execution of this part of the MaLI project, I was in charge of the supervision of one master degree student for the first time. Together, we confirmed proteins expression at all parasite stages (sporozoite, merozoite, schizont), and analysed rhoptry discharge in hepatic forms. We observed that RON2 and RON4 are lost following rhoptry discharge during merozoite and sporozoite entry, whereas RAP2/3 is secreted into the parasitophorous vacuole during infection. We further show that sporozoite rhoptry discharge occurs only in the presence of CD81, providing the first direct evidence of a role of CD81 during

sporozoite productive invasion. After hepatocyte infection, we observed that rhoptry proteins stock was restored late at schizont formation, with a punctuate distribution, consistent with a localization inside the rhoptries of hepatic merozoites (figure 13).



**Figure 12. Expression of RON2-mCherry, RON4-mCherry and RAP2/3-mCherry.** Blood from mice infected with PyGFP expressing RON2::mCherry (A-C), RON4::mCherry (B-C) or RAP2/3::mCherry (D) was collected, fixed and labelled with the nuclear stain Hoechst 33342. RON2- and RON4-mCherry were detected in late schizonts only, where they both display a punctuate pattern typical of rhoptry localisation. RAP2/3::mCherry localised to the PV of early blood stages and to the rhoptries of late schizonts, and shows a typical apical localization. Scale bars, 5  $\mu$ m. In extracellular merozoites (lower panels). Scale bar, 5  $\mu$ m. (C) RON2::mCherry and RON4::mCherry show a typical apical localization in extracellular merozoites. Scale bar, 2.5  $\mu$ m. *P. yoelii* sporozoites expressing RON2::mCherry (E), RON4::mCherry (F) and RAP2/3::mCherry (G), recovered from *A. stephensi* mosquitoes. Transgenic sporozoites were collected from infected mosquito midguts (MG-spz) or salivary gland (SG-spz), 10 or 14 days after mosquito infection, respectively. Parasites were labelled with the nuclear stain Hoechst 33342. Scale bars, 5  $\mu$ m. Sporozoites expressing RON2-, RON4- and RAP2/3-mCherry in the apical complex were detected in both midgut and salivary gland. (H) RON2-, RON4- and RAP2/3-mCherry fusion proteins were not detected in early liver stages (24h) but displayed a punctate pattern in late liver stages (72 h), suggesting localisation in the rhoptries of hepatic merozoites. Scale bars, 10  $\mu$ m.

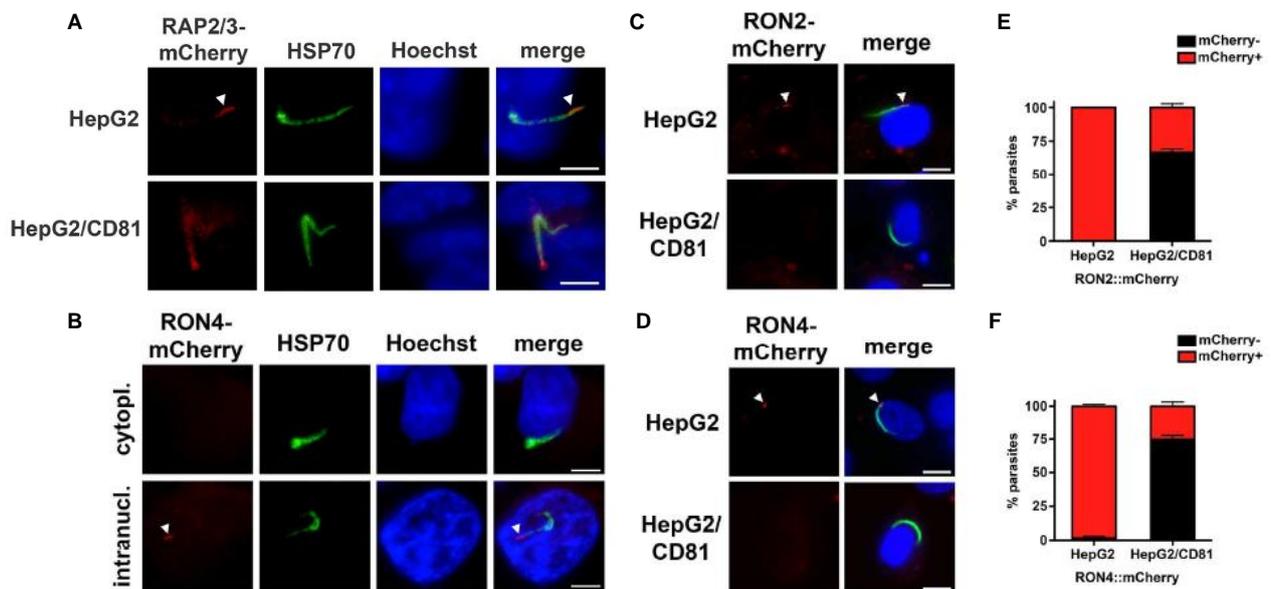


Figure 13. **Secretion of RAP2/3, RON2 and RON4 in sporozoites.** *P. yoelii* sporozoites were incubated for 1 h with HepG2 (upper panels) or HepG2/CD81 cells (lower panels). The upper panels show a sporozoite inside an infected HepG2 cell, where either RAP2/3-mCherry (A), RON2-mcherry (C) or RON4-mcherry (D) remain confined to the parasite rhoptries (arrowhead). In infected HepG2/CD81 cells, RAP2/3 has been secreted into the PV surrounding the invaded sporozoite, while RON2 and RON4 disappear. Note that an intranuclear sporozoite keeps its apical RON4 granules (B). After 2-hour incubation, the proportion of RON2- and RON4-mcherry depleted sporozoites in HepG2/CD81 cells was of 70% and 75%, respectively while RON2 and RON4 stayed visible in all intracellular sporozoites from HepG2 infected cells. Scale bars, 5  $\mu$ m.

The success of malaria transmission relies on the ability of *Plasmodium* sporozoites to actively invade hepatocytes and develop inside a vacuole to produce merozoites. Our results showed that all three proteins are expressed in erythrocytic and hepatic merozoites as well as in sporozoites, with a different fate of RONs and RAP2/3 proteins during erythrocyte and hepatocyte infection, consistent with the proposed role of compartmentalisation of rhoptry proteins. It's suggested that rhoptry neck proteins are involved at an early step during moving junction formation, while bulb rhoptry proteins are secreted later into the nascent parasitophorous vacuole and may have important functions downstream of parasite entry (Zuccala et al., 2012; Counihan et al., 2013). Our results confirm that, in contrast to RON2 and RON4, RAP2/3-mCherry was detected in the parasitophorous vacuoles of early invaded erythrocytes and HepG2/CD81 cells. Our data established for the first time RAP2/3 as an early marker of the vacuole formation following sporozoite entry.

During my post-doc contract, I also joined the multidisciplinary project called MASKIVAC (ANR project). It sought to provide innovative vaccination strategies that will be applicable to infectious diseases for which T cell responses are crucial, by means of developing a transcutaneous (needle-free) mode of vaccine application using innovative particle-based vaccine (Virus-Like particles) for inducing T cell response against Malaria antigens. After comparing several sporozoite-specific antigens, we identified the *Plasmodium* Circumsporozoite protein (CSP) and the Sporozoite Invasion Associated Protein 1 (SIAP1) as VLP vaccine candidates.

After I designed and tested the antigen expression of the *Plasmodium yoelii* CSP VLP, we immunised BALB/c mice by electroporation and followed up their response. Immunisation with plasm-retro VLP induced both cellular and humoral immune responses that were not strong enough for controlling parasitemia after challenge with *P. yoelii* sporozoites (Risco-Castillo et al. 2013). Nevertheless, we observed a 1-day delay in parasitemia with the SIAP1 VLP, which regardless liver parasite load, could be associated to SIAP1 role during

merozoite release, similar to what happens during sporozoite release from oocysts. This would mean a vaccine developed with this gene would likely control parasitemia rather than pre-erythrocytic stages and could be an interesting candidate if associated with a gene such as TRAP (a pre-erythrocytic adhesion protein).

In parallel to my main research activity, I wondered whether *Plasmodium* also required host CD81 after skin inoculation by the mosquito. Previous research showed in a malaria CD81-independent model (*P. berghei*), that the parasite developmental pathway in skin cells, although frequently abortive, leads to the generation of merozoites that are infective to erythrocytes and are released via merozoites, as typically observed in the liver (Gueirard et al., 2010). We decided to set up an *in vivo* model using C57BL/6 CD81<sup>-/-</sup> mice grafted with skin from a C57BL/6 wild type mouse. We observed an increasing parasitemia from day 5 after intradermal inoculation of *P. yoelli* sporozoites, while mice grafted with a using C57BL/6 CD81<sup>-/-</sup> skin graft remained uninfected (figure 14).

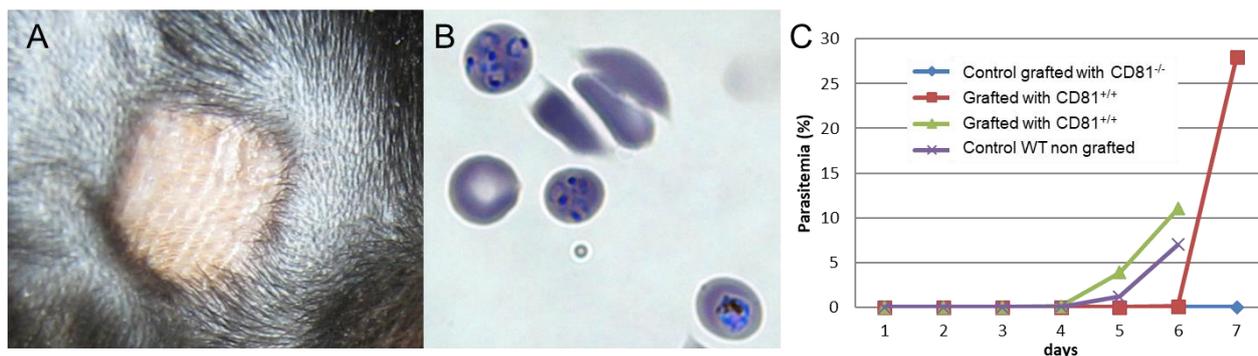


Figure 14. **C57BL/6 CD81<sup>+/+</sup> skin graft into a CD81-deficient mice.** (A) 1cm<sup>2</sup> tail skin graft was posed at the left interscapular area. 10 000 *P. yoelli* sporozoites (265 BY strain) were inoculated by intradermal injection 28 days after graft. (B-C). Parasites were observed from day 5 after infection and either ring forms, trophozoites and schizonts were observed.

In order to continue this project, we applied for ANR financing, that was eligible as pre-proposal but that was not able to pass the final evaluation. Also, several unsuccessful attempts to obtain grafted animals with human skin, together with some infertility issues of the CD81-deficient mouse strain brought the project to a halt. Therefore, important questions such as the possibility of *Plasmodium* to transform into merozoites and directly invade mouse red blood cells remain unanswered. Nevertheless, I was also able to participate to other multiple projects related to the establishment of new malaria mouse models, and their use in the characterisation of human malaria gametocytes, but as those projects were not part of my main work, I think it is not relevant for this report to go into them in more detail.

All together, we were able to publish several articles in high impact scientific journals and to participate to national and international conferences. At the same time of my research activities, I had the opportunity to participate in teaching activities by co-supervising PhD students (GOMO project), master degree students (MaLI and MASKIVAC projects) and other post-graduated students performing short (6 months or less) internships in our research team. This experience, together with my collaboration in the master program supervised by members of the team, gave me a taste for teaching that I decided to explore.

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## 6. Exploring the early dynamics between *Aspergillus conidia* and its host during acute aspergillosis

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After finishing my postdoctoral contract, I decided to seek for a balance where I could reconcile my scientific activity and my desire to participate actively in pedagogical activities, in relation to my experience as a veterinarian, and as a researcher with expertise in protozoology and parasitic diseases of animals and humans. I had the opportunity to apply for a [position](#) as associate professor at the National veterinary College of Alfort (EnvA), and to join the DYNAMYC research team (Dynamics of colonisation and infection by *Aspergillus fumigatus* in the respiratory tract of humans and animals, [UPEC/EnvA](#)).

Under the Direction of Profs. Jacques Guillot and Françoise Botterel, this team gathers, among others, scientists formed to animal science at EnvA and scientists with a human medicine background from the Paris-Est-Créteil University (UPEC). We collaborate in order to provide answers in the field of microbial interactions within the respiratory microbiota, their consequences on colonisation, infection, therapeutics and resistance in humans and in animals. The scientific project of the Dynamyc team focuses on three main strategic objectives:

- Characterisation of the microbial diversity of the airways (bacterial and fungal microbiota) in humans and animals.
- Characterisation of interactions between microorganisms and their physiopathological consequences during respiratory infections in humans and animals.
- Characterisation of the antimicrobial susceptibility of *Aspergillus fumigatus* and *Stenotrophomonas maltophilia*, alone and in combination.

My increasing involvement during my postdoc in local immunological factors inhibiting the ability of the parasite for host invasion, determined me to focus my work on the first two objectives.

Aspergillosis is now recognised as a major opportunistic fungal disease in both humans and animals, particularly birds. Poultry, especially turkeys and waterfowl, are highly susceptible to infection with *A. fumigatus*. Avian aspergillosis is associated with mortality rates that fluctuate between 4.5% and 90%, and produces severe direct and indirect economic losses due to mortality but also to reduced feed conversion, reduced growth rate and increased slaughterhouse condemnations. It can also be responsible for heavy drawbacks to bird conservation programmes (Alley and Gartrell, 2019). Aspergillosis is mainly caused by the ubiquitous fungal species *Aspergillus fumigatus*; susceptible individuals are infected by this opportunistic and major airborne pathogen through inhaling dormant conidia (figure 15) (Arné et al., 2011). Acute and chronic forms of aspergillosis in birds have been known for centuries. Clinical expressions of this mycotic disease include anorexia, aphony, ruffled feathers, gasping, lethargy, lateral recumbency, and often death. Unfortunately, *ante mortem* diagnosis remains difficult (Fischer et al., 2014).

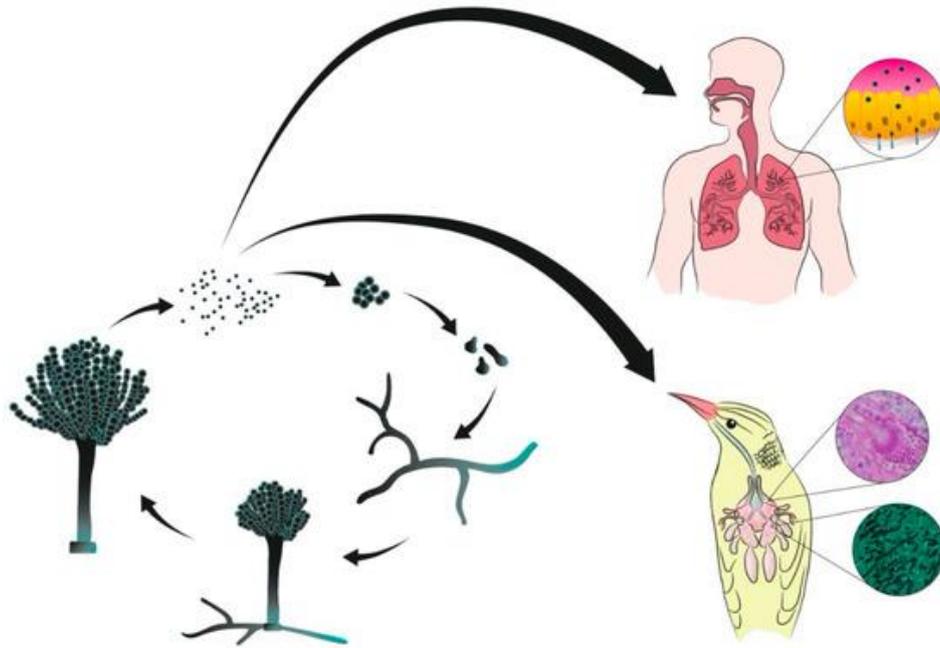


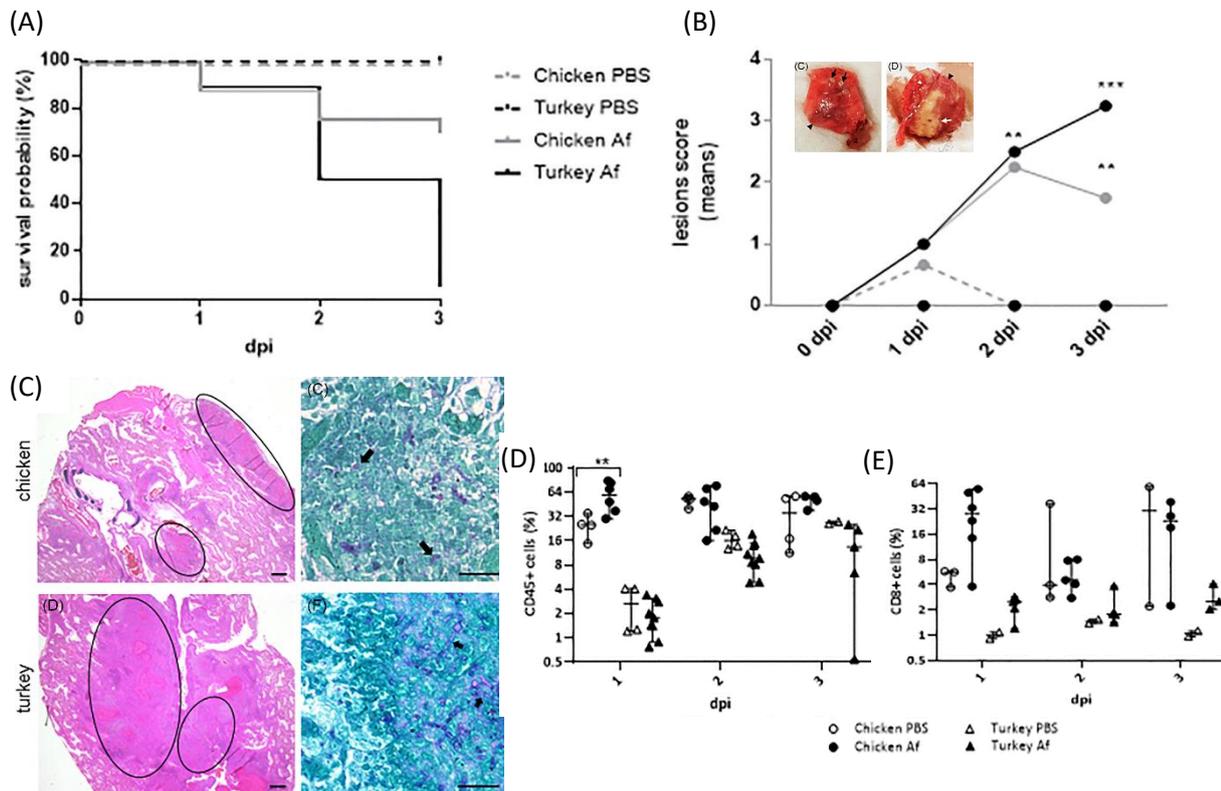
Figure 15. **Representation of vegetative cycle of *Aspergillus* spp. and infection of human and avian hosts by inhalation of conidia.** Humans can develop different forms of aspergillosis, from chronic pneumonia to acute-disseminated aspergillosis, and the severity of the respiratory diseases relies on the host's ability to mount an efficient immune response. In birds, aspergillosis mainly produces respiratory disease and is often fatal (adapted from Melo et al., 2020)

As confirmed *in vitro* and in mouse models, *Aspergillus fumigatus* infection and germination can be detected and limited by the host's innate immune response (Schaffner et al., 1982; Borger et al., 1999; Mehrad et al., 1999; Walsh et al., 2005; Feldmesser, 2006). However, if the immune system fails to clear *Aspergillus* conidia, they swell, germinate, and grow into large hyphal structures that invade surrounding airways and initiate a disseminated infection; by spreading via the bloodstream, the fungus may finally reach the central nervous system (Denning and Stevens, 1990; Kleinschmidt-DeMasters, 2002; Arné et al., 2011). In birds, intrinsic factors affecting susceptibility to *Aspergillus* infection remains scarce. In mammals, host immune response to invasive aspergillosis are mostly mediated by neutrophils and alveolar macrophages, which may inhibit conidia swelling and germination to some extent (Tell, 2005; Blanco and Garcia, 2008). This phenomenon is linked to a switch in cytokine signalling toward a pro-inflammatory profile, leading to the activation of phagocytes, the regulation of epithelial host defence and the setting up of an adequate adaptive immune response through the activation of specific T-cell subsets.

The early detection of fungal elements by pattern recognition receptors (PRRs) and the release of pro-inflammatory cytokines are crucial for controlling *Aspergillus* dissemination (Cunha et al., 2014). The activation of mammalian Toll-like receptors (TLR) 2, 3, and 4, the regulation of inflammatory chemokines (Lebre et al., 2005) and the production of interleukins (ILs) 1- $\beta$  and 17A upon infection are examples for the importance of Th1-associated immune responses against pulmonary aspergillosis (Mambula et al., 2002; Balloy et al., 2005; Bellocchio et al., 2005; Smith et al., 2014). We decided to compare early innate immune response against acute aspergillosis in chickens (*Gallus gallus domesticus*) and turkeys (*Meleagris gallopavo*). The choice of these galliform species enables a side-by-side comparison of their different levels of susceptibility to *Aspergillus* infection, which has already been observed in field conditions but never proved under controlled conditions.

Our study was possible after obtaining EnvA financing of two master degree students and a project funding as young researcher, and we were able to publish our results and to present them at the Conference of I The International Society for Human & Animal Mycology – ISHAM (Vahsen et al., 2020). Our results showed that following *Aspergillus* inoculation, both chickens and turkeys showed body weight loss, mild clinical signs (e.g. sneezing and nasal discharge). After 3 days, the severity of clinical signs was progressive from gasping to death. During necropsy, the presence of congestive and granulomatous tissue lesions in the lungs of all infected birds was observed, independent of the species. Lesion extensions increased over time in both avian

groups, although turkey lungs presented larger lesions from 1 after infection. Moreover, in all infected turkeys, more than half of the lung surface showed lesions compatible with an *Aspergillus* infection 3 after infection, and *Aspergillus* colonisation was confirmed by histopathological analysis and fungal culture from tissue homogenates in all infected birds. Cytometry analysis of respiratory lavages confirmed the presence of the same leukocyte subpopulations. Indeed, heterophils were the dominant leukocyte subpopulation in infected and noninfected birds. However, in both avian species the number of heterophils, thrombocytes, macrophages, and CD4+ lymphocytes in lung lavages were comparable at the time points analyzed. Only chicken CD8+ T lymphocytes showed a slight variation in cell proportions relative to the control group at 1 after infection (figure 16).



**Figure 16. Clinical, pathological and leukocytic differences between chicken and turkey after *A. fumigatus* infection.** Seven days-old chicken and turkey were inoculated by Microsprayer® aerosolization with  $2 \times 10^7$  conidia of *Aspergillus fumigatus* strain CBS144.89, suspended in  $100 \mu\text{l}$  of PBS-tween 20 at 0.01%. (A) Kaplan-Meier survival curves of the four experimental groups 80% (12/17) of infected chicken survived until the end of the experiment while only one turkey survived until 3 dpi. (B) Gross lesions were scored and more severe lung lesions were observed in infected turkeys than in infected chickens. (C) turkey lung sections revealed larger infiltrates and patterns of bronchial damage and peribronchial necrosis than in chicken, and lesions contained many septate hyphae (arrows). Scale bars, 25  $\mu\text{m}$ . (D-E) Flow cytometry analysis of leukocytes from lung lavages. Chickens showed higher modification of cell proportions than turkeys at all days after infection ( $P < .005$ ), and CD8+ lymphocytes fraction at 1 dpi was higher in the infected chicken group but decreased at 2 dpi. \*:  $P < .05$  and \*\*:  $P < .005$  by Dunn's multiple comparisons test.

The early increase of CD8+ T cell proportions in chickens after one day of infection could be interpreted as a consequence of the modulation of this cytotoxic lymphocyte subset by elevated IFN- $\gamma$  levels and by early priming by Toll-like receptors such as TLR3 (Carvalho et al., 2012; Kumaresan et al., 2018). In mammals, a transient expansion of CD8+ T cells has previously been described after sensitization with *Aspergillus* (Tao et al., 2006), while fungal clearance long after infection has been found to be impaired in CD8+ T cell-depleted mice (Carvalho et al., 2012). In this sense, low CD8+ T cell counts in infected turkeys correlate with a poor disease outcome, as has also been described for immunocompromised human patients (Cui et al., 2014).

We also analysed the activation of several cytokines and chemokines known to be secreted soon after *Aspergillus* infection, such as CXCLi2 (the avian ortholog of mammalian IL-8) and IL-1  $\beta$ , which are produced by lung

epithelial cells and activated phagocytic cells, respectively (Steele et al., 2005; Morton et al., 2014). In chickens, both cytokines were strongly upregulated throughout the 3 days of infection, whereas in turkeys IL-1  $\beta$  expression only increased 3 days after infection (figure 17). Although the expression of these cytokines has already been confirmed in chicken lung epithelial cells (Esnault et al., 2011; Mol et al., 2019), this is the first description of CXCL12 and IL-1 $\beta$  production after *Aspergillus* conidia infection; moreover, the delayed expression of IL-1  $\beta$  in turkeys 3 days after infection may suggest a defective recruitment of phagocytic cells, potentially associated with poor PRR interaction in lung epithelial cells (Bidula and Schelenz, 2016).

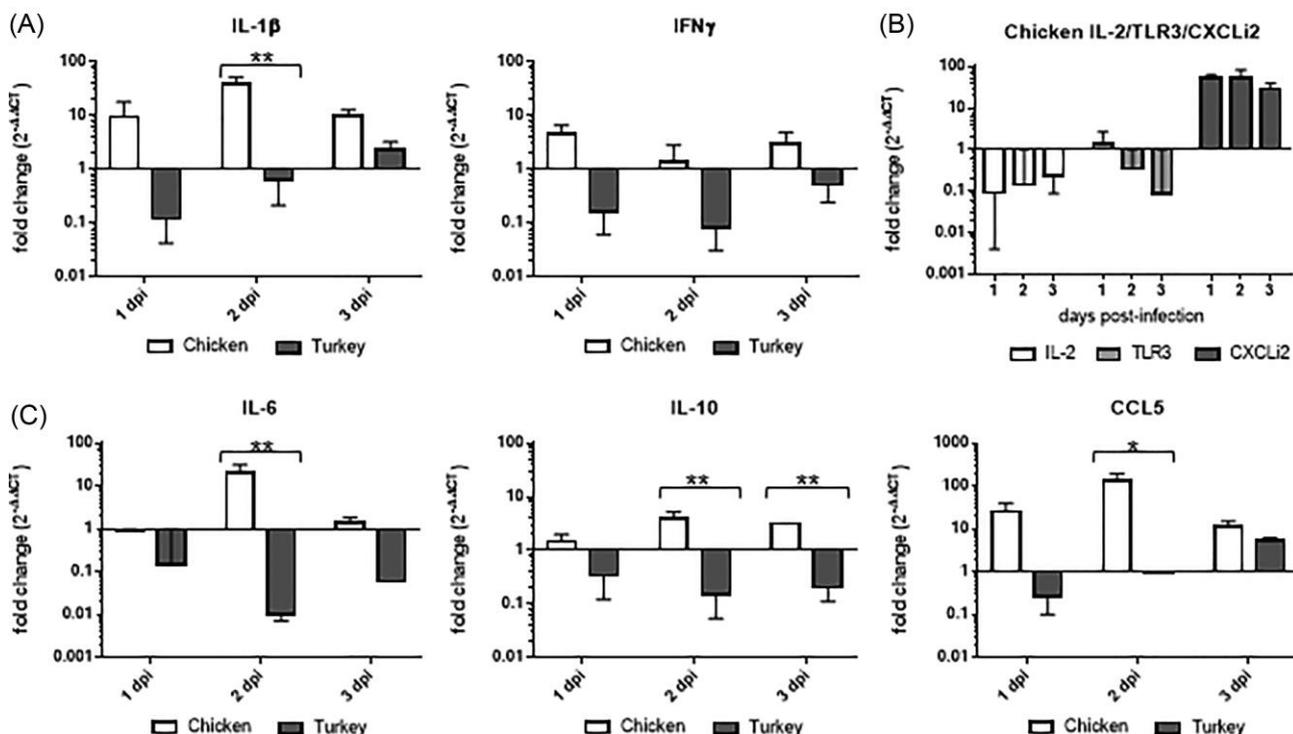


Figure 17. **Relative expression of pulmonary cytokine genes of chickens and turkeys during acute aspergillosis.** Differential expression of chicken and turkey cytokine genes from lungs. The y-axis of each graph represents the relative expression of the respective genes. (A) Upregulation of chicken IL1  $\beta$  and IFN $\gamma$  cytokines contrasts with downregulation of the same cytokines in turkeys. (B) Chicken upregulation of CxCL12 is also observed, while chicken IL-2 and TLR3 cytokines are downregulated. (C) Comparative analysis of chicken and turkey IL-6, IL-10 and CCL5 cytokine genes depicting divergent profiles between chicken and turkey, with oscillating profiles of downregulation and upregulation during the experiment, especially for chicken IL-6 and IL-10 cytokines. (\* $P < .05$ ; \*\* $P < .005$ ).

Furthermore, chicken TLR3 remained downregulated 2 and 3 days after infection. Although early TLR3 upregulation triggers pro-inflammatory cytokine production and controls fungal dissemination, the inhibition of its expression in chickens may be a strategy to reduce *Aspergillus* recognition and to avoid the overproduction of pro-inflammatory cytokines (de Luca et al., 2010; Smith et al., 2014). Divergent IFN- $\gamma$ , IL-6, and IL-10 mRNA regulations were observed between infected chickens and turkeys. As previously described, the upregulation of IFN- $\gamma$  and IL-6 in chickens may ensure a better and faster host immune response (Cheng et al., 2020) and be concomitant with a CD8+ T cell-related inflammation (Farhanah et al., 2018), while their inhibition in turkeys—just as in patients with chronic sinusitis—may be associated with a Th17-like cytokine regulation (Rai et al., 2018). The role of IL-10 as a negative regulator of inflammation during aspergillosis is less known in birds than in mammals (Clemons et al., 2000; Wagener et al., 2014).

Our model also revealed opposed IL-10 expression between infected chickens and turkeys, indicating that the conserved function of this cytokine in chicken may help control aspergillosis-related inflammation (Giansanti et al., 2006; Wu et al., 2016). The direct role of *A. fumigatus* chitin and conidia germination in the activation of host Th1- or Th2-like immune response has also been postulated for mammals and may additionally be true for birds (Lee et al., 2011). Chicken epithelial cells produce CCL5 (Esnault et al., 2011), a pro-inflammatory chemokine developed soon after *Aspergillus* stimulation that may be regulated by anti-inflammatory

IL-10 (Sun et al., 2013; Marischen et al., 2018) and be responsible for exacerbating the disease. Our results confirmed a divergent CCL5 regulation between infected chickens and turkeys one and two days after infection as well as a subsequent upregulation in both avian species. Whereas early CCL5 upregulation in chickens may derive from lung epithelial cells, late expression in turkeys may be associated with natural killer (NK) cells' antifungal response (Ghori and Edgar, 1973).

Mammals' platelets have also been implicated in the response against *Aspergillus* and, in fact *Aspergillus* would be able to potently stimulate the platelets, inducing high expression of the activation markers on the surface, annexin binding to the platelet membrane and significant thrombocyte aggregation, even after few minutes of incubation (Speth et al., 2013). I wondered whether avian platelets would also be able to detect *Aspergillus* and to play a role in antigen presentation. In that sense, we established a protocol for chicken thrombocytes and heterophils purification, and exposed them to GFP- or DsRed-expressing *Aspergillus fumigatus* conidia in a short-term culture.

Thrombocytes recovery rate reached 95.3% purity, while heterophils purification was less efficient (<50%). Following co-culture with conidia, internalization in thrombocytes reached its peak 30min after inoculation (22-28%), while heterophils showed up to 93% of internalization 15 min after inoculation. However, it appeared that heterophils are more efficient in removing conidia due to a more reactive activation of the phagolysosomal machinery (figure 18). Evaluation of the acidification of lysosomal compartments by LysoTracker® staining suggested the presence of lysosomal machinery in both heterophils and thrombocytes.

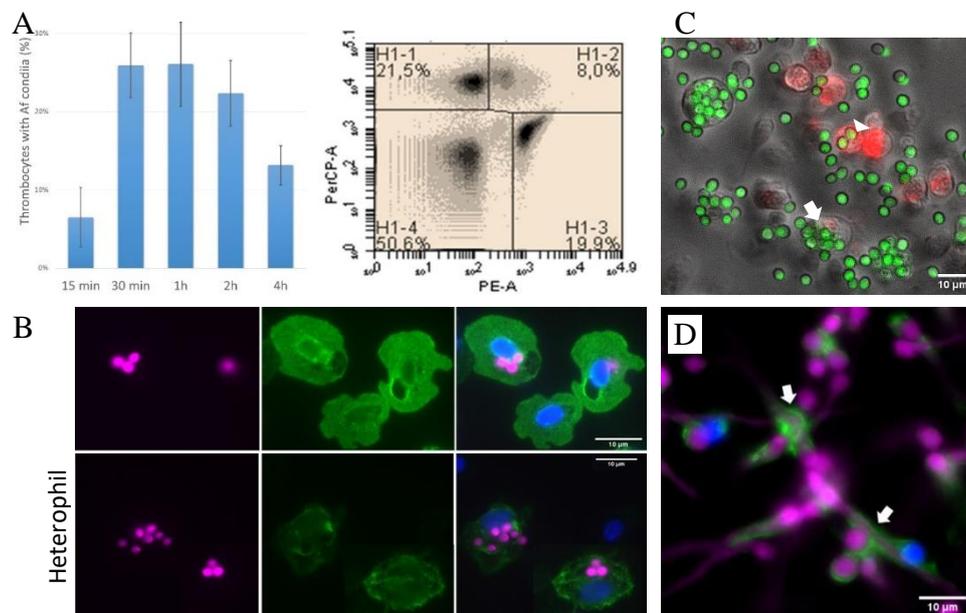


Figure 18. **Internalisation of *Aspergillus fumigatus* GFP or DsRed conidia by chicken thrombocytes and heterophils.** (A) Both manual quantification by microscopy and quantification of individualised thrombocytes by flow cytometry showed a mean of 30% internalisation. (B) heterophils were able to internalise more conidia than thrombocytes 30 minutes after conidia inoculation. (C) Lysosomal machinery (red) in heterophils (arrow) and thrombocytes (arrowhead) 1h post inoculation. (D) *A. fumigatus* DsRed conidia germinating inside of thrombocytes (green, arrows).

We also noticed that chicken thrombocytes are likely to be more efficient than turkey thrombocytes in conidia internalisation. We were able to present our results at the 8th Advances Against Aspergillosis conference (AAA2018) and at the International Society for Human and Animal Mycology (ISHAM 2018) and we are preparing the manuscript for publication.

In the light of our results, it was essential to apply this interesting approach as a predictor of adverse outcomes in birds due to their inherent susceptibility to this fungal infection. In that sense, we decided to have a closer look in the interaction between *Aspergillus* conidia and lung epithelial cells during early encounter *in vitro* in order to target early signals of infection. Thanks to the collaboration with the research unit UMR ISP (INRAe), we were able to examine the interaction between the chicken lung CLEC213 cell strain and *A.*

*fumigatus* conidia *in vitro*. We focused in cellular and molecular interactions between the lung cell line CLEC213 and the fungus *Aspergillus fumigatus* in a transcriptome and secretome approach. To do so, we studied the expression of certain receptors and pro-inflammatory cytokines already recognised as being involved in the immune response in humans *in vitro* and in chicken lungs *in vivo*: TLR4, PTX3, CLEC17AL, IL-6, IL-8 and TNF- $\alpha$ . We also studied the internalization of *Aspergillus fumigatus* conidia by CLEC213 cells, as well as the phagocytosis capacity of the latter.

We first determined the cytotoxicity of *A. fumigatus* against CLEC213 cells, and we observed that cytotoxic effect of *A. fumigatus* became detectable after 15 hours of infection, even if the cellular damage remained moderate and that the cell lysis induced by *A. fumigatus* did not destabilise the cell monolayer (figure 19).

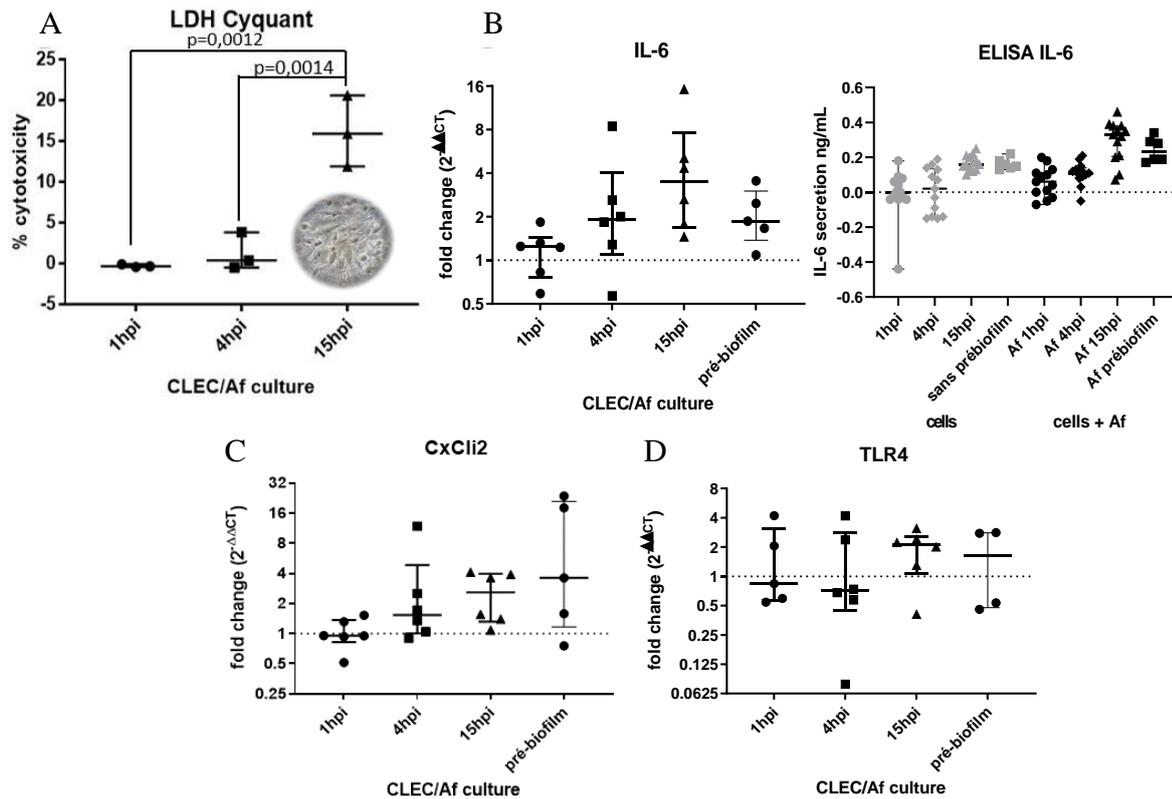


Figure 19. **Response of CLEC213 to *A. fumigatus* infection.** (A) LDH levels remained low at the beginning of the infection but increased after 15 hpi to 16.18% (p-value < 0.05). (B) Relative expression and production of IL-6 . There was significant overexpression of IL-6 at 15hpi (p-value = 0.02) with a moderate IL-6 secretion. Interestingly, infection during 8 hours with a 15-hour pre-biofilm did not stimulate IL-6 as efficiently as the 15h incubation with conidia. (C) Overexpression of Cxcli2 was observed after 15 of infection and after infection with pre-biofilm. (D) Transcription of TLR4 was slightly upregulated after 15 of infection.

With this cell model, our goal to identify early markers of infection was partially achieved since targeted cytokines and PRRs already described in mammals did not seem to be early stimulated in CLEC213 cells. Surprisingly, neither TLR4, PTX3, CLEC17AL, Cxcli2 (orthologous to IL-8) or TNF- $\alpha$  were upregulated as expected during infection. Only IL-6 seemed to reach overexpression levels that increased along time. Nevertheless, we confirmed the ability of CLEC213 cells to internalise *A. fumigatus* conidia, even if CLEC213 cells

were not able to activate their lysosomal machinery properly, and therefore conidia were able to germinate and develop until the formation of a nascent mycelium (figure 20).

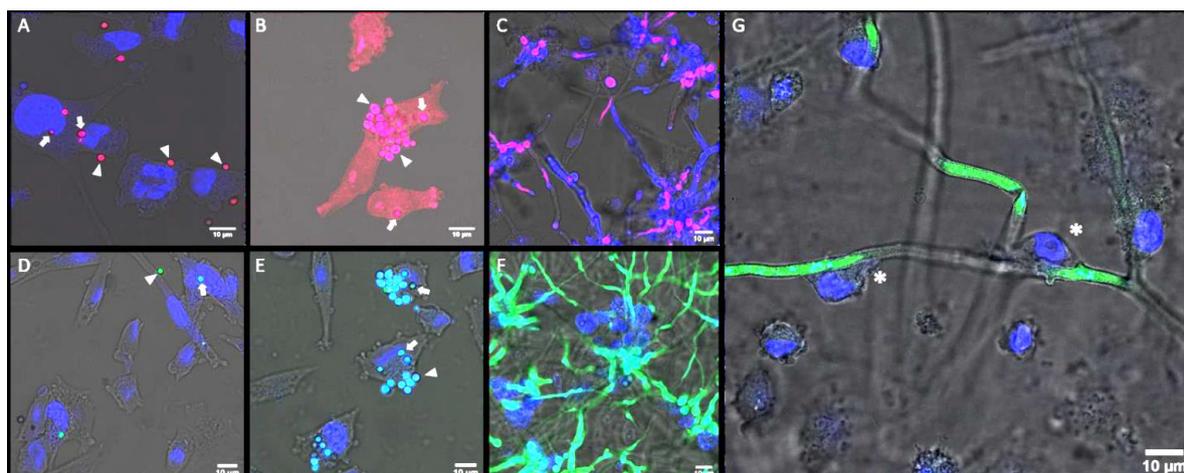
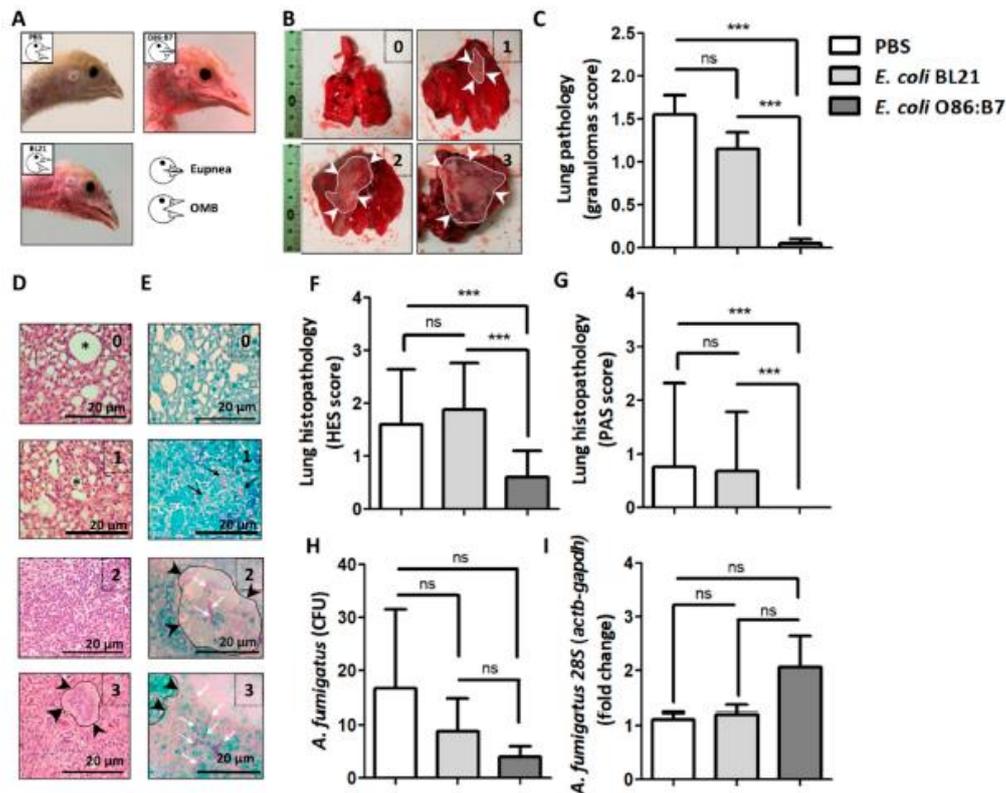


Figure 20. **Interactions between GFP or DsRed *A. fumigatus* conidia and CLEC213 cells.** Cells infected during (A/D) 1 hour, (B/E) 4 hours, (C/F) 15 hpi, and (G) during 8 h with a 15-hour pre-biofilm. At 1hpi, the observation of extracellular conidia was recurrent and only a few CLEC213 cells were able to internalise conidia. At 4hpi, images of cells with numerous intracellular conidia were numerous, but these conidia were often swollen, and very few of the conidia were extracellular. At 15 hpi, the conidia appeared to be able to germinate and begin to form a network despite being internalised by CLEC213 cells. After 8 hours of exposure to an *A. fumigatus* GFP pre-biofilm, the cells appeared to adhere to the hyphae, remaining mainly stuck to the mycelium network. DAPI labelling also showed that only cells exposed for 15 hours to conidia appeared to undergo apoptotic processes (e.g. condensed chromatin, nuclear fragmentation).

In summary, these results highlighted that although chicken lung epithelial cells might play a role in the pro-inflammatory innate immune response to *A. fumigatus*, CLEC213 cells did not appear to be effective efficient enough in producing the pro-inflammatory effectors studied. CLEC213 cells overexpress IL-6, and are able to internalise conidia, but they show a weak chemotaxis during incubation with the pre-biofilm, and a low efficiency in phagocytosis. -and, Other PRRs and cytokines included in the studied did not seem to be stimulated by the *Aspergillus* infection. Quantitative studies of internalisation and phagolysosomal activity are needed, as well as the analysis of other effectors such as TLR2 or other lectins associated with IL-6 overexpression. Complementary experiments will be undertaken in order to confirm the inability of CLEC213 to inactivate internalised conidia before the publication of our results.

Thanks to the advantages of our avian *in vivo* model to study *Aspergillus* infection, we could collaborate with the UMR BIPAR (NeuroPatick team) to establish a proof of concept regarding the protective capacity of anti-Gal antibodies against acute *Aspergillus fumigatus* infection in turkey poults. For this, we evaluated the effector and protective immune response of the animals. The sugar galactose- $\alpha$ -1,3-galactose ( $\alpha$ -Gal) is a molecule found in bacteria and most mammals but it is not produced by Old World primates, humans, reptiles, fishes or birds (Yilmaz et al., 2014). Its antigenic properties have been associated to some allergic reactions in humans, and with protection against infectious diseases such as malaria and tuberculosis (Cabezas-Cruz and de la Fuente, 2017).

Once we confirmed that *Aspergillus fumigatus* was able to produce  $\alpha$ -Gal on its surface (Hodžić et al., 2020), we decided to explore the immunogenicity of  $\alpha$ -Gal in our turkey model. We observed that turkeys with an oral Administration of *E. coli* O86:B7 developed less severe clinical signs, smaller lung granulomas, less inflammation and less fungal colonisation, measured by CFU quantification in culture (figure 21).



**Figure 21. Oral administration of *E. coli* O86:B7 protects turkeys against aspergillosis.** Clinical examination revealed that *A. fumigatus* infection produces open-mouthed breathing (OMB) in turkeys treated with PBS or *E. coli* BL21. Turkeys treated with *E. coli* O86:B7 were protected from developing OMB (A). Pulmonary lesions (i.e., granulomas, delimited area and white arrow heads) were scored (see methods). Examples of lungs with scores 0 to 3 are shown (B). Granuloma score was lower in turkeys treated with *E. coli* O86:B7 (C). Lung samples were processed for histopathology and stained with hematoxylin-eosin-sa<sub>ron</sub> (HES, D) and periodic acid-schiff (PAS, E). Histological lesions were scored (see methods). Examples of histopathology samples with scores 0 to 3 are shown. Visible peribronchial regions (asterisk) and granulomas (delimited area and black arrow heads) are shown (HES score, D). The presence of fungal germ-tube/hyphae (black arrows) and mycelium (white arrows) was scored (see methods). Granulomas associated with fungal hyphae (delimited area and black arrow heads) are shown (PAS score, E). HES and PAS scores were lower in turkeys treated with *E. coli* O86:B7 (F,G). The presence of viable *Aspergillus* in lungs was quantified by colony-forming unit (CFU) counting assay (H). Fungal DNA levels were measured by *A. fumigatus*-specific 28S qPCR normalizing against turkey *actb* and *gapdh* as host genes using the 2ddCt ratio method. Results are relative to 28S levels in the control group (i.e., PBS) (I). No significant change was observed in the amount of CFU and 28S fold change (H,I). Size of bars is indicated. 100X magnification. Results shown are means and standard deviation values. Results were compared by One-way ANOVA with Dunnett's multiple comparison test applied for individual comparisons (\*\*\*)  $p < 0.0001$ ; ns: not significant, 2 experiments,  $n = 30$ ).

Interestingly, we also observed that the oral administration of *E. coli* O86:B7 decreased Anti- $\alpha$ -Gal IgA production in the lungs of *A. fumigatus*-infected turkeys, and this effect was associated with a reduction of lung pathology (figure 20), these results were different in chicken, since immunisation worsened infection outcome, with more clinical signs, lung lesions, lung parasite load and IgA production. Then, we wondered whether oral administration of *E. coli* O86:B7 (oral immunisation) or  $\alpha$ -Gal-BSA (subcutaneous immunisation) had an effect on the expression of genes encoding for pro-inflammatory (i.e., IFN, IL6, IL2) and anti-inflammatory (i.e., IL10) cytokines, as well as on the expression of innate immune receptor genes (i.e., MyD88) in the ceca and lungs of *A. fumigatus*-infected turkeys.

Expression of MyD88, IFN and IL6 genes was significantly upregulated in the ceca of  $\alpha$ -Gal-BSA-immunized turkeys. Immunization also induced a significant upregulation of IL2 in turkey and chicken lungs, and IL6 only in chicken lungs. The oral administration of *E. coli* BL21 in turkeys was associated with the upregulation of IL10, IFN and IL6 expression in ceca and IL6 expression in lungs. Notably, except for IL2, oral administration of *E. coli* O86:B7 was not associated with significant changes in the mRNA levels of the tested genes (figure 22).

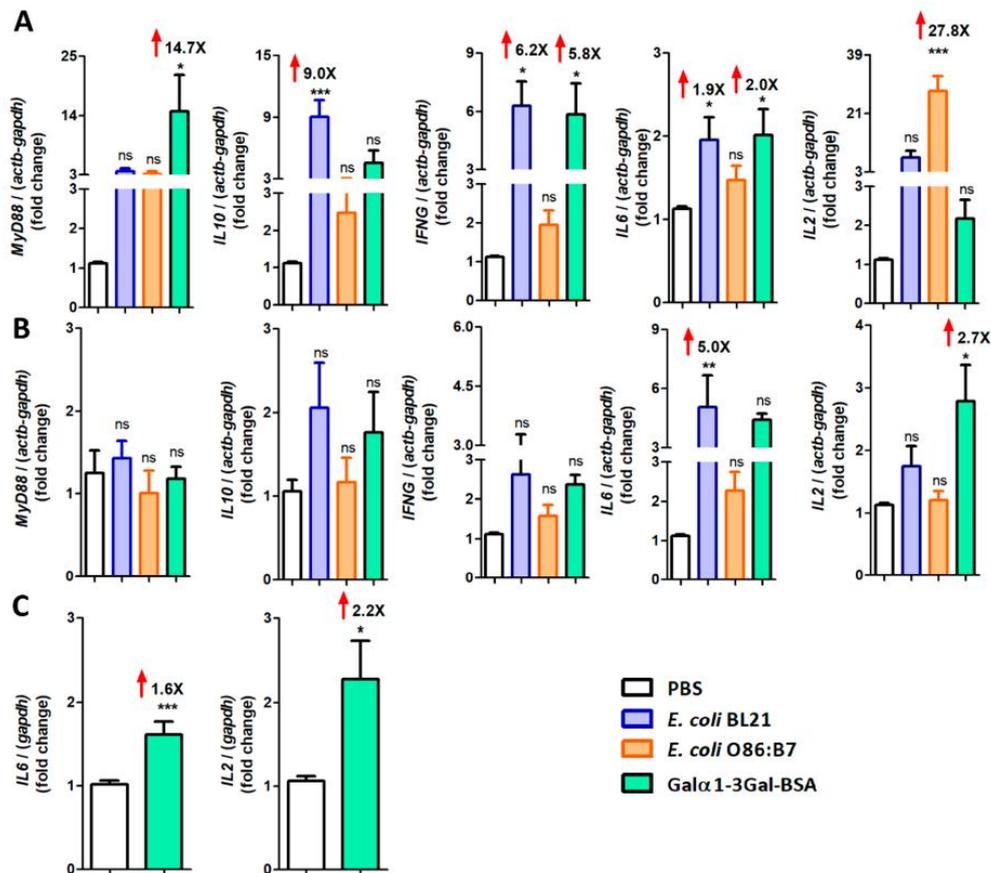


Figure 22. **Expression of turkey and chicken cytokine genes in response to oral administration of *E. coli* O86:B7 and *E. coli* BL21 and  $\alpha$ -Gal-BSA immunization.** The figure displays the mRNA expression levels of INF, IL6, IL2, IL10 and MyD88 in ceca (A) and lungs (B) of turkeys and IL6 and IL2 in lungs of chicken (C). Results shown are means and standard deviation values. Results were compared by One-way ANOVA with Dunnett's multiple comparison test applied for individual comparisons (\*  $p < 0.0001$ , \*\*  $p < 0.001$ , \*\*\*  $p < 0.0001$ ; ns: not significant, the magnitude of significant fold changes compared with the PBS are shown (red arrows), 1 experiment with chicken,  $n = 10$  and 2 experiments with turkeys,  $n = 40$  and three technical replicates per sample).

Anti- $\alpha$ -Gal Abs are immune mediators of inflammation that activate the complement and macrophages, and induce NK cell recruitment and endothelial cell activation (Cooper et al., 1994; Palmetshofer et al., 1998; Katopodis et al., 2002). Oral administration of *E. coli* O86:B7 reduced the occurrence and severity of lung granulomas, with no effect in the fungal burden, suggesting a mechanism in which gut microbiota promotes disease tolerance in the lungs by preventing the uncontrolled upregulation of pro-inflammatory cytokines (i.e., IL2, IL6 and INF), and by decreasing the levels of anti- $\alpha$ -Gal antibodies in response to *A. fumigatus* infection. This study showed that gut microbiota enriched with bacteria expressing high levels of  $\alpha$ -Gal protects turkeys against clinical aspergillosis and the formation of lung granulomas, by reducing lung anti- $\alpha$ -Gal IgA to residual levels. Our results confirmed a reduced risk of infection in orally immunised animals, which would allow an affordable vaccination strategy in poultry. Indeed, we decided to assess the safety and efficacy of an oral probiotic-based vaccine against *Aspergillus* infection in captive-bred Humboldt penguins (*Spheniscus humboldti*).

Yearly fatal *A. fumigatus* cases affect approximately 10% of the penguin colony at ZooParc de Beauval in France and can represent 27% of all cause-mortality in some penguin colonies (Springer and Horton, 1969). Fungal loads in their environment settings are often quite high (Cabezas-Cruz et al., 2014), making immunosuppressed or stressed individuals, particularly at risk of developing clinical aspergillosis (Galili et al., 1987), especially during the summer season (July-September) in the northern hemisphere (Cabezas-Cruz et al., 2014, 2019). Diagnosis in birds is challenging, and often requires a multi-tool diagnostic panel in addition to the anamnesis and clinical evaluation (Steinke et al., 2015; Mateos-Hernández et al., 2017). Besides, treatment of aspergillosis in clinically infected penguins primarily relies on azole drug-based therapy (Mateos-

Hernández et al., 2017; Hodžić et al., 2019; Kiewiet et al., 2020), but the initiation of adequate antifungal therapy is often delayed. Besides, voriconazole toxicity has been described in penguin species (Park et al., 2020) and resistance of *Aspergillus* sp. strains to azole molecules is reported from low (Latgé, 1999; Hodžić et al., 2020) to high (Yilmaz et al., 2014) depending on the zoological institutions. For all these reasons, medical prophylaxis against aspergillosis is paramount in penguins.

Together with the evaluation of the distribution of  $\alpha$ -1,3-galactosyltransferase ( $\alpha$ 1,3GT) genes in the fecal microbiome of Humboldt penguins, immune response after oral administration of gram-negative bacteria expressing high levels of galactose- $\alpha$ -1,3-galactose ( $\alpha$ -Gal) was followed. The occurrence of natural anti- $\alpha$ -Gal antibodies in sera and eggs of healthy Humboldt penguins was also assessed. Immunised animals were followed for one year for aspergillosis detection using a diagnostic panel including computed tomography scans, capillary zone electrophoresis, 3-hydroxybutyrate levels, and anti-*A. fumigatus* antibodies. Anti- $\alpha$ -Gal antibodies were detected in sera (IgM and IgY) and eggs (IgY) of healthy penguins.

Several studies show that the inter-generational transfer of maternal antibodies provides humoral immune defence against pathogens in eggs and early-life offspring. This mechanism is crucial as endogenous production of Abs in chicks occurs only 10-14 days post-hatching (Portillo et al., 2019). Anti-*Aspergillus* antibodies may be transmitted from mother to offspring in penguins (Macher and Galili, 2008). One of our main results is the detection of anti-*Aspergillus* IgY in the two Humboldt penguin eggs tested. Our results also showed the presence of anti- $\alpha$ -Gal antibodies in Humboldt penguin eggs. However, whether anti-*Aspergillus* and/or anti- $\alpha$ -Gal antibodies inter-generationally transferred from the mother to egg to chick, have protective functions against *Aspergillus* or other infectious diseases in penguins remains an open question.

Regarding  $\alpha$ -Gal vaccination, we found a positive correlation between anti-E. coli IgY and anti- $\alpha$ -Gal IgY in the orally-vaccinated group. During the study period, three penguins exhibited respiratory signs consistent with aspergillosis. Two were from the placebo group whose symptoms resolved with specific treatments, while a single vaccinated individual developed fatal respiratory aspergillosis eight months after the trial. We conclude that our vaccine represents a safe potential probiotic with a protective effect against aspergillosis in Humboldt penguins that deserves to be further explored for therapeutic uses in these animals.

In total, collaboration with BIPAR led to the publication of 3 original papers (Hodžić et al., 2020; Mateos-Hernández et al., 2020; Thorel et al., 2022), and opened the gate for further collaboration.

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## 7. Conclusions and Perspectives

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Throughout my professional career, I have sought to maintain coherence between my research and teaching activities. Parasitology, and more particularly the study of host-parasite interactions, has been the guiding thread, first during the development of my doctoral thesis at the Complutense University of Madrid, then during my postdoctoral contracts at INSERM UMR S945 and finally at the National Veterinary College of Alfort. Collaboration with the industrial world, with local authorities and state services, through research projects with national or transnational funding, allow me not only to know how to succeed in financing my research, but above all to remain close to the needs and technical concerns, in order to ensure short-term results that can be applied for the benefit of animal health. This scientific vision is of great importance in order to allow a sorting of fundamental and secondary information to be taught.

My first steps in the world of Apicomplexan parasites, made me discover the amazing interactions and adaptation that these parasites are able to overcome in order to ensure their persistence into the host cell. I am proud of what I accomplished during my PhD and postdoc, since our achievements allowed the improvement of the techniques available for the study of *Neospora caninum* and *Plasmodium*, but also to the development of new diagnostic tools and even vaccine strategies. This amount of work would not have been possible if I had not been lucky enough to have been able to join cutting-edge research teams in the research topics that have guided my career.

Also, the opportunity to participate in teaching and supervision of undergraduate and postgraduate students at the research teams I worked with, allowed me to develop the ability to convey information in an understandable way and to motivate reflection in order to stimulate scientific thinking and to complement medical reasoning. My recent experience in teaching activity has been a double challenge for me: firstly, I had to update my knowledge in the vast field of veterinary parasitology and mycology. At the same time, I had to learn the techniques to transmit information and concepts to veterinary students. The teaching missions defined for the position of lecturer met my aspirations and the Parasitology and Mycology teaching unit of the EnvA is a unit which functioning and objectives correspond to my professional ambitions.

Although my scientific activity had to adapt to a planning in association with my teaching and diagnostic activities, the results obtained within the Dynamyc team allowed me to establish new collaborations with research groups in France (INRAe, BIPAR) and abroad. My results obtained in the field of avian aspergillosis confirmed the need to better characterise avian immune response in target avian species, either poultry or wildlife, in order to better diagnose and protect them. Lung epithelial cells, together with thrombocytes would play a role in antigen presentation that deserves being explored in depth and being compared between avian species sensible and tolerant to *Aspergillus* infection. My results were also helpful during the writing of a review article on Aspergillosis in wild birds.

Also, thanks to the collaboration established with the BIPAR research team, these results have been applied in the context of avian species in confinement, such as in zoos, and have highlighted the possibility of transmitting certain immunological protection to the offspring through the passage of antibodies. This mechanism, which has already been described with other pathogens, deserves to be further investigated, because chicks before the first two weeks of life are the most susceptible group to develop acute aspergillosis, also known as Brooder's pneumonia.

Recently, my duties within the CHUV-FS and the Parasitology and Mycology laboratory of the EnvA have determined a new evolution of my scientific activity. I have been able to supervise (and I am still doing it) several veterinary theses in the field of ectoparasitism. Those theses study parasitism degree in urban and periurban wildlife species hosted by the CHUV-FS, and seek the development of hygienic or surveillance measure to avoid the transmission of vector-borne diseases. My involvement in the identification and diagnosis of parasites and parasite diseases at EnvA, is also evolving towards the implementation of new diagnostic services using proteomic (Maldi TOF) and genomic (real-time PCR) tools. At the intersection between

the two, the possibility has arisen to conceive new research projects to respond to the need to understand and monitor increasingly anthropized ecosystems, and to propose effective tools to prevent the spread of pathogens between wildlife and humans.

My recent affiliation to the UMR Bipar (MiTick team), lead me to new horizons always in search of the understanding of the interaction between the parasite and its host, this time in the context of vector capacity of ixodid ticks and zoonotic hemoparasites such as *Babesia*. Babesiosis is a tick-borne hemoprotozoan disease of domestic and wild animals. The disease is caused by several species of *Babesia* that are host specific. However, some species of *Babesia* harbour zoonotic importance. Human babesiosis has been reported in North America, Europe and Asia (Delbecq, 2022). Human infection is possible after the encounter of a parasitized vector with a susceptible host.

The current epidemiology of human diseases is driven by tick tropism for humans, parasites circulating in the tick population, and frequency of tick bites in shared areas (for leisure, work, or home locations). Species of *Babesia* vary in their infectivity, virulence and pathogenicity for people (Yabsley and Shock, 2012). Various factors (e.g., increased interactions between people and the environment, increased immunosuppression, changes in landscape and climate, and shifts in host and vector species abundance and community structures) have led to an increase in tick-borne diseases in people, including babesiosis.

European hedgehogs (*Erinaceus europaeus*) are small hibernating insectivorous mammals, that have fairly well adapted their behaviour towards synanthropic attitudes and frequent contacts with sympatric wild and domestic species, including humans, these interspecies interactions accounting for its potential involvement (e.g. as a maintenance host, bridge host or dead-end host) in the ecology of several potentially emerging pathogens (Delogu et al., 2020). The CHUV-FS at EnvA receives about 1 000 hedgehogs every year. These animals arrive from the Paris area and its surroundings for different reasons such as malnutrition, trauma or infectious disorders (diarrhoea, coughing). More often than not, these animals arrive parasitized with different kinds of ectoparasites such as hard ticks, fleas and even mites or fly maggots. Even if an ectoparasite treatment were to become systematic, there is always the risk of transmitting tick-borne diseases during the hibernation season, when hedgehogs must wait spring before being released.

Recent studies confirm the role of European hedgehogs in the spread and transmission of tick-borne pathogens -mainly virus and/or bacteria- in urban areas (Jahfari et al., 2017; Barradas et al., 2021), while the information regarding tick-borne transmission of parasites in this species remains scarce (Orkun et al., 2019; Qi et al., 2022). One of my first objectives is to estimate the risk and the frequency of piroplasmid infection in European hedgehogs. The proximity of the CHUV-FS will ensure a collaborative work that will establish the basis for a wider project in coordination with the French network of wildlife care centres. Geographic evolution of hard ticks population and wildlife hosts associated to climatic change will be taken into account to understand the evolution of the risk factors associated to the infection.

The adaptation of previously published protocols for *Babesia* culture (Grande et al., 1997; Singh et al., 2022), will allow us to explore cellular mechanisms associated to parasite invasion and development inside the hard tick. Thanks to my background in the study of host-apicomplexan parasite interactions and the experience of BIPAR on tick-host-pathogen interactions, we will set up an infection protocol that will allow us to identify and target molecules implicated in their transformation into sporozoites and their likely dormancy during molting or even after transovarial transmission (Schnittger et al., 2012). Secreted proteins from the apical complex of the sporozoite will be characterised and modified.

At this time, most of my activity has been focused in the writing of projects proposal that I have send to several projects calls (Ammorçage 2022 – UPE grant, EnvA grant, DIM One Heath- Ile de France grant) and we are now waiting for a response to our applications for funding. Meanwhile, I participate today in the co-supervision of two PhD candidates. One project is related to the application of anti-microbiota vaccines for the control of tick-borne pathogens. The strategy is to develop transmission-blocking vaccines based on the manipulation of keystone taxa in hard ticks by means of vertebrate host's antibody production. Once vertebrate host is "vaccinated", they will transfer antibodies during the blood meal of the vector, in order to target

pathogens and other bacteria within the arthropode. The possibility to find active host IgG lasting after 7 days after engorgement, or even after ovoposition would ensure proper antibody transfer to the tick's progeny. So far, the identification of the best bacterial taxon for this approach is ongoing and some preliminary results have confirmed that the vaccination against the keystone bacteria *E. coli* can modulate the tick microbiome not only at the taxonomic but also at the bacterial community level by shifting the structure, interactions and functional profile of microbial communities.

The second PhD project is related to the activities mediated by cholinergic pathways and associated to peptidergic neurons innervating the salivary glands of *Ixodes ricinus*. Previous results confirmed that tick's Muscarinic acetylcholine receptors prevent *Ixodes* from desiccation during fasting period (Mateos-Hernandéz et al., 2020). My implication in this project will be mainly focused in the manipulation of the cholinergic pathway in order to control tick infection with pathogens such as *Babesia*.

Another team's objective is to identify potential ligands in tick-borne pathogens that could interact with G protein-coupled receptors (GPCR), which regulate physiological processes controlled by neuropeptide hormones. So far, the project is sieving potential bioactive metabolites able to control particular GPCR-driven signal transduction. This objective fits perfectly with the design of control measures against parasite transmission to the vertebrate host. We are confident that these collaborations will allow us a better visibility in new funding attempts (ANR, DIM One Health, ATIP AVENIR). It is worth to point out that, after the pandemics that hit us two years ago, I was able to rebuild my pedagogical and scientific vision in order to adapt as well as possible to this situation. In this sense, the challenges that lie ahead in the short term are both collective and individual.

Even if some side projects I have co-ordinated do not correspond to my main research focus, I consider that it is important to highlight the close communication I have kept with my colleagues at the University of San Marcos, in Lima. Since my PhD studies in Spain and up until now, I have been able to find funding to work in South American camelids health. My first project was financed by the Complutense University of Madrid during my PhD studies, it allowed us to explore the risks of neosporosis and other infectious disease in wild South American Camelids. More recently, I was able to obtain funds thanks to the ECOS Nord program from the French Ministry of Foreign Affairs; this project has opened the gate to establish a PhD co-direction between the University of San Marcos, in Lima, and the Veterinary College of Alfort.

Finally yet importantly, my diagnostic activities at the Laboratory of Parasitology and Mycology will remain a source of field-based information, and the opportunity to set up applied research with veterinary students and post-graduated scholars. With them, I will not only perform routine analysis but we will exploit the data to understand the causes of some infectious problems in order to establish sanitary recommendations or even to ameliorate the techniques for diagnosis and parasite identification. So far, we collaborate with the Service of Parasitology and Mycology from the Pitié Salpêtrière Hospital (PhD Anne-Cécile Normand) in order to propose MALDI TOF identification not only of molds and yeast, but also for tick identification and parasite vectorisation.

Twenty years ago, I started a scientific journey that has never stopped since. I am grateful for all the experiences I have been lucky to accumulate, together with the encounters that I have made all along my scientific formation until my arrival to EnvA. This journey does not stop, to the contrary, the wheel keeps turning and this is why I decide, today, to apply for the accreditation to supervise research (HDR). During these years, I have developed scientific skills that have helped me to succeed in the identification of crucial scientific questions regarding host-parasite interaction. I have also developed project proposals that have later been successful in obtaining project funding. Obtaining the HDR will allow me to carry out my research in complete autonomy, to federate a team around projects that I am passionate about, and to continue supervising master and PhD students. In addition to allowing me to advance in my professional career, it represents a recognition that will give my research team and myself more visibility in the national and international scientific community.

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## 8. Bibliography

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