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Tick research in the uprise of tick-borne diseases

Alejandro Cabezas-Cruz

► **To cite this version:**

Alejandro Cabezas-Cruz. Tick research in the uprise of tick-borne diseases. Life Sciences [q-bio]. Université Paris-Est, 2018. tel-04540835

HAL Id: tel-04540835

<https://hal.science/tel-04540835>

Submitted on 10 Apr 2024

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Public Domain

Tick research in the uprise of tick-borne diseases

**Dossier de candidature
à l'Habilitation à
Diriger des Recherches**

Présenté par

Alejandro CABEZAS-CRUZ

CR1 INRA, UMR BIPAR (ENVA, ANSES)

VECTOTIQ

**Curriculum vitae et
Activités de Recherches**

Université Paris-Est

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Les tiques et les agents pathogènes qu'elles transmettent constituent un fardeau croissant pour la santé humaine et animale dans le monde entier. Pendant leur alimentation, les tiques sécrètent une myriade de protéines pharmacologiquement actives qui constituent un élément majeur des interactions vecteur-hôte. Ces molécules assurent également la transmission des agents pathogènes. Dans la première partie de ce document, je décris brièvement le travail accompli sur la caractérisation des lipocalines salivaires. En particulier, j'ai été impliqué dans la caractérisation fonctionnelle d'une lipocaline de tique, la Japanin, qui a des effets immunomodulateurs. Nous avons montré que la Japanin inhibait la régulation positive de CD86 en réponse à de multiples stimuli de maturation de cellules dendritiques. Récemment, des tiques vectrices (comme *Amblyomma americanum*) ont été impliquées dans une maladie anaphylactique émergente, le syndrome α -Gal. En particulier, la réponse d'anticorps IgE au carbohydrate α -Gal après une morsure de tique est associée à des allergies à la viande rouge, au cetuximab (anticorps monoclonal thérapeutique) et à la gélatine. J'ai été impliqué dans l'identification des protéines de tiques contenant la modification α -Gal. Par ailleurs, l'immunité anti- α -Gal (IgG et IgM) joue un rôle important dans la protection contre les maladies infectieuses et le groupe sanguin B diminue les niveaux d'anticorps anti- α -Gal chez l'homme. Nous avons trouvé une corrélation significative et positive entre la fréquence du groupe sanguin B et l'incidence du paludisme et de la tuberculose en Afrique subsaharienne, en Espagne et au Portugal.

Dans la deuxième partie du document, je présente mes résultats sur la caractérisation d'une nouvelle maladie transmise par les tiques affectant le bétail sur le continent Américain. Anaplasmataceae est une famille d'Alphaproteobacteria qui appartient à l'ordre Rickettsiales et comprend les genres *Anaplasma*, *Ehrlichia*, *Neorickettsia* et *Wolbachia*. Parmi ces genres, *Ehrlichia* et *Anaplasma* sont des agents pathogènes importants pour les animaux et les humains. Les *Ehrlichia* sont des bactéries intestinales gram-négatives obligatoires qui poussent dans des vacuoles liées à la membrane chez les leucocytes humains et animaux provoquant une ehrlichiose. Dans la réorganisation de la famille Anaplasmataceae en 2001, seules cinq espèces d'*Ehrlichia* spp. ont été officiellement reconnues (c'est-à-dire *E. canis*, *E. chaffeensis*, *E. ewingii*, *E. ruminantium* et *E. muris*). En 2012, nous avons isolé une nouvelle espèce d'*Ehrlichia*, acceptée en 2016 par le Comité international sur la systématique des procaryotes sous le nom de *E. minasensis*. Au niveau ultrastructural, cette nouvelle bactérie ressemble à *E. canis*, *E. chaffeensis* et *E. muris*. Le séquençage du génome complet et l'analyse phylogénétique utilisant plusieurs gènes bactériens ont montré que *E. minasensis* est étroitement liée à *E. canis*. Fait intéressant, *E. minasensis* a été initialement isolé de tiques *Rhipicephalus microplus*, tandis que *E. canis* se trouve

principalement dans *R. sanguineus*. Indépendamment, un groupe de chercheurs Brésiliens a signalé que *E. minasensis* était pathogène pour le bétail, tandis que *E. canis* est principalement pathogène pour les chiens. Une analyse évolutive supplémentaire utilisant la glycoprotéine principale TRP36 a suggéré que *E. minasensis* a évolué récemment à partir d'un clade très divergent et variable de *E. canis*.

Ensuite, je présente les principaux résultats de mes recherches sur l'épidémiologie et la diversité génétique d'*Anaplasma marginale* et d'*E. canis* en utilisant les principales protéines de surface MSP1a et TRP36. En collaboration avec des chercheurs d'Afrique du Sud et du Brésil, j'ai étudié l'épidémiologie et la diversité génétique d'*A. marginale* dans ces deux pays à la fois dans les buffles et les bovins. L'une des principales réalisations de ce travail a été de fournir, pour la première fois, des preuves moléculaires sur la prévalence d'*A. marginale* dans 8 des 9 provinces sud-africaines.

Dans la troisième partie du document, j'ai développé l'utilisation des analyses de réseaux pour étudier les interactions écologiques entre les tiques et leurs agents pathogènes. Récemment, nous avons appliqué cette approche pour identifier plus de 14 000 interactions entre les tiques, les hôtes et les agents pathogènes transmis dans le Paléarctique de l'Ouest, en démêlant leurs interactions complexes. Nous avons constaté que: (i) les réseaux naturels de tiques-hôtes-pathogènes sont fortement structurés, modulaires, cohérents et imbriqués, (ii) le grand nombre de vertébrés du réseau contribue à sa robustesse et sa persistance, (iii) la structure du réseau réduit la compétition interspécifique et permet une circulation large mais modulaire des agents pathogènes transmis chez les vertébrés, (iv) remarquablement, la prise en compte des hôtes domestiques effondre la structure modulaire du réseau, reliant des groupes d'hôtes qui n'étaient pas liés auparavant, et augmente la circulation des agents pathogènes. Dans plusieurs articles, nous avons montré que l'analyse des réseaux écologiques est un outil exceptionnel pour étudier les associations écologiques des micro-organismes et de leurs hôtes.

Une partie importante de mes recherches porte sur les interactions des agents pathogènes avec les tiques. La compétence vectorielle est une composante de la capacité vectorielle et dépend des facteurs génétiques affectant la capacité d'un vecteur à transmettre un agent pathogène. Ces facteurs génétiques affectent des traits comme la susceptibilité d'être infecté par un agent pathogène. Par conséquent, l'élucidation des mécanismes impliqués dans les interactions entre les tiques et les agents pathogènes qui affectent la compétence du vecteur, est essentielle pour l'identification des facteurs génétiques liés aux maladies transmises par les tiques chez l'homme et les animaux. Dans ce cadre, je détaille mes recherches portant sur la caractérisation des interactions moléculaires entre *A. phagocytophilum* et sa tique vectrice, *Ixodes scapularis* ; plus précisément, sur le rôle de la réponse au stress bactérien (protéines GroEL et HSP70) et des protéines de surface (MSP4) dans les cellules de tiques et les

glandes salivaires, au cours de l'infection et son implication fonctionnelle dans les interactions tique-pathogène.

Enfin, je présente une partie de mes recherches sur l'épigénétique des tiques. Dans un travail pionnier, nous avons identifié 39 gènes modifiant les histones (HME) dans le génome / transcriptome de *I. scapularis* et nous avons caractérisé phylogénétiquement et structurellement 5 histones et 34 HME, y compris des histones acétyltransférases, des histones désacétylases, des sirtuines, des histones méthyltransférases et des histones déméthylases.

À l'avenir, je souhaite développer quatre principaux domaines de recherche sur les tiques et les agents pathogènes transmissibles par les tiques: (i) les interactions tiques-agents pathogènes, (ii) l'allergie induite par les tiques (iii) l'écologie des tiques et des agents pathogènes transmissibles par les tiques et (iv) l'épigénétique des tiques. En ce qui concerne le premier point, nous utilisons l'agent pathogène *Anaplasma phagocytophilum* comme modèle pour étudier la manipulation des tiques par les agents pathogènes. Pour cela, nous utilisons une approche System Biology qui combine la génomique, la transcriptomique et la protéomique pour démêler les voies (par exemple métabolisme, cytosquelette, épigénétique, réponse immunitaire et stress oxydatif) affectées lors de l'infection par un agent pathogène (cette année, nous avons publié 2 manuscrits sur ce sujet). En ce qui concerne le deuxième point, notre laboratoire est leader sur ce sujet au sein de la communauté « tique ». Cela se reflète dans nos publications dans des revues de premier plan telles que *Allergy* (2017) et *Trends in Parasitology* (2017). Notamment, cette année, nous avons publié le premier rapport sur les protéines de tiques qui peuvent être impliquées dans l'allergie induite par les tiques, un problème de santé croissant en Europe et aux États-Unis (cette année, nous avons publié 4 manuscrits sur ce sujet). Le troisième point reflète notre intérêt pour la dimension écologique des interactions entre les tiques, les agents pathogènes et leurs hôtes. Pour cela, nous utilisons une combinaison d'analyse phylogénétique et de réseau. Dans ce point, nous avons publié cette année nos premiers résultats suggérant que *Ixodes ricinus*, le vecteur le plus important en Europe, a évolué pour maximiser son habitat pour qu'il se chevauche avec certains hôtes qui sont des épandeurs d'agents pathogènes (cette année, nous avons publié 2 manuscrits sur ce sujet). Enfin, notre laboratoire développe un nouveau domaine de recherche au sein de la communauté « tiques », l'épigénétique des tiques. Dans ce sens, je supervise la thèse de trois étudiants dont les résultats qui donneront les premières étapes vers une meilleure compréhension du rôle de l'épigénétique sur la biologie des tiques et les interactions tique-agent pathogène.

CURRICULUM VITAE

CABEZAS-CRUZ Alejandro

CR1 INRA
UMR BIPAR VECTOTIQ
ANSES (Laboratoire de Santé Animale de Maisons Alfort)
14 Rue Pierre et Marie Curie
94701 Maisons-Alfort Cedex
Tel 01 49 77 46 77
Email: alejandro.cabezas@vet-alfort.fr
Date of Birth: 14.01.1983

EDUCATION

– **10.2013 – 09.09.2016.** PhD in molecular and cellular aspects of biology

Institutions: University of Lille 2, Lille, France.

Thesis: *Schistosoma mansoni* sirtuins: characterization of their role in schistosome energy metabolism. **Très honorable avec félicitations**

Supervisor: Dr. Raymond Pierce

– **01.2014 – 10.10.2014.** PhD in basic and applied research in natural resources ¹

Institution: University of Castilla-La Mancha (UCLM), Ciudad Real, Spain.

Thesis: Evolution and molecular characterization of tick-borne Anaplasmataceae and implications for pathogen diagnostics and control. **Honors Degree (Cum Laude)**

Supervisor: Professor Jose de la Fuente

– **04.2012 – 09.2013.** Master in Parasitology, MSc. (Marie Curie fellowship)

Institution: University of South Bohemia, České Budějovice, Czech Republic.

Thesis: Cellular and molecular characterization of *Ehrlichia mineirensis* (UFMG-EV), a new organism isolated from *Rhipicephalus (Boophilus) microplus* ticks. **Honors Degree**

Supervisor: Professor Libor Grubhoffer

– **09.2001 – 06.2006.** Doctor of Veterinary Medicine, DVM

Institution: Agricultural University of Havana (UNAH), Havana, Cuba.

Thesis: Effect of tumor host microenvironment on growth, morphology and spontaneous metastases in a breast cancer mouse model. **Honors Degree**

Supervisor: Dr. Dasha Fuentes

¹ **Explanatory note:** After I finished my Master studies in September 2013, I matriculated in a PhD program in France to join my family (University Lille-2, Institute Pasteur of Lille, Lille, France). During January 2014, I matriculated in a second PhD program in Spain (Universidad de Castilla-La Mancha, Ciudad Real, Spain) under the supervision of Professor José de la Fuente and with approval of my supervisor in France, Dr. Raymond Pierce. That the two PhDs covered unrelated topics was a compulsory request from the University in France: The PhD at Universidad de Castilla-La Mancha, Spain, was related to the molecular characterization, evolution and epidemiology of tick-borne bacteria of the family Anaplasmataceae, while the second PhD degree at the Institute Pasteur of Lille, France, was related to metabolism regulation in the trematode *Schistosoma mansoni*. I finished the PhD in Spain last October 10, 2014 (day of PhD exam). I performed most of the work of the PhD in Spain during my Master studies as a side project. However, during the 9 months of PhD in Spain, I performed new experiments and published new manuscripts to complete the curricular requirements of this PhD. The PhD project in France has complemented my skills and knowledge in parasitology and epigenetics.

RESEARCH EXPERIENCE

- **2013 – 2016. Topic 1:** Evolution and molecular characterization of tick-borne Anaplasmataceae and study of host-pathogen interactions. Laboratory of Professor José de la Fuente: bacterial genetic diversity analysis (*Anaplasma marginale* and *Ehrlichia canis*), ecology and epidemiology of tick-borne pathogens, evolutionary analyses, tick-pathogen interactions (*Ixodes scapularis* - *Anaplasma phagocytophilum*), transcriptomics, proteomics. **Topic 2:** Metabolic regulation in the trematode *Schistosoma mansoni*. Laboratory of Dr. Raymond Pierce: qPCR, comparative genomics, transcriptional regulation, activity of histone deacetylases.
- **2012 – 2013.** Cellular and molecular characterization of *Ehrlichia minasensis*, a new organism isolated from *Rhipicephalus microplus* ticks. Laboratory of Professor Libor Grubhoffer: tick cell culture, in vitro culture of tick-borne pathogens (intracellular pathogens of the family Anaplasmataceae), electron microscopy, phylogenetic and sequence analysis.
- **2011.** Role of the innate and adaptive immunity in the pathogenesis of non-ischemic heart failure with main interest in inflammatory cardiomyopathy, diabetic cardiomyopathy and heart failure. Berlin-Brandenburg Center for Regenerative Therapies, Berlin, Germany. Laboratory of Dr. Sophie Van Linthout: experimental immunocardiology, mammalian cell culture, isolation of immune cells, qPCR.
- **2010.** Functional characterization of a novel immunomodulator from hard ticks that selectively reprogramme human dendritic cell responses. University of Oxford, Oxford, UK. Laboratory of Professor Pat Nuttall: molecular biology, recombinant protein expression in insect cells, in vitro culture of human dendritic cell, analysis of cytokines profiles.
- **2006 – 2010.** Recombinant vaccines against ticks. Identification and isolation of protective antigens from the dog tick *Rhipicephalus sanguineus*. Use of heterologous protein expression systems (i.e. *Escherichia coli* and *Pichia pastoris*). Cuban Center for Genetic Engineering and Biotechnology (CIGB), Havana, Cuba. Tick laboratory: molecular biology, biotechnology, biochemical analyzes, PCR, cloning, sequence analysis, recombinant protein expression and purification, vaccine preparation and immunization.
- **2001 – 2006.** Development of a murine model of breast cancer. Effect of age on the growth of breast cancer. Centre of Molecular immunology (CIM), Havana, Cuba. Laboratory of Dr. Enrique Montero: Immunology, cancer biology, animal models.

TEACHING AND THESIS DIRECTION

- **2017 – Present. Co-director of PhD thesis** of Pedro Jose Espinosa Prados.
Institution: University of Castilla-La Mancha, Spain
Thesis: Tick-pathogen interactions in *Anaplasma phagocytophilum*.
- **2016 – Present.** Director of **Undergraduate thesis** of Sewmi Madu, Hellen Chemtai and Julia Gobl
Institution: University of South Bohemia, Czech Republic

Thesis: Role of histone modifying enzymes in the embryogenesis of the soft tick *Ornithodoros moubata*.

- **2016.** Training on phylogenetic analysis for **Master 2** project of Jordi Nelis.
- **2012 – 2013.** Assistance and training on bioinformatics and molecular biology for **PhD thesis** of Miray Tonk.
- **2007 – 2010.** Director of **Undergraduate thesis** of Pedro Enrique.
Institution: Agricultural University of Havana, Cuba
Thesis: Genetic and biological characterization of a Cuban tick strain from *Rhipicephalus sanguineus* complex.
- **2002 – 2003.** Teaching assistant at the Agricultural University of Havana for first year undergraduate students (60h per year) courses in 'General Anatomy' and 'Comparative Anatomy'.

AWARDS

- **2017.** Award, Odile Bain Memorial Prize (OBMP)
Award, Marie Skłodowska-Curie Actions Seal of Excellence.
- **2015.** Award, Outstanding Reviewer Ticks and Tick-borne Diseases
- **2012.** Scholarship, Marie Skłodowska-Curie Early Stage Researcher
- **2010.** Award, Cuban Academy of Sciences

COMPETITIVE FUNDING

- **2017.** "Gratifications de Master" grant, Ecole Nationale Veterinaire de maisons alfort, ENVA (3 300 €)
- **2013 – 2016.** Doctoral Scholarship, University of Lille 2 (46 800 €)
- **2012 – 2013.** Early Stage Researcher, Marie Skłodowska-Curie Scholarship (81 022 €)

CONTRIBUTION AS REVIEWER

- **2014 – present.** Vaccine; BioMed Research International; Infection Genetics and Evolution; Ticks and Tick-borne Diseases; Revista brasileira de parasitologia veterinaria; Veterinary Parasitology; Letters in Applied Microbiology; Parasites & Vectors; Medical and Veterinary Entomology; BMC Veterinary Research; Acta tropica; Comparative Immunology, Microbiology & Infectious Diseases, Frontiers in Cellular and Infection Microbiology

INVITED PRESENTATIONS

- **2017.** Justus Liebig University Giessen, Giessen, Germany
Institute of Hygiene and Tropical Medicine, Lisbon, Portugal
Veterinary Research Institute, Brno, Czech Republic
South Bohemia University, České Budějovice, Czech Republic
- **2016.** Federal Research Institute for Animal Health, Jena, Germany
- **2015.** Institute of Hygiene and Tropical Medicine, Lisbon, Portugal

PUBLICATIONS

2017

1. **Cabezas-Cruz A.**, et al. 2017. Immunological basis of tick-induced allergy to red meat. *Trends in Parasitology*. 33(7): 495-498. doi: 10.1016/j.pt.2017.03.004.
2. **Cabezas-Cruz A.**, de la Fuente J. 2017. Is the blood type an overlooked risk factor in epidemiological studies of the α -Gal syndrome? *Allergy*.
3. **Cabezas-Cruz A.**, et al. 2017. Effect of blood type on anti- α -Gal immunity and the incidence of infectious diseases. *Experimental and Molecular Medicine*. 49(3): e301.
4. **Cabezas-Cruz A.**, et al. 2017. *Anaplasma phagocytophilum* infection subverts carbohydrate metabolic pathways in the tick vector, *Ixodes scapularis*. *Frontiers in Cellular and Infection Microbiology*. 7:23.
5. **Cabezas-Cruz A.**, et al. 2017. Remodeling of tick cytoskeleton in response to infection with *Anaplasma phagocytophilum*. *Frontiers in Bioscience*. 22(11):1830-1844.
6. **Cabezas-Cruz A.**, et al. 2017. Tick-pathogen ensembles: do molecular interactions lead ecological innovation? *Frontiers in Cellular and Infection Microbiology*. 7:74.
7. Estrada-Peña A., de la Fuente J., **Cabezas-Cruz A.** 2017. Functional redundancy and ecological innovation shape the communities of tick-transmitted pathogens. *Frontiers in Cellular and Infection Microbiology*. 7:234.
8. de la Fuente J., Antunes S., Bonnet S., **Cabezas-Cruz A.**, et al. 2017. Tick-pathogen interactions and vector competence: identification of molecular drivers for tick-borne diseases. *Frontiers in Cellular and Infection Microbiology*. 7:114.
9. Mateos-Hernández L., Villar M., Moral A., Rodríguez C G., Arias T A., de la Osa V., Brito F F., Fernández de Mera I G., Alberdi P., Ruiz-Fons F., **Cabezas-Cruz A.**, et al. 2017. Tick-host conflict: Immunoglobulin E antibodies to tick proteins in patients with anaphylaxis to tick bite. *Oncotarget*. doi: 10.18632/oncotarget.15243.
10. Contreras M., Alberdi P., Mateos-Hernandez L., Fernández De Mera I G., Garcia-Perez A L, Vancova M., Villar M., Ayllón N., **Cabezas-Cruz A.**, et al. 2017. *Anaplasma phagocytophilum* MSP4 and HSP70 proteins are involved in pathogen interaction with host cells but are only partially protective against pathogen infection in sheep. *Frontiers in Cellular and Infection Microbiology*. doi: 10.3389/fcimb.2017.00307.

2016

11. **Cabezas-Cruz A.**, et al. 2016. Antiplasmodial activity is an ancient and conserved feature of tick defensins. *Frontiers in Microbiology*. 7:1682.
12. **Cabezas-Cruz A.**, et al. 2016. Be aware of ticks when strolling through the park. *Frontiers for Young Minds*. 4(24).
13. **Cabezas-Cruz A.**, et al. 2016. Control of vector-borne infectious diseases by human immunity against α -Gal. *Expert Review of Vaccines*. 15(8):953-955.
14. **Cabezas-Cruz A.**, et al. 2016. *Anaplasma phagocytophilum* increases the levels of histone modifying enzymes to inhibit cell apoptosis and facilitate pathogen infection in the tick vector, *Ixodes scapularis*. *Epigenetics*. 11(4):303-19.
Figure selected for "Cover art" of Vol. 11, 2016, Epigenetics.
15. **Cabezas-Cruz A.**, et al. 2016. *Ehrlichia minasensis* sp. nov., isolated from the tick *Rhipicephalus microplus*. *International Journal of Systematic and Evolutionary Microbiology*. 66:1426-1430.
16. Valdés J J*, **Cabezas-Cruz A***, et al. 2016. Substrate prediction of *Ixodes ricinus* salivary lipocalins differentially expressed during *Borrelia afzelii* infection. *Scientific Reports*. 6:32372.
***Joint first authors.**
17. Tellier G., Lenne A., Cailliau-Maggio K., **Cabezas-Cruz A.**, et al. 2016. Identification of *Plasmodium falciparum* translation initiation 1 eIF2 β subunit: direct interaction with Protein Phosphatase type 1. *Frontiers in Microbiology*. 7:777.
18. de la Fuente J., Villar M., **Cabezas-Cruz A.**, et al. 2016. Tick-host-pathogen interactions: conflict and cooperation. *PLoS Pathogens*. 12(4): e1005488.
19. Estrada-Peña A., de la Fuente J., **Cabezas-Cruz A.** 2016. A comparison of the performance of regression models of *Amblyomma americanum* (L.) (Ixodidae) using life cycle or landscape data from administrative divisions. *Ticks and tick-borne diseases*. 7(4):624-30.
20. Ferrolho J., Antunes S., Santos A S., Velez R., Padre L., **Cabezas-Cruz A.**, et al. 2016. Detection and phylogenetic characterization of *Theileria* spp. and *Anaplasma marginale* in *Rhipicephalus bursa* in Portugal. *Ticks and tick-borne diseases*. 7(3): 443–448.
21. de la Fuente J., Villar M., Estrada-Peña A., **Cabezas-Cruz A.**, et al. 2016. *Anaplasma phagocytophilum* uses similar strategies to establish infection in tick vectors and vertebrate hosts. *Trends in Microbiology*. 24(3): 173–180.
22. Almazán C., González-Álvarez H., García-Fernández I., **Cabezas-Cruz A.**, et al. 2016. Molecular identification and characterization of *Anaplasma platys* and *Ehrlichia canis* in dogs in Mexico. *Ticks and tick-borne diseases*. 7(2):276-283.
23. Noda A A., Rodríguez I., Miranda J., Mattar S., **Cabezas-Cruz A.** 2016. First Report of Spotted Fever Group Rickettsia in Cuba. *Ticks and Tick-borne Diseases*. 7 (2016): 1057–1058.
24. Espinosa P J., Alberdi P., **Cabezas-Cruz A.**, et al. 2016. *Anaplasma phagocytophilum* manipulates host cell apoptosis by different mechanisms to establish infection. *Veterinary Sciences*. 3(15): vetsci3030015.
25. Villar M., López V., Ayllón N., **Cabezas-Cruz A.**, et al. 2016. The intracellular bacterium *Anaplasma phagocytophilum* selectively manipulates the levels of vertebrate host proteins in the tick vector *Ixodes scapularis*. *Parasites & Vectors*. 9:467.
26. Estrada-Peña A., Sprong H., **Cabezas-Cruz A.**, et al. 2016. Nested coevolutionary networks shape the ecological relationships of ticks, hosts and the Lyme disease bacteria, *Borrelia burgdorferi*. *Parasites & Vectors*. 9:517.

2015

27. **Cabezas-Cruz A.**, et al. 2015. Regulation of the immune response to α -Gal and vector-borne diseases. *Trends in Parasitology*. 31(10): 470-476.
 28. **Cabezas-Cruz A.**, et al. 2015. Fast evolutionary rates associated with functional loss in class I glucose transporters of *Schistosoma mansoni*. *BMC Genomics*. 16(1):980.
 29. **Cabezas-Cruz A** & de la Fuente J. 2015. *Anaplasma marginale* major surface protein 1a: a marker of strain diversity with implications for control of bovine anaplasmosis. *Ticks and tick-borne diseases*. 6(2015):205-210.
 30. **Cabezas-Cruz A.**, et al. 2015. Complete Genome Sequence of *Ehrlichia mineirensis*, a Novel Organism Closely Related to *Ehrlichia canis* with a New Host Association. *Genome Announcement*. 3(1):e01450-14.
 31. Tonk M*, **Cabezas-Cruz A***, et al. 2015. *Ixodes ricinus* defensins attack distantly-related pathogens. *Developmental & Comparative Immunology*. 53(2):358-365.
- *Joint first authors.**
32. Lancelot J., **Cabezas-Cruz A.**, et al. 2015. Schistosome sirtuins as drug targets. *Future Medicinal Chemistry*. 7(6): 765-782.
 33. Alberdi P., Ayllón N., **Cabezas-Cruz A.**, et al. 2015. Infection of *Ixodes* spp. tick cells with different *Anaplasma phagocytophilum* isolates induces the inhibition of apoptotic cell death. *Ticks and tick-borne diseases*. 6(6):758-767.
 34. Tonk M., Knorr E., **Cabezas-Cruz A.**, et al. 2015. *Tribolium castaneum* defensins are primarily active against Gram-positive bacteria. *Journal of invertebrate pathology*. 132: 208-215.
 35. Kocan K M., de la Fuente J., **Cabezas-Cruz A.** 2015. The genus *Anaplasma*: New challenges after reorganization. *Revue Scientifique et technique (International Office of Epizootics)*. 34(2):577-586.
 36. Estrada-Peña A., de la Fuente J., Ostfeld R., **Cabezas-Cruz A.** 2015. Interactions between tick and transmitted pathogens evolved to minimise competition through nested and coherent networks. *Scientific Reports*. 5: 10361.
 37. Ayllón N., Villar M., Galindo R C., Kocan K M., Šíma R., López J., Vázquez J., Alberdi P., **Cabezas-Cruz A.**, et al. 2015. Systems Biology of Tissue-Specific Response to *Anaplasma phagocytophilum* Reveals Differentiated Apoptosis in the Tick Vector *Ixodes scapularis*. *PLoS Genetics*. 11(3): e1005120.
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CONFERENCES/PRESENTATIONS/POSTERS

- **2017**: IX International Conference on Ticks and Tick-borne Pathogens (TTP-9), Cairns, Australia (ORAL PRESENTATION)
International Congress on Rickettsia and other Intracellular Bacteria (ESCCAR) 2017, Marseille, France (ORAL PRESENTATION)
- **2016**: Workshop on Arthropod-Borne Diseases, Jena, Germany (ORAL PRESENTATION)
- **2015**: Molecular and Cellular Biology of Helminths IX, Hydra, Greece (POSTER)
- **2014**: VIII International Conference on Ticks and Tick-borne Pathogens (TTP-8), Cape Town, South Africa (POSTER)
- **2013**: II Tick-borne diseases workshop, Berlin, Germany (ORAL PRESENTATION)
International Symposium on Ectoparasites of Pets (ISEP) conference 2013, Munich, Germany (POSTER)
XII. International Jena Symposium on Tick-Borne Diseases, Jena, Germany (POSTER)
- **2012**: POSTICK immunology workshop, Research Center Borstel, Borstel, Germany (ATTENDING)
Joint conference on emerging and reemerging epidemics affecting global health, Orvieto, Italy (POSTER)

Euroscience Open Forum (ESOF) and Marie Curie Actions Conferences, Dublin, Ireland
(ATTENDING)

I Tick-borne diseases workshop, Berlin, Germany (ORAL PRESENTATION)

POSTICK bioinformatics workshop, University of Glasgow Veterinary School, Glasgow, UK
(ATTENDING)

RESEARCH ACTIVITY

Introductory note.

Ticks transmit more pathogens to humans and animals than any other arthropod (Gulia-Nuss et al., 2016). At the beginning of February 2016, the first tick genome was published (Gulia-Nuss et al., 2016). This is a major breakthrough in the study of ticks and related microorganisms. Genome analysis revealed amazing adaptations contributing to the parasitic lifestyle of the tick and its success as a vector of multiple disease-causing organisms. Whereas much information has been collected in the last years regarding the ecological association of ticks and major tick-borne pathogens, we lack knowledge about the particular tick-pathogen interactions that contribute to pathogen persistence in nature. More importantly, we have yet to understand how pathogen coinfections (Moutailler et al., 2016) impacts pathogen survival within the tick and affect the epidemiology of particular tick-borne pathogens. The **Pathobiome** concept is a theoretical framework in which ecology, molecular biology, microbiology and epidemiology are integrated to better understand tick-host-pathogen interactions and zoonotic pathogen circulation (Vayssier-Taussat et al., 2015). Combining tick and microbial “omics” technologies, next-generation sequencing (NGS), experimental evolution and ecological network analysis we aim to decipher the tick-host-pathogen entanglement to ultimately develop control measures to improve animal and human health.

Relevant experience in ticks and tick-borne pathogens research:

1- Ticks and tick-borne pathogens.

From 2006 to 2010, after finishing a **DVM (Doctor of Veterinary Medicine)** degree with **First Class Honours** in the University of Havana, I worked in the Center for Genetic Engineering and Biotechnology (CIGB) in Cuba (López et al., 2007). The CIGB is one of the most important research and production facilities in Cuba and it produces the only registered recombinant vaccine against cattle ticks in the world, called Gavac (de la Fuente et al., 1999). **My main duties were to find new antigens to improve Gavac and to establish a laboratory strain of tick for preclinical assays.** Beyond the benefits of being part of a tick research group with considerable experience in the topic, I developed relevant molecular biology skills. In the search for new antigens, I constructed cDNA

libraries from tick gut and used them as a source of vaccine candidates. After literature mining and identification of proteins involved in key physiological processes, the putative candidates were amplified by Polymerase Chain Reaction (PCR). Cloning and genetic manipulation of *Escherichia coli* was then used to obtain plasmids containing genes of interest for further protein expression in the yeast *Pichia pastoris*. At the same time, I was performing field collection of ticks and with the collected specimens, I established the *Rhipicephalus sanguineus* life cycle in laboratory conditions. Therefore, at this stage, **while mastering standard molecular biology techniques, I familiarized myself with the ecology and life cycle of ticks in tropical environments.**

2- Internship in the laboratory of Professor Pat Nuttall, Oxford.

In 2010, I spent six months in the laboratory of Professor Pat Nuttall who is a leading scientist in the field of tick research. She made a decisive contribution to the so-called Saliva-Activated Transmission (SAT) concept (Jones et al., 1987). During feeding, ticks secrete a myriad of pharmacologically active proteins that constitute a major component of vector-host interactions (Nuttall and Labuda, 2004). In addition, these molecules assist pathogen transmission (Nuttall and Labuda, 2004). In this lab, I worked in the production and functional characterization of a tick lipocalin with immunomodulatory effects, Japanin. In a *PLoS Pathogens* publication (Preston et al., 2013), **we established the role of the tick lipocalin Japanin in inhibiting human dendritic cell (DC) maturation.** Remarkably, we showed that Japanin inhibits CD86 upregulation in response to multiple DC maturation stimuli (Figure 1). In another attempt to identify novel tick lipocalins, we mined the *Ixodes ricinus* salivary gland transcriptome for specific, uncharacterized lipocalins and three were identified (Valdés et al., 2016). Both structural analysis and biophysical simulations generated robust predictions showing these *I. ricinus* lipocalins have the potential to bind monoamines similar to other tick species previously reported (Valdés et al., 2016). Differential expression of these *I. ricinus* lipocalins during feeding at distinct developmental stages and in response to *Borrelia afzelii* infection suggests a role in transmission of this Lyme disease spirochete (Valdés et al., 2016).

We cannot understand the impact of the tick pathobiome on pathogen transmission without understanding its contribution to the vector-host interface. The tick pathobiome contribution to pathogen transmission could go beyond specific proteins with anti-haemostatic, anti-inflammatory and immunomodulatory effects.

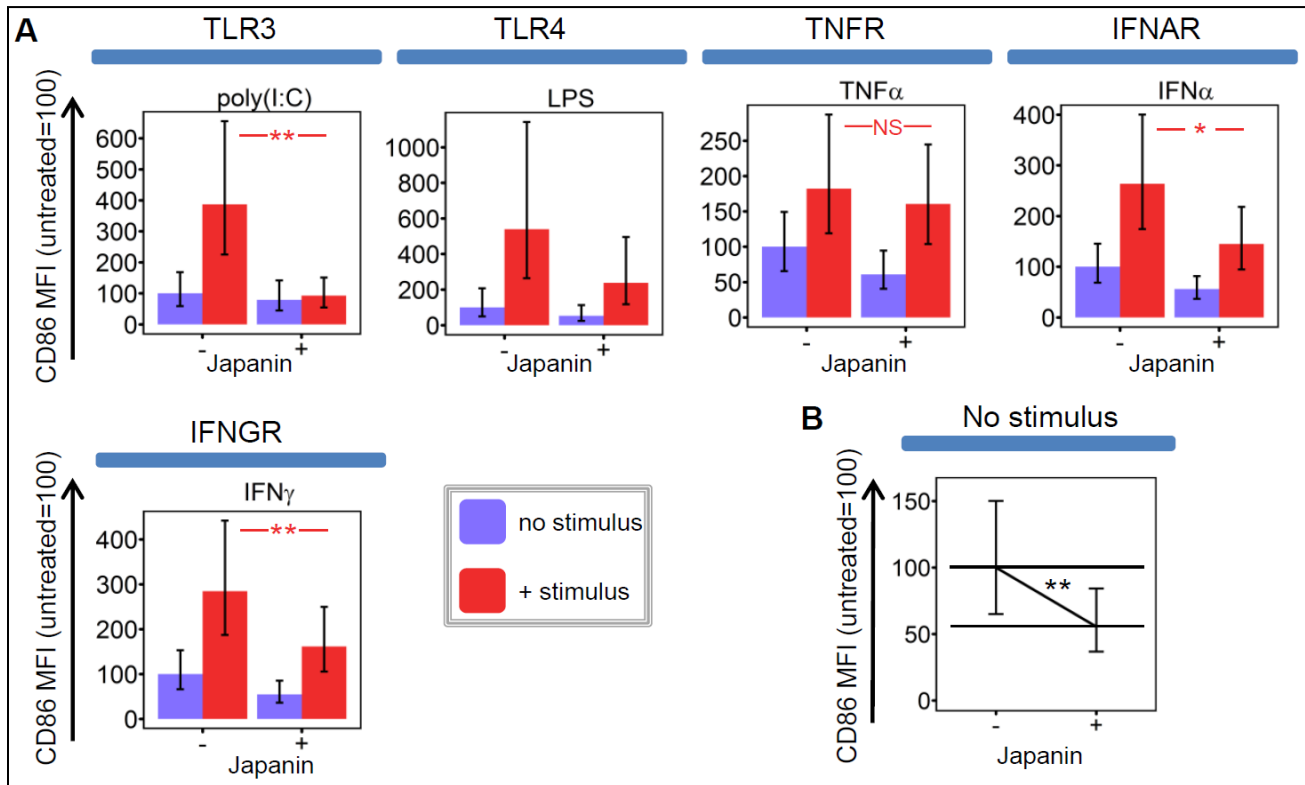


Figure 1. Japanin inhibits CD86 upregulation in response to multiple DC maturation stimuli. (A) Dendritic cells were cultured for 18–20 hours in the presence or absence of Japanin (500 ng/ml) and stimuli: (25 mg/ml Poly(I:C), via TLR3; 100 ng/ml LPS, TLR4; 4 mg/ml CL097, TLR7/8; 20 ng/ml IFN α 2, IFNAR; 10–12.5 ng/ml TNF α , TNFR; 20 ng/ml IFN γ , IFNGR). CD86 expression was then assessed by flow cytometry. Modelled means \pm 95% confidence intervals using data from at least four experiments are shown, except for CL097 for which three experiments were performed, using cells from a total of five human donors. (B) Data from all these experiments was used to assess the effect of Japanin on CD86 expression in the absence of stimuli. ** $P < 0.01$, * $P < 0.05$, NS $P > 0.05$. Figure from (Preston et al., 2013).

3- Tick-induced allergy and anti- α -Gal immunity

Recently, arthropod vectors (i.e. tick species, e.g. *Amblyomma americanum*) have been involved in emerging anaphylactic diseases. In particular, the IgE antibody response to the carbohydrate α -Gal after tick bite was associated with allergies to red meat, Cetuximab (therapeutic monoclonal antibody) and gelatin. We proposed that particular tick microbiota bacteria could be producing α -Gal that is secreted to the host inducing an IgE antibody response that may assist pathogen

transmission (Cabezas-Cruz et al., 2015a, 2017a). My expertise in this area is relevant as I can transfer my knowledge relative to SAT and contribute in the debate and demonstration of novel hypothesis regarding the role of α -Gal-producing tick microbiota on pathogen transmission. **In the current debate on this topic, I contributed with three opinions published in leading journals, *The LANCET infectious diseases* (Cabezas-Cruz et al., 2014a) and *Trends in Parasitology* (Cabezas-Cruz et al., 2015a, 2017a). In addition, recently we identified the tick proteins containing the α -Gal modification (Mateos-Hernández et al., 2017).** Another very interesting output of my research, related to the anti- α -Gal immunity, is the discovery that blood group B has a major role in driving malaria and tuberculosis epidemiology. We found a significant, **positive correlation between the frequency of blood group B and the incidence of malaria and tuberculosis in Sub-Saharan Africa and Spain and Portugal, respectively.** However, no correlation was found between blood type and dengue fever incidence (Figure 2).

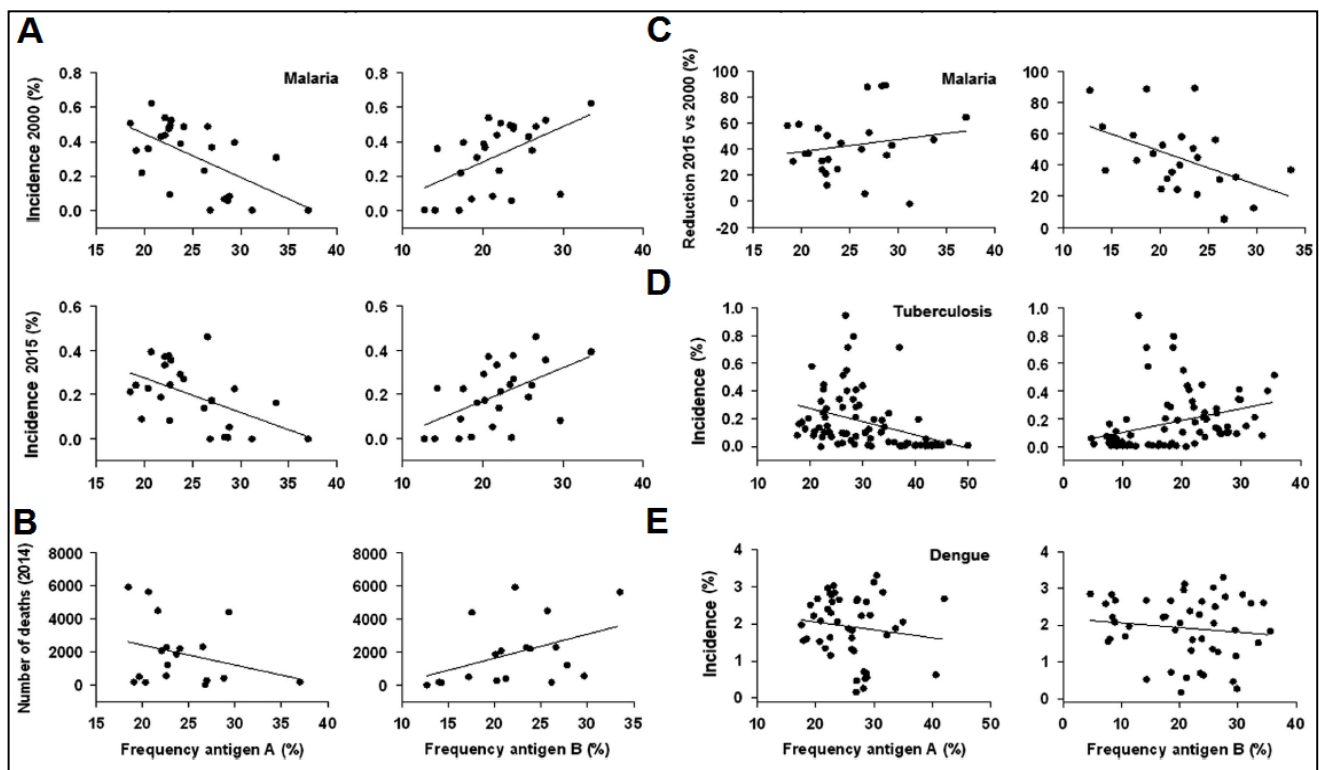


Figure 2. Correlation between ABO blood types and disease. (A) Correlation analysis between the frequencies of blood types A and B and malaria incidence in Africa in 2000 and 2015. Spearman's rank correlation coefficient tests were significant for blood types A (Spearman $r \leq -0.50$, $P \leq 0.004$) and B (Spearman $r \geq 0.55$, $P \leq 0.002$) in both years 2000 and 2015. (B) Correlation between the number of deaths due to malaria in 2014 and the frequencies of blood types A (Spearman $r = -0.19$,

$P=0.21$) and B (Spearman $r=0.50$, $P=0.01$) in Africa. (C) Correlation between the percentage of reduction in malaria incidence (2015 vs 2000) and the frequencies of blood types A (Spearman $r=0.28$, $P=0.09$) and B (Spearman $r=-0.46$, $P=0.01$) in Africa. (D) Correlation between the frequencies of blood types A (Spearman $r=-0.54$, $P<0.0001$) and B (Spearman $r=0.51$, $P<0.0001$) and the incidence of tuberculosis in Africa, Asia, America and Europe. (E) Correlation between the frequencies of blood types A and B and the incidence of dengue fever in Africa, Asia, America and Europe. Figure from (Cabezas-Cruz et al., 2017b).

My hypothesis, and that of my collaborators, is that the presence of blood group B decreases the levels of antibodies against the antigen α -Gal that were recently shown to protect against *Plasmodium falciparum* transmission by *Anopheles* spp. mosquitoes (Yilmaz et al., 2014). Group B antigen is very similar to α -Gal, therefore the possibility exist that the presence of blood antigen B induces tolerance to antigen B-like antigens, such as α -Gal. We further hypothesized that anti- α -Gal immunity may have a broader effect on pathogens expressing the antigen α -Gal on their surfaces. To further test this hypothesis, we collected serum samples from *P. falciparum* and *Mycobacterium tuberculosis* infected and uninfected individuals from Senegal and Western Europe, respectively. Surprisingly, we found that individuals infected with these pathogens in Africa (*P. falciparum*) and Europe (*M. tuberculosis*) had significantly lower anti- α -Gal antibodies titers compared to the uninfected individuals (Cabezas-Cruz et al., 2017b). We are currently following this lead with promising results and further interesting hypothesis to test. This line of research may result in promising control measures against infectious diseases. We have recently published some of these ideas in a special editorial published in *Expert Review of Vaccines* (Cabezas-Cruz et al., 2016a).

4- Getting into the European context and connecting with the tick community: POSTICK.

In 2012, I obtained an Early Stage Researcher (ESR) fellowship for 18 months upon applying to a highly competitive Marie Skłodowska-Curie action in Czech Republic as part of a project called POSTICK (Post-graduate training network for capacity building to control ticks and tick-borne diseases)². From the beginning of the project, I devoted myself to research to obtain the MSc in Parasitology within 1.5 years, much quicker than average, and **received my master with Honours**. More importantly, the project gave me the possibility to be at hand with many of the leaders of tick

² http://cordis.europa.eu/result/rcn/165816_en.html (accessed 26-06-2017)

research in Europe like Dr. Lesley Bell-Sakyi (tick cell lines, UK), Professor José de la Fuente (anti-tick vaccine development and tick proteomics and transcriptomics, Spain), Professor Libor Grubhoffer (tick carbohydrate research, Czech Republic) and Professor John K Fazakerley (Director of The Pirbright Institute, UK). **My collaborative network includes tick research leaders in Europe. During my master, as a Marie Curie fellow, I isolated (Cabezas-Cruz et al., 2012), cultured (Zweygarth et al., 2013; Cabezas-Cruz et al., 2013a) and characterized at the molecular (Cabezas-Cruz et al., 2012) and ultrastructure (Cabezas-Cruz et al., 2013a) levels a new species of pathogenic tick-borne bacterium from Brazil. In addition, in collaboration with Professor de la Fuente, I sequenced and annotated the genome of this bacterium (Cabezas-Cruz et al., 2015b) that was officially recognized by the International Committee on Systematics of Prokaryotes as *Ehrlichia minasensis* (Cabezas-Cruz et al., 2016b).** These bacteria have morphological features typical of intracellular pathogens of the family (Figure 3).

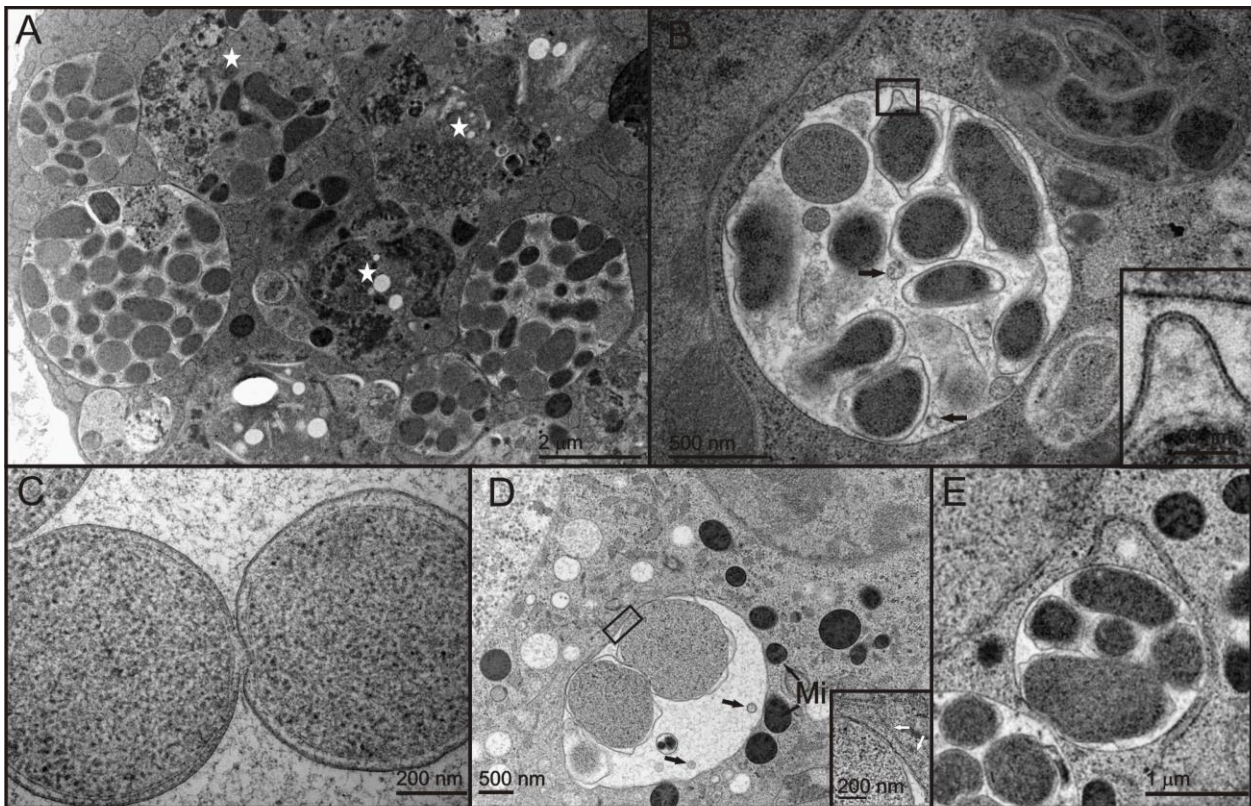


Figure 3. Electron micrograph of *Ehrlichia minasensis* – infected IDE8 cells. (A) Cells containing phagolysosomes/secondary lysosomes (white asterisk) and numerous vacuoles with bacteria. (B) Electron-dense bodies and small vesicles (black arrows) inside membrane-lined vacuoles. The inset shows in detail the rippled membrane. (C) Reticulate cells undergoing binary fission. (D) The vacuole shows in detail the rippled membrane. (E) The vacuole

containing reticulate bodies that have ruffled outer membrane and small vesicles (black arrows) is surrounded with mitochondria (Mi) and microtubules (detail in inset, white arrows). (E) A cisterna of endoplasmic reticulum in tight contact with the membrane of the morulae. Figure from (Cabezas-Cruz et al., 2013a).

After POSTICK

The major goal of POSTICK was to create a training network for capacity building to control ticks and tick-borne diseases in Europe. During and after POSTICK, I built a fruitful collaborative network, my interest for aspects related to ticks and tick-borne diseases increased substantially and I proved my capacity to conduct and lead original research in many collaborative projects.

1- Tick innate immunity collaborative project.

Tick innate immunity is based on an orchestrated action of humoral and cellular immune responses. The direct antimicrobial defense in ticks is accomplished by a variety of small molecules such as the antimicrobial peptides called defensins. In collaboration with Dr. Miray Tonk (a Marie Curie fellow from POSTICK) at the Institute for Insect Biotechnology, Giessen, Germany, we identified a novel multigene family of defensins in *I. ricinus*, the most common tick species in Europe (Tonk et al., 2014a). These defensins were found to be active against distantly-related pathogens: fungi, gram-negative and gram-positive bacteria, i.e. they have a wide antimicrobial spectrum (Tonk et al., 2015a). We also characterized the antimicrobial activity of defensins from *Ixodes scapularis* (Tonk et al., 2014b) and the model organism *Tribolium castaneum* (Tonk et al., 2015b). A long standing question in the evolution of defensins is the contribution of amino acid variability to antimicrobial activity. Recently, we addressed this question by deciphering the amino acid sequence of an ancestral tick defensin (a chelicerate which existed approximately 444 million years ago), producing the synthetic protein, testing its antimicrobial activity and comparing it with that of extant tick defensins (Cabezas-Cruz et al., 2016c). Our results showed that this ancestral protein (named Scorpions-Ticks Defensins Ancestor, STiDA) has a conserved defensin fold (determined by 3D modelling) and has antimicrobial activity. However, the antimicrobial spectrum is reduced compared to extant defensins. This infers that amino acid variability has a major impact in increasing the antimicrobial spectrum of tick defensins (Cabezas-Cruz et al., 2016c). In addition, we

discovered that antiplasmodial activity is an ancient and conserved feature of tick defensins (Cabezas-Cruz et al., 2016c) (Figure 4).

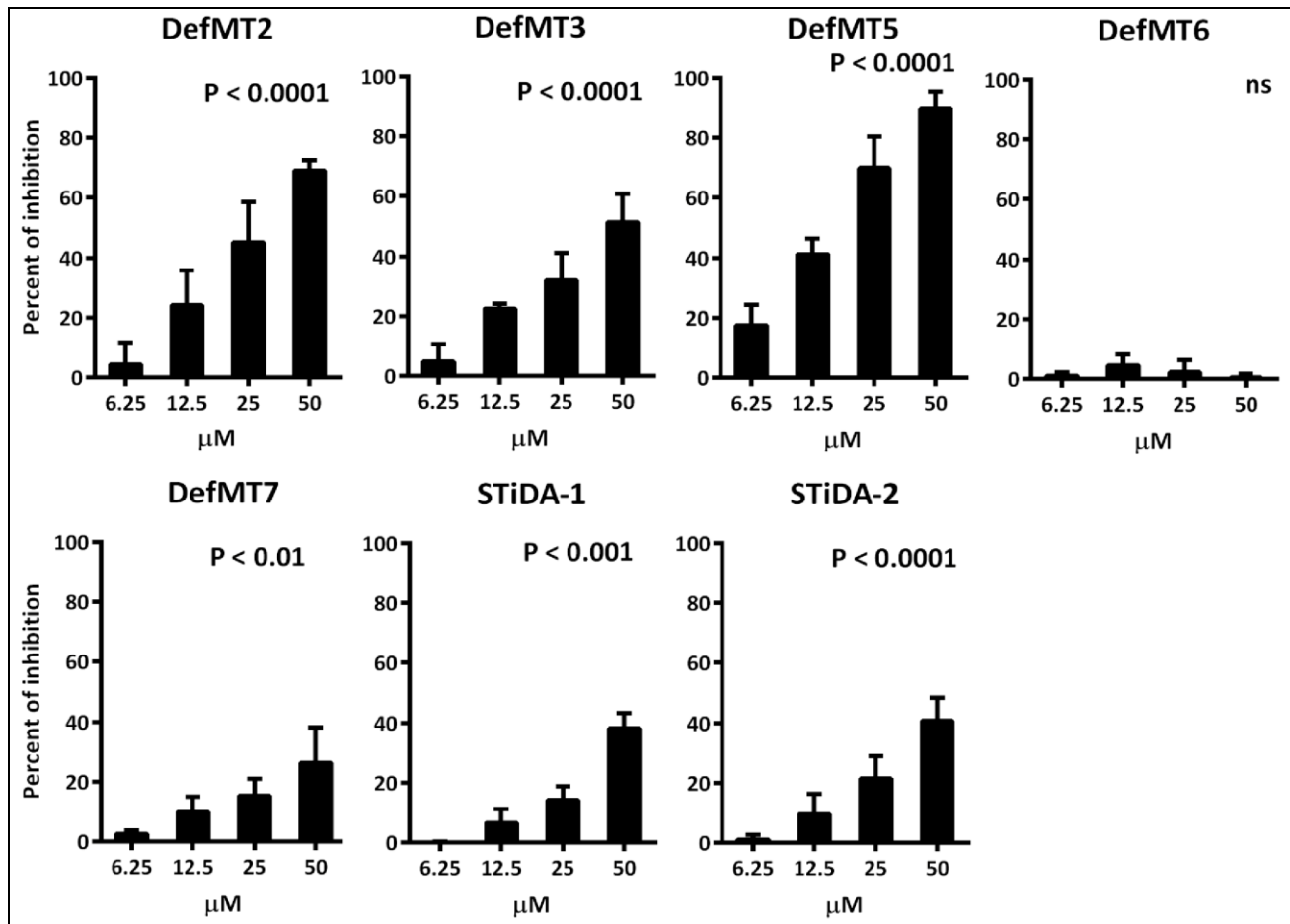


Figure 4. Anti-*Plasmodium falciparum* activity of STiDA and *Ixodes ricinus* defensins. The percentage of *P. falciparum* growth inhibition induced by STiDA and *I. ricinus* defensins was calculated. Increasing concentrations (6.25–50 mM) of STiDA (1 and 2), DefMT2, DefMT3, DefMT5, and DefMT7 correlate with higher percentage of inhibition. No effect was observed using DefMT6. The non-parametric Kruskal–Wallis test was used to test the null hypothesis, i.e., that the medians of all groups were equal. Differences were considered significant when $P < 0.05$. Figure from (Cabezas-Cruz et al., 2016c).

This collaborative project on tick innate immunity involved researchers from at least two European countries and has produced five publications in peer-reviewed journals. Further studies should clarify whether tick defensins have antiplasmodial activity *in vivo*.

Remarkably, none of the pathogens found to be susceptible to tick defensins (Tonk et al., 2014b, 2015a; Cabezas-Cruz et al., 2016c) is transmitted by ticks, suggesting that **tick defensins control the growth of microorganisms encountered during off-host periods or those present in the tick microbiota as occurs in other organisms (Giorgetti et al., 2015).**

2- Epidemiology and genetic diversity of tick-borne bacteria, the first PhD project.

The family Anaplasmataceae (Class Alphaproteobacteria) is composed by two major genera of tick-borne bacteria that include *Anaplasma* and *Ehrlichia*. Particularly, *A. marginale*, the agent of bovine anaplasmosis, is an important constraint in the cattle industry in tropical countries and *E. canis* is an important pathogen of dogs that can affect humans. I applied a phylogenetic approach to study the epidemiology and characterize the genetic variability of *A. marginale* (Cabezas-Cruz et al., 2013b; Pohl et al., 2013; Silva et al., 2014ab; Mutshembele et al., 2014; Baêta et al., 2015) and *E. canis* (Zweygarth et al., 2014) using surface proteins as genetic markers. **This resulted in a “Cum Laude” PhD degree under the supervision of Professor de la Fuente at the University of Castilla-La Mancha, Ciudad Real, Spain.** As result of the analysis from the doctoral thesis, we proposed the use of the major surface protein 1 alpha (MSP1a) of *A. marginale* as a way to study the epidemiology, strain diversity of this pathogen, and to develop potential control measures for bovine anaplasmosis (Cabezas-Cruz and de la Fuente, 2015). In collaboration with researchers from South Africa and Brazil, I studied the epidemiology and genetic diversity of *A. marginale* in these two countries in both water buffalo (Silva et al., 2014ab) and cattle (Pohl et al., 2013; Mutshembele et al., 2014; Baêta et al., 2015) herds. **One of the main achievements of the thesis was to provide, for the first time, molecular evidence regarding the prevalence of *A. marginale* in 8 of the 9 South African provinces (Mutshembele et al., 2014).** In the thesis, I placed a major emphasis on the genetic variability of *A. marginale* strains in relation to different tick vector species. From the results obtained in the thesis, I concluded that in ecoregions where *Dermacentor* spp. are the main *A. marginale* vectors (e.g. USA), the genetic diversity is lower than in ecoregions where *Rhipicephalus* spp. are the main vectors (e.g. South Africa). Interestingly, we found that the amino acid variability of MSP1a tandem repeats, which have been implicated in tick-pathogen interactions (de la Fuente et al., 2003), is different among *A. marginale* strains from different countries (Figure 5).

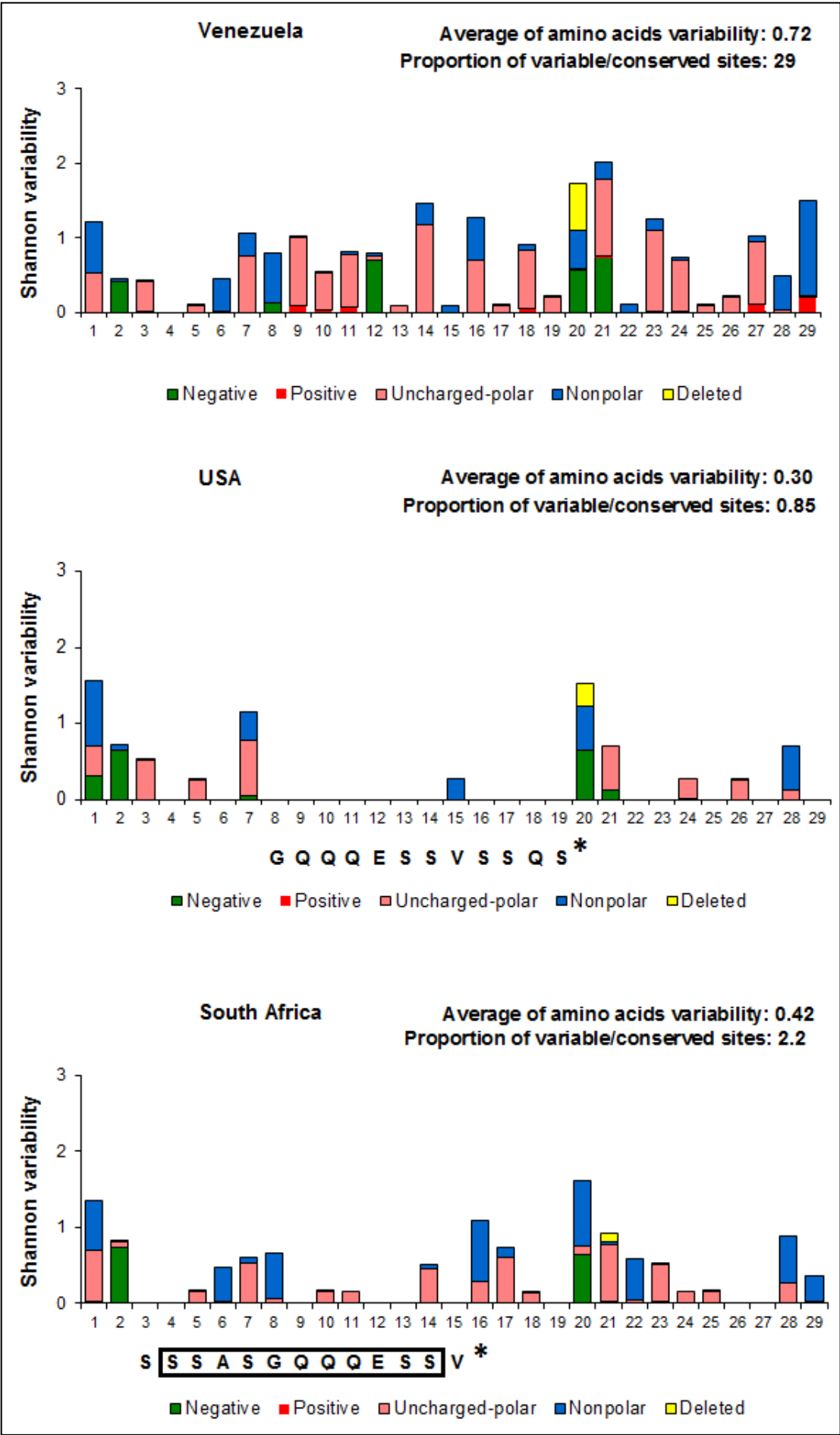


Figure 5. MSP1a amino acid variability, composition, and low-variable peptides. The figure shows the amino acid variability and composition among the tandem repeats in 3 different countries: Venezuela, USA, and South Africa. Venezuela shows a high proportion of variable/conserved sites and a high average of amino acid variability while South Africa and the USA show middle and lower values, respectively. Different colours in columns depict different biochemical properties in the amino acid composition: negative (green), positive (red), uncharged-polar (beige), and non-polar (blue); proportion of deleted positions is shown in yellow. Consensus sequences of low-variable peptides are shown (*) for the USA and South Africa. The region of the immunodominant B-cell epitope from *A. marginale* (Garcia-Garcia et al., 2004) is boxed in the low-variable peptide from South Africa. Figure from (Mutshembele et al., 2014).

Although my main experience in genetic classification of *A. marginale* strains is using MSP1a, I have also used a multilocus sequence analysis (MLSA) approach using a combination of low variable genes (i.e. *hsp70* and *sodB*) and other major surface protein genes (i.e. *msp4* and *msp5*) (Lis et al., 2015). Regarding *E. canis*, using the glycoprotein TRP36, a major immunogenic protein, we proposed that *E. canis* strains were divided into 2 well-defined phylogenetic clusters (Zweygarth et al., 2014). Resuming, **this doctoral thesis included eleven publications in specialized peer-reviewed journals.**

After finishing the first PhD, I strengthened my collaborative network and started to deepen the acquired knowledge and to find new applications for it.

1- Emergence of new tick-borne pathogens

After characterizing *E. minasensis* (Cabezas-Cruz et al., 2012; Zweygarth et al., 2013; Cabezas-Cruz et al., 2013a; Cabezas-Cruz et al., 2015b; Cabezas-Cruz et al., 2016b), I was intrigued by the fact that it is closely related to *E. canis*, but it was isolated from *R. microplus* ticks (Cabezas-Cruz et al., 2012; Carvalho et al., 2016), while *E. canis* is mainly found in *R. sanguineus*. Independently, another group in Brazil reported *E. minasensis* to be pathogenic for cattle (Aguiar et al., 2014). However, *E. canis* can be considered as a dog-specific pathogen and has not been found to be pathogenic in cattle. Accordingly, we hypothesized that *E. minasensis* recently evolved from *E. canis*. The emergence of new pathogens is frequently associated to mutations that confer the ability to infect novel hosts, known as “host shift”. We performed phylogenetic and evolutionary analysis using all available *E.*

canis TRP36 nucleotide and protein sequences and its ortholog in *E. minasensis* (Cabezas-Cruz et al., 2014b; Aguiar et al., 2013). From these analyses, we concluded that *E. minasensis* evolved from a highly variable clade of *E. canis* under adaptive diversifying selection which resulted in a host shift (Cabezas-Cruz et al., 2014b) (Figure 6).

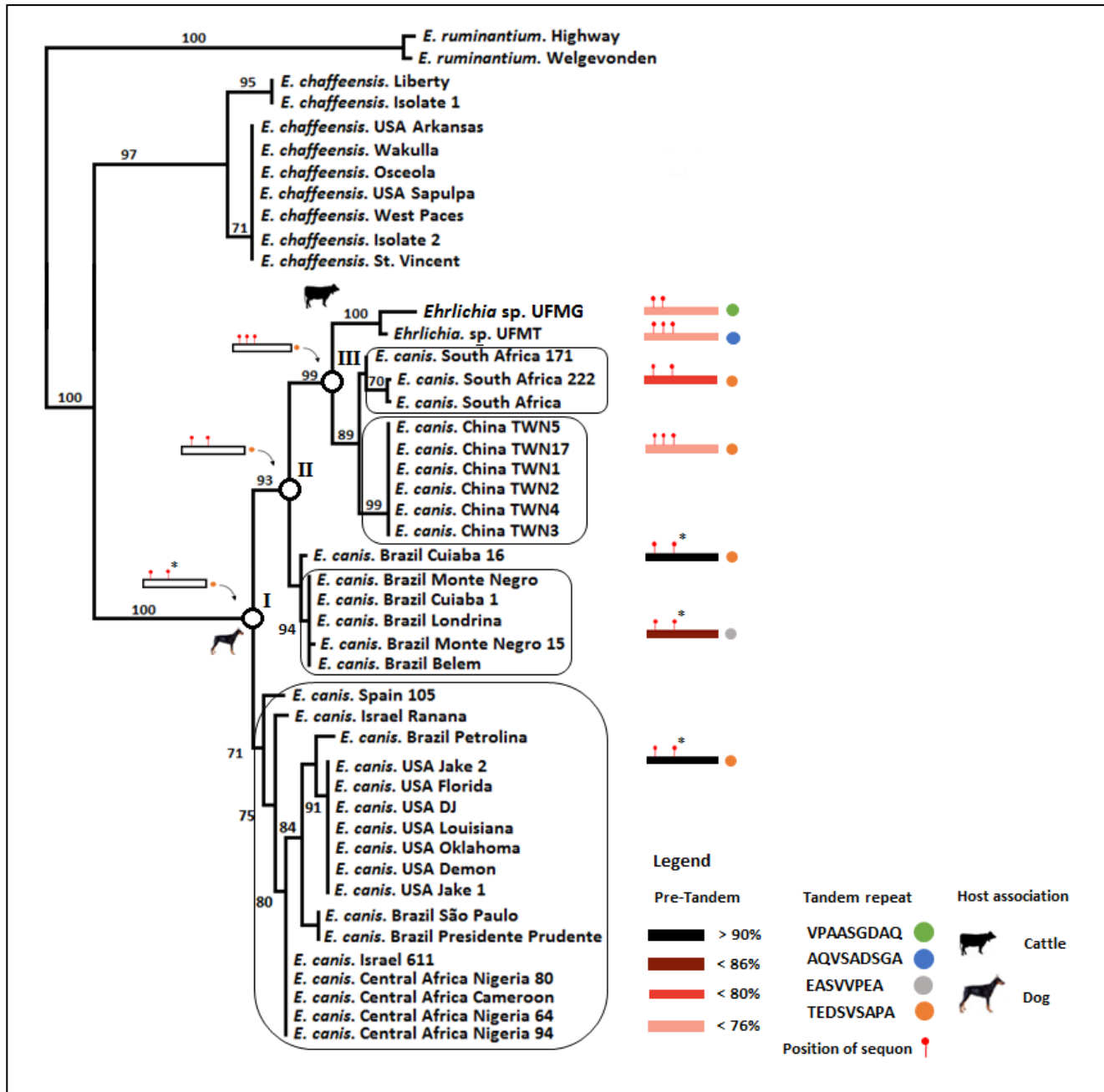


Figure 6. *Ehrlichia minasensis* belongs to a variable clade within *E. canis*. The *trp36* (*E. canis*), *trp47* (*E. chaffeensis*) and mucin-like protein gene (*E. ruminantium*) nucleotides sequences were aligned and gap regions removed. Phylogenetic analyses were conducted using maximum likelihood (ML)

and neighbor joining (NJ). The figure shows that *Ehrlichia* sp. UFMG-EV and *Ehrlichia* sp. UFMT-BV fall in a divergent clade of *E. canis trp36* having low homology (less than 80%: red and pink boxes) compared to the isolate *E. canis* USA Jake 2 that was taken as a reference. The amino acid sequence of the different TRP36 tandem repeats variants are shown (Coloured circles). The positions of the sequons are shown (red sticks on the boxes). The position of TRP36 ancestor clades I, II and III at internal branches (white circles) and position of sequons on ancestors (red sticks on white boxes) are also shown. The topologies obtained with the two methods, ML and NJ, were similar. The numbers above the internal branches represent bootstrap values. Only bootstrap values higher than 70 are shown. Figure from (Cabezas-Cruz et al., 2014b).

The host shift in this context may have occurred in a scenario where dogs infected with a variable *E. canis* strain, as previously found in Brazil (Aguiar et al., 2013), were the source of infection for *R. microplus* or *R. sanguineus* ticks that later infested cattle. Both tick species are able to infect dogs and cattle (Dantas-Torres, 2009; Szabó et al., 2010; Mirzaei and Khedri, 2014). The scenario involving *R. microplus* is unlikely as this is a one-host tick species that infests only one host during its life cycle. However, *R. sanguineus* is a three-host tick species, thereby increasing the chances of horizontal pathogen transmission among different hosts. Changes in evolutionary pressures on *E. canis*, related to new host association, may have selected a completely new species. These results have major implication for the epidemiology of tick-borne bacteria because it is generally assumed that the only *Ehrlichia* causing bovine ehrlichiosis is *E. ruminantium*. However, **according to our results and those by the group of Dr. Daniel Aguiar in Brazil (Aguiar et al., 2014; Carvalho et al., 2016), *E. minasensis* and associated bacterial strains may constitute an emerging cause of bovine ehrlichiosis in the Americas.**

Interestingly, it was suggested that *Coxiella burnetii*, the causative agent of Q fever, recently evolved from an inherited symbiont of ticks (Duron et al., 2015) such as those that constitute the microbiota. This supports the hypothesis that under certain conditions, tick microbiota bacteria could become pathogenic (“host shift”-like).

2- Studying ecological associations between ticks, hosts and pathogens using networks analysis.

Recently, in collaboration with Professor Agustin Estrada-Peña at the University of Zaragoza, Spain, we applied **ecological networks methodology to unravel the complex interactions between ticks,**

their vertebrate hosts and pathogens in the western Palearctic (Estrada-Peña et al., 2015). Using data mining, we searched keywords that included the name of the genera of ticks reported from western Palearctic (e.g. *Argas*, *Boophilus*, *Dermacentor*, *Haemaphysalis*, *Hyalomma*, *Ixodes*, *Ornithodoros*, and *Rhipicephalus*) combined with a logical “OR” with the names of the genera of pathogens reported from the same territory (e.g. *Anaplasma*, *Babesia*, *Borrelia*, *Ehrlichia*, *Haemolivia*, *Hepatozoon*, *Neoehrlichia*, *Rickettsia*, *Theileria*, Crimean-Congo hemorrhagic fever virus, Tick-borne encephalitis virus) combined with a logical “OR” with the names of European or northern African countries. Using this approach **we collected and quantified > 14,000 interactions among ticks, vertebrates, and pathogens in the western Palearctic (Estrada-Peña et al., 2015).** Several indices (e.g. Nestedness, Centrality and Modularity) were used to measure the network properties and quantify the relevance of the associations. We found that: (i) the tick-host-pathogen natural networks are highly structured, modular, coherent, and nested to some degree, (ii) the large number of vertebrates in the network contributes to its robustness and persistence, (iii) the network structure reduces interspecific competition and allows ample but modular circulation of transmitted pathogens among vertebrates, (iv) remarkably, accounting for domesticated hosts collapses the network’s modular structure, linking groups of hosts that were previously unconnected and increasing the circulation of pathogens (Figure 7). **We concluded that ticks and vertebrates interact along the shared environmental gradient, while pathogens are linked to groups of phylogenetically close reservoirs (Estrada-Peña et al., 2015).**

In another work, using ecological network analysis we explored the role of birds in the dissemination of tick-borne pathogens. **The study of tick-host-pathogen associations reveals a prominent role for birds in the dissemination of *Borrelia* spp. (e.g. *B. burgdorferi* the causative agent of Lyme disease in humans) and *A. phagocytophilum* (the causative agent of human granulocytic anaplasmosis), with little contribution to the possible dissemination of other tick-borne pathogens (de la Fuente et al., 2015).**

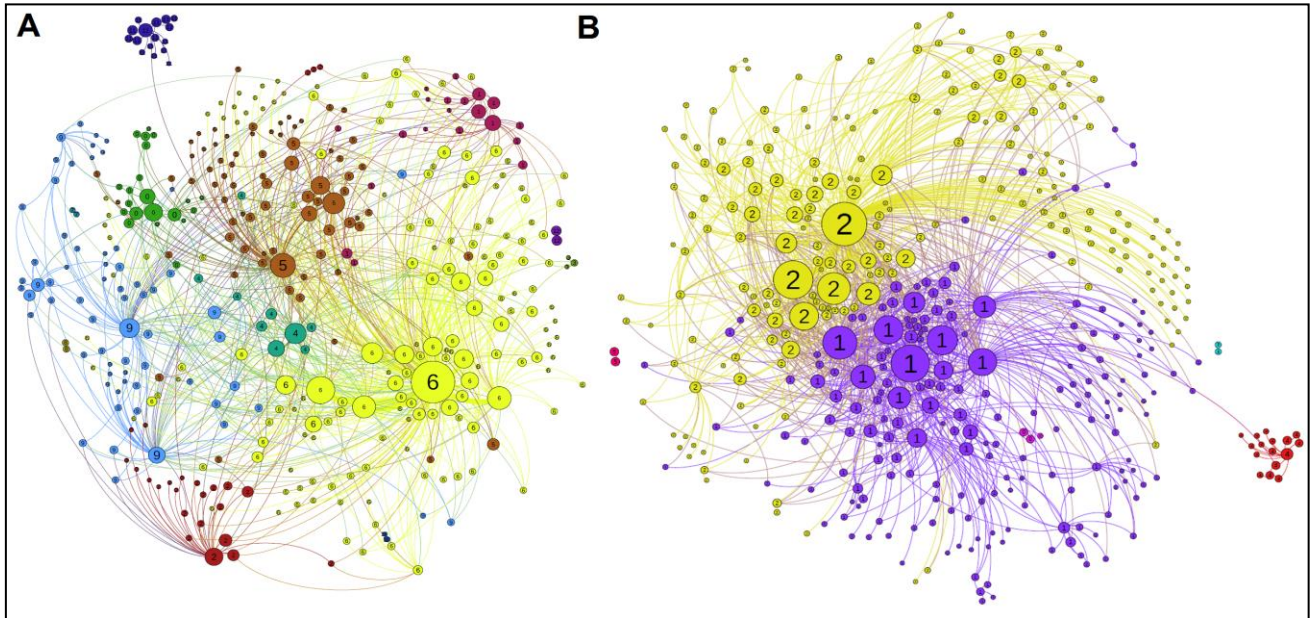


Figure 7. The network of ticks, vertebrates and pathogens in the Western Palearctic. (A) The network without domesticated animals. The 13 clusters found by a modularity algorithm, represented according to the ForceAtlas2 scheme, randomly coloured, and numbered from 0 to 12 (the agglomerative algorithm include minimum values of 0). Each circle is a partner of the network, a vertebrate, tick, or pathogen. The size of each circle is proportional to its NBC. Each line is a link between two nodes, and its colour is the same as that of the cluster. The width of each line is proportional to the weighted degree, a measure of the strength of the link between two nodes. (B) Changes in the structure modularity of the network of ticks, pathogens, and vertebrates in the western Palearctic after the inclusion of domestic vertebrates. These changes were detected by the modularity algorithm, are represented according to the ForceAtlas2 scheme, and are randomly coloured. Figure modified from (Estrada-Peña et al., 2015).

We have shown in four manuscripts (Estrada-Peña et al., 2015,2016,2017 de la Fuente et al., 2015) in peer-reviewed journals (including the journal *Scientific Reports*) that ecological network analysis is an exceptional tool to study tick-microorganism and tick-host ecological associations. We can adapt this methodology to quantify the relevance of the microbiota in pathogen circulation. NGS projects, such as those performed currently to study the ecology of tick associated microorganisms (Vayssier-Taussat et al., 2013; Bonnet et al., 2014; Razzauti et al., 2015) generate large data sets that can be combined with ecological networks methodology.

3- Host-pathogen interactions.

I was involved in the characterization of molecular interactions between *A. phagocytophilum* and its tick vector *I. scapularis* (Ayllón et al., 2015ab; Villar et al., 2015; Alberdi et al., 2015; Contreras et al., 2017) in collaboration with Professor de la Fuente in Spain. Recently, in a ***PLoS Genetics*** publication (Ayllón et al., 2015a), we applied a Systems Biology approach combining transcriptomic and proteomics to dissect a host-parasite system in which the tick-borne bacteria ***A. phagocytophilum* manipulates the apoptotic pathways of the tick host to establish infection**. In addition, we found that **different *A. phagocytophilum* isolates inhibit apoptosis in various *Ixodes* spp. tick cells (Alberdi et al., 2015)**. These results suggested an impact of vector-pathogen co-evolution on the adaptation of *A. phagocytophilum* isolates to grow in tick cells as each isolate grew better in the tick cell line derived from its natural vector species (Alberdi et al., 2015). We further established that *A. phagocytophilum* increases the level of certain bacterial stress response (i.e. GroEL and HSP70) and surface (i.e. MSP4) proteins during tick cells and salivary glands infection with functional implication in tick-pathogen interactions (Villar et al., 2015). **We further showed that *A. phagocytophilum* MSP4, GroEL and HSP70 interact and bind to tick (Villar et al., 2015, Figure 8) and human (Contreras et al., 2017, Figure 9) cells**, thus playing a role in rickettsia-host interactions.

In an attempt to understand tick cellular immunity to intracellular pathogens, we characterized the role of Tudor-SN in dsRNA-mediated RNA interference (RNAi) in response to infection with *A. phagocytophilum*. Interestingly, we found that **tick Tudor-SN dsRNA-mediated RNAi do not affect *A. phagocytophilum* infection (Ayllón et al., 2015b)**. This is an interesting finding considering that RNAi mechanisms protect the host against intracellular pathogens in other host-pathogen systems. After collecting additional data, in a ***PLoS Pathogens*** publication (de la Fuente et al., 2016a) we concluded that **tick-host-pathogen ensembles should be understood as evolutionary units with conflicting, but also cooperative interactions**.

This has been a very fruitful collaborative project resulting in seven publications in peer-reviewed journals, including *PLoS Genetics* (Ayllón et al., 2015a), *PloS Pathogens* (de la Fuente et al., 2016a) and *Trends in Microbiology* (de la Fuente et al., 2016b).

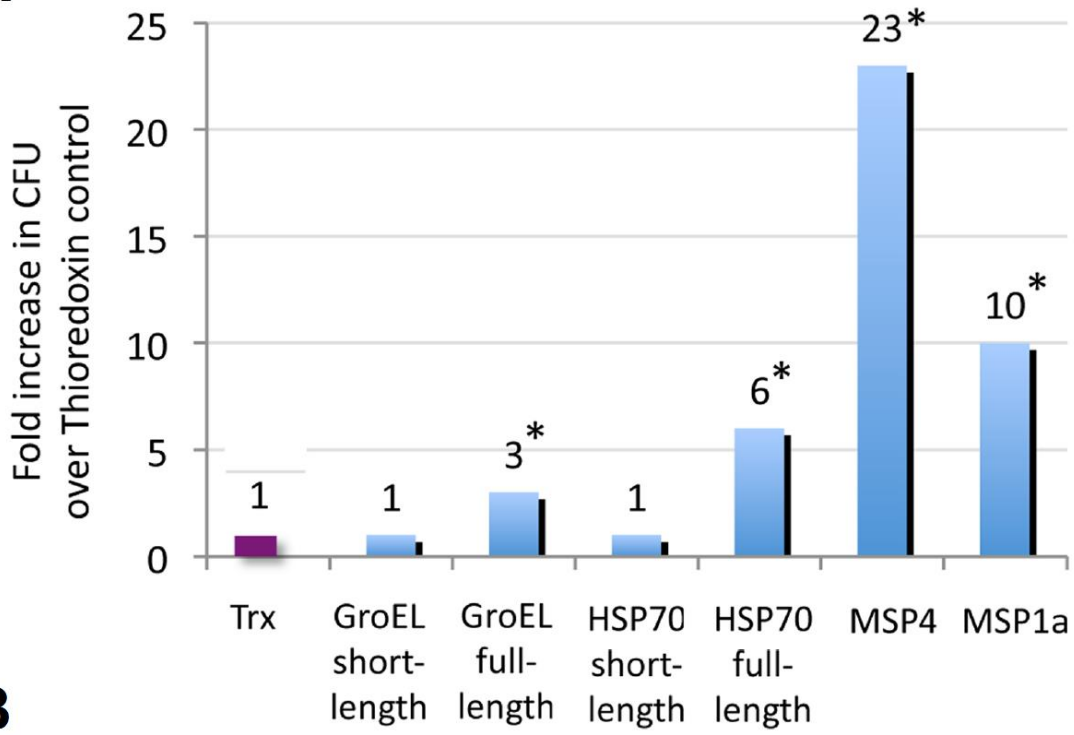
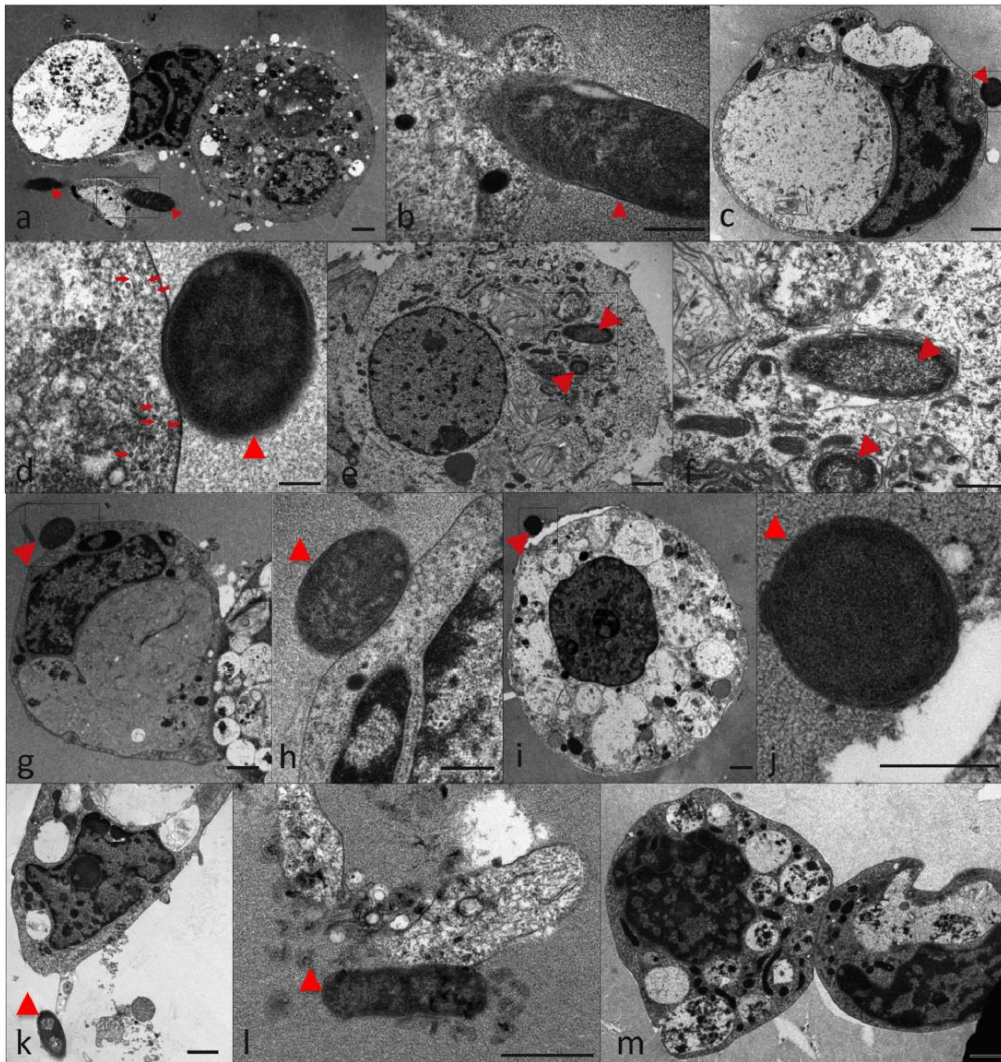
A**B**

Figure 8. Characterization of *A. phagocytophilum* protein adhesion to tick cells. (A) *E. coli* strains were grown and induced for the production of recombinant proteins. *E. coli* strains with expression vector alone producing recombinant Thioredoxin and *A. marginale* MSP1a were used as negative and positive control, respectively. Adhesive bacteria were quantitated as the number of colony forming units (CFU) recovered from each test, shown as fold increase and compared to the Thioredoxin (Trx) control values by Student's t-test for paired samples (* $P < 0.05$). (B) Electron micrographs of ISE6 tick cells reacted with recombinant *E. coli* producing *A. phagocytophilum* recombinant proteins. Representative images of recombinant *E. coli* shown by arrowheads adhered to tick cells. (a, b) *A. marginale* MSP1a positive control; (c-f) MSP4; (g-k) HSP70; (l) GroEL; (m) Thioredoxin negative control. Microtubules are shown by arrows in (d). Scale bars, 1 μm (a, c, e, g, i, k-m), 500 nm (b, d, f, h, j). Figure modified from (Villar et al., 2015).

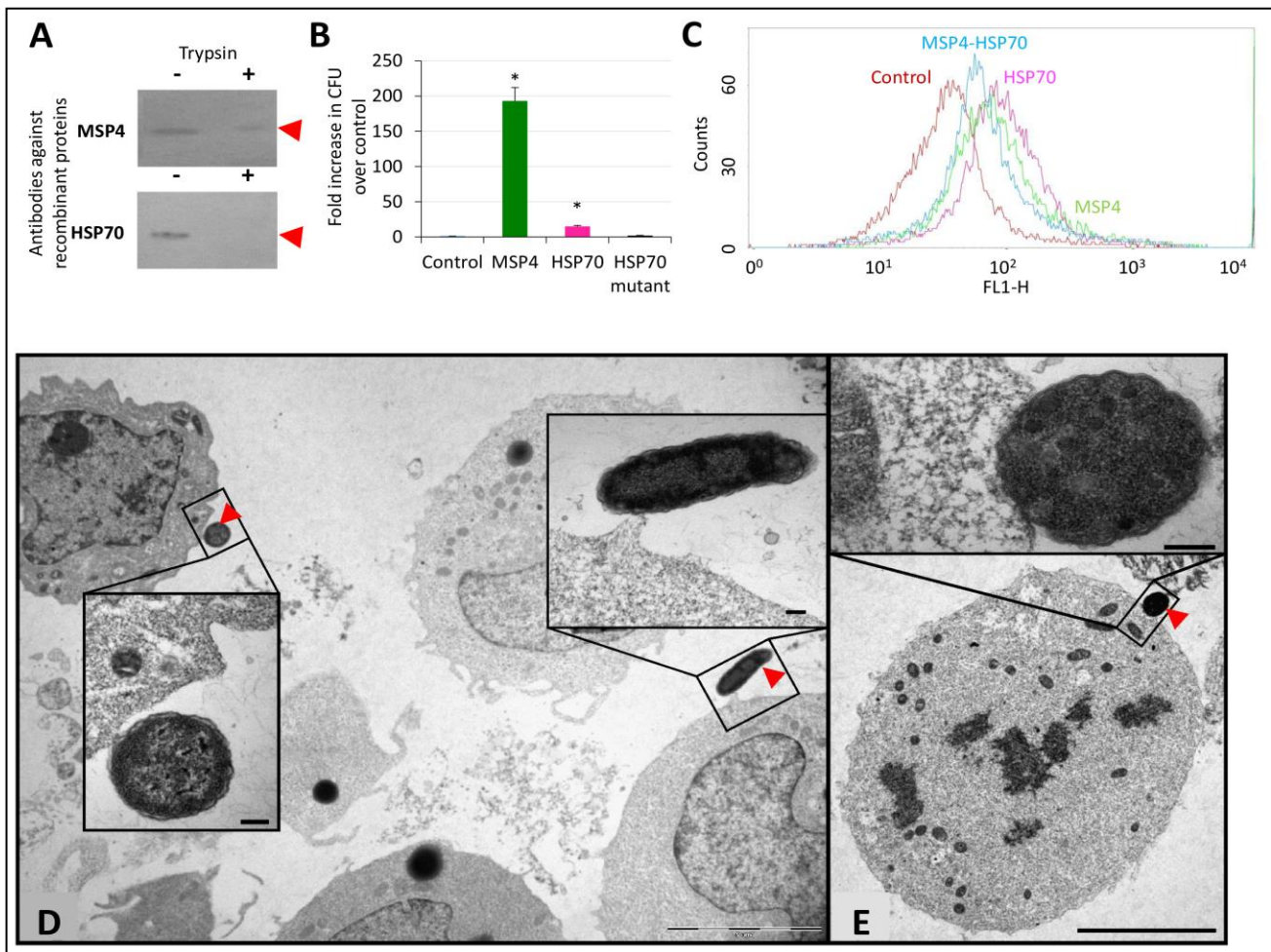


Figure 9. Role of *A. phagocytophilum* MSP4 and HSP70 proteins in interactions with HL60 human cells. (A) *A. phagocytophilum* (NY18) purified from infected HL60 human cells were mock treated (–) or surface digested with trypsin (+) and 10µg protein loaded onto polyacrylamide gels for Western blot analysis using rabbit antibodies produced against recombinant proteins. These experiments have been repeated three times with similar results. (B) *E. coli* strains were grown and induced for the production of recombinant proteins. *E. coli* cells transformed with expression vector alone were used as negative control (Control). Bacteria adhesion to human HL60 cells was quantitated as the number of colony forming units (CFU) recovered from each test and compared to the control values by Chi2 test (**P* < 0.001; N = 2 replicates per treatment). (C) HL-60 cells were incubated with *A. phagocytophilum* human NY18 isolate in the presence of recombinant MSP4, HSP70 and their combination. HL60 cells incubated with PBS served as negative controls. After washing to remove unbound bacteria and proteins, host cells were incubated for 48 h and binding of recombinant proteins to human host cells was analyzed by flow cytometry. The viable cell population was gated according to forward scatter and side scatter parameters. (D, E) Representative images of the adhesion of recombinant *E. coli* producing membrane exposed *A. phagocytophilum* proteins to human HL60 cells. (D) *E. coli* producing MSP4 or (E) HSP70 were incubated with HL60 cells and revealed by TEM to show adhesion to human cells (arrows). Cells incubated with control bacteria did not show adhesion to HL60 cells. Details of both interacting cells are shown in insets. Scale bars: 5µm (D, E) and 200 nm (insets). Figure from (Contreras et al., 2017).

New horizons in ticks and tick-borne pathogen interactions research: Tick epigenetics.

During my 10 years (2006-present) working on ticks, I noticed the lack of knowledge on the epigenetic machinery of ticks as well as on the epigenetic mechanisms that tick-borne pathogens use to manipulate tick physiology. **Epigenetics has bloomed in the last 20 years as a promising area of research.** When “epigenetics” was used to search PubMed³, 3498 publications were found in 2015 compared to 16 publications in 2000. However, when the terms “epigenetics AND ticks” or “histone modifying enzymes AND ticks” were used to search PubMed, only 1 publication was found. This probably reflects a lack of researchers with formation in epigenetics in the tick community. Therefore, during my PhD in Spain, **I matriculated for a second PhD on parasite epigenetics in France.** This PhD was devoted to the study the role of histone modifying enzymes (HMEs) in the

³ <http://www.ncbi.nlm.nih.gov/pubmed> (accessed 28–06–2017)

regulation of *Schistosoma mansoni* metabolism with Dr. Raymond Pierce, *Institut Pasteur de Lille*. This second PhD on epigenetics has complemented my formation in parasitology and further led me to apply various skills. I will not detail my *Schistosoma* PhD project here as I want to focus on my tick skills, but **I was able to publish three manuscripts during this second PhD (Cabezas-Cruz et al., 2014c,2015c; Lancelot et al., 2015)**. Epigenetic modifications of histones (mainly tails) impact gene expression and have major implications in the response of organisms to environmental changes. **In a pioneer work on tick epigenetics, we identified 39 histones and HMEs genes in the *I. scapularis* genome/transcriptome and characterized phylogenetically and structurally 5 histones and 34 HMEs, including histone acetyltransferases (HATs), histone deacetylases, sirtuins, histone methyltransferases and histone demethylases (Cabezas-Cruz et al., 2016d)**. As an example, Figure 10 displays the molecular characterization of the HATs that were found in the *I. scapularis* genome. Then, we used transcriptomics and proteomics data to analyze the mRNA and protein levels of these *I. scapularis* histones and HMEs in response to *A. phagocytophilum* infection of tick tissues and cultured cells (Figure 11). Finally, selected HMEs were functionally characterized by pharmacological studies in cultured tick cells. The results suggested that *A. phagocytophilum* manipulates histones and HMEs and by this epigenetic mechanism this pathogen simultaneously regulates several defense mechanisms such as apoptosis to increase infection and multiplication in tick cells (Cabezas-Cruz et al., 2016d). **This is a groundbreaking work for the tick community!**

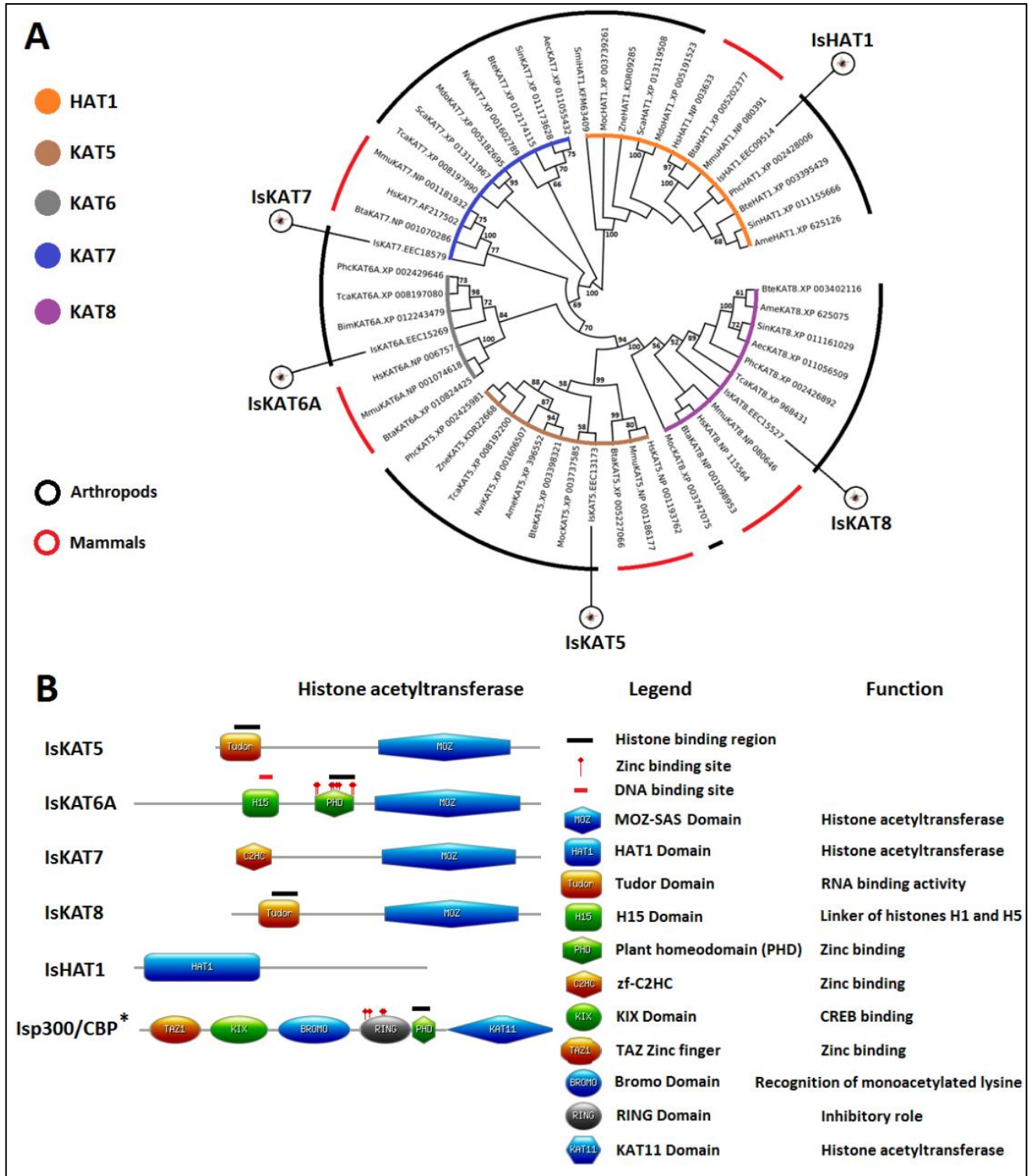


Figure 10. Characterization of *I. scapularis* HATs. (A) Phylogenetic analysis of HAT amino acid sequences. A neighbor joining phylogenetic tree was built using the amino acid sequences of the HATs identified in *I. scapularis* (IsHAT1, IsKAT5, IsKAT6A, IsKAT7 and IsKAT8) and their homologs in *Homo sapiens* (Hs), *Mus musculus* (Mm), *Bos Taurus* (Bta), *Apis mellifera* (Ame), *Acromyrmex*

echinator (Aec), *Bombus terrestris* (Bte), *Bombus impatiens* (Bim), *Musca domestica* (Mdo), *Nasonia vitripennis* (Nvi), *P. h. corporis* (Phc), *Stomoxys calcitrans* (Sca), *Solenopsis invicta* (Sin), *T. castaneum* (Tca), *Zootermopsis nevadensis* (Zne), *Megasoma occidentalis* (Moc), and *Stegodyphus mimosarum* (Smi). Bootstrap values for internal branches and GenBank accession numbers are shown. (B) Major protein domains found in *I. scapularis* HATs.

Histones	Genome accession	GenBank ID	Length (aa)	mRNA				Protein					
				N	G	SG	ISE6	N	G	SG	ISE6		
IsH1	ISCW016230	EEC00176	172										
IsH2A	ISCW019497	EEC09557	124										
IsH2B	ISCW019496	EEC09556	124										
IsH3	ISCW002300	EEC02656	136										
IsH4	ISCW019498	EEC09558	103										
Histone acetyltransferase													
IsKAT5	ISCW020584	EEC13173	454										
IsKAT6A	ISCW021150	EEC15269	1659										
IsKAT7	ISCW013774	EEC18579	427										
IsKAT8	ISCW020926	EEC15527	441										
IsHAT1	ISCW007480	EEC09514	411										
IsP300/CBP	ISCW021822	EEC13782	1851										
Histone deacetylases class I/II													
IsHDAC8	ISCW007923	EEC09382	413										
IsHDAC1	ISCW007830	EEC10597	484										
IsHDAC3	ISCW012528	EEC17638	392										
IsHDAC4	ISCW014481	EEC18867	900										
Histone deacetylases class III (Sirtuins)													
IsSirt2	ISCW023257	EEC19211	430										
IsSirt1	ISCW006267	EEC07078	276										
IsSirt6	ISCW014656	EEC19029	363										
IsSirt5	ISCW011143	EEC14061	304										
IsSirt7	ISCW021135	EEC15416	403										
Histone methyltransferases													
IsSETDB1-B	ISCW005550	EEC08546	1043										
IsSETDB1-A	ISCW001711	EEC01716	744										
ISEHMT	ISCW019618	EEC11008	1033										
IsSETD7	ISCW020034	EEC12894	184										
IsSETD2	ISCW004542	EEC05044	729										
IsSETD4	ISCW020125	EEC12062	429										
IsDOT1L	ISCW021226	EEC14772	325										
IsSMYD4	ISCW000409	EEC01028	770										
IsSMYD3	ISCW012780	EEC16757	374										
IsSMYD5	ISCW000706	EEC00979	413										
Histone demethylases													
IsLSD1-A	ISCW004580	EEC06619	666										
IsLSD1-B	ISCW019383	EEC11164	772										
IsUTY	ISCW011899	EEC17277	1166										
IsJARID2-A	ISCW006964	EEC10887	713										
IsJMJD1B	ISCW018588	EEC07701	754										
IsJARID1A	ISCW013765	EEC18990	1356										
IsJMJD2B	ISCW019624	EEC10206	952										
IsJARID2-B	ISCW006962	EEC10886	173										

Up in infected ticks No difference Down in infected ticks No found

Figure 11. *I. scapularis* histone and HME mRNA and protein levels in response to *A. phagocytophilum* infection. Comparison of histone and HME mRNA and protein levels in *I. scapularis* nymphs (N), female midguts (G), female salivary glands (SG), and ISE6 cells (ISE6) in response to *A. phagocytophilum* infection. Figure from (Cabezas-Cruz et al., 2016d).

CONCLUSIONS

The research activities I have conducted so far had let me to a better understanding of tick biology and ecology, the epidemiology of tick-borne pathogens as well as tick-host-pathogen interactions.

- The results show that tick saliva is a complex mixture of pharmacologically active molecules. The identification of novel tick salivary lipocalins may provide alternative treatment for human diseases. The capacity of Japanin to inhibit human dendritic cell maturation can be used to treat autoimmune or inflammatory diseases.
- The anti- α -Gal immunity plays an important role in the incidence of infectious diseases such as malaria and tuberculosis. There is a positive correlation between blood group B and the incidence of malaria and tuberculosis, presumably due to the low anti- α -Gal antibody levels associated to blood group B individuals.
- *Ehrlichia minasensis* is a novel species of *Ehrlichia* closely related to *E. canis* that constitute an undiagnosed and overlooked cause of bovine ehrlichiosis in the Americas. The evolutionary context in which *E. minasensis* emerged offers the importunity to use *E. minasensis* and *E. canis* genome comparisons to uncover the genetic basis of host-shift within the genus *Ehrlichia*.
- Tick defensins have a wide antimicrobial spectrum and therefore are promising molecules to develop antibacterial, antifungal and antiplasmodial drugs.
- Tick vectors influence the evolution of the genetic diversity of tick-borne bacteria of the family Anaplasmataceae.
- Network analysis is a promising tool for the study of tick-host-pathogen interactions at the ecological level. The combination of high throughput sequencing and network analysis may be used to disentangle the complexity of microbial communities of ticks and hosts within and between ecosystems.
- *Anaplasma phagocytophilum* is an excellent model for the study of tick-pathogen interactions. Our results show that *A. phagocytophilum* uses *I. scapularis* HMEs to manipulate the tick response to bacterial infection.

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UMR BIPAR ANSES, ENVA, INRA

