

THÈSE

Pour obtenir le grade de
Docteur

Délivré par l'**Université de Montpellier**

Préparée au sein de l'école doctorale
CBS2 Et de l'unité mixte de recherche
MIVEGEC (Centre IRD 911 avenue
agropolis 34394 Montpellier)

Spécialité : **Biologie-Santé**

Présentée par Jorian PRUDHOMME

**Phlébotomes et écosystèmes : impact des
facteurs biotiques et abiotiques sur la
structure génétique et phénotypique des
populations.**

Soutenue le 18 décembre 2015 devant le jury composé de

Mr. Gérard DUVALLET, Professeur émérite, Université Paul-Valéry-Montpellier III	Président
Mr. Jérôme DEPAQUIT, Professeur des Universités, Faculté de Pharmacie, Université de Reims	Rapporteur
Mr. Thierry BALDET, Chargé de recherche, International Development Research Center	Rapporteur
Mr. Rémi CHARREL, Professeur des Universités, Faculté de Médecine de Marseille	Examineur
Mme Anne-Laure BAÑULS, Chargée de recherche, Institut de Recherche pour le Développement	Co-directrice
Mr. Denis SERENO, Chargé de recherche, Institut de Recherche pour le Développement	Co-directeur



Je dédie cette thèse à ma mère

"Avec l'amour maternel, la vie vous fait à l'aube une promesse qu'elle ne tient jamais."
Romain Gary, La promesse de l'aube

Remerciements

Cette thèse a été financée par le projet européen EDENext mais aussi par Mr. Lacoste, à qui j'exprime ma profonde gratitude pour m'avoir fait confiance dans ce travail et m'avoir permis de finir cette thèse ;

Thierry Baldet d'avoir accepté d'être le rapporteur de ma thèse et pour sa présence durant la réalisation de mon master ;

Jérôme Depaquit pour m'avoir tant appris sur les phlébotomes, pour nos discussions, son accueil au sein de son équipe et d'avoir accepté d'être le rapporteur de ma thèse ;

Remi Charrel pour avoir accepté de juger ce travail, pour tous les congrès passés ensemble et pour m'avoir appris sa célèbre technique de networking aux pauses-café de ces mêmes congrès ;

Gérard Duvallet pour tout ce que vous avez fait pour moi, pour votre soutien durant mon master, pour m'avoir fait découvrir le monde des stomoxes et d'avoir accepté d'être examinateur de ma thèse ;

Denis Sereno, mon co-directeur, pour avoir accepté de m'encadrer, m'avoir permis de faire cette thèse, de m'avoir soutenu dans son accomplissement et pour m'avoir fait confiance durant ces trois ans ;

Anne-Laure Bañuls, ma co-directrice, pour la confiance qu'elle m'a accordée, pour son soutien tout au long de ces trois années, pour sa rigueur scientifique et ses précieux conseils (même pour les lancers de stylo... Pour ma défense, en français ou en anglais, les « s » c'est jamais facile à placer...) ;

Bulent my dear father-brother ! I will never thank you enough for everything. And for sure, also to make me meet the wonderful Turkish team, my dear friends, Ozge, Aslı the little dictator, Filiz, Karo, Çağaçan, and all the students Gizem, Yasemen and Mert, just some last words for you “ölüyorum! Çalış çalış!! Hadi hadi!!!!” ;

Pr. Rioux, pour nos longues discussions toujours très intéressantes, votre disponibilité, votre aide quand j'avais des questions à vous poser et pour ce que vous m'avez fait découvrir ;

Didier Fontenille et Frédéric Simard pour m'avoir accueilli dans leur laboratoire, ainsi que toute l'équipe MIVEGEC pour leur aide et leurs conseils avisés ;

La famille EDENext, pour leur accueil chaleureux, leur aide et leur disponibilité, leur gentillesse et pour les rencontres qu'ils m'ont permis de faire ;

A toutes les personnes de l'IRD (Romain, Manon, Gildas, Daniel...) qui m'ont soutenu dans cette dernière année ;

Et plus particulièrement, Val, Mallo, David, Yvon, Fred, Audrey et Marie pour leur soutien et les multiples pauses-café-clopes. Pour m'avoir fait relativiser et tellement rire (le tuning, les dragées Fuca, le fantôme de La Merci, les parties de morpions, beIN SPORTS, la robe-nappe de Marie, One Health, Batcat, le féminisme vs la galanterie...) même si évidemment je ne comprenais pas tout dû à mon jeune âge, n'est-ce pas Val ?

Toutes les personnes venues sur le terrain et surtout Baptiste, Cécile, Céline et Mag, pour tous les barbecues («ANKARA») et toutes les bières bues autour de grandes discussions ;

Pascal, Agnès et leur petite famille pour leur accueil à Roquedur, leur gentillesse, les apéro-discussions et les repas partagés ;

Claude, Josette, Eric ainsi que tous les habitants des communes de Roquedur, Saint-Julien-de-la-Nef et le Vigan pour leur accueil toujours chaleureux, leur gentillesse et leur aide ;

Nil pour m'avoir tant appris sur les phlébotomes et pour tous nos craquages après des heures et des heures de dissections, pour les pissettes d'alcool et le bleu de méthylène ;

A la meute au grand complet (Nicolas, Max, Diane, Charaf, Marie, Anis, Adrien, Céline, Greg, Lucie, Flo, Hadrien, Psychocailoux et clem) pour m'avoir écouté me plaindre et m'avoir supporté et soutenu au cours de cette thèse. Je ne vous remercierai jamais assez ;

« Les amis sont une seconde existence »

A tous mes amis rencontrés durant les congrès pour m'avoir tant apporté et soutenu. Et plus particulièrement Aurélien, mon jumeau diabolique et la petite Julie ;

Bien sûr à mes parents (Michel et Françoise), à mon frère Moe, ces personnes chères à mon existence, pour leur patience et leur soutien et sans qui je ne serais jamais arrivé là où j'en suis ;

Merci énormément à Margaux, de m'avoir soutenu, encouragé et rassuré quand j'en avais besoin (même quand tu étais loin). D'avoir été présente même dans les moments difficiles de ma thèse, même au sommet de mon râlage, parce qu'en définitive une thèse c'est difficile mais aussi pour la personne qui partage notre vie ;

Et pour finir je remercie aussi chaleureusement le phlébotome, le plus bel insecte connu, pour son amour sans faille et son besoin de me sauter dessus à chaque terrain même en pleine journée. Quoi de mieux qu'une petite chanson adaptée par ma chef pour ça ?

*Permettez-vous que j'emprunte votre oreille ?
Histoire de vous raconter l'histoire de Tom
Tom est un phlébotome, comme tous les phlébotomes
Le soir il se réveille, à l'aube il se couche (normalement !!)*

*Un matin, il atterrit sur le front d'une crapule
Jorian était son nom, il tirait 2 ans d'IRD
Bosseur, chahuteur, casseur de voiture, Jorian attend son heure
Abruti par l'ennui, le phlébotome le surprie*



*Jorian: Bonjour, le phébotome ! Tom: Bonjour, Jorian !
Soyons de bons amis, des amis pour de bon
La brute apprivoisée, passant toute sa journée
À jouer avec Tom, pour bonne conduite a remplié*

*Qu'est-ce qu'on peut bien faire, quand on sort de l'IRD
Dans une poche un phlébotome, dans l'autre aucun rond ?
"Si je me faisais dompteur de puces, de cafards et d'abeilles
Je me ferais manager, la bête de scène serait Tom"*

*"Voyons ce que ça donne, voyons si tu étonnes
Les collègues de boulot, Tom, va faire ton numéro"
"Tiens, un phlébotome ? Pardon", dit Anlo
Et d'un pouce farouche...*

Oh oué !

Ah zoum zoum... zoumahzoumzoum... zoum...

Résumé

Les phlébotomes sont des insectes hématophages appartenant à la famille des Psychodidae et à la sous-famille des Phlebotominae. Cet insecte diptère, jaunâtre, relativement petit (2 à 3 mm) peut transmettre différents pathogènes dont les principaux sont les leishmanies et les phlébovirus.

Ce travail de thèse est focalisé sur les phlébotomes vecteurs du parasite *Leishmania*. Les leishmanioses sont des maladies parasitaires causées par un protozoaire du genre *Leishmania*. Elles touchent un large panel d'hôtes vertébrés, dont l'homme et le chien. Elles sont toujours un problème de santé publique majeur dans de nombreux pays et sont actuellement en expansion. Bien que cette maladie soit largement étudiée, nous avons encore beaucoup à apprendre sur son vecteur. Par exemple, l'organisation des populations dans les écosystèmes et les paramètres qui les structurent sont, à l'heure actuelle, très peu étudiés.

Dans ce contexte, le but de cette thèse est d'étudier l'écologie et la structure des populations de phlébotomes dans un foyer connu de leishmaniose et l'impact des facteurs biotiques et abiotiques sur leur organisation. Pour atteindre cet objectif, nous avons réalisé une collecte de phlébotomes le long d'un transect de 14km localisé dans la région de Montpellier, présentant une diversité altitudinale, climatique et environnementale. Cette aire d'étude a été choisie en raison de la disponibilité de données collectées il y a plus de 30 ans par Rioux et al. [1]. Les populations de phlébotomes ont été caractérisées d'un point de vue taxonomique, spatio-temporel, génétique (microsatellites), et morphométrique (morphométrie géométrique). Les résultats génétiques, morphométriques et de distribution des espèces ont été ensuite confrontés à des paramètres climatiques (température, humidité) ou environnementaux (altitude, versant, station, micro-habitat).

Durant ce travail, 4 espèces ont été capturées : *Phlebotomus ariasi* (93,23%), *P. perniciosus* (0,48%), *P. mascittii* (0,11%) et *Sergentomyia minuta* (6,18%). Parmi ces espèces, *P. ariasi* est le vecteur majeur local de *Leishmania*. Elles ont une activité saisonnière de Mai à Octobre avec un pic d'abondance en Juillet-Août quand les températures moyennes sont optimales pour les phlébotomes (20-30°C). Bien que l'environnement ait été relativement transformé dans notre zone d'étude en 30 ans, l'abondance des phlébotomes ne semble pas avoir changé de façon significative, soulignant leur capacité d'adaptation aux modifications de l'écosystème à court et long terme. La présence et l'abondance des deux espèces prédominantes (*P. ariasi* et *S. minuta*) sont significativement influencées par l'altitude, la température, l'humidité relative, le versant ainsi que l'orientation des murs.

Les analyses génétiques et morphométriques n'ont été réalisées que sur *P. ariasi* pour des raisons d'effectifs. Les analyses génétiques montrent que la diversité est conservée à toutes les échelles d'études et qu'il existe une structuration des phlébotomes en populations micro-géographiques. Les données de géométrie morphométrique révèlent un dimorphisme sexuel bien connu chez les insectes mais également une structuration phénotypique en fonction des facteurs climatiques et environnementaux (mois, versant, altitude et station).

Ces deux types d'approches permettent, grâce à leur complémentarité, d'apporter des informations sur l'écologie et l'organisation des populations de phlébotomes à une échelle locale et de discuter des conséquences sur la transmission de la leishmaniose.

Mot clés : *Phlébotomes, Structure micro-géographique des populations, Plasticité phénotypique, Microsatellites, Géométrie morphométrique, Ecologie, Leishmaniose, France*

Sandflies and ecosystems: impact of biotic and abiotic factors on the genetic and phenotypic population structure.

Summary

Sandflies are hematophagous insects belonging to the family Psychodidae and the subfamily phlebotominae. This diptera, yellowish, relatively small (2-3 mm) can transmit different pathogens; the main ones are *Leishmania* and phlebovirus.

This thesis focuses on sandflies, vectors of *Leishmania*. Leishmaniasis are parasitic diseases caused by protozoa of the genus *Leishmania*. They affect a wide range of vertebrate hosts, including humans and dogs. They are still a major problem of public health in many countries and are currently expanding. Although this disease is widely studied, we still have a lot to learn about its vector. For example, the organization of populations in ecosystems and the parameters which structure them are very little studied up to now.

In this context, the aim of this thesis is to study the ecology and the structuring of sandfly populations in a known endemic area of leishmaniasis and the impact of biotic and abiotic factors on their organization. To reach this objective, we performed captures of sandflies along a 14km transect located in the Montpellier region presenting an altitudinal, climate and environmental diversity. This study area was selected because data collected more than 30 years ago are available. Sandfly populations have been characterized by taxonomic, spatio-temporal, genetic (microsatellites) and morphometric (geometric morphometry) approaches. The genetic, morphometric and species distribution results were then confronted with climatic (temperature, relative humidity) or environmental parameters (altitude, slope, station, micro-habitat).

During this work, four species were captured: *Phlebotomus ariasi* (93.23%), *P. perniciosus* (0.48%), *P. mascittii* (0.11%) and *Sergentomyia minuta* (6.18%). Among these species, *P. ariasi* is the main local vector of Leishmaniasis. They have a seasonal activity from May to October with an abundance peak in July-August when average temperatures are optimal for sandflies (20-30°C). Although the environment has been relatively modified in our study area in 30 years, the abundance of sandflies does not seem to have changed significantly, highlighting their ability to adapt to ecosystem modifications in short and long-term. The presence and abundance of the two predominant species (*P. ariasi* and *S. minuta*) are significantly influenced by altitude, temperature, relative humidity, slope, and wall orientation.

Genetic and morphometric analyzes were performed only on *P. ariasi* due to the low number of samples for the other species. The genetic analyses show that diversity is maintained at all scales of study and that sandflies are organized in microgeographic populations. The morphometric geometry data reveal a sexual dimorphism, well known in insects but also a phenotypic structuring correlated to climatic and environmental factors (month, slope, altitude and station).

Both of these approaches, because of their complementarity, help provide information on the ecology and organization of sandfly populations at local scale and to discuss about the consequences in terms of leishmaniasis transmission.

Keywords: Sandflies, Microgeographic population genetic structure, Phenotypic plasticity, Microsatellites, Geometric Morphometry, Ecology, Leishmaniasis, France

Table des matières

TABLE DES ILLUSTRATIONS - FIGURES.....	11
TABLE DES ILLUSTRATIONS - TABLEAUX.....	13
LISTE DES PUBLICATIONS.....	14
LISTE DES COMMUNICATIONS.....	15
INTRODUCTION ET OBJECTIFS DE L'ETUDE.....	16
I. GENERALITES.....	18
I.1. LES PHLEBOTOMES : ETAT DE L'ART.....	18
I.1.1. TAXONOMIE.....	18
I.1.2. MORPHOLOGIE.....	18
I.1.3. BIOLOGIE ET ECOLOGIE.....	19
I.1.4. REPARTITION GEOGRAPHIQUE.....	20
I.2. IMPORTANCE MEDICALE DES PHLEBOTOMES.....	20
I.2.1. BARTONELLOSE.....	20
I.2.2. ARBOVIROSES.....	21
I.2.3. LEISHMANIOSES.....	21
I.3. LES LEISHMANIOSES EN FRANCE.....	24
I.3.1. HISTORIQUE.....	24
I.3.2. DISTRIBUTION.....	24
I.3.3. LES PRINCIPAUX ACTEURS.....	25
I.4. LES PRINCIPAUX PHLEBOTOMES VECTEURS EUROPEENS.....	26
I.4.1. DISTRIBUTION.....	26
I.4.2. ACTIVITE SAISONNIERE.....	27
I.4.2.1. <i>Résumé</i>	27
I.4.2.2. <i>Article 1 : Seasonal dynamics of phlebotomine sand fly species proven vectors of Mediterranean leishmaniasis caused by Leishmania infantum.</i>	27
I.5. LES PHLEBOTOMES EN FRANCE.....	58
I.5.1. HISTORIQUE ET ESPECES PRESENTES EN FRANCE.....	58
I.5.2. ESPECES IMPLIQUEES DANS LA TRANSMISSION DE LA LEISHMANIOSE.....	59
I.6. TECHNIQUES DE CAPTURES.....	60
I.6.1. RESUME.....	60
I.6.2. ARTICLE 2 : SAMPLING STRATEGIES FOR PHLEBOTOMINE SAND FLIES (DIPTERA: PSYCHODIDAE) IN EUROPE.....	60
I.7. CARACTERISATION DES POPULATIONS DE PHLEBOTOMES.....	76
I.7.1. LES OUTILS MOLECULAIRES.....	76

I.7.2. LES OUTILS PHENOTYPIQUES	76
I.7.3. STRUCTURE DES POPULATIONS DE <i>P. ARIASI</i>	77
II. MATERIELS ET METHODES	78
II.1. PRESENTATION DE LA ZONE D'ETUDE	78
II.1.1. CARACTERISTIQUES GENERALES	78
II.1.2. CONTEXTE CLIMATIQUE	81
II.1.3. VEGETATION	81
II.1.4. DONNEES DEMOGRAPHIQUES	81
II.2. ENQUETE ENTOMOLOGIQUE	82
II.2.1. TECHNIQUES DE CAPTURE ET CONSERVATION	82
II.2.2. IDENTIFICATION	82
II.2.3. RELEVÉ DES DONNEES CLIMATIQUES	82
II.2.4. ANALYSES DES DONNEES	83
II.3. ETUDE DE MORPHOMETRIE GEOMETRIQUE	84
II.3.1. PREPARATION DES AILES	84
<i>II.3.1.1. Résumé</i>	<i>84</i>
<i>II.3.1.2. Article 3: Wing size and shape variation of Phlebotomus papatasi (Diptera: Psychodidae) populations from the south and north slopes of the Atlas Mountains in Morocco.</i>	<i>85</i>
II.3.2. ANALYSES DES DONNEES	97
II.4. CARACTERISATION GENETIQUE	98
II.4.1. EXTRACTION D'ADN	98
II.4.2. DEVELOPPEMENT DES MARQUEURS MICROSATELLITES	99
II.4.3. AMPLIFICATION D'ADN ET GENOTYPAGE	99
II.4.4. ANALYSES DES DONNEES	101
II.5. DETECTION DES LEISHMANIES PAR QPCR	101
III. ANALYSES DES DONNEES CLIMATIQUES	102
IV. IMPACT DE L'ECOSYSTEME SUR L'ORGANISATION SPATIO-TEMPORELLE DES POPULATIONS DE PHLEBOTOMES	106
IV.1. RÉSUMÉ	106
IV.2. ARTICLE 4 : ECOLOGY AND SPATIOTEMPORAL DYNAMICS OF SANDFLIES IN THE MEDITERRANEAN LANGUEDOC REGION (ROQUEDUR AREA, GARD, FRANCE).....	107
V. PLASTICITE PHENOTYPIQUE ET CAPACITE D'ADAPTATION DE <i>P. ARIASI</i>	141
V.1. ECHANTILLONS.....	141
V.2. RÉSUMÉ.....	141

V.3. ARTICLE 5 : PHENOTYPIC VARIATION OF <i>PHLEBOTOMUS ARIASI</i> IN THE REGION OF ROQUEDUR (GARD, FRANCE).	142
VI. UNE STRUCTURATION GENETIQUE EN SOUS-POPULATIONS MICRO-GEOGRAPHIQUES	173
VI.1. DEVELOPPEMENT DES MARQUEURS MICROSATELLITES	173
VI.1.1. <u>RÉSUMÉ</u>	173
VI.1.2. <u>ARTICLE 6 : NEW MICROSATELLITE MARKERS FOR MULTI-SCALE GENETIC STUDIES ON <i>PHLEBOTOMUS ARIASI</i> TONNOIR, VECTOR OF <i>LEISHMANIA INFANTUM</i> IN THE MEDITERRANEAN AREA.</u>	173
VI.2. ECOSYSTEME ET STRUCTURE GENETIQUE DES POPULATIONS DE <i>P. ARIASI</i>	181
VI.2.1. <u>RÉSUMÉ</u>	181
VI.2.2. <u>ARTICLE 7 : MOLECULAR ADVANCES IN UNDERSTANDING THE GENETIC POPULATION STRUCTURE OF <i>PHLEBOTOMUS ARIASI</i> IN THE MEDITERRANEAN LANGUEDOC REGION (ROQUEDUR AREA, GARD, FRANCE).</u>	181
DISCUSSION GENERALE	220
CONCLUSION ET PERSPECTIVES	223
REFERENCES BIBLIOGRAPHIQUES	225
ANNEXE 1	232
ANNEXE 2 - ARTICLE 8	238
ANNEXE 3 - ARTICLE 9	248

Table des illustrations - Figures

Figure 1 : Phlébotome femelle gorgée (A) et phlébotome mâle dans de l'alcool à 96% (B)....	18
Figure 2 : Cycle de vie du phlébotome (Photos Gizem Oğuz et Jorian Prudhomme).	19
Figure 3 : Exemples de gîtes de repos, enclos (A), barbacanes (B) et chenil (C) (Photos Jorian Prudhomme).	20
Figure 4 : Nodule cutané dû à <i>Bartonella bacilliformis</i> sur un patient péruvien (http://www.nejm.org/doi/full/10.1056/NEJM199808133390706).	21
Figure 5 : Cycle de développement du parasite <i>Leishmania</i> [32].	22
Figure 6 : Manifestations cliniques des leishmanioses : formes cutanées (A et B), forme muco-cutanée (C) et forme viscérale (D) (http://www.who.int/leishmaniasis/clinical_forms_leishmaniasis/en/index2.html).	22
Figure 7 : Distribution géographique des leishmanioses cutanées (A) et viscérales (B) en 2013 (WHO-Global Health Observatory data).	23
Figure 8 : Distribution des 268 cas autochtones répertoriés de LV entre 1999 et 2012 dans les départements français les plus touchés [34].	25
Figure 9 : Manifestations cliniques de la leishmaniose canine : lésions cutanées (A), dépigmentation autour des yeux (B), griffes anormalement longues (C) et amaigrissement généralisé (D) (Photos Pr. Jean-Antoine Rioux).	25
Figure 10 : Cartes européennes de répartition de <i>Phlebotomus ariasi</i> (A), <i>P. perniciosus</i> (B), <i>P. neglectus</i> (C), <i>P. perfiliewi</i> (D) et <i>P. tobbi</i> (E) (European Centre for Disease Prevention and Control).	26
Figure 11 : Cartes de répartition en France métropolitaine de <i>Phlebotomus ariasi</i> (A), <i>P. perniciosus</i> (B), <i>P. papatasi</i> (C) et <i>P. sergenti</i> (D) (European Centre for Disease Prevention and Control).	59
Figure 12 : Carte de la zone d'étude. (●) indiquent les sites d'échantillonnage	79
Figure 13 : Coupe transversale de la zone d'étude. NW : Nord-Ouest ; SE : Sud-Est ; S1 : Vallée de l'Arre versant nord, stations entre 200 et 300 m, S2 : mi-pente versant nord, stations entre 300 et 500 m, S3 : sommet versant nord, stations entre 500 et 600 m, S4 : mi-pente versant sud, stations entre 300 et 500 m, S5 : Vallée de l'Hérault versant sud, stations entre 300 et 100 m. Figure adaptée de Rioux et al. [1]	80
Figure 14 : Localisation des 16 points de repère sur une aile de <i>Phlebotomus ariasi</i>	97
Figure 15 : Schéma de l'élimination des variations entre deux ailes (◀ points de repère sur l'aile 1 ; ● points de repère sur l'aile 2). (A) configuration d'origine de deux ailes ; (B) Elimination des différences de position ; (C) Elimination des différences d'échelle ; (D) Elimination des différences d'orientation. Ces trois ajustements permettent de mettre en avant uniquement les variations de forme. Figure provenant de Zelditch et al. [57].	98
Figure 16 : Boxplot de la température (°C) et de l'humidité relative (HR%) par station et par année. Les valeurs manquantes représentent les enregistreurs perdus. Le boxplot (ou boîte à moustache) représente une distribution des valeurs avec la médiane (trait épais), une boîte s'étendant du quartile 0,25 au quartile 0,75 et un intervalle de confiance de 95% pour la médiane.	103

Figure 17 : Boxplot montrant les variabilités de température (A) et d'humidité relative (B) par mois. Le boxplot (ou boîte à moustache) représente une distribution des valeurs avec la médiane (trait épais), une boîte s'étendant du quartile 0,25 au quartile 0,75 et un intervalle de confiance de 95% pour la médiane. 103

Table des illustrations - Tableaux

Tableau 1 : Descriptif des stations échantillonnées (PL : Pièges lumineux ; PC : Pièges collants)	78
Tableau 2 : Données démographiques de (1) Saint-Julien-de-la-Nef, (2) Roquedur-le-Haut et (3) Le Vigan (Institut National de la Statistique et des Etudes Economiques).	81
Tableau 3 : Descriptif des 16 marqueurs microsatellites utilisés pour <i>Phlebotomus ariasi</i> (Th : température d'hybridation ; Gp : groupe de multiplexage).	100
Tableau 4 : Significativité des tests post-hoc (tests de Mann-Whitney avec correction de Bonferroni) réalisés sur la température et l'humidité relative par altitude et par mois (*: significatif, NS: Non Significatif).	104
Tableau 5: Nombre d'ailes de <i>P. ariasi</i> utilisées par station pour l'étude de morphométrie géométrique (SE: Sud-Est; NO: Nord-Ouest; Groupe d'altitude: A1 (100-200m), A2 (200-300m), A3 (300-400m), A4 (400-500m), A5 (>500); ♂: male; ♀: femelle).	141

Liste des publications

Prudhomme J., Toty C., Cassan C., Rahola N., Vergnes B., Alten B., Sereno D.*, Bañuls A-L*. Advances in understanding the genetic population structure of *Phlebotomus ariasi* in the Mediterranean Languedoc region (Roquedur area, Gard, France). En cours de rédaction pour Molecular Ecology.

Prudhomme J., Cassan C., Hide M., Toty C., Rahola N., Vergnes B., Dujardin J-P., Alten B., Sereno D.*, Bañuls A-L*. Phenotypic variation of *Phlebotomus ariasi* in the region of Roquedur (Gard, France). En cours de rédaction pour Infection, Genetics and Evolution.

Prudhomme J., Rahola N., Toty C., Cassan C., Roiz D., Vergnes B., Thierry M., Rioux J-A, Alten B.*, Sereno D.*, Bañuls A-L*. Ecology and spatiotemporal dynamics of sandflies in the Mediterranean Languedoc region (Roquedur area, Gard, France). Parasites & Vectors, accepted with minor revisions.

Alten B, Maia C, Afonso O, Campino L, Jiménez M, González E, Molina R, Bañuls A-L, **Prudhomme J**, Vergnes B, Toty C, Cassan C, Thierry M, Sereno D, Bongiorno G, Bianchi R, Khoury C, Tsirigotakis N, Dokianakis E, Antoniou M, Christodoulou V, Mazeris A, Karakus M, Ozbel Y, Arserim S K, Erisoz Kasap O, Gunay F, Oguz G, Kaynas S, Tsertsvadze N, Tskhvaradze L, Giorgobiani E, Gramiccia M, Volf P, Gradoni L. Seasonal dynamics of phlebotomine sand fly species proven vectors of Mediterranean leishmaniasis caused by *Leishmania infantum*, PLoS Neglected Tropical Disease, sous presse.

Mathis A., Depaquit J., Dvořák V., Tuten H., Bañuls AL., Halada P., Zapata S., Lehrter V., Hlavačková K., **Prudhomme J.**, Volf P., Sereno D., Kaufmann C., Pflüger V., Schaffner F. Identification of phlebotomine sand flies using one MALDI-TOF MS reference database and two mass spectrometer systems. Parasites & Vectors. 2015, 8(266).

Alten B., Ozbel Y., Ergunay K., Erisoz Kasap O., Antoniou M., Velo E., **Prudhomme J.**, Molina R., Bañuls A-L, Schaffner F., Hendrickx G., Van Bortel W. and Medlock J.: Sampling strategies for phlebotomine sand flies (Diptera: Psychodidae) in Europe. Bulletin of Entomological Research. 2015, 105(6): 664-78.

Prudhomme J., Toty C., Erisoz Kasap O., Rahola N., Vergnes B., Maia C., Campino L., Antoniou M., Jimenez M., Molina R., Cannet A., Alten B., Sereno D. and Bañuls A-L.: New microsatellite markers for multi-scale genetic studies on *Phlebotomus ariasi* Tonnoir, vector of *Leishmania infantum* in the Mediterranean area. Acta Tropica. 2015, 142: 79-85.

Rioux J.-A., Carron S, Dereure J, Périères J, Zeraia L, Franquet E, Babinot M, Gállego M & **Prudhomme J**: Ecology of leishmaniasis in the South of France. 22. Reliability and representativeness of 12 *Phlebotomus ariasi*, *P. perniciosus* and *Sergentomyia minuta* (Diptera: Psychodidae) sampling stations in Vallespir (eastern French Pyrenees region). Parasite, 2013, 20, 35: doi: 10.1051/parasite/2013035

Prudhomme J., Gunay F., Rahola N., Ouanaimi F., Guernaoui S., Boumezzough A., Bañuls A-L., Sereno D., & Alten B.. Wing size and shape variation of *Phlebotomus papatasi* (Diptera: Psychodidae) populations from the south and north slopes of the Atlas Mountains in Morocco. Journal of Vector Ecology. 2012, 37(1): 137-47.

Liste des communications

Avril 2015 (Grèce) – EDENext annual meeting. Présentation orale “Sandflies in the Cevennes Region: 3-year to investigate the parameters affecting their spatiotemporal dynamics” **J. Prudhomme**, N. Rahola, C. Toty, C. Cassan, D. Roiz, B. Vergnes, M. Thierry, B. Alten, D. Sereno* & A-L Bañuls*.

Octobre 2014 (Grèce) - E-SOVE meeting. Présentation orale “Spatiotemporal dynamics and bio-ecology of sandflies in the region of Montpellier” **J. Prudhomme**, N. Rahola, C. Toty, D. Roiz, B. Vergnes, C. Cassan, M. Thierry, B. Alten, D. Sereno* & A.L. Bañuls*.

Septembre 2013 (USA) - SOVE meeting. Présentation orale “Microsatellites development for population structure analysis of *Phlebotomus ariasi*, main vector of *L. infantum* in the region of Montpellier (France)” **J. Prudhomme**, C. Toty, N. Rahola, B. Vergnes, C. Maia, B. Alten, D. Sereno & A.L. Bañuls.

Juin 2013 (France) - AG Mivegec. Présentation poster “Seasonal dynamics and spatial structure of sandflies in the region of Montpellier (Southern France)” **J. Prudhomme**, N. Rahola, B. Vergnes, M. Senghor, E. Elguero, M. Choisy, C. Toty, B. Alten, D. Sereno & A.L. Bañuls.

Mars 2013 (Espagne) - EDENext annual meeting. Second prix de présentation orale “Spatiotemporal dynamics and bio-ecology of sandflies in the region of Montpellier” **J. Prudhomme**, N. Rahola, B. Vergnes, M. Senghor, E. Elguero, B. Alten, D. Sereno & A.L. Bañuls.

Novembre 2012 (Italie) - 3rd European PhD Network Meeting. Présentation orale “Spatiotemporal dynamics and bio-ecology of sandflies in the region of Montpellier” **J. Prudhomme**, N. Rahola, B. Vergnes, M. Senghor, E. Elguero, B. Alten, D. Sereno & A.L. Bañuls.

Octobre 2012 (France) - E-SOVE meeting. Présentation orale “Spatiotemporal dynamics and bio-ecology of sandflies in the region of Montpellier” **J. Prudhomme**, N. Rahola, B. Vergnes, M. Senghor, E. Elguero, B. Alten, D. Sereno & A.L. Bañuls.

Mai 2012 (France) - 7ème symposium de morphométrie. Présentation poster “Wing size and shape variation of *Phlebotomus papatasi* (Diptera: Psychodidae) populations from the south and the north slopes of the Atlas Mountains in Morocco” **J. Prudhomme**, F. Gunay, N. Rahola, F. Ouanaimi, S. Guernaoui, A. Boumezzough, A.L. Bañuls, D. Sereno & B. Alten

Mars 2012 (Turquie) - EDENext annual meeting. Présentation orale “Wing size and shape variation of *Phlebotomus papatasi* (Diptera: Psychodidae) populations from the south and the north slopes of the Atlas Mountains in Morocco” **J. Prudhomme**, F. Gunay, N. Rahola, F. Ouanaimi, S. Guernaoui, A. Boumezzough, A.L. Bañuls, D. Sereno & B. Alten

Introduction et objectifs de l'étude

Les phlébotomes sont des insectes hématophages, vecteurs de pathogènes responsables de maladies humaines et animales. Parmi ces maladies, ils sont responsables de la transmission en France, des leishmanioses cutanée et viscérale ainsi que de certains arbovirus. Ce travail de thèse se concentre spécifiquement sur les phlébotomes, vecteurs de *Leishmania*. Bien que ces dernières soient classées comme maladies négligées, elles présentent un problème de santé publique majeur dans de nombreux pays et sont actuellement en expansion. En effet, on assiste à une augmentation des situations à risque due en particulier aux changements anthropiques (migrations massives, déforestations, urbanisation, etc.). Ces changements peuvent entraîner une plus grande susceptibilité des hôtes mais également des modifications des aires de distribution des vecteurs et des réservoirs du parasite et par conséquent peuvent augmenter de façon importante l'exposition de l'homme aux phlébotomes infectés [2]. Bien que les caractéristiques biologiques du parasite *Leishmania* commencent à être relativement bien décrites, celles de son vecteur, telles que la capacité vectorielle, la localisation des gîtes larvaires ou les préférences alimentaires, restent assez méconnues [3]. Il en est de même pour la structure des populations des phlébotomes dans les écosystèmes et les paramètres qui influencent leur organisation qui sont très peu étudiés. Il est pourtant primordial de bien connaître la biologie au sens large des différents acteurs du cycle épidémiologique pour mieux cerner la transmission du parasite, évaluer les risques de transmission et enfin lutter efficacement contre ces parasitoses. La transmission des *Leishmania* est conditionnée par différents facteurs environnementaux (altitude, versant, etc.) et climatiques (température, humidité relative, etc.) soit directement (effet de la température sur le développement du parasite chez le vecteur) soit indirectement (changement dans le comportement et dans l'aire de distribution des vecteurs ou bien des réservoirs) [4].

Dans ce contexte, le but de ce projet de thèse est d'étudier pour la première fois la structure des populations de phlébotomes à une échelle locale et l'impact des facteurs écologiques (environnement, température, humidité, altitude, etc.) sur l'organisation spatio-temporelle de ces insectes. Notre première hypothèse de travail est que les phlébotomes sont organisés en populations micro-géographiques expliquant le fonctionnement des foyers de transmission [5, 6]. Notre deuxième hypothèse est que les facteurs environnementaux influencent fortement l'organisation dans l'espace de ces populations et expliquent l'expansion des phlébotomes et des maladies qu'ils transmettent. En parallèle, nous tentons également de répondre à des questions telles que : comment évoluent les populations de phlébotomes dans le temps ou encore existe-t-il un lien entre phénotype (taille et forme des ailes) et génotype ?

Ainsi, pour tester ces hypothèses et répondre à ces questions, nous étudions dans ce projet des populations de phlébotomes de la région de Montpellier, foyer bien connu de leishmaniose, d'un point de vue environnemental, spatial et temporel mais également génétique et phénotypique. La première partie de ce travail porte sur la dynamique spatiale et temporelle des différentes espèces de phlébotomes le long d'un transect de 14 km et sur les caractéristiques écologiques (altitude, température, hygrométrie, etc.) associées à cette organisation. La deuxième partie est focalisée sur *Phlebotomus ariasi* Tonoir, 1921, vecteur connu de *Leishmania infantum* Nicoll, 1908, qui est l'espèce la plus abondante dans notre région. Le but, ici, est de définir la structure génétique et phénotypique des populations au niveau intraspécifique.

Ce travail s'intègre dans le projet européen EDENext (Biology and control of vector-borne infections in Europe) et, plus particulièrement, dans le groupe de travail sur les

phlébotomes (PhBD group). Ce projet est fondé sur les concepts, les méthodes, les outils et les résultats du précédent projet EDEN (Emerging diseases in a changing European environment). Alors que le projet EDEN portait sur l'étude des répercussions des changements environnementaux sur l'émergence des maladies vectorielles, le projet EDENext cherche à expliquer et modéliser les processus conduisant à l'introduction, l'établissement et la propagation des vecteurs et / ou des maladies vectorielles et ainsi d'évaluer les stratégies de lutte permettant d'interrompre les cycles épidémiologiques des maladies infectieuses transmises par des arthropodes.

I. Généralités

I.1. Les phlébotomes : état de l'art

I.1.1. Taxonomie

Les Phlébotomes appartiennent à l'ordre des Diptères Nématocères, famille des Psychodidae Bigot, 1854 et sous-famille des Phlebotominae Kertész, 1904. Les phlébotomes comptent environ 900 espèces dont 70 ont été identifiées comme vecteurs potentiels dont une quarantaine comme vecteurs prouvés de protozoaires du genre *Leishmania* [7]. Ces vecteurs appartiennent principalement au genre *Phlebotomus* Rondani & Berté, 1840 pour l'Ancien Monde et *Lutzomyia* França, 1924 pour le Nouveau Monde. Cependant, d'autres taxa sont actuellement suspectés d'être vecteurs de *Leishmania* tels que le genre *Sergentomyia* Franca & Parrot, 1920 [8-13]. L'identification des phlébotomes est généralement réalisée sur des critères morphologiques. Cependant d'autres techniques sont en cours de développement telles que l'identification par un instrument de type MALDI-TOF (Matrix-Assisted Laser Desorption/Ionisation-time-of-flight mass spectrometry) [14] (Annexe 2 - Article 8).

I.1.2. Morphologie

Les phlébotomes possèdent une seule paire d'ailes (Diptères), des antennes filiformes (Nématocères), une présence abondante de soie sur la tête, le thorax, les 6 pattes, les ailes (Psychodidae) et des pièces buccales aptes à prendre un repas sanguin (Phlebotominae). De plus, ils sont relativement petits (2 à 3 mm), abondamment poilus, avec une couleur pouvant varier de blanc ou beige clair à noir. Ils sont pourvus d'ailes lancéolées qui, en position de repos, sont repliées au-dessus du corps formant ainsi un V [15] (Figure 1).

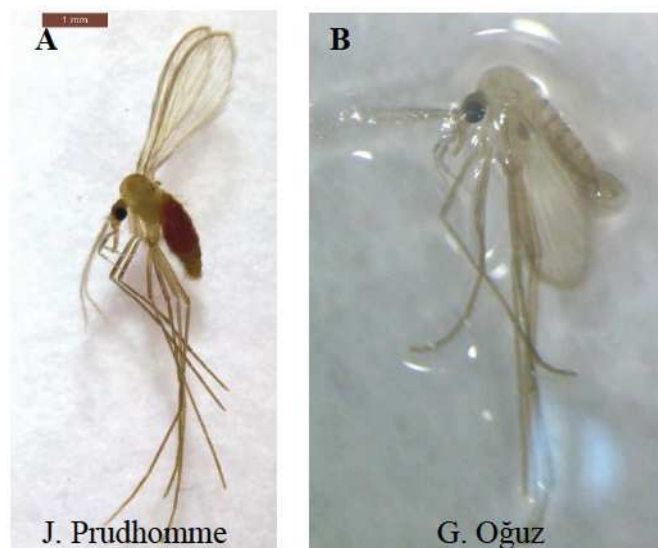


Figure 1: Phlébotome femelle gorgée (A) et phlébotome mâle dans de l'alcool à 96% (B).

I.1.3. Biologie et écologie

Les deux sexes se nourrissent de jus sucrés mais seulement les femelles prennent un repas sanguin. Celui-ci est nécessaire pour la maturation des œufs. Néanmoins, l'autogénie (capacité à pondre sans prise de repas sanguin) est observée chez certaines espèces [16]. Pour la reproduction, le mâle effectue une parade nuptiale qui se caractérise par des soubresauts de l'abdomen et des mouvements oscillatoires tandis que la femelle reste immobile [17]. Il s'en suit un accouplement de durée variable (de 2 à 25 min) pouvant avoir lieu avant ou après le repas sanguin en fonction des espèces [17]. La durée du cycle gonotrophique varie en fonction des espèces et des facteurs environnementaux comme par exemple la température et la quantité de sang ingérée [18]. La ponte des œufs intervient généralement entre 6 à 8 jours après le repas sanguin. Les gîtes larvaires ne sont actuellement pas identifiés, mais les terriers de rongeurs sont fortement suspectés car le développement des larves nécessite des milieux riches en matière organique [19]. Les femelles pondent plusieurs dizaines d'œufs qui éclosent environ 6 à 10 jours après la ponte [18]. Après l'éclosion, quatre stades larvaires vont se succéder (durant environ 3 semaines) avant de donner une pupe et enfin un adulte (Figure 2). Le stade pupal dure entre 7 à 10 jours. Cependant, une diapause des œufs ou des larves peut être observée chez certaines espèces de phlébotomes afin de survivre à l'hiver. La diapause larvaire du quatrième stade étant la plus fréquente [7].

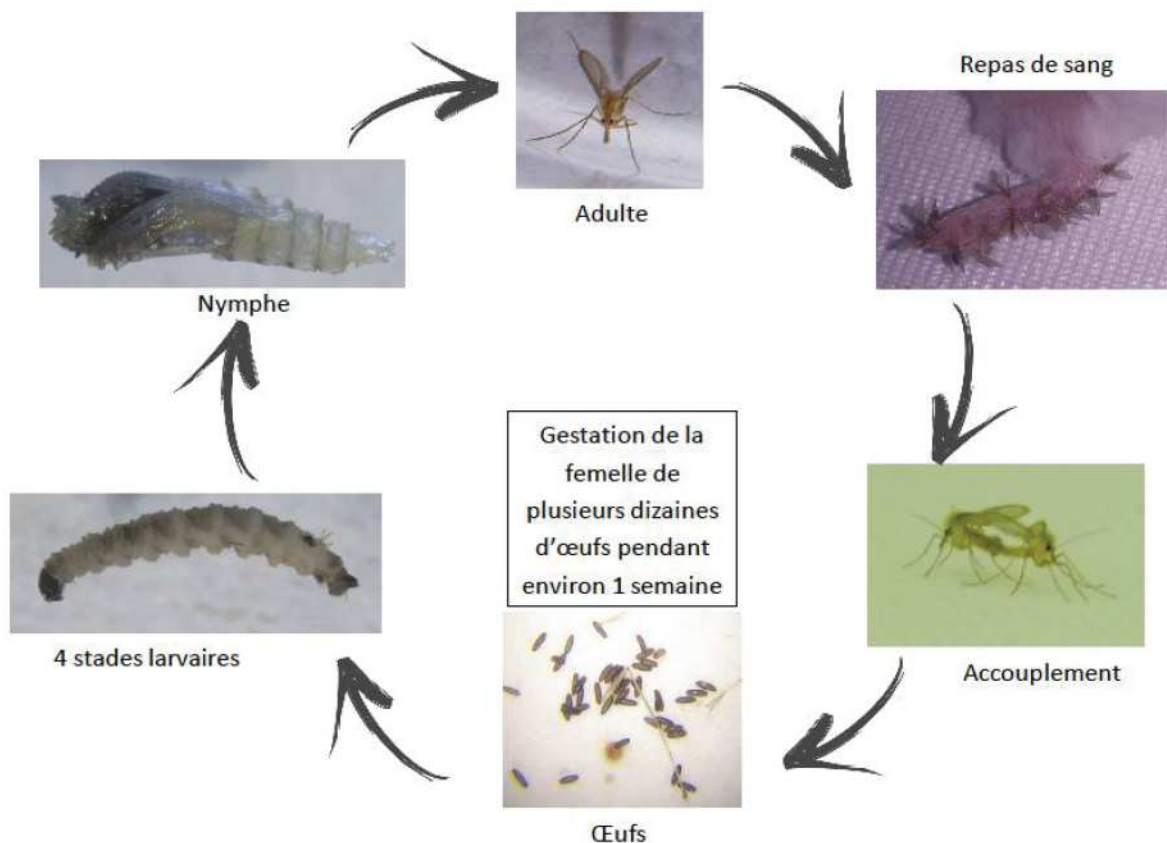


Figure 2 : Cycle de vie du phlébotome (Photos Gizem Oğuz et Jorian Prudhomme).

La majorité des espèces de phlébotomes ont une activité crépusculaire et nocturne. Dans la journée, les lieux de repos sont généralement des environnements frais et humides [20]. Les gîtes de repos possibles sont donc les étables (intérieur / extérieur), les maisons (intérieur / extérieur), les poulaillers (intérieur / extérieur), les grottes, les crevasses, les terriers, etc. (Figure 3). Les terriers et les crevasses rocheuses ont été identifiés comme étant des sites de

reproduction ou de repos. Dans le Sud de la France, les trous dans les murs (nommés barbacanes) à proximité des routes ou dans les villages sont des lieux de repos très utilisés par ces insectes.

Le vol des phlébotomes est silencieux et en général de courte portée. Ils avancent par bonds dans une série de vols courts entrecoupés de périodes de repos. Très fragiles, ils ne sortent que par temps sec et calme (vent inférieur à 1 m/s). Les distances de vol varient selon les espèces mais la dispersion maximale excède rarement un kilomètre [17].

Néanmoins, cet insecte reste assez méconnu d'un point de vue biologique (capacité et compétence vectorielle, etc.) et écologique (gîtes larvaires inconnus, préférences alimentaires mal connues, etc.).



Figure 3 : Exemples de gîtes de repos, enclos (A), barbacanes (B) et chenil (C) (Photos Jorian Prudhomme).

I.1.4. Répartition géographique

Les phlébotomes sont très abondants dans les régions chaudes (ex : Bassin méditerranéen, Asie, Afrique, Amérique du Sud), cependant leur aire de distribution est très étendue (entre 50°N et 40°S). Ils sont présents sur tous les continents mais n'ont pas été signalés en Nouvelle-Zélande et dans les îles du Pacifique. Leur répartition altitudinale est comprise entre le niveau de la mer et 3500 mètres d'altitude (Afghanistan : *Phlebotomus rupester*) [20-22]. Des études de modélisation suggèrent une expansion de l'aire de distribution de différentes espèces de phlébotomes en Europe liée à l'influence des changements environnementaux et climatiques (*Phlebotomus ariasi*, *P. mascittii*, *P. perniciosus*, *P. neglectus* and *P. perfiliewi*) leur permettant ainsi de coloniser de nouveaux habitats [23, 24].

I.2. **Importance médicale des phlébotomes**

I.2.1. Bartonellose

La bartonellose, aussi appelée Verruga Peruana, la fièvre d'Oroya ou maladie de Carrión est causée par *Bartonella bacilliformis*. C'est un bacille gram négatif transmis par *Lutzomyia verrucarum* s.l.. Cette affection est présente dans le nouveau monde et plus particulièrement dans les montagnes des Andes, dans les régions du Pérou, de l'Équateur et de la Colombie [25]. Cette maladie présente deux phases distinctes. La première phase, la fièvre d'Oroya, durant laquelle le patient peut présenter de la fièvre, des maux de tête, des douleurs musculaires / abdominales associés à une anémie sévère. Elle peut être mortelle si non traitée. La seconde phase, la verruga du Pérou, se caractérise par l'apparition de lésions cutanées qui régressent et guérissent sur plusieurs mois sans laisser de cicatrices (Figure 4).



Figure 4 : Nodule cutané dû à *Bartonella bacilliformis* sur un patient péruvien (<http://www.nejm.org/doi/full/10.1056/NEJM199808133390706>).

Même s'il existe des preuves démontrant que cette maladie est présente au Pérou depuis l'époque pré-inca, l'épidémiologie et la dynamique de transmission demeurent toujours mal connues [26].

I.2.2. Arboviroses

Différentes espèces de phlébotomes sont connues pour être des vecteurs compétents d'un grand nombre d'arbovirus comme par exemple : le virus Toscana, le virus Sicilien ou le virus Naples. Cependant, mis à part le virus Toscana qui a un tropisme pour le système nerveux central et entraîne des cas de méningites et d'encéphalites, les autres virus actuellement connus pour être transmis par les phlébotomes causent généralement des symptômes modérément sévères [27-30]. Le cycle de développement des arbovirus chez les phlébotomes est actuellement inconnu.

I.2.3. Leishmanioses

Les leishmanioses sont des maladies parasitaires causées par un protozoaire de l'ordre des Kinetoplastidés de la famille des Trypanosomatidae appartenant au genre *Leishmania*. Elles touchent un large panel d'hôtes vertébrés tels que les rongeurs, les canidés, les primates, les félins et l'homme. Le parasite est transmis par les phlébotomes femelles durant le repas sanguin indispensable pour le développement des œufs. Lors de ce repas sanguin, les phlébotomes injectent à l'hôte vertébré des parasites au stade promastigote infectant (promastigote métacyclique) ; ces parasites sont mobiles grâce à un flagelle. Une fois chez l'hôte, ils vont être phagocytés par les macrophages (cellules immunitaires), dans lesquelles ils vont se transformer en forme amastigote, ronde et immobile. Une fois dans ces cellules, les parasites vont se multiplier et entraîner l'éclatement de ces dernières. Le cycle se poursuit par la colonisation de nouveaux macrophages entraînant la progression de l'infection dans les divers tissus dépendamment des espèces de *Leishmania* mais aussi de l'état physiologique et immunologique de l'hôte. Lorsqu'un phlébotome vient prendre un repas sanguin sur un hôte infecté, celui-ci ingère des formes amastigotes majoritairement présentes dans les macrophages circulants et résidents. Ces formes amastigotes se différencient en promastigotes de division dans l'intestin du vecteur et après maturation migrent vers la partie antérieure de l'intestin, elles pourront alors être transmises durant le repas sanguin suivant (Figure 5). Le temps nécessaire pour le développement du parasite varie en fonction des espèces de phlébotomes mais il est d'environ 6 à 9 jours [31].

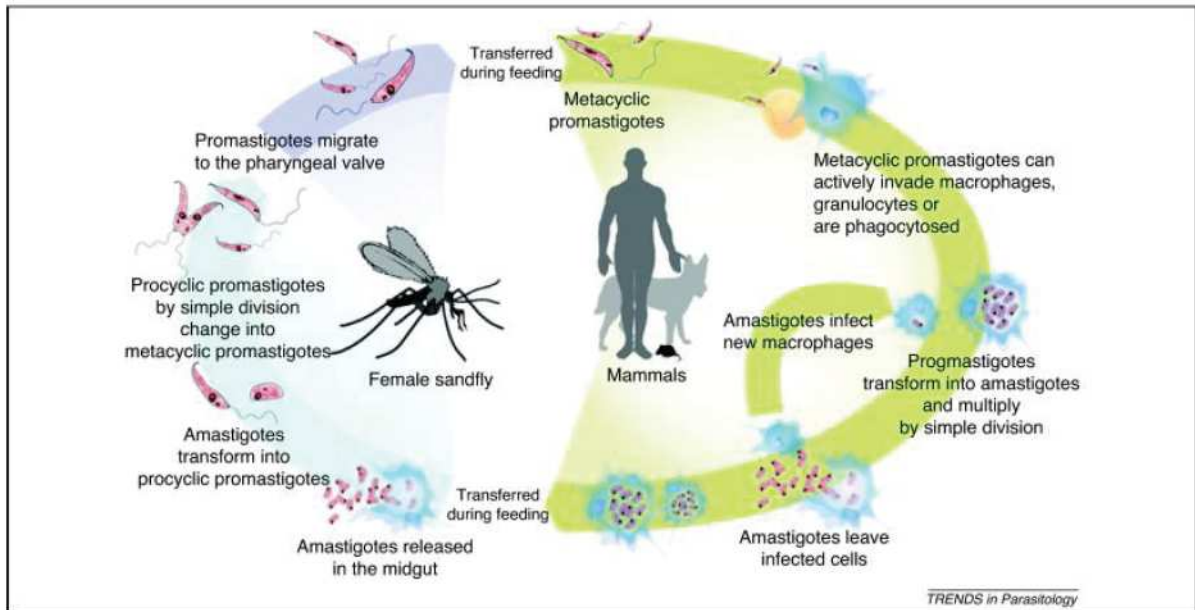


Figure 5 : Cycle de développement du parasite *Leishmania* [32].

Chez l'homme cette affection existe sous une grande variété d'expressions cliniques allant de l'asymptotisme aux formes cutanées bénignes et viscérales [33]. Les leishmanioses sont subdivisées en trois groupes cliniques majoritaires : leishmaniose cutanée (LC), leishmaniose cutanéomuqueuse (LCM) et leishmaniose viscérale (LV). Les LC sont les formes les plus fréquentes et peuvent présenter un large spectre de signes cliniques (Figures 6A et 6B). La LCM, quant à elle, provoque des lésions pouvant partiellement ou totalement détruire les muqueuses du nez, de la bouche, de la gorge ainsi que les tissus environnants produisant parfois des défigurations importantes chez les patients (Figure 6C). Enfin, la LV est une affection grave touchant certains organes vitaux tels que la rate et le foie, pouvant entraîner la mort en l'absence de traitement (Figure 6D).



Figure 6 : Manifestations cliniques des leishmanioses : formes cutanées (A et B), forme muco-cutanée (C) et forme viscérale (D)
(http://www.who.int/leishmaniasis/clinical_forms_leishmaniasis/en/index2.html).

La majorité des cas de LC sont généralement observés en Europe centrale, Amérique du Sud, Afrique, Asie et dans la région Méditerranéenne, tandis que les LV ont une incidence

importante en Asie, Afrique de l'Est, Amérique du Sud ainsi qu'autour du bassin méditerranéen. Certains cas ont également été reportés en Afrique de l'Ouest.

Selon l'Organisation Mondiale de la Santé, approximativement 310 millions de personnes vivent dans les zones d'endémie, 98 pays sont considérés à risque et 20 000 à 40 000 personnes meurent de LV chaque année. De plus, 1 million de nouveaux cas de LC ainsi que 300 000 de LV sont déclarés tous les ans. Ces chiffres seraient sous-estimés car de nombreux cas ne sont pas diagnostiqués ou bien ne sont pas déclarés, en particulier dans les pays où la population n'a pas accès facilement aux soins (Figure 7).

Depuis 1993, un accroissement de l'aire de répartition des leishmanioses ainsi qu'une augmentation du nombre de cas ont été observés [2]. Cependant, la déclaration de ces affections n'étant obligatoire que dans 33 pays, il est difficile de chiffrer de façon précise l'ampleur de cette expansion. Enfin, l'infection par le virus de l'immunodéficience humaine (VIH) a entraîné une augmentation de la susceptibilité aux leishmanioses. Depuis 2012, 35 pays, dans lesquels les leishmanioses sont endémiques, ont signalé des cas de co-infection VIH/*Leishmania*. De plus, on observe une augmentation de la prévalence de la LV dans les régions où l'accès aux thérapies antirétrovirales est difficile [2].

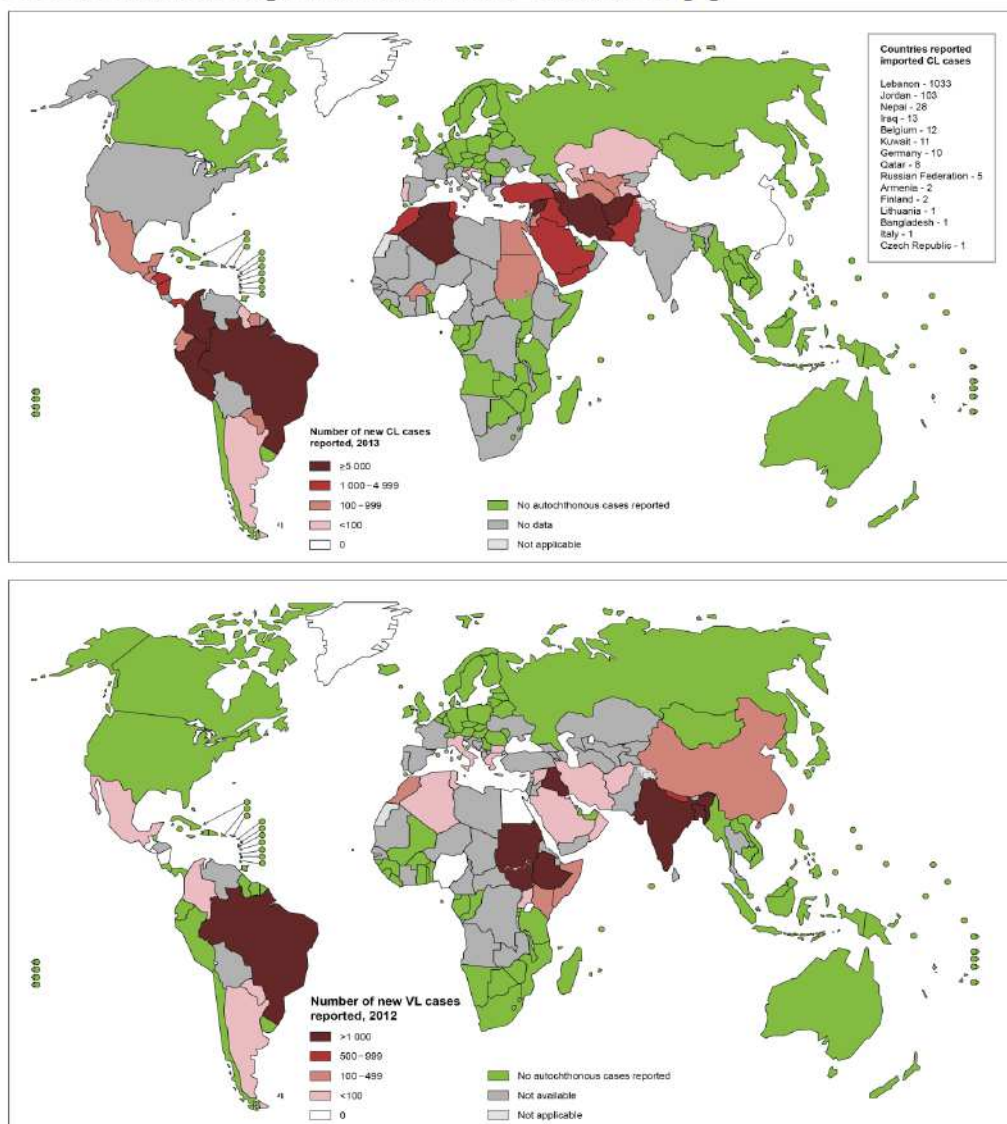


Figure 7 : Distribution géographique des leishmanioses cutanées (A) et viscérales (B) en 2013 (WHO-Global Health Observatory data).

I.3. Les leishmanioses en France

I.3.1. Historique

Comme dans beaucoup de pays, la leishmaniose est présente en France. Le réservoir principal est le chien et cette parasitose représente un problème important en santé vétérinaire, particulièrement dans le Sud de la France. Cependant, elle touche aussi l'homme et en particulier les personnes immunodéficientes [34]. Les formes cliniques de leishmaniose présentes en France sont la forme viscérale (LV) et cutanée (LC). L'espèce responsable est *L. infantum*. Bien que la leishmaniose soit endémique et dépistée en France dès 1918 pour la LV et en 1920 pour la LC, ces affections sont restées pendant longtemps peu connues des cliniciens français [35]. Les cas humains sont répertoriés depuis 1999. Néanmoins, entre 1989 et 1995, l'incidence annuelle moyenne des LV autochtones a été estimée à environ 1,3, 0,66 et 0,22 cas pour 100000 habitants dans les foyers des Pyrénées Orientales, des Alpes-Maritimes et des Cévennes, respectivement [34].

Entre 1999 et 2012, selon le Centre National de Référence des Leishmanioses (CNRL), 317 cas autochtones et 1154 cas importés ont été déclarés dans le Sud de la France. Soit en moyenne 22,6 cas autochtones par an (84,5% de LV) et 82,4 cas importés par an. Les cas importés (91% de LC) proviennent principalement d'Afrique du Nord, d'Afrique subsaharienne et de Guyane Française. Les LC n'étant pas à déclaration obligatoire, un certain nombre de cas n'est sûrement pas comptabilisé. Les études épidémiologiques montrent que la séroprévalence canine peut varier de 3 à 66% en fonction des régions étudiées et de la méthode utilisée [34]. Il est important de noter, qu'une tendance à l'accroissement du nombre de cas et à l'extension territoriale a été observée en France [36]. La leishmaniose canine, mais également humaine, représente donc un problème de santé publique à surveiller.

I.3.2. Distribution

La leishmaniose à *L. infantum* est endémique mais principalement limitée au Sud du pays [34]. Plusieurs foyers de leishmaniose sont clairement identifiés le long de la côte méditerranéenne. Ils sont présents dans les Pyrénées-Orientales, les Cévennes, la Provence, les Alpes-Maritimes et la Corse (Figure 8). La transmission est généralement rurale et deux grandes villes, Nice et Marseille, sont connues comme foyers endémiques dans certains quartiers [34]. Cependant, d'autres régions présentent aussi des cas autochtones tel que le département du Lot et Garonne.



Figure 8 : Distribution des 268 cas autochtones répertoriés de LV entre 1999 et 2012 dans les départements français les plus touchés [34].

I.3.3. Les principaux acteurs

Le chien est le principal réservoir et *L. infantum* est la seule espèce responsable de tous les cas autochtones français [34]. Les vecteurs dans le Sud de la France sont *P. ariasi* et *P. perniciosus* (Voir ci-dessous - Espèces impliquées dans la transmission de la leishmaniose). Les chiens malades présentent une grande diversité de symptômes dont un amaigrissement progressif, des problèmes cutanés (lésions, dermatites) et des griffes anormalement longues (Figure 9).



Figure 9 : Manifestations cliniques de la leishmaniose canine : lésions cutanées (A), dépigmentation autour des yeux (B), griffes anormalement longues (C) et amaigrissement généralisé (D) (Photos Pr. Jean-Antoine Rioux).

I.4. Les principaux phlébotomes vecteurs européens

I.4.1. Distribution

Parmi les espèces présentes sur le pourtour méditerranéen, 8 ont été décrites comme les principaux vecteurs de *L. infantum* : *P. ariasi*, *P. perniciosus*, *P. balcanicus* Theodor, 1958, *P. kandelakii* Shurenkova, 1929, *P. langeroni* Nitzulescu, 1930, *P. neglectus* Tonnoir, 1921, *P. perfiliewi* Parrot, 1930, et *P. tobbi* Adler, Theodor & Lourie, 1930 [37]. Les cartes de répartition disponibles pour 5 de ces espèces (*P. ariasi*, *P. perniciosus*, *P. neglectus*, *P. perfiliewi* et *P. tobbi*) sont présentées dans la figure 10 (European Centre for Disease Prevention and Control (ECDC)).

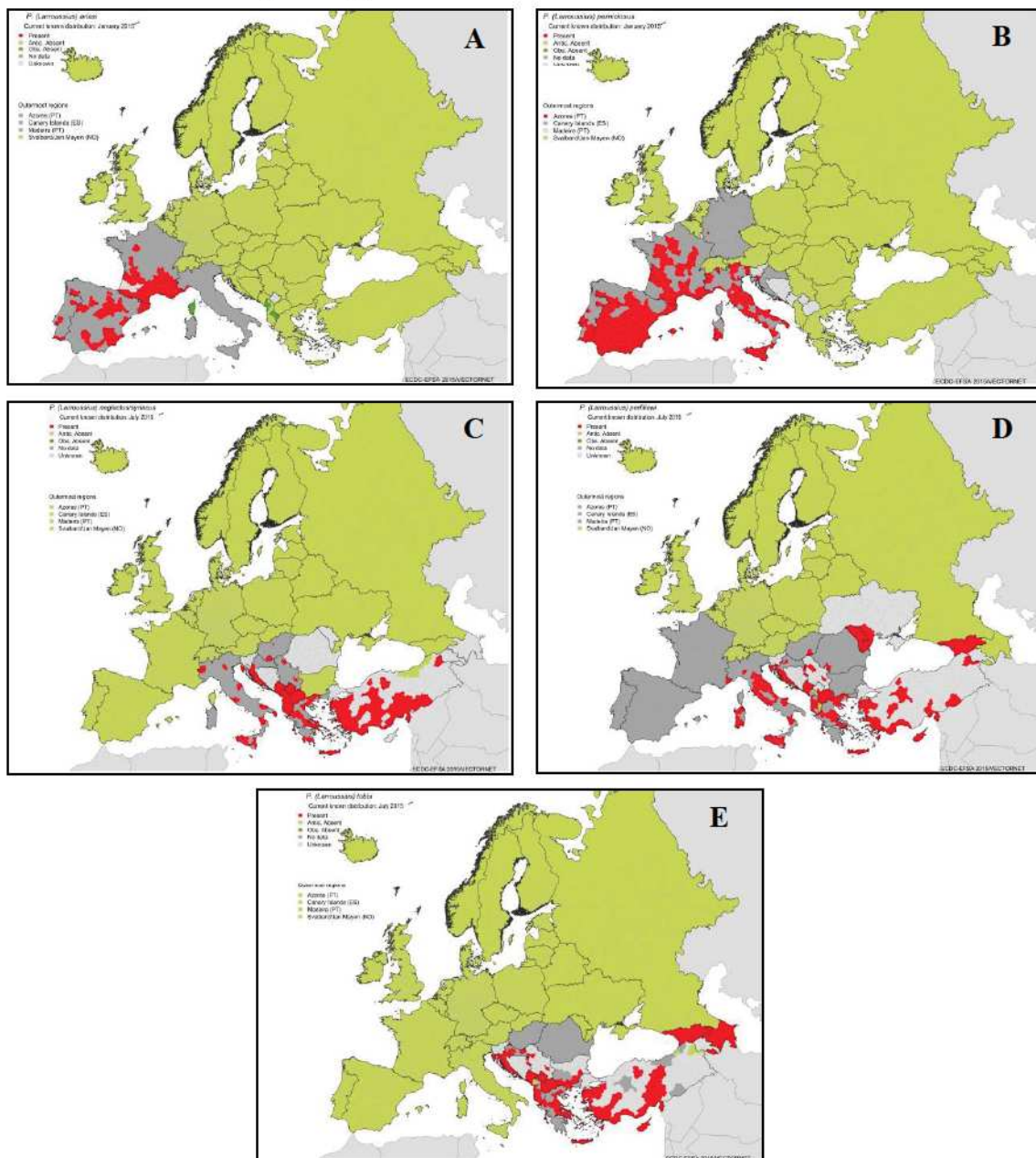


Figure 10 : Cartes européennes de répartition de *Phlebotomus ariasi* (A), *P. perniciosus* (B), *P. neglectus* (C), *P. perfiliewi* (D) et *P. tobbi* (E) (European Centre for Disease Prevention and Control)

I.4.2. Activité saisonnière

L'activité saisonnière des principaux phlébotomes vecteurs en Europe est détaillée dans : « Alten B, Maia C, Afonso O, Campino L, Jiménez M, González E, Molina R, Bañuls A-L, **Prudhomme J**, Vergnes B, Toty C, Cassan C, Thierry M, Sereno D, Bongiorno G, Bianchi R, Khoury C, Tsirigotakis N, Dokianakis E, Antoniou M, Christodoulou V, Mazeris A, Karakus M, Ozbel Y, Arserim S K, Erisoz Kasap O, Gunay F, Oguz G, Kaynas S, Tsertsvadze N, Tskhvaradze L, Giorgobiani E, Gramiccia M, Volf P, Gradoni L. *Seasonal dynamics of phlebotomine sand fly species proven vectors of Mediterranean leishmaniasis caused by Leishmania infantum*, PLoS Neg. Trop. Dis, sous presse » [24].

I.4.2.1. *Résumé*

L'activité des phlébotomes est saisonnière dans les pays du pourtour méditerranéen en raison des basses températures en hiver. Elle est dépendante des conditions environnementales et climatiques et peut varier en fonction du vecteur ainsi que du lieu de capture. Il est donc important d'étudier les spécificités pour chacun des pays et espèces pour mieux comprendre l'actuelle expansion de *L. infantum* et de ses vecteurs sur le pourtour méditerranéen.

Dans ce contexte, cette publication a donc pour but de présenter une base de référence sur la dynamique saisonnière des principaux vecteurs de *L. infantum* du pourtour méditerranéen.

Entre 2011 et 2014, dans le cadre du projet Européen EDENext (www.edenext.eu) 119 partenaires provenant de 8 pays méditerranéens ont effectué des enquêtes entomologiques dans des sites représentatifs de leur pays pendant au moins 2 années consécutives. L'altitude des sites de captures était comprise entre 3m (Portugal) et 691m (Espagne). Sur les 99000 spécimens capturés, 56000 ont permis d'établir la dynamique saisonnière des 6 principales espèces vectrices de ces différents pays, à savoir *P. perniciosus* (Portugal, Espagne et Italie), *P. ariasi* (France), *P. neglectus* (Grèce), *P. tobbi* (Chypre et Turquie), *P. balcanicus* et *P. kandelakii* (Géorgie). Les données récoltées dans le cadre de cette thèse ont permis d'aider à la réalisation de cette publication.

Sur le pourtour méditerranéen, les phlébotomes sont présents de Mai à Octobre. Les températures moyennes annuelles ainsi que la latitude vont influencer les périodes d'émergence (d'Avril à Juin) mais aussi les dynamiques saisonnières de ces insectes (allant d'un pic en Juillet / Août à plusieurs pics). Ces données mettent en évidence des distributions saisonnières homogènes localement et par conséquent permettent d'identifier des périodes à risque pour la transmission de la leishmaniose dans chacun des pays mais également à l'échelle de la région. La période à risque pour la transmission de *L. infantum* dans la région méditerranéenne est comprise entre juin et octobre. La densité d'adulte ainsi que la probabilité de femelles infectées par le parasite sont faibles au début de la période d'activité. Le risque reste cependant dépendant de la géographie du pays et du vecteur.

I.4.2.2. *Article 1 : Seasonal dynamics of phlebotomine sand fly species proven vectors of Mediterranean leishmaniasis caused by Leishmania infantum.*

1 **Seasonal dynamics of phlebotomine sand fly species proven vectors of**
 2 **Mediterranean leishmaniasis caused by *Leishmania infantum***

3
 4 **Short Title:** Seasonal dynamics of Mediterranean *Leishmania infantum* vectors

5
 6 Bulent Alten¹, Carla Maia², Odete Afonso², Lenea Campino², Maribel Jiménez³, Estela González³,
 7 Ricardo Molina³, Anne Laure Bañuls⁴, Jorian Prudhomme⁴, Baptiste Vergnes⁴, Celine Toty⁴,
 8 Cécile Cassan⁴, Magali Thierry⁴, Denis Sereno⁴, Gioia Bongiorno⁵, Riccardo Bianchi⁵, Cristina
 9 Khoury⁵, Nikolaos Tsirigotakis⁶, Emmanouil Dokianakis⁶, Maria Antoniou⁶, Vasiliki
 10 Christodoulou⁷, Apostolos Mazeris⁷, Mehmet Karakus⁸, Yusuf Ozbel⁸, Suha K. Arserim⁹, Ozge
 11 Erisoz Kasap¹, Filiz Gunay¹, Gizem Oguz¹, Sinan Kaynas¹⁰, Nikoloz Tsertsvadze¹¹, Lamzira
 12 Tskhvaradze¹¹, Ekaterina Giorgobiani¹¹, Marina Gramiccia⁵, Petr Volf¹², Luigi Gradoni^{5*}

13
 14 ¹Department of Biology, Ecology Division, HUESRL-VERG laboratories, Hacettepe University, Ankara,
 15 Turkey; ²Instituto de Higiene e Medicina Tropical, Unidade Parasitologia Médica, Universidade Nova de
 16 Lisboa, Portugal; ³Centro Nacional de Microbiología, Servicio de Parasitología, Unidad de Entomología
 17 Médica, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain; ⁴Institut de Recherche pour le
 18 Développement, MIVEGEC, Montpellier, France; ⁵M.I.P.I. Department, Unit of Vector-borne Diseases and
 19 International Health, Istituto Superiore di Sanità, Rome, Italy; ⁶Laboratory of Clinical Bacteriology,
 20 Parasitology, Zoonoses and Geographical Medicine, University of Crete, Heraklion, Greece; ⁷Veterinary
 21 Services of Cyprus, Nicosia, Cyprus; ⁸Department of Parasitology, Ege University, Izmir, Turkey;
 22 ⁹Vocational School of Health Sciences, Cela Bayar University, Manisa, Turkey; ¹⁰Veterinary Faculty,
 23 Mehmet Akif Ersoy University, Burdur, Turkey; ¹¹National Center for Disease Control and Public Health,
 24 Tbilisi, Georgia; ¹²Department of Parasitology, Faculty of Science, Charles University, Prague, Czech
 25 Republic.

26
 27 *Corresponding author

28 Email: luigi.gradoni@iss.it

29
 30 **Keywords:** Mediterranean phlebotomine vectors of *Leishmania infantum*; seasonal dynamics;
 31 circadian activity rhythm; *Phlebotomus perniciosus*; *Phlebotomus ariasi*; *Phlebotomus neglectus*;
 32 *Phlebotomus tobbi*; *Phlebotomus balcanicus*; *Phlebotomus kandelakii*; Portugal; Spain; Italy;
 33 France; Greece; Cyprus; Turkey; Georgia

35 **Abstract**

36 **Background**

37 The recent geographical expansion of phlebotomine vectors of *Leishmania infantum* in the
38 Mediterranean subregion has been attributed to ongoing climate changes. At these latitudes, the
39 activity of sand flies is typically seasonal; because seasonal phenomena are also sensitive to general
40 variations in climate, current phenology data can provide a baseline for continuing investigations on
41 sand fly population dynamics that may impact on leishmaniasis transmission. With this aim, a
42 consortium of partners from eight Mediterranean countries carried out multiannual entomological
43 investigations in sites where *L. infantum* transmission was recently reported.

44 **Methods/Principal findings**

45 A common protocol for sand fly collection included monthly captures by CDC light traps which
46 were complemented by sticky traps in most of the sites. Collections were replicated for more than
47 one season in order to reduce the effects of annual climate variations and local weather events. Data
48 from 99,000 collected specimens were analyzed, resulting in the descriptive seasonal dynamics of
49 56,000 sand flies belonging to *L. infantum* vector species throughout a wide geographical area,
50 namely *P. perniciosus* (Portugal, Spain and Italy), *P. ariasi* (France), *P. neglectus* (Greece), *P. tobbi*
51 (Cyprus and Turkey), *P. balcanicus* and *P. kandelakii* (Georgia). The seasonal trend cumulative for
52 3 years, 37 sites and 6 vector species consisted in a bell-shaped curve falling between May and
53 October for 99% of values; no sand flies were collected in March or December. Significant
54 correlations were found between latitude or mean annual temperature of trapping sites and i) the
55 first month of sand fly appearance, ranging from April to June, and ii) the type of density trend,
56 ranging from a single peak in July/August to multiple peaks increasing in magnitude from May
57 through September.

58 **Conclusions**

59 Multiple collections performed over consecutive years can provide homogeneous patterns of the
60 potential behavior of leishmaniasis vectors. In the investigated years, higher potential risk for *L.*
61 *infantum* transmission in the Mediterranean was identified in the June-October period. Such risk
62 was not equally distributed throughout the region, since density waves of adults were more frequent
63 in southern territories.

65 **Author summary**

66 Recent projections on global warming indicate a constant rise of temperatures in the Mediterranean
67 subregion in the near-mid future. While this phenomenon already caused geographical expansion of
68 several arthropod-borne diseases, it is likely to affect also temporal parameters of seasonally

69 transmitted diseases such as leishmaniasis, a protozoan infection spread by the bite of phlebotomine
70 sand flies. Phenology of sand flies consists in the periods of emergence of adults and their
71 disappearance from collections following an activity period during warm months, which can be
72 characterized by peaks of abundance. Current phenological observations can be important for
73 continuing investigations on sand fly dynamics that may impact on leishmaniasis transmission in
74 the future. With this aim, partners from eight Mediterranean countries identified sites with
75 documented *Leishmania infantum* transmission carried by six vector species and performed
76 multiannual trappings. From the high number of 56,000 specimens collected throughout an area
77 spanning from Portugal at west to Georgia at east, a descriptive seasonal dynamics of
78 Mediterranean vectors was obtained. Both, period of adults emergence and type of abundance trend,
79 were found to be significantly correlated with latitude or mean annual temperature of trapping sites.

80

81 **Introduction**

82 Phlebotomine sand flies (Diptera, Psychodidae) are the unique haematophagous insects proven to
83 transmit leishmaniasis [1]. Of approximately 900 species estimated to exist [2] less than a hundred,
84 belonging to *Phlebotomus* and *Lutzomyia* (*sensu* Young and Duncan, 1994 [3]) genera are proven
85 or suspected vectors of human disease in the Old and New Worlds, respectively [4,5]. *Leishmania*
86 *infantum* is the main causative agent of zoonotic visceral leishmaniasis (VL) and also responsible
87 for cases of cutaneous leishmaniasis (CL) across the Mediterranean subregion that includes
88 southern Europe, northern Africa and parts of Asia [6,7]. Domestic dogs are primary reservoirs of
89 infection for humans and may suffer from a severe chronic disease (canine leishmaniasis, CanL).
90 The annual incidence of human VL is estimated to range from 1500 to 2700 cases; available figures
91 on *L. infantum* CL incidence are no reliable because of poor reporting [8]. A dozen of *Phlebotomus*
92 species have been implicated in the transmission of Mediterranean *L. infantum*, of which eight have
93 been incriminated as vectors according to conventional criteria [4,9]: *Phlebotomus ariasi* [10], *P.*
94 *balcanicus* [11], *P. kandelakii* [11], *P. langeroni* [12], *P. neglectus* [13], *P. perfiliewi* [14], *P.*
95 *perniciosus* [15] and *P. tobbi* [16]. All these species but *P. balcanicus*, which belongs to the
96 subgenus *Adlerius*, are members of the *Larroussius* subgenus.

97 Effects of long-term climate changes on the geographical expansion of Mediterranean *L.*
98 *infantum* vectors towards northern latitudes or higher altitudes have been predicted [17] and
99 actually observed in some places, such as in northern Italy [18, 19], French and Spanish Pyrenees
100 [20,21] and in Rhineland-Palatinate, Germany [22]. As a consequence, spread of human and canine
101 disease associated with progressive increase of *Leishmania* seroprevalence rates in dogs were
102 reported at altitudes and latitudes higher than might be expected in several Mediterranean countries

103 [reviewed in [23]]. The activity period of Mediterranean adult sand flies is typically seasonal.
104 Because seasonal phenomena are also very sensitive to variations in temperature, phenological
105 observations may provide high resolution of ongoing climate changes in addition to geographical
106 dispersion parameters [24]. However the available information on sand fly seasonal dynamics in the
107 Mediterranean region is patchy, being disperse in time and space. Although several investigations
108 on this subject were performed over the past 50 years, they included mostly one season - rarely 2 or
109 3 - and were performed in single sites at different periods [11,25-31]. Hence, recent and accurate
110 knowledge of Mediterranean vectors dynamics would be required as a starting baseline for
111 continuing investigations on changes that may have an impact on leishmaniasis transmission.
112 Because investigations of this type are prone to several confounding parameters, prerequisites
113 would be: a) to perform studies in a relatively short period (i.e. a time range of a few years) in order
114 to minimize possible effects of the ongoing slow temperature increase [32]; b) to replicate studies in
115 the same sites for more than one season, with a view to reduce as far as possible the effects of
116 annual climate variations and local weather events; and c) to carry out studies over a wide
117 geographical area, in order to provide general information from different latitudes and longitudes.

118 In the frame of EDENext EU FP7 (www.edenext.eu) project, in 2011-2014 a consortium of
119 partners from 8 Mediterranean countries endemic for *L. infantum* have carried out entomological
120 investigations in representative sites for at least 2 consecutive years. In this paper, we report
121 observational data from about 99,000 collected specimens, resulting in the description of seasonal
122 dynamics and nocturnal activity of *L. infantum* vectors over a wide geographical range spanning
123 from Portugal at west to Georgia at east, namely: *P. perniciosus* (Portugal, Spain and Italy); *P.*
124 *ariasii* (France); *P. neglectus* (Greece); *P. tobbi* (Cyprus and Turkey); *P. balcanicus* and *P.*
125 *kandelakii* (Georgia).

126

127 **Materials and methods**

128

129 **Study areas and vectors**

130 In each country, one or more locations were identified on the basis of the historical presence of
131 proven phlebotomine vector(s) and the evidence of human and/or animal leishmaniasis transmission
132 in the area. Geographical coordinates and altitude of study regions and sites are shown in Table 1.
133 The southernmost site was located in Cyprus (latitude 34°59'54''N) and the northernmost in
134 Languedoc-Roussillon, France (latitude 43°58'23''N). The westernmost site was in Setúbal district,
135 Portugal (longitude 9°16'52''W) and the easternmost in Tbilisi, Georgia (longitude 44°49'30''E).
136 All sites were at low-mid altitudes above sea level: the lowest altitude was in Portugal (3 m), the

137 highest in Spain (691 m). On overview of the collecting sites location in the Mediterranean region is
 138 shown in Fig. 1.

139

140 **Table 1. Geographical coordinates and elevation of 37 sand fly collecting sites.**

141

Country	Region/District/Site*	No. of sites	Latitude	Longitude	Elevation (m a.s.l.)
Portugal	Lisbon Metropolitan Region/Setúbal and Lisbon	11	From 38°28'37''N to 38°44'51''N	From 9°16'52''W to 8°45'2''W	3 – 330
	Algarve/Faro	11	From 37°3'27''N to 37°14'20''N	From 8°37'45''W to 7°26'34''W	10 – 74
Spain	Autonomous Community of Madrid/Fuenlabrada	1	40°17'53''N	3°47'31''W	635 – 691
France	Languedoc-Roussillon/Gard/Roquedur-le-haut	1	43°58'23''N	3°39'26''E	603
Italy	Latium/Rome/Frascati	1	41°50'34''N	12°41'57''E	192
Greece	Crete/Heraklion/Fodele	1	35°22'52''N	24°57'29''E	40 – 70
Cyprus	Paphos/Steni	1	34°59'54'' N	32°28'17'' E	200
Turkey	Aegean/Aydin/Bascayir	1	37°57'35''N	28°04'03''E	427
	Cukurova/Adana	6	From 37°17'59''N to 37°26'01''N	From 35° 31'01''E to 35°39'27''E	150 – 280
Georgia	Tbilisi/Gldani-Nadzaladevi and Isani-Samgori	3	From 41°42'01''N to 41°44'08''N	From 44° 48'59''E to 44°49'30''E	495 – 603

142 *Named when only one site was investigated

143

144

145 **Figure 1. Location of the sand fly collecting sites in the Mediterranean region**

146

147 In Portugal, the Lisbon Metropolitan Region in the centre and the Algarve Region in the
 148 south are endemic for VL, CL and CanL, predominantly caused by the *L. infantum* zymodeme
 149 MON-1 and transmitted by two vectors, *P. perniciosus* and *P. ariasi* [33,34]. The investigations
 150 required collections in multiple sites of the two Regions, because of the known low density of local
 151 vectors in most places. Thus, 11 collecting sites from the Setúbal and Lisbon Departments in Lisbon
 152 Metropolitan Region were investigated in 2011 and 2012; 11 sites from the Department of Faro in
 153 Algarve Region were investigated in 2011, 2012 and 2013.

154 In Spain, an outbreak of both VL and CL has occurred since mid 2009 in four towns
 155 southwest of Madrid, with nearly 600 cases mainly recorded in Fuenlabrada [35]. In contrast to a
 156 low prevalence of CanL, lagomorphs – the hare *Lepus granatensis* and the wild rabbit *Oryctolagus*
 157 *cuniculus* - were identified as unusual reservoir in a large urban park. These animals have been
 158 found infected by *L. infantum* at high rates and proved to transmit the parasite to reared *P.*

159 *perniciosus* [36]. Wild *P. perniciosus*, the only proven vector in the area, was found to harbor the
160 same strain of *L. infantum* causing human and animal infections [37]. Locations bordering the urban
161 center of Fuenlabrada were surveyed in this endemic setting in 2012 and 2013.

162 In southern France, a traditional endemic focus of human leishmaniasis and CanL caused by
163 *L. infantum* is represented by a hilly area located between the Hérault and Arre valleys in the
164 Languedoc-Roussillon Region. The dominant phlebotomine species in the area is *P. ariasi*,
165 incriminated as *L. infantum* vector in late 1970s [10]. A representative site (Roquedur-le-haut) was
166 investigated in 2011, 2012 and 2013.

167 In Italy, the central Latium Region is ranked as the third most endemic territory for human
168 VL and CanL, both caused by *L. infantum* MON-1 [38]. *P. perniciosus* is the proven vector in
169 Rome province [29] and this species is found abundant in hills surrounding the capital city. A site
170 from this area (Frascati) was surveyed in 2011 and 2012.

171 In Greek islands and mainland, leishmaniasis have re-emerged during the past three decades
172 [39]. In Crete, both *L. infantum* zymodemes MON-1 and MON-98 were found to cause human VL
173 and CanL. Among 9 *Phlebotomus* species endemic in the island, *P. neglectus*, a proven *L. infantum*
174 vector in Greece [13], is the most abundant and geographically widespread [40]. A representative
175 site (Fodele, Heraklion region) was investigated from 2011 through 2013.

176 In Cyprus, leishmaniasis has re-emerged in recent years; apparently no human cases caused
177 by *L. infantum* occur in the island, despite this parasite (predominantly zymodeme MON-1) is
178 widespread among dogs [41]. *P. tobbi* is the proven *L. infantum* vector in Cyprus [16], whereas the
179 role of this species in the transmission of the recently introduced *Leishmania donovani* MON-37
180 (apparently the only cause of human VL and CL in the island [23]) remains to be determined. A
181 representative collection site (Steni, Paphos district) was investigated in 2012 and 2013.

182 Different entities of human leishmaniasis are endemic in Turkey, including zoonotic VL and
183 CL caused by *L. infantum*, and anthroponotic CL by *L. tropica* [42]. *L. infantum* was traditionally
184 prevalent in the Aegean and Mediterranean coasts of western Turkey, however it has been recently
185 detected as an agent of CL in Cukurova region, south Anatolia [43]; genome sequencing of strains
186 isolated from *P. tobbi* and human patients revealed *L. infantum*/*L. donovani* hybrids [44]. Among
187 at least 21 *Phlebotomus* species described in Turkey, a few potential *L. infantum* vectors have been
188 suspected throughout the country, but only *P. tobbi* has been incriminated conclusively [43]. Six
189 collecting sites were selected in villages of the northwest part of the Cukurova region, Adana
190 province, for seasonal dynamics investigations in 2011 and 2012. In western Turkey, a
191 representative site (Bascayr village, Aydin province) endemic for human VL and CanL and

192 showing predominance of the vector *P. tobbi* [45], was selected for investigations on nocturnal
193 activity in 2012.

194 In Georgia, the disease has emerged as a significant public threat in the capital city of Tbilisi
195 beginning from the years 1990s. The infections, caused by *L. infantum*, were also found widespread
196 in domestic and stray dogs [46]. In this endemic setting, 2 phlebotomine species have been recently
197 incriminated as *L. infantum* vectors, *P. kandelakii* and *P. balcanicus* [11]. Two collecting sites were
198 selected for phenological investigations performed from 2011 through 2013 in each of the two
199 urban districts where VL mostly occurs: Lotkini in Gldani-Nadzaladevi district, and Elia in Isani-
200 Samgori district, respectively. Investigations on nocturnal activity were performed in 2014 in the
201 Vera area of Tbilisi.

202

203 **Sand fly collection, habitat and species identification**

204 A common standardized protocol for trapping methods and periodicity of sand fly collections was
205 established. Both attraction and interception methods were employed, and the number of traps per
206 site was left unchanged over the years. Priority was given to the use of CDC miniature light traps
207 (the same model was purchased from different traders) which were used by all investigators and
208 placed in each collection site for a minimum of 2 consecutive nights per month, with replacement of
209 traps after every night. Previous investigator's experience in each setting showed that trapping
210 yields may vary greatly depending whether sand flies concentrate in hot spots, thus requiring the
211 use of a few traps, or they are dispersed at low density over a territory, which requires a greater
212 sampling effort. Thus, a range of 2-12 CDC traps were set per site per night, operating 1-2 hours
213 before sunset until 1-2 hours after sunrise. Light-attraction collections were complemented by
214 sticky trap collections in most of the surveyed sites, the choice depending on previous investigator's
215 experience on sand fly yields by both methods. For example, in sites of Cukurova region and
216 Tbilisi interception methods were found to be low productive so that only CDC traps were
217 employed there. Sticky traps consisted of 20x20 cm white paper coated with castor oil; a variable
218 number of sticky traps were set monthly for at least 2 consecutive days. For specific investigations
219 on the nocturnal activity of sand flies, CDC light traps operated for at least one night in selected
220 sites, and manual or automatic trap replacement was performed every 1 or 2 hours. In each site,
221 temperature and humidity were recorded daily during the trapping period.

222 The common protocol established that within a month, setting of traps had to be made in a
223 period having day 15 as median. Within a year, the start of trapping activity was based on previous
224 local experience. It was established that investigators had to bring collections forward by one month
225 compared to historical observations on the local sand fly appearance. Trapping was stopped when

226 no more sand flies were captured by any method. A large variety of domestic and peri-domestic,
227 rarely sylvatic, habitats were surveyed in rural, village or urban areas by means of indoor and/or
228 outdoor collections. In general, they were fairly representative of typical *L. infantum* transmission
229 biotopes in the Mediterranean region. Traps were set in human dwellings, cellars, courtyards,
230 livestock sheds, hen houses, kennels, gardens, shady areas in various types of orchards, olive
231 groves, and vineyards. The large diversity of potential blood meal sources included humans and
232 domestic animals such as dogs, cats, cattle, sheep, goats, horses, rabbits, and poultry. A number of
233 wild mammals of potential interest in *L. infantum* epidemiology were also present in surrounding
234 areas (e.g. black rats, hares, wild rabbits and foxes).

235 Collected sand flies were preserved in ethanol pending species identification. This was
236 largely performed morphologically using published keys and descriptions [47-52]. Species identity
237 of some specimens belonging to taxonomically challenging species (e.g. *P. transcausicus*/*P.*
238 *galilaeus*; *P. sergenti*/*P. similis*; or members of the *Adlerius* subgenus) was also confirmed by
239 morphometric measurements [53] and/or sequencing analysis of two gene markers, namely
240 cytochrome b [54] and internal transcribed spacer 2 (ITS2) [55].

241

242 **Data analysis and output**

243 The final outcome of this observational study was the production in practical graphic format of
244 phenological and abundance parameters that may have an impact on *L. infantum* transmission by
245 the relevant vector species, namely: a) the beginning and the end of adults activity during a year; b)
246 the variation in abundance during the activity period; c) the nocturnal activity . Various data levels
247 were combined *a priori* (e.g. collections from multiple sites within a small territory; sexes; trapping
248 methods) or in course of data analysis. Cumulative number of specimens rather than averages were
249 considered in most graphical presentations. Correlation analyses were generated by Pearson
250 correlation coefficient; statistics and graph production were performed using GraphPad Prism
251 Software, version 5.00 for Windows (San Diego, California, USA).

252

253 **Results**

254

255 **Sand fly fauna**

256 A total of 99195 sand fly specimens (59.8% males) were collected from 2011 through 2014, of
257 which 95384 were morphologically identified at species level. Specimens that presented
258 problematic identification (mainly females of the *Larroussious* subgenus) were excluded from
259 further analysis. The sand fly fauna included both *Phlebotomus* and *Sergentomyia* genera;

260 cumulatively, males were more abundant than females for most species, sites and years of
 261 collection. Furthermore, the male/female ratio was usually higher during the first period of
 262 collection, whereas females outnumbered males in the middle of the warm season (S1-S9 Tables).

263 In sites where a combination of the two trapping methods was regularly used, 26482/47873
 264 specimens (55.3%) were collected by sticky traps. However large variations were recorded
 265 depending on sand fly species and sites: for example, yields of *P. perniciosus* in Spain and Portugal
 266 were almost identical by both trapping methods, whereas specimens of *P. perniciosus* in Italy, *P.*
 267 *neglectus* in Crete and *P. tobbi* in Cyprus were mostly collected by CDC light traps. On the other
 268 hand, *Sergentomyia* specimens were almost exclusively collected by sticky traps (>90% in all sites)
 269 confirming low light attractiveness by the members of this subgenus (S10 Table).

270 Table 2 summarizes the distribution by country of *Phlebotomus* species identified regardless
 271 their relative abundance. From 1 to 3 species were collected in western Mediterranean sites, and
 272 from 3 to 9 species in eastern Mediterranean sites. As expected per study design, higher densities
 273 were recorded for the proven *L. infantum* vector species in each locality. Where two proven vector
 274 species were identified (e.g. *P. perniciosus* and *P. ariasi* in Portuguese and French sites), only the
 275 most abundant species was considered in the descriptive seasonal dynamics.

276

277 **Table 2. *Phlebotomus* species collected in selected sites of 8 countries of the Mediterranean**
 278 **region.** Exclusive or predominant *Leishmania infantum* vector species are marked with an asterisk

279

<i>Phlebotomus</i> species	Portugal	Spain	France	Italy	Greece	Cyprus	Turkey	Georgia
Subgenus <i>Larroussius</i>								
<i>P. ariasi</i>	X		X*					
<i>P. perniciosus</i>	X*	X*	X	X*				
<i>P. neglectus</i>					X*			
<i>P. neglectus/syriacus</i> ^a							X	
<i>P. major</i> s.l. ^a							X	
<i>P. tobbi</i>						X*	X*	
<i>P. galilaeus</i> ^b						X		
<i>P. transcaucasicus</i> ^b							X	
<i>P. kandelakii</i>								X*
<i>P. wenyoni</i>								X
Subgenus <i>Paraphlebotomus</i>								
<i>P. sergenti</i>	X	X				X	X	X
<i>P. similis</i>					X			
<i>P. alexandri</i>							X	
Subgenus <i>Adlerius</i>								
<i>P. balcanicus</i>								X*
<i>P. halepensis</i>								X
<i>P. simici</i>							X	
Subgenus <i>Phlebotomus</i>								
<i>P. papatasi</i>		X			X	X	X	

Subgenus <i>Transphlebotomus</i>								
<i>P. mascittii</i>			x				x	

280 ^a Exhibiting intermediate morphological characters within the *Phlebotomus major* group

281 ^b Both species are also considered subspecies within the *Phlebotomus perfiliewi* group

282

283

284 **Overview of the seasonal dynamics of Mediterranean *L. infantum* vectors**

285 The six *L. infantum* vectors marked with an asterisk in Table 2 accounted for a total of 56101
 286 specimens collected from 2011 through 2013. They consisted of 8665 specimens cumulatively
 287 collected in 2011 in six countries (Portugal, France, Italy, Greece, Turkey and Georgia); 28168
 288 specimens collected in 2012 in all eight countries; and 19268 specimens collected in 2013 in six
 289 countries (Portugal, Spain, France, Greece, Cyprus and Georgia). Graphs of Fig. 2 show seasonal
 290 trends for each year, pooled for sites (n=37) and vector species (n=6); an additional graph reports
 291 such data combined for the 3 years. The latter graph consists in a regular bell-shaped curve having a
 292 wide peak center falling between July and September for 80% of values, and between May and
 293 October for 99% of values. Flat tails (0.5% of values each) are found in April and November; no
 294 specimens were collected in March or December. This homogeneous curve results from the
 295 combination of 3 yearly patterns that are markedly different from each other: in 2011, probably
 296 because of low total catches, no sand flies were collected in April and November, nor major
 297 abundance peak(s) were recorded during the activity period. The 2012 trend was mostly mono-
 298 modal with a sharp peak in August; by contrast, the 2013 trend was bi-modal including two sharp
 299 peaks in June-July and in September.

300

301 **Figure 2. Seasonal dynamics of *Leishmania infantum* vectors (n= 56101) recorded in the**
 302 **Mediterranean region in the 2011-2013 period.** Specimen number is pooled for sites (n=37) and
 303 vector species (n=6), separately for each year and for the whole period.

304

305 **Phenology by species and site**

306 Two major parameters for vector phenology, the month of appearance of adults in collections and
 307 the last month in which they were still collected, have been examined separately by target species
 308 and sites. The relationship between the period of appearance/disappearance of sand flies and
 309 latitude or average annual temperature of sites, was evaluated using data from cumulative years.
 310 When used separately in the analysis, latitude and average annual temperature gave comparable
 311 results. Fig. 3 shows the significant correlation found between latitude/temperature and the first
 312 month of sand fly collection ($r^2= 0.6$; $p= 0.02$). In the two southernmost sites of Cyprus and Crete,

313 specimens of *P. tobbi* and *P. neglectus*, respectively, were collected as early as April; in four
314 territories of Portugal, Turkey and Spain, located at intermediate latitudes, the first specimens of *P.*
315 *pernicius* and *P. tobbi* have been captured in May; at more elevated latitudes such as those of the
316 Italian and Georgian sites, *P. perniciosus*, *P. kandelakii* and *P. balcanicus* were collected starting
317 from June. An exception to this correlation was *P. ariasi* from the northernmost site in France,
318 which was collected since May - although only in 2011 and involving a low number of specimens
319 (S4 Table). Some species appeared earlier at lower latitude, and later at higher latitude: this is the
320 case of *P. tobbi*, which was collected in April in Cyprus and in May in Turkey, and *P. perniciosus*,
321 collected in May in Portugal and Spain, and in June in Italy.

322

323 **Figure 3. Relationship between latitude of collecting sites and the month of appearance of**
324 **sand flies in collections.** Each species is shown by a different color, and the average annual
325 temperature of each site is reported in parenthesis. By excluding the few catches of *Phlebotomus*
326 *ariasi* from the French site in May 2011 (S4 Table), then $r^2 = 0.9$ and $p < 0.001$.

327

328 By contrast, a similar analysis performed using the last month of sand fly collection as a variable,
329 showed that the disappearance of adults was not dictated by latitude or average annual temperature:
330 as shown in Fig. 4, each species per site appeared to be ending the activity in a period from
331 September through November without significant correlation with the above parameters ($r^2 < 0.01$;
332 $p = 0.92$).

333

334 **Figure 4. Relationship between the latitude of collecting sites and the last month of positive**
335 **sand fly collection.** Each species is shown by a different color.

336

337 **Monthly abundance by species and site**

338 The absolute and relative abundance of sand flies varied between species, sites and year of
339 collection (see S1-S9 Tables for details). The most abundant catches were those of *P. perniciosus*
340 from Fuenlabrada, Spain, in 2012 (14101 specimens); *P. ariasi* from Roquedur-le-haut, France, in
341 2013 (7065 specimens); and *P. tobbi* from Cukurova, Turkey, in 2012 (5189 specimens). Seasonal
342 trends of vectors by site and year are shown individually in graphs of S1 Fig. Most of the target
343 species showed annual variations in both magnitude and periodicity of density peaks in each site.
344 Peaks were rarely recorded in May (e.g. *P. neglectus* in Crete, 2013) or in June (e.g. *P. tobbi* in
345 Turkey, 2011), whereas they were frequently observed from July through September. Broadly, four
346 types of density trends could be identified when cumulative years were considered for each species

347 and location (Fig. 5): 1) A sharp mono-modal trend, with a single peak occurring in July or in
348 August; 2) A trend much probably resulting from the confluence of 2 density peaks encompassing
349 the June-September period; 3) A sharp bi-modal trend, with a first minor peak in May-June and a
350 major peak in August-September; 4) A tri-modal trend, with peaks increasing in magnitude from
351 May through September.

352

353 **Figure 5. Types of abundance trends recorded in 2011-2013 for Mediterranean vectors of**
354 ***Leishmania infantum*.** In each graph, values of Y axis represent the number of specimens pooled
355 for the years of trapping

356

357 The relationship between the type of abundance trend and the latitude or the average annual
358 temperature of sites, was evaluated using data from cumulative years. Again, when analyzed
359 separately, latitude and average annual temperature gave comparable results. As shown in Fig. 6, a
360 highly significant correlation was detected between latitude decrease/temperature increase and the
361 increase in number of density peaks ($r^2= 0.85$; $p <0.001$. For example, the trend of *P. tobbi* was tri-
362 modal in the southernmost site of Cyprus, whereas it was bi-modal in Cukurova, Turkey.

363 Analogously, the two distinct peaks observed for *P. perniciosus* in southern Portugal turned to be
364 confluent at higher latitudes in the same country, and similar to what observed in Spanish and
365 Italian sites. The abundance patterns of French and Georgian vectors were confirmed to be typically
366 mono-modal, as previously reported [11,56].

367

368 **Figure 6. Relationship between latitude of sand fly collecting sites and type of abundance**
369 **trend.** 1: mono-modal; 2: confluence of 2 density peaks; 3; bi-modal; 4; tri-modal. Each species is
370 shown by a different color, and the average annual temperature of each site is reported in
371 parenthesis.

372

373 Daily temperatures recorded during the trapping periods have been analyzed for each site
374 and year in relation to the abundance trends, both within the same season and between different
375 years. We did not detect any meaningful association between temperatures registered at the
376 beginning of the sand fly activity and the occurrence and magnitude of density peaks, and neither
377 between average temperatures registered from June to September of different years, and the
378 respective fly abundances.

379

380

381 **Nocturnal activity**

382 Hourly or bi-hourly collections were performed by CDC light traps in one site per country, selected
383 among those investigated for the seasonal dynamics except for a new site in the Aegean region of
384 Turkey (Bascayir, Aydin province) and an additional site in Tbilisi, Georgia. They were typical
385 sites for *P. perniciosus* (Portugal and Italy), *P. ariasi* (France), *P. tobbi* (Aegean and Cukurova
386 regions of Turkey), *P. kandelakii* and *P. balcanicus* (Georgia). Collections were performed once a
387 month from May or June through September or October in 2011 and 2012, respectively, in
388 Cukurova, Turkey; once a month from June through August 2013 in Portugal; 7 days distributed in
389 July and August 2014 in Georgia; 1 day in France in July 2011, and 1 day in Italy and Aegean
390 Turkey in July 2012. Results from each site are shown in graphs of S2 Fig. In Fig. 7, the trend of
391 hourly activity expressed as a percentage of total collected specimens is shown cumulatively for all
392 vectors. A total of 1167 sand flies (40.5% females) were collected, with a range by site of 18-349
393 specimens. The activity of both males and females started at twilight (around 7-8 p.m.) and ended at
394 dawn (before 7-8 a.m.), with slight variations among species and sites. The main peak of activity
395 (19% of all specimens) was between 10 and 11 p.m., and a second peak (12% of specimens)
396 between midnight and 2 a.m. This activity trend was found similar in multiple collections
397 performed in different months or years in the same site.

398

399 **Figure 7. Hourly abundance of *Leishmania infantum* vectors in 6 selected Mediterranean sites.**

400 Data are presented as percentage of cumulative specimens collected per hour from all sites, with
401 ranges representing the lowest and highest percentage values per hour.

402

403

404 **Discussion**

405

406 A large retrospective meta-analysis of phenology data sets collected on animals and plants over the
407 past few decades has indicated an advance of spring/summer in Europe [24]. In particular,
408 invertebrates have shown an earlier shift in phenological indicators of about 4 days over 30 years of
409 observations, at latitudes of northern hemisphere that include the Mediterranean basin [57]. Because
410 the global average temperature is projected to rise at a rapid rate [32], it is expected that earlier
411 shifts will increase in magnitude in the near-mid future. As regards Mediterranean phlebotomine
412 sand flies, a retrospective analysis of phenology data sets published over the past 50 years did not
413 result in meaningful conclusions due to the limitations highlighted in the introduction of this paper.
414 Of our investigated sites, much of previous information was left unpublished or the data were
415 relatively recent, such as those from Georgia [11] or eastern Turkey sites [58]. As expected, in these
416 sites the main parameters of seasonal dynamics were found unchanged. In the French location of

417 Roquedur-le-haut, no phenological variations have occurred since the collections performed in 1977
418 by Rioux et al. [59] as compared to our 2011 trapping: in both years, the emergence of adults was
419 recorded in mid May. Hence, our data from several georeferenced sites may represent a robust
420 starting point for continuing surveillance on sand fly dynamics that may impact on leishmaniasis
421 transmission in the near future. Among basic entomological parameters, the unequivocal
422 identification of species and their ascertained role as leishmaniasis vectors is important. Therefore
423 specimens improperly identified, or species so far unproven to transmit *L. infantum* were excluded
424 from our analyses.

425 Our findings confirm historical observations that temperature – whose magnitude is
426 negatively correlated with latitude – is a major determinant for the activity start of leishmaniasis
427 vectors. It is well established that during cold months sand flies undergo diapause as fourth larval
428 stage (L4). In laboratory-reared species, at low temperature conditions a diapause period may run
429 even 9 months, and only after the temperature is raised to a certain level L4 larvae pupate and adults
430 emerge within two or three weeks [60]. Thus, in our Mediterranean sites the periods of pupation and
431 adults emergence were much probably dependent on generalized temperature increase to which L4
432 larvae were exposed in their natural environment, approximately in March, April or May in sites
433 where adults were firstly collected in April, May or June, respectively. On the other hand, the end
434 of adults activity was shown to occur at different months independently from latitude, average
435 annual temperature, or temperatures recorded in the sites during the activity season, suggesting that
436 for this stage of the sand fly life cycle the dependence on this parameter is less pronounced.
437 Laboratory observations have shown that adults are not much sensitive to temperature changes as
438 they are larval stages: lower temperatures slow down sand fly metabolism and increase survival
439 time especially of blood-fed females; periods of developmental phases become longer as regards
440 defecation, oviposition and eggs hatch [61]. These processes may be variable from one site to the
441 other, and may depend on the initial larval/adult population size, local environmental variables and
442 on less investigated parameters interfaced with temperature such as endogenous clocks sensitive to
443 photoperiod [62,63].

444 Patterns of sand fly abundance were found to vary greatly between years in the same sites. A
445 recent analysis of historical *P. ariasi* data has confirmed that large differences in abundance can be
446 found between close collecting stations within the same territory, without appreciable relationship
447 with major phytoecological variables [56]. Local weather events limited in time, such as strong
448 wind and/or heavy rain, were found to affect the productivity of our adults collections; this fact
449 could have offered a biased picture of potential phlebotomine dynamics if the investigations had
450 been performed during a single season only.

451 We did not find significant correlations between monthly temperatures recorded during the
452 collection period and the concomitant abundance patterns of adult sand flies. This would suggest,
453 rather, that winter or the whole annual mean temperature may affect the relative density of adults
454 emerging in the next season, most probably having an impact on the survival rate of overwintering
455 larval populations. Actually, the occurrence of multiple density peaks was significantly correlated
456 with sites located at lower latitudes and having higher annual temperatures; the early peaks,
457 representing the first emerging adult generation, have always been smaller than the subsequent
458 peak(s), which may indicate a sequence of summer generations with increasing abundance.

459 The nocturnal activity of phlebotomine vectors is generated by the circadian clock that
460 coordinates feeding activities important for the dynamics of *Leishmania* transmission [62]. While
461 we do not expect that the circadian rhythm will be much influenced by the rise in global average
462 temperature, we consider our findings as a useful complement to evaluate and predict leishmaniasis
463 risk associated with sand fly dynamics and abundance. Adult sand fly activity increased shortly
464 after sunset and continued until just after sunrise; the greatest peak of activity was early in the
465 evening (10-11 p.m.) and then declined, with another smaller peak, toward morning. The adult
466 female population was collected by light traps, so that it included specimens with different activity
467 behaviors such as sugar feeding, host seeking, blood feeding, mating, and oviposition. We did not
468 determine the parous rate of females, which can be important to evaluate the behavior of potentially
469 *Leishmania*-infected flies. Recently, it was shown that parous and nulliparous females of *P.*
470 *orientalis* (a member of the *Larroussius* subgenus) exhibit different periodicity of nocturnal
471 abundance [64].

472 In conclusions, we found that cumulative collections performed over consecutive years in
473 the same site can provide homogeneous patterns of the potential behavior of local leishmaniasis
474 vectors. Considering both, the relative adults density and the low probability of females harboring
475 parasite infections in early periods of activity, the highest potential risk for *L. infantum* transmission
476 in the Mediterranean region may be identified during the months from June through October in the
477 investigated period. We can also conclude that such risk was not equally distributed throughout the
478 region, since density waves of adults were more frequent in southern territories.

479
480

481 **References**

- 482 1. Killick-Kendrick R. The biology and control of Phlebotomine sand flies. Clin Dermatol
483 1999; 17: 279-289

- 484 2. Ready PD. Biology of phlebotomine sand flies as vectors of disease agents. *Annu Rev*
485 *Entomol* 2013; 58: 227-250
- 486 3. Young DG, Duncan MA. Guide to the identification and geographic distribution of
487 *Lutzomyia* sandflies in Mexico, the West Indies, Central and South America (Diptera:
488 Psychodidae). *Mem Am Entomol Inst* 1994; 54: 1-881.
- 489 4. World Health Organization. Control of the leishmaniasis. Report of a Meeting of the WHO
490 Expert Committee on the Control of Leishmaniases, 22-26 March 2010. Geneva: WHO
491 Technical Report Series 949
- 492 5. Maroli M, Feliciangeli MD, Bichaud L, Charrel RN, Gradoni L. Phlebotomine sandflies and
493 the spreading of leishmaniases and other diseases of public health concern. *Med Vet Entomol*
494 2013; 27: 123-147
- 495 6. Pratlong F, Lami P, Ravel C, Balard Y, Dereure J, Serres G, et al. Geographical distribution
496 and epidemiological features of Old World *Leishmania infantum* and *Leishmania donovani*
497 foci, based on the isoenzyme analysis of 2277 strains. *Parasitology* 2013; 140: 423-434
- 498 7. Gouzelou E, Haralambous C, Antoniou M, Christodoulou V, Martinković F, Živičnjak T, et
499 al. Genetic diversity and structure in *Leishmania infantum* populations from southeastern
500 Europe revealed by microsatellite analysis. *Parasit Vectors* 2013; 6:342
- 501 8. Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis worldwide
502 and global estimates of its incidence. *PLoS One* 2012; 7: e35671
- 503 9. Killick-Kendrick R. Phlebotomine vectors of the leishmaniases: a review. *Med Vet Entomol*
504 1990; 4: 1-24
- 505 10. Rioux JA, Killick-Kendrick R, Leaney AJ, Young CJ, Turner DP, Lanotte G, et al. [Ecology
506 of leishmaniasis in the south of France. 11. Canine leishmaniasis: successful experimental
507 transmission from dog to dog by the bite of *Phlebotomus ariasi* Tonnoir, 1921]. *Ann*
508 *Parasitol Hum Comp* 1979; 54 :401-407. French
- 509 11. Giorgobiani E, Lawyer PG, Babuadze G, Dolidze N, Jochim RC, Tskhvaradze L, et al.
510 Incrimination of *Phlebotomus kandelakii* and *Phlebotomus balcanicus* as vectors of
511 *Leishmania infantum* in Tbilisi, Georgia. *PLoS Negl Trop Dis* 2012; 6: e1609
- 512 12. Doha S, Shehata MG. *Leishmania infantum* MON-98 isolated from naturally infected
513 *Phlebotomus langeroni* (Diptera: Psychodidae) in El Agamy, Egypt. *J Med Entomol* 1992;
514 29: 891-893
- 515 13. Léger N, Gramiccia M, Gradoni L, Madulo-Leblond G, Pesson B, Ferté H, et al. Isolation
516 and typing of *Leishmania infantum* from *Phlebotomus neglectus* on the island of Corfu,
517 Greece. *Trans R Soc Trop Med Hyg* 1988; 82: 419-420

- 518 14. Maroli M, Gramiccia M, Gradoni L. Natural infection of *Phlebotomus perfiliewi* with
519 *Leishmania infantum* in a cutaneous leishmaniasis focus of the Abruzzi region, Italy. Trans R
520 Soc Trop Med Hyg 1987; 8: 596-598
- 521 15. Bettini S, Gramiccia M, Gradoni L, Atzeni MC. Leishmaniasis in Sardinia: II. Natural
522 infection of *Phlebotomus perniciosus* Newstead, 1911, by *Leishmania infantum* Nicolle,
523 1908, in the province of Cagliari. Trans R Soc Trop Med Hyg 1986; 80: 458-9
- 524 16. Léger N, Depaquit J, Ferté H, Rioux JA, Gantier JC, Gramiccia M, et al. [Phlebotomine
525 sandflies (Diptera-Psychodidae) of the isle of Cyprus. II-Isolation and typing of *Leishmania*
526 (*Leishmania infantum* Nicolle, 1908 (zymodeme MON 1) from *Phlebotomus (Larroussius)*
527 *tobbi* Adler and Theodor, 1930]. Parasite 2000; 7: 143-146. French
- 528 17. Fischer D, Moeller P, Thomas SM, Naucke TJ, Beierkuhnlein C. Combining climatic
529 projections and dispersal ability: a method for estimating the responses of sandfly vector
530 species to climate change. PLoS Negl Trop Dis 2011; 5: e1407
- 531 18. Maroli M, Rossi L, Baldelli R, Capelli G, Ferroglia E, Genchi C, et al. The northward spread
532 of leishmaniasis in Italy: evidence from retrospective and ongoing studies on the canine
533 reservoir and phlebotomine vectors. Trop Med Int Health 2008; 13: 256-264
- 534 19. Morosetti G, Bongiorno G, Beran B, Scalone A, Moser J, Gramiccia M, et al. Risk
535 assessment for canine leishmaniasis spreading in the north of Italy. Geospat Health 2009; 4:
536 115-127
- 537 20. Dereure J, Vanwambeke SO, Malé P, Martinez S, Pratlong F, Balard Y, et al. The potential
538 effects of global warming on changes in canine leishmaniasis in a focus outside the classical
539 area of the disease in southern France. Vector Borne Zoonotic Dis 2009; 9: 687-694
- 540 21. Ballart C, Barón S, Alcover MM, Portús M, Gállego M. Distribution of phlebotomine sand
541 flies (Diptera: Psychodidae) in Andorra: first finding of *P. perniciosus* and wide distribution
542 of *P. ariasi*. Acta Trop 2012; 122: 155-159
- 543 22. Naucke TJ, Menn B, Massberg D, Lorentz S. Sandflies and leishmaniasis in Germany.
544 Parasitol Res 2008; 103: S65-68
- 545 23. Antoniou M, Gramiccia M, Molina R, Dvorak V, Volf P. The role of indigenous
546 phlebotomine sandflies and mammals in the spreading of leishmaniasis agents in the
547 Mediterranean region. Euro Surveill 2013 25; 18: 20540
- 548 24. Menzel A, Sparks TH, Estrella N, Koch E3, Aasa A, Ahas R et al. European phenological
549 response to climate change matches the warming pattern. Glob Chang Biol 2006; 12: 1969-
550 1976

- 551 25. Rioux JA, Golvan YJ, Croset H, Houin R, Juminer B, Bain O, et al. [Ecology of
552 leishmaniasis in Southern France. 1. *Phlebotomus*. Sampling-ethology]. Ann Parasitol Hum
553 Comp 1967; 42: 561-603. French
- 554 26. Maroli M, Bettini S. Leishmaniasis in Tuscany (Italy): (I) An investigation on phlebotomine
555 sandflies in Grosseto Province. Trans R Soc Trop Med Hyg 1977; 71: 315-321
- 556 27. Pesson B, Leger N, Madulo-Leblond G. [Leishmaniasis in Greece: the sandflies of the Ionian
557 islands and Aegean Sea]. Ann Parasitol Hum Comp 1984; 59: 277-295. French
- 558 28. Morillas Márquez F, Sanchís Marín MC, Martín Sánchez J, Acedo Sánchez C. On
559 *Phlebotomus perniciosus* Newstead, 1911 (Diptera, Phlebotomidae) in the Province of
560 Almeria in southeastern Spain. Parassitologia 1991; 33: 437-444
- 561 29. Rossi E, Bongiorno G, Ciolli E, Di Muccio T, Scalone A, Gramiccia M, et al. Seasonal
562 phenology, host-blood feeding preferences and natural *Leishmania* infection of *Phlebotomus*
563 *perniciosus* (Diptera, Psychodidae) in a high-endemic focus of canine leishmaniasis in Rome
564 province, Italy. Acta Trop 2008; 105: 158-165
- 565 30. Maia C, Afonso MO, Neto L, Dionísio L, Campino L. Molecular detection of *Leishmania*
566 *infantum* in naturally infected *Phlebotomus perniciosus* from Algarve region, Portugal. J
567 Vector Borne Dis 2009;46: 268-272
- 568 31. Lisi O, D'Urso V, Vaccalluzzo V, Bongiorno G, Khoury C, Severini F, et al. Persistence of
569 phlebotomine *Leishmania* vectors in urban sites of Catania (Sicily, Italy). Parasit Vectors
570 2014; 9:560
- 571 32. IPCC - Intergovernmental Panel on Climate Change. Climate Change 2014 - Impacts,
572 Adaptation, and Vulnerability. Part B: Regional Aspects. New York: Cambridge Press; 2014
- 573 33. Pires CA. [Phlebotomus of Portugal. I-Natural infestation of *Phlebotomus ariasi* Tonnoir,
574 1921 and *Phlebotomus perniciosus* Newstead, 1911, by *Leishmania* in the zoonotic focus of
575 Arrábida (Portugal)]. Ann Parasitol Hum Comp 1984; 59: 521-524. French
- 576 34. Campino L, Pratlong F, Abranches P, Rioux JA, Santos-Gomes G, Alves-Pires C, et al.
577 Leishmaniasis in Portugal: enzyme polymorphism of *Leishmania infantum* based on the
578 identification of 213 strains. Trop Med Int Health 2006; 11: 1708-1714
- 579 35. Arce A, Estirado A, Ordobas M, Sevilla S, García N, Moratilla L, et al. Re-emergence of
580 leishmaniasis in Spain: community outbreak in Madrid, Spain, 2009 to 2012. Euro Surveill
581 2013 25;18: 20546
- 582 36. Jiménez M, González E, Martín-Martín I, Hernández S, Molina R. Could wild rabbits
583 (*Oryctolagus cuniculus*) be reservoirs for *Leishmania infantum* in the focus of Madrid,
584 Spain? Vet Parasitol 2014 28; 202: 296-300

- 585 37. Domínguez-Bernal G, Jiménez M, Molina R, Ordóñez-Gutiérrez L, Martínez-Rodrigo A,
586 Mas A, et al. Characterisation of the ex vivo virulence of *Leishmania infantum* isolates from
587 *Phlebotomus perniciosus* from an outbreak of human leishmaniasis in Madrid, Spain. Parasit
588 Vectors 2014 7; 7: 499
- 589 38. Gramiccia M, Scalone A, Di Muccio T, Orsini S, Fiorentino E, Gradoni L. The burden of
590 visceral leishmaniasis in Italy from 1982 to 2012: a retrospective analysis of the multi-annual
591 epidemic that occurred from 1989 to 2009. Euro Surveill 2013 18;18: 20535
- 592 39. Ntais P, Sifaki-Pistola D, Christodoulou V, Messaritakis I, Pralong F, Poupalos G, et al.
593 Leishmaniasis in Greece. Am J Trop Med Hyg 2013 ;89: 906-915
- 594 40. Christodoulou V, Antoniou M, Ntais P, Messaritakis I, Ivovic V, Dedet JP, et al. Re-
595 emergence of visceral and cutaneous leishmaniasis in the Greek Island of Crete. Vector
596 Borne Zoonotic Dis 2012; 12: 214-222
- 597 41. Mazeris A, Soteriadou K, Dedet JP, Haralambous C, Tsatsaris A, Moschandreas J, et al.
598 Leishmaniasis and the Cyprus paradox. Am J Trop Med Hyg 2010; 82: 441-448
- 599 42. Ozensoy Toz S, Culha G, Zeyrek FY, Ertabaklar H, Alkan MZ, Vardarli AT, et al. A real-
600 time ITS1-PCR based method in the diagnosis and species identification of *Leishmania*
601 parasite from human and dog clinical samples in Turkey. PLoS Negl Trop Dis 2013 9; 7:
602 e2205
- 603 43. Svobodová M, Alten B, Zídková L, Dvorač V, Hlavacková J, Mysková J, et al. Cutaneous
604 leishmaniasis caused by *Leishmania infantum* transmitted by *Phlebotomus tobbi*. Int J
605 Parasitol 2009; 39: 251-256
- 606 44. Rogers MB, Downing T, Smith BA, Imamura H, Sanders M, Svobodova M, et al. Genomic
607 confirmation of hybridisation and recent inbreeding in a vector-isolated *Leishmania*
608 population. PLoS Genet 2014; 10: e1004092
- 609 45. Ozbel Y, Balcioğlu IC, Olgen MK, Simsek FM, Ozensoy Toz S, Ertabaklar H, et al. Spatial
610 distribution of phlebotomine sand flies in the Aydin Mountains and surroundings: the main
611 focus of cutaneous leishmaniasis in western Turkey. J Vector Ecol 2011; 36: S99-S105
- 612 46. Giorgobiani E, Chitadze N, Chanturya G, Grdzeldze M, Jochim RC, Machablishvili A, et al.
613 Epidemiologic aspects of an emerging focus of visceral leishmaniasis in Tbilisi, Georgia.
614 PLoS Negl Trop Dis 2011; 5: e1415
- 615 47. Theodor O. Psychodidae–Phlebotomine. In: Lindner E, editor. Die Fliegen der
616 Palaearktischen Region, 9c. Stuttgart: E. Schweizerbart'sche Verlagsbuchhandlung; 1958.
617 pp. 1-55

- 618 48. Perfil'ev PP. [Phlebotominae]. Jerusalem: Israel Program of Scientific Translations; 1968.
619 Russian
- 620 49. Lewis DJ. A taxonomic review of the genus *Phlebotomus* (Diptera: Psychodidae). Bull Br
621 Mus Nat Hist Entomol 1982; 45: 121-209
- 622 50. Artemiev MM. A revision of the sandflies of the subgenus *Adlerius* (Diptera, Psychodidae,
623 *Phlebotomus*). Zool Zh 1980; 59: 1177-1193
- 624 51. Leger N, Pesson B, Madulo-Leblond G, Abonnenc E. Sur la differenciation des femelles du
625 sous-genre *Larroussius* Nitzulescu, 1931(Diptera-Phlebotomidae) de la region
626 mediterraneenne. Ann Parasitol Hum Comp 1983; 58: 611-623
- 627 52. Killick-Kendrick R, Tang Y, Killick-Kendrick M, Sang DK, Sirdar MK, Ke L, et al. The
628 identification of female sandflies of the subgenus *Larroussius* by the morphology of the
629 spermathecal ducts. Parasitologia 1991; 33: 335-347
- 630 53. Depaquit J, Bounamous A, Akhoundi M, Augot D, Sauvage F, Dvorak V, et al. A taxonomic
631 study of *Phlebotomus (Larroussius) perfiliewi* s. l. Infect Genet Evol 2013; 20:500-508
- 632 54. Essegir S, Ready PD, Killick-Kendrick R, Ben-Ismaïl R. Mitochondrial haplotypes and
633 geographical vicariance of *Phlebotomus* vectors of *Leishmania major*. Insect Mol Biol 1997;
634 6: 211–225
- 635 55. Depaquit J, Ferté H, Léger N, Lefranc F, Alves-Pires C, Hanafi H, et al.. ITS 2 sequences
636 heterogeneity in *Phlebotomus sergenti* and *Phlebotomus similis* (Diptera, Psychodidae):
637 possible consequences in their ability to transmit *Leishmania tropica*. Int J Parasitol 2002;
638 32: 1123–1131
- 639 56. Rioux JA, Carron S, Dereure J, Périères J, Zeraia L, Franquet E, et al. Ecology of
640 leishmaniasis in the South of France. 22. Reliability and representativeness of 12
641 *Phlebotomus ariasi*, *P. perniciosus* and *Sergentomyia minuta* (Diptera: Psychodidae)
642 sampling stations in Vallespir (eastern French Pyrenees region). Parasite 2013; 20: 34
- 643 57. Root TL, Price JT, Hall KR, Schneider SH, Rosenzweig C, Pounds JA. Fingerprints of global
644 warming on wild animals and plants. Nature 2003 2;421: 57-60
- 645 58. Belen A, Bulent A. Seasonal dynamics and altitudinal distributions of sand fly (Diptera:
646 Psychodidae) populations in a cutaneous leishmaniasis endemic area of the Cukurova region
647 of Turkey. J Vect Ecol 2011; 36 (Suppl 1): S87-S94
- 648 59. Rioux JA, Killick-Kendrick R, Périères J, Turner DP, Lanotte G. Écologie des leishmanioses
649 dans le Sud de la France. 13. Les sites de « flanc de coteau », biotopes de transmission
650 privilégiés de la leishmaniose viscérale en Cévennes. Ann Parasitol Hum Comp 1980; 55,
651 445–453. French

- 652 60. Volf P, Volfova V. Establishment and maintenance of sand fly colonies. J Vector Ecol 2011;
653 36: S1-9
- 654 61. Benkova I, Volf P. Effect of temperature on metabolism of *Phlebotomus papatasi* (Diptera:
655 Psychodidae). J Med Entomol 2007; 44: 150-154
- 656 62. Meireles-Filho AC, Kyriacou CP. Circadian rhythms in insect disease vectors. Mem Inst
657 Oswaldo Cruz 2013; 108: 48-58
- 658 63. Rivas GB, de Souza NA, Peixoto AA, Bruno RV. Effects of temperature and photoperiod on
659 daily activity rhythms of *Lutzomyia longipalpis* (Diptera: Psychodidae). Parasit Vectors 2014
660 19; 7: 278
- 661 64. Gebresilassie A, Kirstein OD, Yared S, Aklilu E, Moncaz A, Tekie H, Balkew M, Warburg
662 A, Hailu A, Gebre-Michael T. Nocturnal periodicity of *Phlebotomus (Larroussius) orientalis*
663 (Diptera: Psychodidae) in an endemic focus of visceral leishmaniasis in Northern Ethiopia.
664 Parasit Vectors 2015; 8: 186
665

666 **Supporting Information captions**

667

668 **S1 Fig. Annual seasonal trends recorded for Mediterranean *L. infantum* vectors in the 2011-**
669 **2013 period**

670

671 **S2 Fig. Hourly or bi-hourly CDC-light trap collection of *L. infantum* vector specimens in 6**
672 **selected Mediterranean sites**

673

674 **S1 Table. Phlebotomine sand fly species collected in 11 sites of the Lisbon Metropolitan**
675 **region, Portugal**

676

677 **S2 Table. Phlebotomine sand fly species collected in 11 sites of the Algarve region, Portugal**

678

679 **S3 Table. Phlebotomine sand fly species collected in Fuenlabrada, Spain**

680

681 **S4 Table. Phlebotomine sand fly species collected in Roquedur-le-haut, France**

682

683 **S5 Table. Phlebotomine sand fly species collected in Frascati, Italy**

684

685 **S6 Table. Phlebotomine sand fly species collected in Fodele, Crete, Greece**

686

687 **S7 Table. Phlebotomine sand fly species collected in Steni, Cyprus**

688

689 **S8 Table. Phlebotomine sand fly species collected in 6 sites of Cukurova region, Turkey**

690

691 **S9 Table. Phlebotomine sand fly species collected in 2 sites of Tbilisi, Georgia**

692

693 **S10 Table. Results of phlebotomine sand fly collections in sites where miniature CDC light**
694 **trapping (CDC) was regularly associated with sticky paper trapping (ST)**

Figure 1



Figure 2

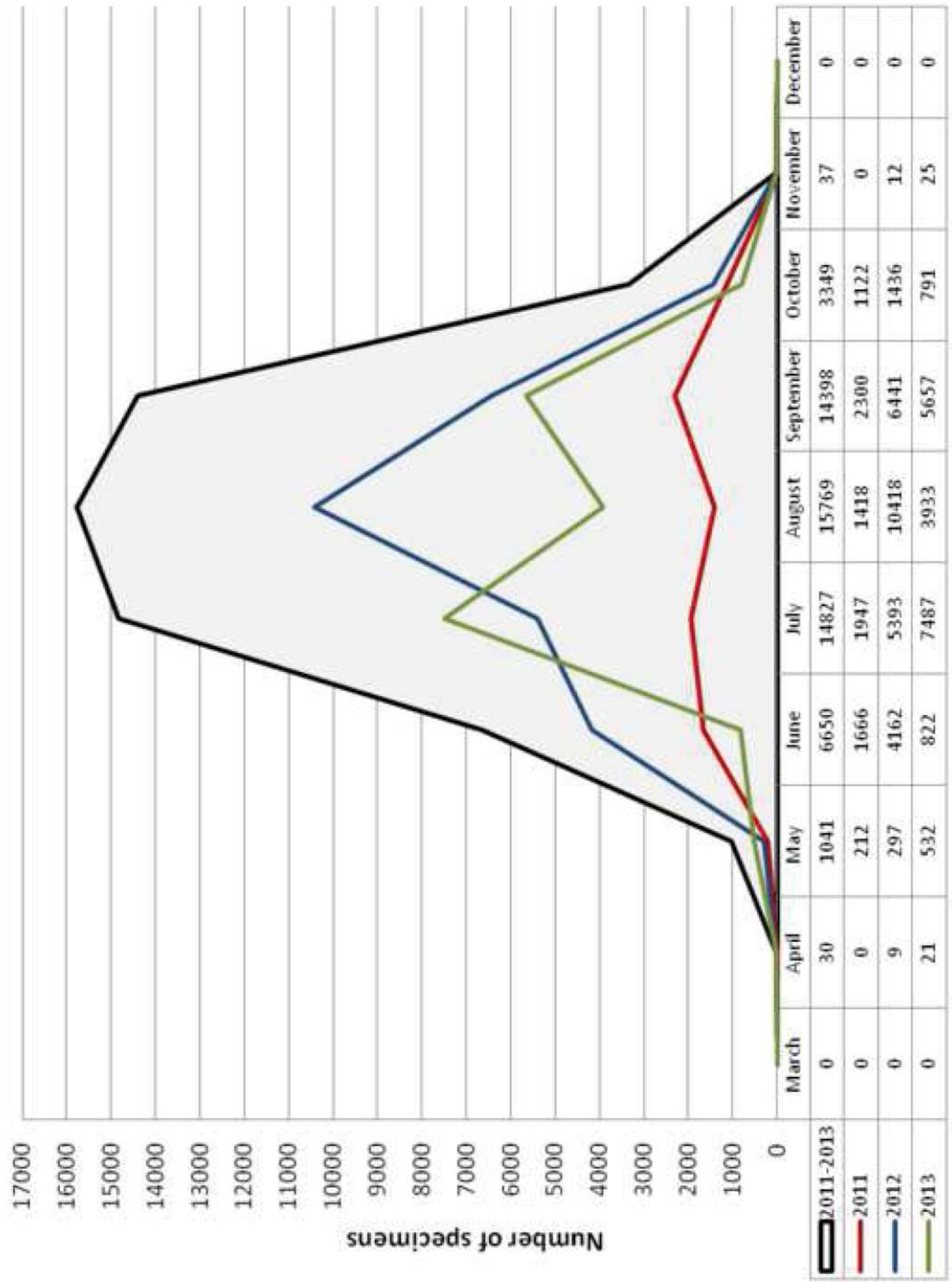


Figure 3

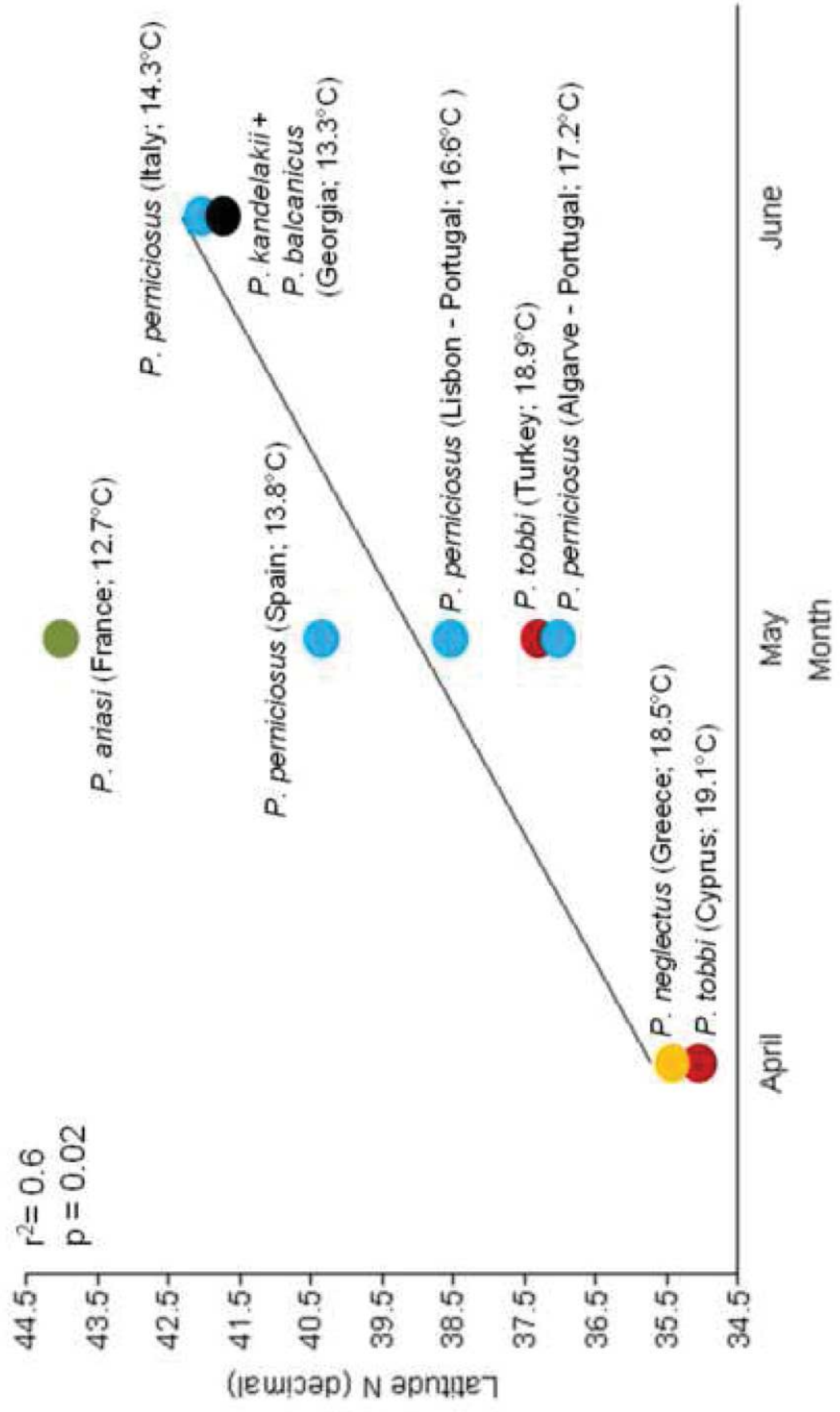


Figure 4

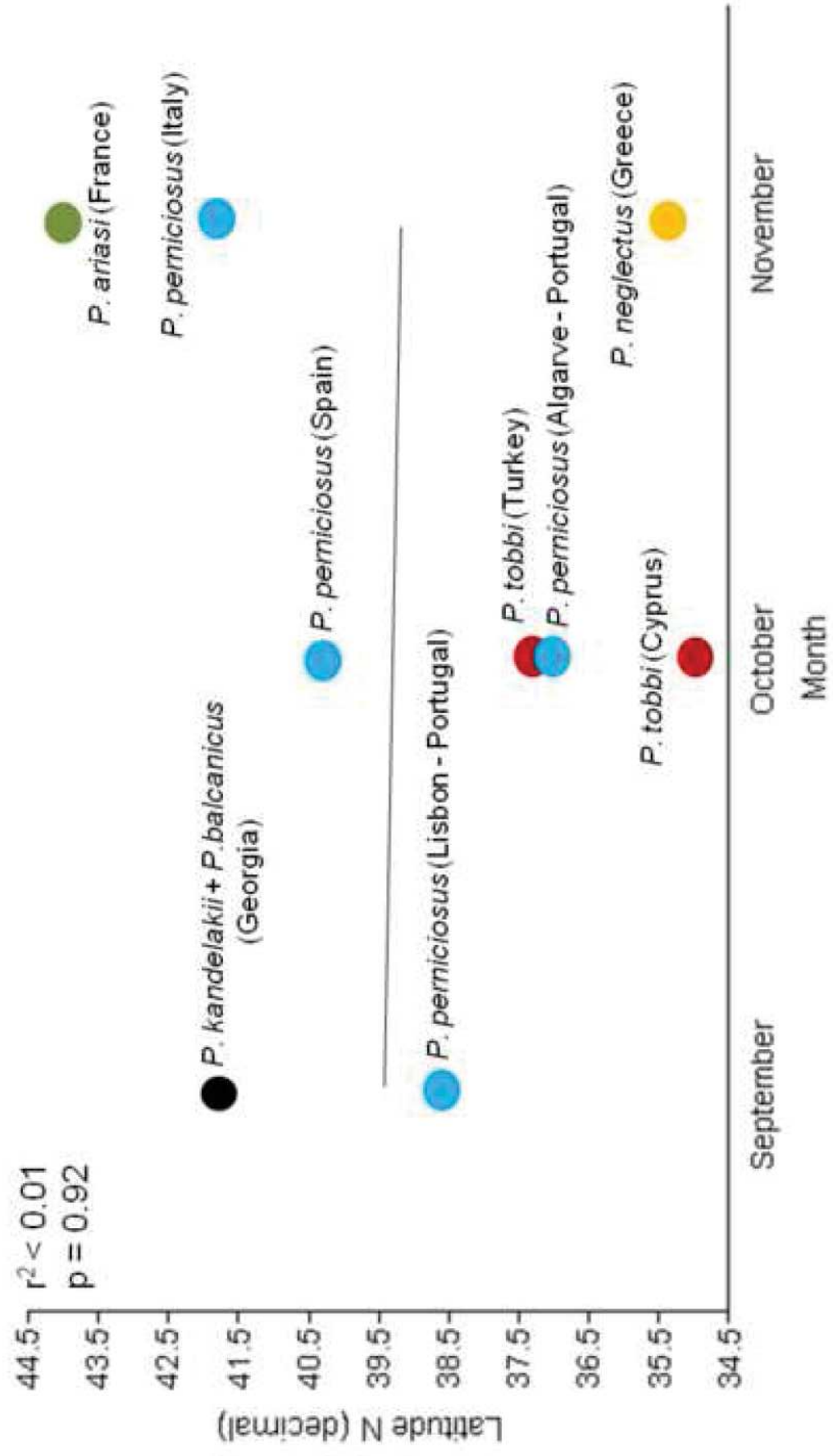


Figure 5

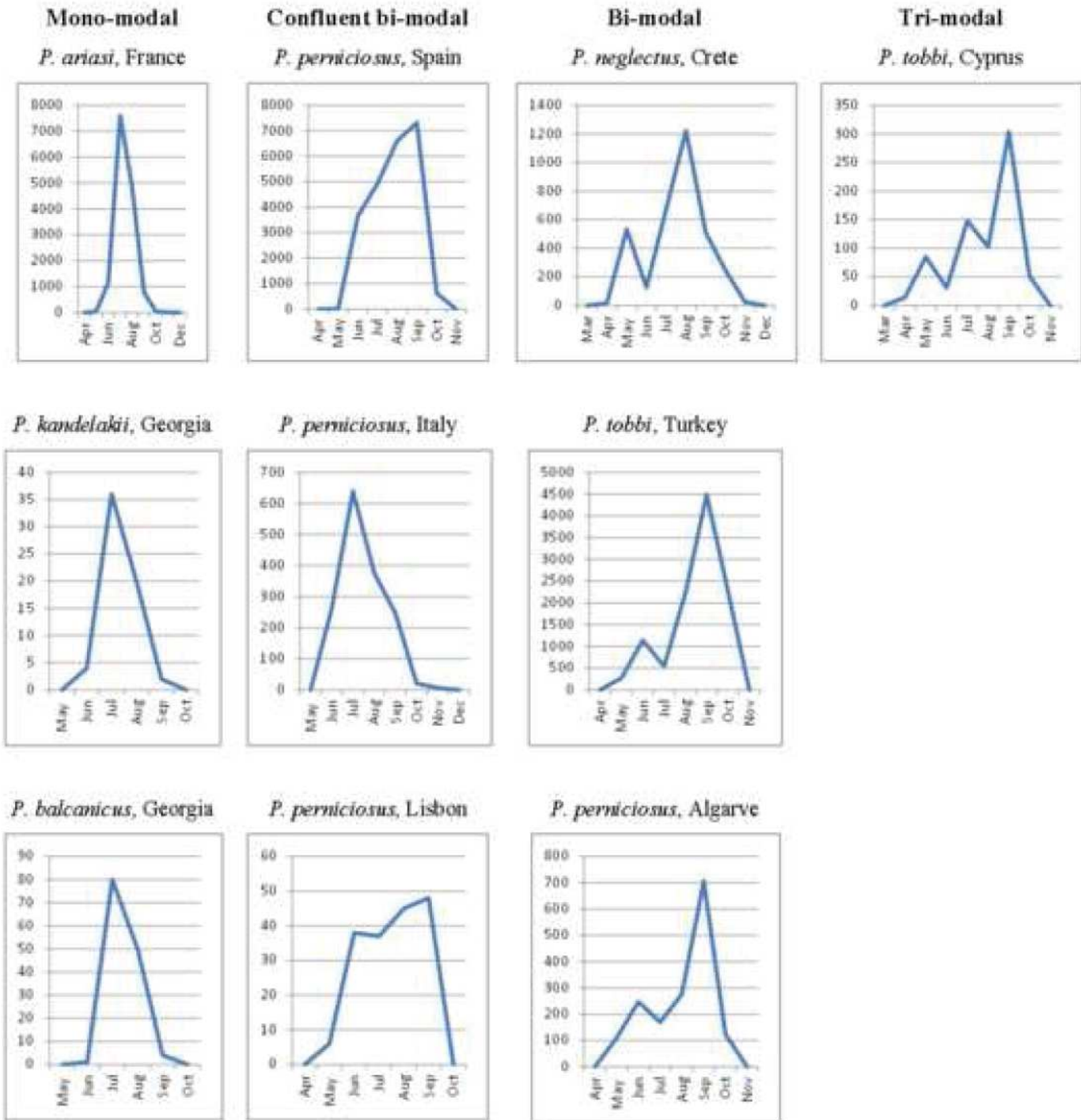


Figure 6

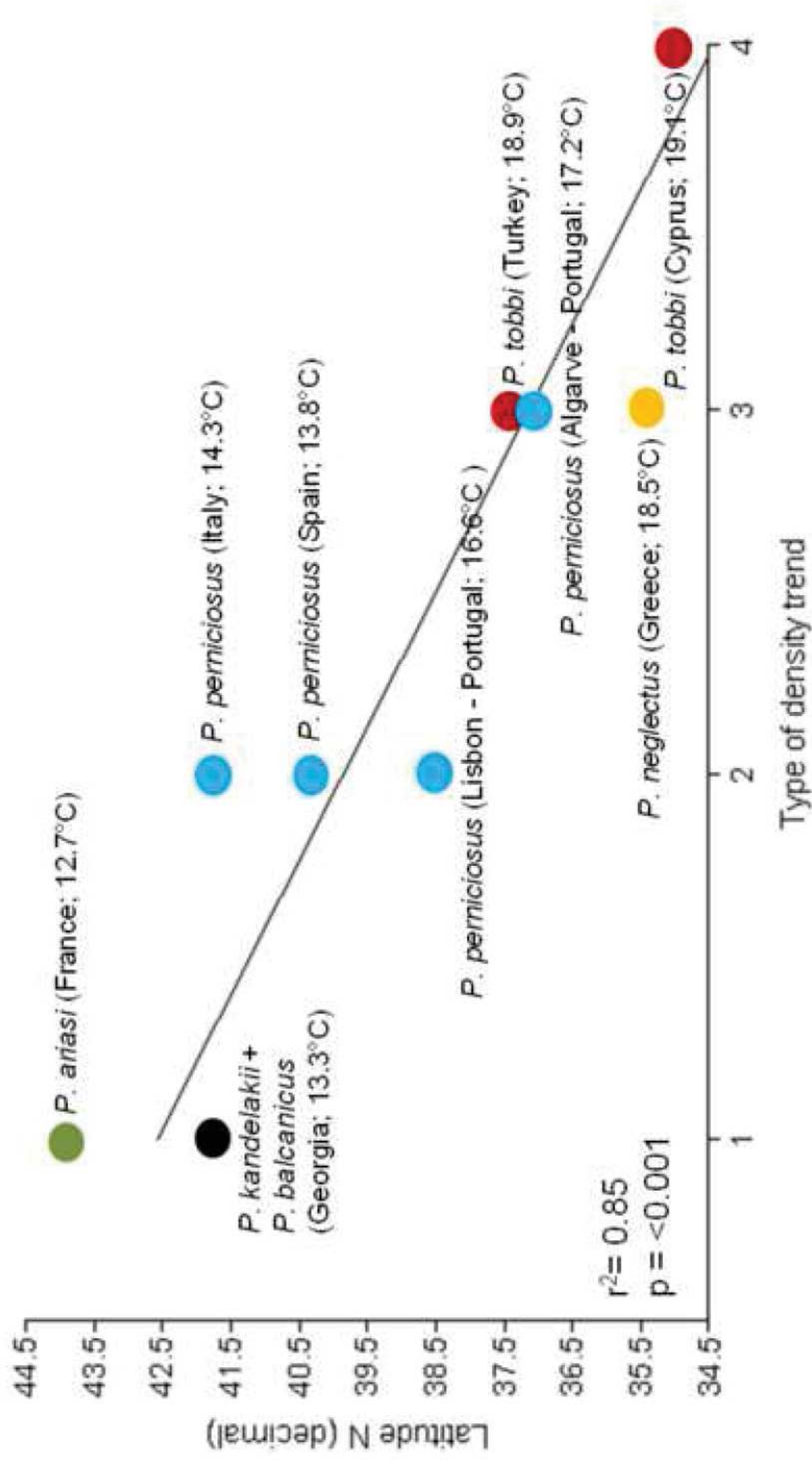
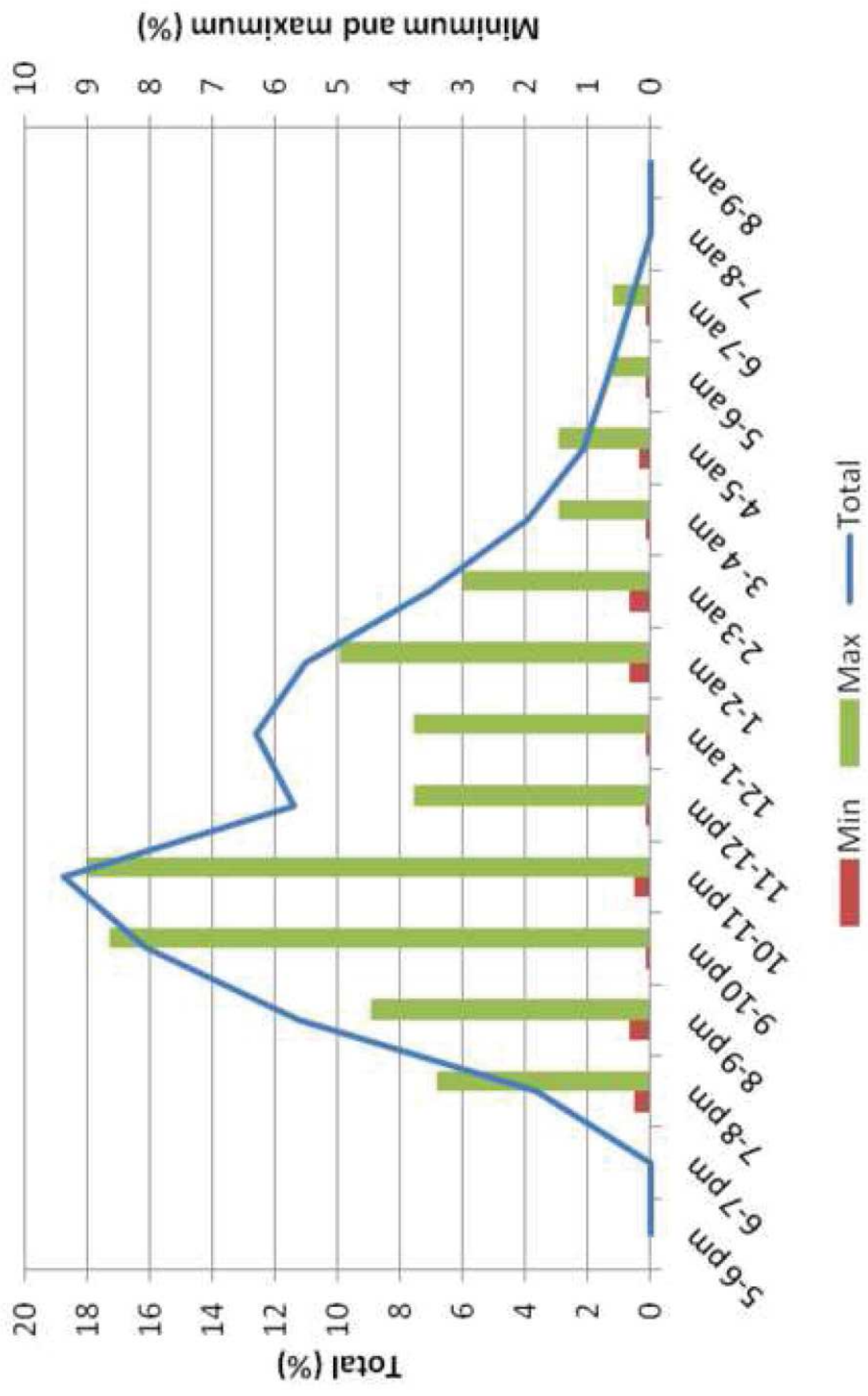
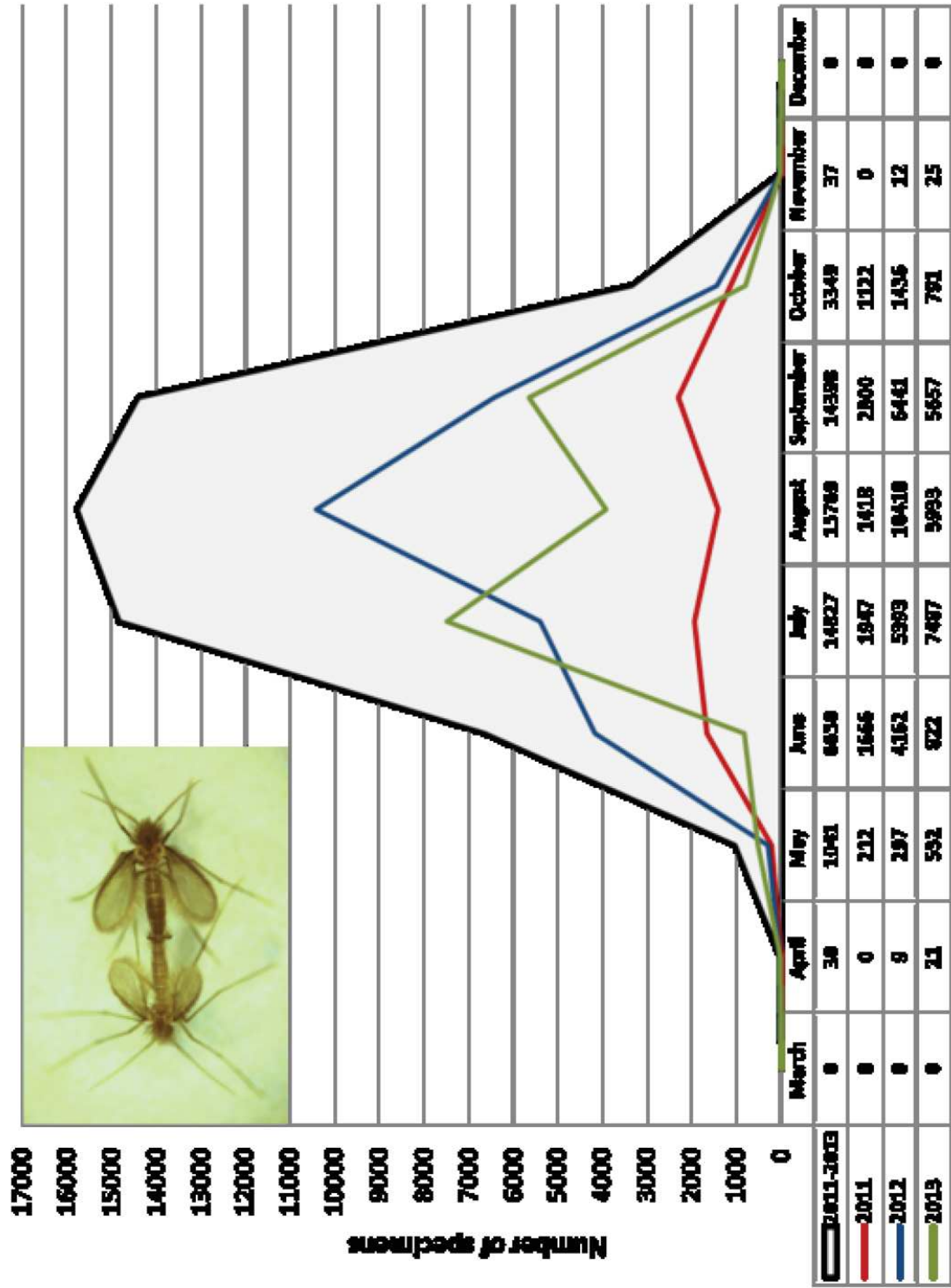


Figure 7





I.5. Les phlébotomes en France

I.5.1. Historique et espèces présentes en France

Le premier inventaire de la faune phlébotomienne française a été réalisé par Langeron et Nitzulescu [38, 39] et a permis de dénombrer six espèces. De nombreux auteurs ont ensuite contribué à l'étude de ces espèces [35, 40-43](non exhaustif) (Annexe 3 - Article 9). Selon la littérature, 6 espèces sont présentes en France :

Genre *Phlebotomus*

Sous-genre *Larroussius*

Phlebotomus (Larroussius) ariasi Tonnoir, 1921

P. (L.) perniciosus Newstead, 1911

Sous-genre *Phlebotomus*

P. (Phlebotomus) papatasi (Scopoli, 1786)

Sous-genre *Paraphlebotomus*

P. (Paraphlebotomus) sergenti Parrot, 1917

Sous-genre *Transphlebotomus*

P. (Transphlebotomus.) mascittii Grassi, 1980

Genre *Sergentomyia*

Sous-genre *Sergentomyia*

Sergentomyia (Sergentomyia) minuta (Rondani, 1843)

Les cartes de répartition en France métropolitaine disponibles pour 4 de ces espèces (*P. ariasi*, *P. perniciosus*, *P. papatasi* et *P. sergenti*) sont présentées dans la figure 11 (European Centre for Disease Prevention and Control). Durant ce travail, 4 espèces ont été collectées *P. ariasi*, *P. perniciosus*, *P. mascittii* et *S. minuta*.

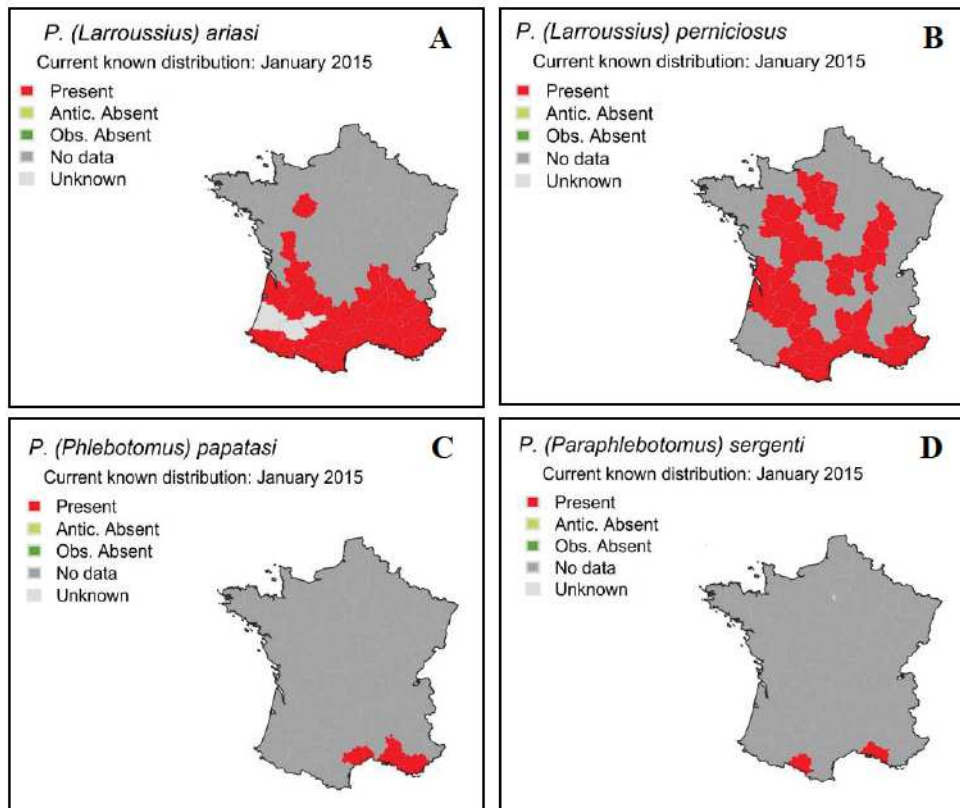


Figure 11 : Cartes de répartition en France métropolitaine de *Phlebotomus ariasi* (A), *P. perniciosus* (B), *P. papatasi* (C) et *P. sergenti* (D) (European Centre for Disease Prevention and Control).

I.5.2. Espèces impliquées dans la transmission de la leishmaniose

P. ariasi, *P. perniciosus*, *P. papatasi* et *P. sergenti* sont des vecteurs prouvés de *Leishmania*. Cependant, dans le Sud de la France, la transmission est assurée principalement par *P. perniciosus* et *P. ariasi*. *P. perniciosus* est présent dans tous le Sud de la France à une altitude inférieure à 600 m, tandis que *P. ariasi* est présent préférentiellement dans les chênaies mixtes et entre 200 et 1400 m d'altitude. De plus, cette espèce est moins abondante sur la plaine littorale méditerranéenne [35, 44]. Dans les Cévennes, *L. infantum* est transmise par *P. ariasi* du fait de sa forte abondance [35, 45].

P. mascittii est connu pour être cavernicole mais cette espèce se capture toujours en faible densité et sa biologie est méconnue [46-49]. Cependant, des études témoignent de son caractère anthropophile et de sa présence près de cas autochtones de leishmaniose dans certaines régions d'Europe [22, 50].

De l'ADN de *L. major* a récemment été mis en évidence dans un spécimen de *S. minuta* capturé au Portugal [10]. Cependant, le possible rôle vecteur de cette espèce doit encore être étudié.

I.6. Techniques de captures

Les techniques de capture sont détaillées dans : « Alten B, Ozbel Y, Ergunay K, Kasap OE, Cull B, Antoniou M, Velo E, **Prudhomme J**, Molina R, Bañuls AL, Schaffner F, Hendrickx G, Van Bortel W, Medlock JM. *Sampling strategies for phlebotomine sand flies (Diptera: Psychodidae) in Europe. Bull Entomol Res. 2015 Aug 14: 1-15* » [22].

I.6.1. Résumé

Les phlébotomes adultes (mâles et femelles) peuvent être capturés à l'aide de différentes méthodes dans leurs gîtes de repos ou durant leurs déplacements nocturnes. Les gîtes larvaires n'étant pas connus, les stades immatures sont, quant à eux, très difficiles à trouver et capturer.

La majorité des études de terrain sur les phlébotomes sont basées sur la nécessité d'identifier les vecteurs potentiels dans une zone de transmission de la maladie à l'homme ou l'animal (Voir ci-dessus - Importance médicale des phlébotomes). Ces études impliquent l'échantillonnage et la caractérisation des différentes espèces de phlébotomes présentes dans la région de transmission. Contrairement aux moustiques, les phlébotomes restent encore peu étudiés en Europe. Les maladies transmises par ces derniers présentant une faible prévalence, la surveillance de ces vecteurs ne représente pas une priorité. Pourtant, dans le contexte des changements climatiques actuels, le risque d'émergence et de ré-émergence de la leishmaniose augmente en raison de l'expansion de l'aire de distribution des phlébotomes.

Cet article a pour objectif de résumer les étapes importantes pour l'étude des phlébotomes sur le terrain et en laboratoire : collecte, interprétation des données de terrain, conservation des spécimens obtenus par chaque méthode de capture, techniques moléculaires utilisées dans l'identification des différentes espèces, détection et isolement des agents pathogènes associés aux phlébotomes. Les techniques et stratégies de capture utilisées dans le cadre de cette thèse ont permis d'aider à la réalisation de cette publication technique.

I.6.2. Article 2 : Sampling strategies for phlebotomine sand flies (Diptera: Psychodidae) in Europe.

Sampling strategies for phlebotomine sand flies (Diptera: Psychodidae) in Europe

B. Alten¹, Y. Ozbel², K. Ergunay³, O.E. Kasap¹, B. Cull⁴,
M. Antoniou⁵, E. Velo⁶, J. Prudhomme⁷, R. Molina⁸,
A.-L. Bañuls⁷, F. Schaffner⁹, G. Hendrickx⁹, W. Van Bortel¹⁰
and J.M. Medlock^{4*}

¹Hacettepe University (HUESRL), Ankara, Turkey; ²Ege University (EGE), Izmir, Turkey; ³Hacettepe University, Medical Faculty (HU-MED), Ankara, Turkey; ⁴Medical Entomology group, Emergency Response Department, Public Health England, Salisbury, UK; ⁵University of Crete (UoC), Heraklion, Greece; ⁶Institute of Public Health (IPH), Tirana, Albania; ⁷UMR MIVEGEC (IRD 224 – CNRS 5290 – Universités Montpellier 1 et 2), Institut de Recherche pour le Développement (IRD), Montpellier, France; ⁸Instituto de Salud Carlos III (ISCIII), Madrid, Spain; ⁹Avia GIS, Zoersel, Belgium; ¹⁰ECDC, Stockholm, Sweden

Abstract

The distribution of phlebotomine sand flies is widely reported to be changing in Europe. This can be attributed to either the discovery of sand flies in areas where they were previously overlooked (generally following an outbreak of leishmaniasis or other sand fly-related disease) or to true expansion of their range as a result of climatic or environmental changes. Routine surveillance for phlebotomines in Europe is localized, and often one of the challenges for entomologists working in non-leishmaniasis endemic countries is the lack of knowledge on how to conduct, plan and execute sampling for phlebotomines, or how to adapt on-going sampling strategies for other haematophagous diptera. This review brings together published and unpublished expert knowledge on sampling strategies for European phlebotomines of public health concern in order to provide practical advice on: how to conduct surveys; the collection and interpretation of field data; suitable techniques for the preservation of specimens obtained by different sampling methods; molecular techniques used for species identification; and the pathogens associated with sand flies and their detection methods.

Keywords: Phlebotomus, sand flies, Europe, sampling

(Accepted 24 January 2015)

Introduction

The phlebotomine sand flies (Diptera: Psychodidae, Phlebotominae) are vectors of several infectious pathogens

causing leishmaniasis, bartonellosis and arbovirus infections due to phlebotomines. Several of these diseases have wide geographical distributions around the world, and give rise to occasional epidemic outbreaks. In numerous countries, increasing risk factors are making sand fly-borne diseases a major public and veterinary health problem (Dedet, 2008). Among the 800 known species of sand fly, about 70, belonging to the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World, are proven or suspected vectors of pathogens.

*Author for correspondence

Phone: +44 (0) 1980612100

E-mail: jolyon.medlock@phe.gov.uk

Table 1. Guide to sand fly sampling strategy.

I want to	Purpose	Suggested trapping method(s)	Also see
Collect adults	Catalogue species present in the study area	Light traps, sticky traps, human landing catches, flight traps	Sampling strategy; handling and processing; identification
	Test for pathogens	Light traps, sticky traps, resting site collections	Sampling strategy; pathogen detection
	Preservation and display	Resting site collections, animal-baited traps	Sampling strategy; handling and processing; identification
	Collect live specimens	Resting site collections, animal-baited traps, human landing catches	Sampling strategy; handling and processing
	Determine seasonality	Light traps, sticky traps	Sampling strategy; identification
	Identify breeding areas	Emergence traps, soil screening and sugar flotation	Sampling strategy
Collect immature stages	Determine host preference	Animal/human-baited traps, human landing catches	Identification; handling and processing
		Soil screening and sugar flotation, emergence traps	Sampling strategy; handling and processing

Although sand flies are principally found in the warmer parts of the world including southern Europe, Asia, Africa, Australia and South America, their distribution extends northwards to just above latitude 50°N in southwest Canada, northern France and Mongolia. Their southernmost distribution is at about latitude 40°S, but they are absent from New Zealand and the Pacific islands. Their altitudinal distribution is from below sea level (by the Dead Sea) to 3500 m above sea level in Afghanistan (*Phlebotomus rupester*) (Artemiev, 1980; Lane, 1993; Killick-Kendrick, 1999). Sand flies are generally associated with desert and semi-arid ecosystems in the Old World and forests in the New World. They breed in a wide variety of habitats in domestic, peridomestic and sylvatic environments, including abandoned buildings, cracks in floors and walls, animal burrows, domestic animal shelters, garbage, under stones, soil, caves, termite mounds, tree roots and leaf litter, among others (Feliciangeli, 2004).

According to literature, among the species resident in Europe, ten are medically important, namely *Phlebotomus (Larroussius) ariasi*, *Phlebotomus (Larroussius) perniciosus*, *Phlebotomus (Larroussius) perfiliewi*, *Phlebotomus (Larroussius) major* s.l., *Phlebotomus (Larroussius) tobbi*, *Phlebotomus (Phlebotomus) papatasi*, *Phlebotomus (Paraphlebotomus) sergenti*, *Phlebotomus (Paraphlebotomus) similis* and *Phlebotomus (Paraphlebotomus) alexandri*. Most of them are proven vectors of leishmaniasis and several phleboviruses. Although the vectorial role of *Phlebotomus (Transphlebotomus) mascittii* has not been confirmed, correlation between its distribution and the autochthonous cases of leishmaniasis in some parts of Europe has led to research on its biology and distribution. Additional information on the distribution of these species in Europe is updated on the ECDC website VBORNET project: http://ecdc.europa.eu/en/activities/diseaseprogrammes/emerging_and_vector_borne_diseases/Pages/VBORNET_maps_sandflies.aspx, and the VBORNET Project web site: <http://www.vbornet.eu>

Studies of sand fly–pathogen relationships and vector behaviour, taxonomy or ecology require sampling methods that maintain the insects alive or in an adequate state of preservation for transportation to the laboratory and processing. This review prepared by ECDC-VBORNET PROJECT provides a critical guide to sampling methods used for sand flies. The review covers the collection and interpretation of field data, describes suitable techniques for the preservation of specimens obtained by each method of collection, provides advice

on how to conduct surveys, indicates molecular techniques used in identification of species and discusses pathogens associated with sand flies and their detection methods. Advantages and disadvantages of each type of method are also considered with regard to practicality, safety and cost.

It is important to be aware that each method has its own limitation in relation to environmental factors and the vector's natural or induced behaviour. As adequate sampling and processing of material are essential for obtaining significant information, the epidemiologist, entomologist and/or researcher should agree together the extent of the entomological investigations to be undertaken in each situation in order to achieve the study objectives (Table 1), with adjustments made to meet local requirements.

Sampling methods of adult sand flies

Vector surveillance aims to detect the presence of a vector in a given population or to determine vector abundance for estimating the risk of disease transmission, and hence the sampling methods required will depend upon the questions to be addressed. Sampling methods exist for both the adult and the immature stages of sand flies (Table 2). The most used trapping methods are light traps for host-seeking females and sticky traps or aspirator collection for the resting population, although depending upon the specific research objective, a range of other traps and techniques are available and may be used in the field.

Light traps

Light traps are used extensively in field studies on sand flies. The most widely used trap is a battery-operated light-suction trap, with the main advantage being that it can be left overnight to collect sand flies (fig. 1, Table 2). The New Jersey Mosquito Light Trap, first developed in 1932, has been the most widely used light trap for sand fly work. The trap operates by sucking sand flies into a funnel with a mesh screen adapted to the size of the sand flies, terminating in a killing bottle. For special investigations in which live caught sand flies are required, the killing bottle is replaced by a holding cage, which can be changed several times during the same night, as required. Although the range over which this trap attracts sand flies is not large, it is highly efficient if located among resting or breeding places and if the right light source is used (Table 3). Light traps attract host-seeking sand flies, but

Table 2. Overview of the sand fly trapping methods indicating advantages and limitations.

Stage	Target population	Collection method	Advantages	Disadvantages
Adult	<ul style="list-style-type: none"> Resting population, both males and females Feeding population from animals 	Aspirator collections	<ul style="list-style-type: none"> Collection of live sand flies Reveal where sand flies rest during the day, to aid control measures Resting population: tend to target male sand flies, which can permit identification to species where females are morphologically indistinguishable Can be used to measure population changes 	<ul style="list-style-type: none"> Labour intensive, difficult to standardize Sand flies easily damaged
	<ul style="list-style-type: none"> Resting population, both males and females 	Sticky traps	<ul style="list-style-type: none"> Inexpensive and easy to make Large numbers can be used May be used for diurnal collections Rows of traps at floor to ceiling can be used to sample intra-domiciliary adult activity Can be used in burrows or rock crevices by hanging across entrances, or sail-like or rolled to intercept exiting sand flies 	<ul style="list-style-type: none"> Only dead specimens are collected Ineffective in areas with high relative humidity
	<ul style="list-style-type: none"> Host-seeking females (but also gravid females and males are attracted) 	Light trap	<ul style="list-style-type: none"> Standardization is possible Overnight collections 	<ul style="list-style-type: none"> Attracts only specific species Distance/range of attraction is limited Specimens often damaged and dead Dead specimens collected Large by-catch Specimens may be damaged by other insects Logistically heavy Number of traps that can be used simultaneously is limited
	<ul style="list-style-type: none"> Flying males and females 	Flight trap	<ul style="list-style-type: none"> Catches both males and females 	<ul style="list-style-type: none"> Dead specimens collected Large by-catch Specimens may be damaged by other insects Logistically heavy Number of traps that can be used simultaneously is limited
	<ul style="list-style-type: none"> Host-seeking females attracted to animals 	Animal baited traps	<ul style="list-style-type: none"> Large catches of live sand flies Collection of live sand flies 	<ul style="list-style-type: none"> Logistically heavy Number of traps that can be used simultaneously is limited
	<ul style="list-style-type: none"> Host-seeking females attracted to humans 	Human landing collections	<ul style="list-style-type: none"> Collection of anthropophilic sand flies 	<ul style="list-style-type: none"> Not recommended for ethical reasons Logistically heavy Inter-person variation
Immature	<ul style="list-style-type: none"> Immature stages 	Emergence trap	<ul style="list-style-type: none"> Live sand flies can be collected 	<ul style="list-style-type: none"> Exact breeding sites are difficult to locate
	<ul style="list-style-type: none"> Immature stages 	Soil screening	<ul style="list-style-type: none"> Live larvae can be collected 	<ul style="list-style-type: none"> Exact breeding sites are difficult to locate Labour intensive

also yield gravid females and males. A potential disadvantage of light traps is that they preferentially sample females of some groups (such as *Lutzomyia* subgenus *Psychodopygus*) that are highly phototropic and do not attract some other groups or species such as *Sergentomyia minuta* in southern France. Although they are useful in catching large numbers of certain species and measuring relative changes in abundance of these species over time and space, light traps have somehow limited value in ecological studies of sand flies. Some species may be relatively more active at greater heights above the ground, even at tree canopy height, and therefore tend to be under-represented in samples taken at ground level. Care must be taken in interpreting results of vertical distribution studies based on light trapping, as sand flies may fly upwards in response to the unusual light source, because these movements constitute part of their normal behaviour.

Sticky traps

Sticky traps constitute a method of sampling sand flies by interception rather than attraction. Standardized pieces of

white un-waxed paper or cards are soaked in castor oil and placed in sand fly resting places overnight (at least two or three consecutive days) (fig. 2, Table 3). To obtain quantitative results, either one or both sides of the paper must be completely exposed. The number of papers used at each sampling event is normally constant. These traps are generally inexpensive and easy to manufacture in large numbers. They can be prepared in bulk before field studies and stored until required (Table 3). Because of the viscosity of castor oil they are ineffective in habitats with high relative humidity and only dead specimens are collected.

Flight traps

Malaise traps are designed to catch flying insects, and consist of a tent-like nylon structure hung across trees or bushes in likely insect flight paths. Insects entering the trap are directed by the sloped roof into two transparent plastic or glass cylinders at either end of the structure, which contain a killing agent such as ethyl acetate, carbon tetrachloride or potassium cyanate in a plaster of Paris vial. Modifications can be made to



Fig. 1. Light trapping for sand flies in Turkey (Photo credit: Filiz Gunay & Gizem Oguz).

the entrance of the cylinders, e.g., reducing the opening size or fitting a wire mesh, to prevent the entry of larger flying insects, which may damage or eat fragile sand flies. Malaise traps can be assembled from everyday materials (Gressitt & Gressitt, 1962; Townes, 1962). Sand flies are likely to be trapped in small numbers, but an advantage is that both male and females will be caught, required for the identification of some species (Alexander, 2000). In addition, this method allows the detection of all sand fly species present within a habitat, while reducing sampling bias.

Aspirator collections

Sand flies can be collected by an aspirator from animals, and when resting on walls in buildings or animal shelters, with the main advantage of collecting live specimens. Sand flies are fragile and therefore the body of the aspirator should be wider than the opening. Plastic aspirators are light and strong but can damage sand flies because of static electricity. Therefore, a glass aspirator is recommended for most studies both in the field and in the laboratory. However, they are not commercially available. The Castro aspirator, a modification of the mouth aspirator, is made from a length of rubber tubing attached to the glass/plastic tube, with fine mesh gauze sealing the connection between the two components (Alexander, 2000). This allows sand flies to be sucked up rapidly and then quickly blown into holding containers, the principal

advantage being that insects collected from different microhabitats during a single session can be separated to provide more data, rather than being stored together in a single aspirator. Additionally, the Castro aspirator can be cleared regularly so that hairs shed by sand flies inside the tube are not inhaled by the operator. Holding containers can be made from 100 ml urine cups: plaster of Paris is applied to the base, a circle is cut from the lid and covered with gauze, and another hole is cut into the container's side then covered with two pieces of rubber, one slit vertically and the other horizontally, to produce a seal with a cross-like opening through which the aspirator tube can be inserted to blow collected sand flies inside. When working with a large number of sand flies, a reservoir type is favoured, with the body wider than the entrance. It consists of a tube sealed at one end by rubber, except for a narrow tube through which the sand flies enter. This makes it difficult for the insects to escape and helps to capture more specimens without the necessity to transfer them into a cage or other type of container. Emptying the reservoir aspirator may involve dismantling it within a cage. Hand-held power-operated aspirators are also available and these should be used in the field for sand fly collections from crevices, stables, or even from traps. Sand flies are easily captured in the periphery of direct light.

Animal baited traps

If the objective of the study is to detect the presence and the relative density of sand flies biting animals in a given area and/or to collect material (both females and males) for observation, animal-baited traps are the most appropriate tools. They are also useful for host preference studies. The bed net trap consists of a large mosquito net with smaller mesh size (200 meshes per square inch) for sand flies suspended from four poles, with a second smaller net inside under which the bait animal is placed. The amount of free space should be determined by experience with local sand fly species. The trap net should be made as tight and as rigid as possible, the edge of the net being tied with string and pegged into the ground. This prevents the net from flapping in the wind, which deters and disturbs the sand flies. Animal-baited traps have also been used for indoor collections.

Many kinds of animal-baited traps have been used in the field such as the Disney trap, the cone trap and animal-baited box trap, each with advantages or disadvantages depending on the aim of the study. A range of animals have been used as bait including cattle, donkeys, small mammals, chickens, ducks and dogs. To prepare the Disney trap, a cage containing an animal is placed in the centre of a metal tray, which is then coated in castor oil, and the apparatus is covered by a plastic/canvas roof to protect it from the rain (Disney, 1996). Female sand flies approaching the bait animal are trapped in the sticky oil, due to the fact that sand flies tend to land near to their host and approach in a few short hops rather than landing directly on the host. Blood-fed females that avoided the oil on their way to the animal may also be trapped after feeding. As with sticky traps, only dead specimens are collected. The cone trap consists of a large tent of semi-transparent material arranged over the bait, usually a large animal such as a horse or donkey (Montoya-Lerma & Lane, 1996). The material used allows the sand flies to sense the animal by sight, but also via other stimuli: CO₂, body heat and odour (Alexander, 2000). Blood-seeking sand flies enter the trap through funnels ending in small apertures, making escape difficult, and can be

Table 3. Overview of technical and operational requirements of two sand fly collection methods.

LIGHT TRAP – fig. 1

Technical requirements

- It is possible to adapt and/or modify light traps for mosquitoes. In the case of the CDC miniature light trap, it is possible to change from the larger mesh size of funnel used for mosquitoes, to a smaller mesh size type (preferentially 150–200 meshes per square inch).
- The distance at which these traps are attractive to sand flies appears to be limited. Killick-Kendrick *et al.* (1985) concluded that *P. ariasi* was attracted to CDC light traps from a maximum distance of only two metres. Valenta *et al.* (1995) determined that the maximum range of attraction to a 60 W light bulb for *Lutzomyia youngi* was 6 m. This information is important when considering efficient collections in the field. Light traps require the correct light source. Latest generation light traps generally require the following bulb types (recommended by several studies): (1) Type CM-47; voltage 6.3; mA h⁻¹ 150; Candlepower 0.52; Lifespan (h) 3000; (2) Type CM-44; voltage 6.3; mA h⁻¹ 250; Candlepower 0.90; Lifespan (h) 3000.

Operational requirements

- Light traps also require ca. 320 mA (0.320 A) per hour to operate at 6.0–6.3 V DC. Four D-size flashlight batteries (preferably alkaline) in series will provide power for one night's operation. However, motor cycle, lead-acid, 6 V batteries provide many nights worth of power on one charge, and these are the most common power source. A 6 V battery capacity of 10 AH is a good size for these traps. Although a light source can be efficient, an additional lure is often required. All of which can be used with light traps.
- Traps should be placed before dusk and emptied in the morning (after dawn). Collections should be rapidly removed from the trap to limit damage from heat and/or desiccation.
- Traps should be suspended 1.5–2 m above the ground, preferably at least 10 m or more from an external light source. Background light intensity negatively affects the efficiency of trapping capacity of light traps. Hence, it is better to avoid areas near other sources of artificial light, as well as sites exposed to strong winds, places near buildings housing animals, or those areas exposed to industrial fumes and smoke.
- A single trap usually reflects sand fly flight activity within a few metres of its location. A sufficient number of traps must be utilized to ensure a representative sample. If a site fails to produce the expected number of sand flies, judging from collections in other traps in the area, the trap should be relocated. Sometimes a shift of only a few metres makes a considerable difference in the number of sand flies attracted.
- The sand fly catch can be augmented by an additional source of carbon dioxide (CO₂), such as dry ice (approximately 700 g per night). According to Service (1993), mosquito response to CO₂ becomes saturated at levels close to that of human exhaled breath (4.5%) and gas released from a trap would therefore remain above the background atmospheric level (0.03%) until dispersed downwind and diluted by a factor of 100. Therefore, CO₂ is usually a long-range attractant for sand flies as for mosquitoes, whereas light is probably perceived by sand flies at much closer range. The addition of a source of CO₂ can therefore improve catches by increasing the effective range of the sampling area of a light trap (Veronesi *et al.*, 2007; Erisoz Kasap *et al.*, 2009; Kline *et al.*, 2011). Depending on the species, adding olfactory attractants (such as octenol), replacing incandescent bulbs with a long wave ultra-violet light source or inverting the light traps were found to increase the collection size (Faiman *et al.*, 2009; Kline *et al.*, 2011).

STICKY TRAPS – fig. 2

Technical requirements

- There is no commercially available standard template form. Many studies suggest the use of 20 × 20 cm or 25 × 20 cm standard papers, but it is not obligatory, so A4 plain paper can be used. The catch results by sticky traps are expressed as 'number of sand flies per m² of sticky paper per night'. The formula is: total number of collected specimens/total surface of sticky traps used (m²) (if both sides of the sticky paper are exposed, both have to be considered in the surface calculation formula).
- Preparation of oil-impregnated papers is simple: papers (different sizes mentioned above) are dipped into a sufficient amount of castor oil in a plastic container (uptake rate of 20 × 20 cm paper: 1 l oil / at least 250 papers). Using plastic gloves vigorously move the papers in the oil. When the papers take up no further oil, they are removed from the container and allowed to drip dry, and then placed in plastic bags. It is possible to apply oil using a paint roller to both sides; however these strategies can be time consuming. Alternatively, prepared papers can be placed in a lockable plastic container with an appropriate amount of castor oil. In the field, oil-coated papers can be removed and used as required.
- If sticky traps are intended to be left exposed to the sun during very hot days, high viscosity oils such as castor oil are suggested. Castor oil-impregnated sticky traps can be used productively in dry areas such as desert and dry mountain valleys, even though they are also effective to collect sand fly adults in habitats with different conditions. Sand flies are removed from the oiled paper (e.g., with a small brush) washed in saline or a drop of alcohol (70%) and then identified. Only dead specimens can be collected with these traps.

Operational requirements

- The papers are fixed in the proximity of resting places with tools (e.g., wooden or bamboo support, wire, hook, staple or paper clip) according to the shape of the resting place or applied directly onto the wall.
- Sticky traps can be located around resting places in large numbers. Possible resting sites include animal barns (inside/outside), houses (inside/outside), poultry houses (inside/outside), caves, tree holes, leaf litter, between or under rocks and animal burrows, rock crevices, holes of walls and among vegetation. Rows of traps hung at floor level, 20–50 cm above the ground or ceiling level can be used in, for example, animal barns or houses. Animal burrows and rock crevices are used as diurnal resting or breeding sites by many species and sticky traps hung across the entrances. For instance, in Mediterranean France, wall holes (barbacanes) near roads or in villages are very important resting places for *P. ariasi* so that, mounted sail-like on small sticks or rolled, sticky traps can be used to collect sand fly specimens in the holes. For these types of resting places, a good place to locate traps is in the vicinity of a hole with a thin layer of moist soil and vegetation, as these can be good resting sites.
- More information on different methods on how to use sticky papers efficiently or where to place them during field studies are described in Alexander (2000).



Fig. 2. Sticky traps used in Switzerland (Photo credit: Francis Schaffner).

collected from inside the trap by aspiration at the end of the trapping period. The animal-baited box trap (Espinola *et al.*, 1983) comprises a wooden box divided into two chambers which are connected by hinges and separated by a mesh screen. The large bottom chamber holds the bait animal while the upper chamber collects sand flies which are attracted to the animal and enter through two funnels in the box's lid.

Human landing catches

Catching host-seeking flies landing on people they intend to bite, the so-called 'human landing collections', are the most productive method of collecting anthropophilic Diptera. Because of the danger of contracting leishmaniasis and other infections, the flies should not be allowed to actually bite. For ethical reasons, 'human biting collections' are officially discouraged or prohibited by many funding agencies. It is recommended that human landing catches be performed for pre-determined periods of time so that data are comparable (e.g., no. flies per man-hour), and so that risk to collectors is reduced.

Maroli *et al.* (1997) describe a bed net trap (mentioned above) in which a human volunteer lying on a bed and protected by fine-mesh mosquito net is enclosed within a second net, elevated a few centimetres above the ground to permit access of sand flies attracted to the human bait. The insects that enter have a tendency to move towards the upper limits

of this second net, from where they can be aspirated at the end of the observation period.

Sampling methods of immature sand flies

Sand flies oviposit in terrestrial microhabitats where their developmental stages are seldom found. For this reason control measures directed against immature stages are not considered as an option in leishmaniasis control programmes. There are two main approaches to look for breeding sites. One involves placing emergence traps in places where adult insects have been found resting (Table 2). These are inspected at regular intervals and newly emerged adults are collected. Depending on the type of microhabitats, different kinds of emergence traps can be used (Moncaz *et al.*, 2012). Empty pupal cases at the soil-air interface and resting adult male flies with their genitalia in the teneral position (at 180° to that of the mature specimens) are indications of a breeding site. The second approach to finding immature phlebotomines involves a variation of the sugar flotation technique (McCombie-Young *et al.*, 1926; Hanson, 1961; Thatcher, 1968; Rutledge & Mosser, 1972). Soil and leaf litter samples from potential breeding sites are mixed with water and the supernatant (containing material such as dead leaves and exuviae of insects) is poured off. The remaining material is mixed with a saturated sugar solution and sand fly larvae float to the surface, from which they are collected and placed in



Fig. 3. *P. similis* habitat in Crete (Photo credit: Maria Antoniou).

plaster-lined pots containing some suitable larval diet. This method has the advantage of preserving the larvae alive so that they can be reared to adults, this being of critical importance in areas where more than one sand fly species occurs, because specific identification of immature stages is generally impossible.

Sampling strategy

Several factors affect the success of sampling methods. In the European region of the Old World sand fly activity is limited to the summer months. By contrast, New World leishmaniasis have a distribution that is almost exclusively tropical. Precipitation rather than temperature is the principal climatic factor that determines the sand fly seasons (Alexander, 2000). Decreases in the populations of anthropophilic species are preceded by corresponding peaks in rainfall, which also reduces the amount of suitable diurnal resting sites for the adult insects, as well as limiting adult flight activity. Hence, the timing of the survey programme is dependent on climatic, ecological and the geographical situation of the study site. For example, the active sampling period of sand fly species in southern Europe is between April and late October. Moreover, different sand fly species have different seasonal activity periods and different peaks of activity. It is possible however, using various sampling methods, to collect a number of species together during field visits; however the number of specimens per species will be variable. Ideally, a variety of different sampling methods should be employed during each survey, as particular methods may favour a single sand fly species, which may not be the most abundant or important from a public health perspective.

There are a number of health and safety risks that should be noted when sampling in sand fly habitats. Firstly, phlebotomines often share microhabitats with dangerous animals, such as scorpions and snakes. Secondly, there is a potential risk of acquiring respiratory infections such as histoplasmosis from aspirating in humid environments where sand flies rest (Alexander, 2000). Also, the inhalation of sand fly hairs during aspiration may cause respiratory irritation.

Trapping density and frequency

The trapping density and frequency are dependent on several factors such as the study objectives, capacity of the team

and finances. First the researchers have to make a decision on the number of samples per habitat unit. Aspects to consider include: (a) whether different regions of the unit need to be sampled separately, for instance according to altitude, vegetation, or microhabitats and (b) the number of samples within each unit/subunit for maximum efficiency. The distribution of the population throughout the habitat can be biased towards certain subdivisions. It is sometimes important that if the distribution of the population throughout the habitat is biased towards certain subdivisions but the samples are taken randomly, 'systematic errors' will arise. This can be overcome either by sampling so that the differential number of samples from each subdivision reproduces the gradient in the habitat or by regarding each part separately and subsequently correcting. To select the sampling unit and its size and shape it is important to decide the trapping density and frequency. The criteria for the sample unit must: (a) have an equal chance of selection by sampling, (b) have stability (or, if not, the changes should be easily and continuously measured) by regarding, (c) lend itself to conversion to unit areas, (d) be easily delineated in the field, (e) be of such a size as to provide a reasonable balance between the variance and the cost, (f) not be too small in relation to the species' population size, as this would increase edge-effect errors. The objectives of the study will also determine the sampling pattern. If the aim is to obtain estimates of the mean density then variance should be minimized. It is preferable that both the trapping density and trapping frequency are high. Trapping twice a month with a sufficient amount of traps (for sticky traps it is unlimited because of their very low cost and ease of preparation; for CDC light traps, the unit is 10–15 traps per 500 m) is required to keep variance errors at a minimum level.

Generally, light traps are operated on a regular basis of 1–7 nights/week; with four nights giving as valid an index as 7 nights/week. Therefore, trap collections should be made on four consecutive nights, such as Monday through Thursday of each week. For more information refer to Southwood & Henderson (2000).

Different sand fly species breed and rest in different habitats (figs 3–7), prefer urban and/or rural areas, sheltered and/or open areas. For instance, *S. minuta* prefers spaces between small rocks, while *P. mascittii* prefers caves as resting sites. In general, adult sand flies shelter during the day in dark locations in various resting sites, humid places such as tree-holes, or arid places such as under rocks, or animal burrows, barns,



Fig. 4. *P. neglectus* habitat in (a) Albania (Photo credit: Enkelejda Velo) and (b) Crete (Photo credit: Maria Antoniou).



Fig. 5. *P. tobbi* habitat in Albania (Photo credit: Enkelejda Velo).



Fig. 6. *P. perniciosus* habitat (a) in Ticino, Switzerland, (b) CO₂-baited light trap (Photo credit: Francis Schaffner).



Fig. 7 *P. mascittii* habitat in (a) Alsace, France and (b) Ticino, Switzerland; combination of light and sticky traps (Photo credit: Francis Schaffner).

inside houses or under leaves. Resting site collections may be the best method for obtaining male sand flies, which are required for the identification of some species. Generally there are no differences between trapping techniques but some modification according to resting place may be needed.

Local people may be able to provide useful information to researchers, which may be important in identifying areas of sand fly abundance, seasonal activity, etc. and could help direct sampling efforts. If sand flies are perceived locally as a public health problem, people living in the area are likely to engage with any control measures that are implemented in the future, and may even assist with sampling activities.

Factors influencing the collections

Sand flies are small, fragile, nocturnally active insects with weak direct flight capability. Several factors may affect the success of sampling methods. Sand fly flight activity is limited by rain, wind or low temperatures and attraction to bait animals depends on the direction in which the odour plume is wafted. Moonlight reduces the distance at which CDC light trap illumination equals background levels and thus the effective sampling area and the number of sand flies are likely to be caught. There is considerable difference in light intensity between moonless nights and those with a full moon; Kuiper (1938) calculated a 140-fold difference in radiation between the two extremes. Bowden & Morris (1975) expressed the relationship as 'catch constant \times (W/I)' where W is the trap illumination and I is background light intensity. Although strong moonlight may interfere with light trap collections, it is sometimes associated with increased activity in mosquito populations, and may be similar for sand flies. Nevertheless, the relationship between moonlight or the lunar cycle and the abundance and activity of sand flies has been controversial (Santos-De Marco *et al.*, 2002).

Several types of trap such as sticky traps and flight interception traps catch insects that are carried into or onto them by the wind; consequently their practical use varies with wind speed. Other variables noted by Martin *et al.* (1994) as

influencing sticky trap sampling efficiency for three species of black fly, were wind speed, light, temperature, saturation deficit and time of day. A similar list would be found for sand flies. In general, adult sand flies do not fly in wind of more than $2\text{--}3\text{ m s}^{-1}$.

For the reasons discussed above, researchers measure the variation of climatic conditions such as temperature, relative humidity and wind speed throughout the field study or representative area(s) of the study area with sensitive devices such as data-loggers or portable climate stations. The measurement of climatic factors is meaningful when you measure for the entire area. The data obtained from these measurements allow comparisons between population fluctuations and climatic factors that may affect sand fly abundance.

Sand flies are highly susceptible to contact insecticides and spraying programmes directed against mosquitoes or agricultural pests, which are likely to reduce phlebotomine activity in the same habitats.

Handling and processing sampled sand flies

The preservation technique used for transportation of sand flies depends on the purpose for which the specimens were obtained. Those to be used in taxonomic studies can be preserved dry in layers of tissue paper prior to being cleared in lactophenol for identification and subsequent slide mounting in Canada balsam, Berlese, or Eupharal fluids (Moore & Gage, 1996; Alexander, 2000; Marquardt *et al.*, 2005; Volf & Volfova, 2011). Storage in 70% alcohol can improve the chances of specimens remaining intact but complicates the clearing process by hardening the muscles of the insects and obscuring internal structures such as the female spermathecae used in identification. Uncleared specimens should not be kept in alcohol for more than 6 months. The best way to transport adult specimens to the laboratory for identification or establishment of a colony is to keep them alive with larval pots which have a layer of plaster of Paris on the sides and bottom and closed with a snap cap top and fine gauze, placed into a plastic ice-container at 4°C .



Fig. 8. Specimen preservation and laboratory rearing of *P. papatasi* (Photo credit: Filiz Gunay & Gizem Oguz).

The presence of sympatric, morphologically identical species in a particular habitat can be detected using a variety of sampling methods and regimes to reveal differences in activity and other behavioural aspects. These observations can subsequently be confirmed by techniques such as isoenzyme electrophoresis or DNA studies.

Because enzymes used in studies of population genetics must retain their activity after electrophoresis, sand flies must be kept alive (fig. 8) until processed in the laboratory or stored at temperatures below -40°C , for example, at -80°C or in liquid nitrogen. Specimens to be examined for natural *Leishmania* infections should be immobilized and immersed in a detergent solution to dissolve the oil on the water-repellent hairs that cover their bodies and ensure that the insects do not remain floating on the surface of the solution in which they are to be preserved. This should contain a cryoprotectant such as 10% dimethyl sulphoxide in phosphate-buffered saline. The vials containing the insects are submitted to a 30 min cooling period in a styrofoam container placed in the neck of a liquid nitrogen tank or dry shipper prior to total immersion. Yaghoobi-Ershadi & Javadin (1996), working in Iran, were able to recover natural infections of sand flies captured on sticky traps in rodent burrows and retrieved the next morning. According to Moore & Gage (1996) however, sticky traps are unlikely to produce specimens suitable for virus isolation because the oil used on the surface of traps interferes with cell culture.

Studies involving DNA-based protocols can use dried, fresh, frozen or alcohol-preserved specimens whereas those for pheromone analysis should be placed fresh in a Pasteur pipette or glass tube containing hexane and heat-sealed. Insects intended for establishment of colonies, behavioural studies or immediate dissection on return to the laboratory should be transported alive in larval pots placed inside styrofoam boxes supplied with a damp sponge or cloth and a sugar source such as an apple slice or cotton wool pad saturated in sucrose solution.

For virology studies, collected sand fly adults must be transported to the laboratory on dry ice, or alive into larval pots mentioned above. If adults are transported alive to the laboratory, they should be placed and killed in a freezer (-80°C) immediately. This process will preserve the virus RNA.

Although several age-grading methods have been developed for bloodsucking insects, probably the only fairly reliable technique for sand flies involves the examination of the

ovarioles for follicular dilations to determine the number of gonotrophic cycles females have undergone (Detinova, 1962). This requires that the insects be kept alive or preserved in liquid nitrogen with a cryoprotectant until they can be transported to the laboratory and dissected. Anez & Tang (1997), however, claimed that sand fly ovaries were unsuitable for the application of these techniques because they show no obvious and reliable changes in tracheolar coiling or follicular dilations that can be correlated with parity.

For the detailed microscopic examination of external or internal structures, sand flies may be preserved on slides in mounting media, of which the following are recommended: chloralhydrate medium gelatin, lactophenol, Canada balsam, Berlese, euparal, CMCP-10, Hoyer. The latter three media in this list have been frequently used in recent studies with great success. However, these preparations generally do not survive long-term museum storage. In preparing the slides, careful clearing, dissection and arrangement of each specimen is essential to enable identification to species. Folded wings, rotated heads, collapsed spermathecae, or lost antennae can prevent a secure final identification (WHO, 1975, 1979, 1980; Alexander, 2000; Marquardt *et al.*, 2005).

Identification

The family Psychodidae is very old and maintains some of the most ancient dipteran characters. Members of the family are distinguished by a dense covering of narrow scales on head, thorax, legs and wing veins. Of the five psychodid sub-families, only the Phlebotominae have piercing mouthparts capable of taking blood. Furthermore, the phlebotomines tend to have an elongate and more fragile structure, in contrast to a squatter and more robust appearance of the other psychodid flies. Phlebotomine sand flies are small with a body length seldom exceeding 3 mm. Their colour ranges from almost white to almost black. Three features of phlebotomines are diagnostic to distinguish them from other Psychodidae: (1) when at rest, they characteristically hold their wings at an angle above the abdomen (fig. 9); (2) they are hairy; and (3) when alighting to engorge, they typically hop around on the host before settling down to bite.

The most important keys used for the morphological identification of sand flies are Theodor (1958), Artemiev (1980), Lewis (1982), Lane (1993), Abonnenc (1972) and the digital key entitled 'Sand Flies of the Afrotropical Region' prepared by Niang *et al.* (2004). There are also many incomplete (in



Fig. 9. *Phlebotomus papatasi* female: Photo credit: David Modry, Brno, Czech Republic.

many cases obsolete) written keys in the literature, and some of them are on local sand fly fauna for studied countries and/or studied area(s). In fact, for most of the sand fly species, morphological identification is very difficult and needs experience, particularly for female specimens. The taxonomy is not always resolved and there are important gaps in our knowledge. In many cases, morphological identification using written keys is not sufficient, and molecular confirmation is additionally required. The best approach is to conduct a morphological identification and then to use molecular methods for confirmation. Even if most sand fly species can be distinguished morphologically it requires expertise to discern these differences. For instance, it is difficult to distinguish *P. perniciosus* from *Phlebotomus longicuspis* (Pesson *et al.*, 2004) or *Phlebotomus neglectus* from *Phlebotomus syriacus* (Badakhshan *et al.*, 2011; Erisoz Kasap *et al.*, 2013). For damaged specimens and immature stages, molecular methods will be required for accurate identification.

One method is a DNA barcoding approach, involving PCR amplification and sequencing of a genetic region (e.g., ribosomal ITS2, nuclear EF-1 α , mitochondrial cytochrome b, mitochondrial cytochrome oxidase subunit 1) which can be compared with sequences in an online database to identify the sand fly species (Latrofa *et al.*, 2011; Erisoz Kasap *et al.*, 2013; Gutiérrez *et al.*, 2014). However, this is time consuming and requires access to specialist equipment. More rapid molecular methods for the identification and differentiation of sand fly species include (polymerase chain reaction) PCR of the ribosomal ITS2 region (Khalid *et al.*, 2010; Latrofa *et al.*, 2012) and PCR-RFLP (restriction fragment length polymorphism) of the mitochondrial DNA fragment *cytb* – *nd1* (Latrofa *et al.*, 2012) or 18S ribosomal RNA gene (Tiway *et al.*, 2012). Both methods allow the separation of species based on differences in fragment sizes, and allow the identification of a large number of samples in a short time with limited resources. However, published protocols for these methods are usually region-specific and only cover a few common species. Recently, a MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight) mass spectrometry-based method for the rapid identification of phlebotomine species has been developed (Dvorak *et al.*, 2014), although

currently this only covers five species and would require access to specialized equipment.

Pathogen detection

The phlebotomine sand flies are vectors of several zoonotic, or even anthroponotic, diseases such as leishmaniasis, bartonellosis and sandfly fever (Table 4). It must be recognized that the importance of the leishmaniasis has eclipsed the possible role of sand flies in the transmission of other pathogens. The recent emergence of some of these diseases has led to a change in the view of sand flies as important disease vectors.

There are various methods that can be used to detect pathogens in sand flies. These methods include dissections (for *Leishmania* spp.), multiple locus electrophoresis (for *Leishmania* spp., *Bartonella* spp.), molecular detection including PCR, reverse transcriptase PCR, real-time PCR (for *Leishmania* spp., phlebovirus and bacteria) and *in vitro* cultivation and cell culture inoculation (*Leishmania* spp., phlebovirus and bacteria). Cultivation is cumbersome since the prevalence is very low for *Leishmania* spp. and phleboviruses. Prevalence can also show significant variation between different sand fly species and across an area. For example, in Italy *Leishmania* spp. prevalence in sand flies has been found to be 2.9% in Sicily (Gómez-Saladín *et al.*, 2005) and 47.2% in Rome province (Rossi *et al.*, 2008), whereas prevalence rates range from 0 to 78% in different regions of Spain (Martín-Sánchez *et al.*, 2006; Alcover *et al.*, 2012; Jiménez *et al.*, 2013), and from 0 to 4% in Portugal (Branco *et al.*, 2013; Maia *et al.*, 2013). It is worth noting that *Leishmania* was detected more often in unfed versus blood-fed females (Jiménez *et al.*, 2013). Phlebovirus infection rates have been recorded at 0.5% in Italy (Verani *et al.*, 1988) and a study in France found that 9.1% of 66 pools of 30 sand flies were PCR-positive (Charrel *et al.*, 2009).

Viral RNA detection via PCR and inoculation of cell cultures are the two most widely-used techniques for virus detection in sand flies. Once safe transportation is ensured, many methods including standard PCR or real-time PCR targeting a single pathogen (or many serotypes) can be employed according to the specific aims. See Marquardt *et al.* (2005) for detailed information.

If the pathogen has to be isolated, the viability of the organism in question has to be strictly preserved. To ensure this, samples must be kept cool as much as possible and must be transported in dry ice or liquid nitrogen. For nucleic acid detection, commercially available preservation solutions that inactivate nucleases can be employed. The best way to ensure viability is to keep the specimens alive until frozen in -80°C , if possible.

Pooling is frequently performed to increase the probability of detection in an endemic setting, to process a high number of specimens and to decrease screening costs. However, pooling may also decrease detection sensitivity if very few infected vectors are present in individual pools. Therefore, the decision of whether to use a pooling strategy should be based on the estimated prevalence of the pathogen in sand flies in the study region.

Blood-fed, gravid, or non-fed female sand flies are analysed for the detection of *Leishmania* spp. Nevertheless, the gravid and non-fed stages are more informative since they reflect the parasite's ability to survive in the sand fly after blood digestion, and thus the vectorial capacity of the sand fly species can be studied at the same time. For viruses with

Table 4. Summary of diseases transmitted by sand flies.

Disease	Pathogen details	Clinical perspective
Leishmaniasis	<ul style="list-style-type: none"> Flagellate protozoa of the genus <i>Leishmania</i> (Kinetoplastida, Trypanosomatidae), which infect numerous mammalian species, including humans. Thirty different species: can be morphologically indistinguishable, other characters have been used for their taxonomy, mainly multilocus isoenzyme electrophoresis, and more recently molecular techniques (Bañuls <i>et al.</i>, 2007; Schönian <i>et al.</i>, 2011). Widely distributed occurring in 88 countries, ranging over the intertropical zones of America and Africa, and extending into temperate regions of South America, southern Europe and Asia. The limits of the disease are latitudes 45°N and 32°S. Geographical distribution is governed by the limitations of the sand fly vector or mammalian host species. 	<ul style="list-style-type: none"> In humans, the disease may be visceral (VL), cutaneous (CL), of localized (LCL) or diffuse (DCL) type, or mucocutaneous leishmaniasis (MCL) (Bañuls <i>et al.</i>, 2011). The leishmaniases are diseases with natural focality. They include several noso-epidemiological units, which can be defined as the conjunction of a particular <i>Leishmania</i> species, circulating in specific natural hosts, evolving in a natural focus with specific ecological patterns, and having a particular clinical expression.
Carrión's disease	<ul style="list-style-type: none"> A human infection caused by <i>Bartonella bacilliformis</i>, a bacterium that invades primarily the blood cells and secondarily the phagocytic mononuclear system, particularly the endothelial cells. The bacteria are transmitted to humans by the bite of the sand fly species <i>Lutzomyia verrucarum</i> (Maroli <i>et al.</i>, 2013). 	<ul style="list-style-type: none"> The disease is limited to small Andean communities in Peru, Colombia and Ecuador and, to date, a natural non-human vertebrate reservoir has not been identified.
Phleboviruses	<ul style="list-style-type: none"> Phleboviridae is one of the five genera of the family Bunyaviridae. They are members of the class V virus, with segmented ambisense RNA genomes. Isolated in southern Europe, Africa, Central Asia and the Americas (Alkan <i>et al.</i>, 2013; Maroli <i>et al.</i>, 2013). They include two groups: the <i>Phlebotomus</i> fever virus, transmitted by phlebotomine sand flies (Alenquer, Candiru, Chagres, sand fly fever Naples/Toscana, sand fly fever Punta Toro, sand fly fever Sicilian, sand fly fever Turkey virus) or by mosquitoes (Rift Valley fever virus) and the Uukuniemi group, transmitted by ticks. Twenty-five phleboviruses have been isolated on the American continent, reflecting the great diversity of the New World sand flies. 	<ul style="list-style-type: none"> Phleboviruses cause diseases ranging from short self-limiting fevers to encephalitis and fatal haemorrhagic fever. The phleboviruses Sicily and Toscana are responsible for 'sand fly fevers', and are present in the Mediterranean basin (Papa <i>et al.</i>, 2006). Sand fly fevers are serious, but non-fatal, diseases. Most common clinical presentation is flu-like symptoms, with fever, headache, muscular pain, nausea, conjunctivitis and photophobia. Meningitis and meningoencephalitis can occasionally occur. Asymptomatic cases are frequent in endemic country populations, particularly in populations exposed to risk of sand fly bites, as shown in the Tuscany region of Italy, where the seroprevalence in forestry workers reached 77.2%. The Toscana serotype of the Naples virus has regularly been associated with central nervous system infections in southern Italy and Sardinia, southern France, Spain, and Portugal (Dionisio <i>et al.</i>, 2001; Echevarria <i>et al.</i>, 2003; Di Nicuolo <i>et al.</i>, 2005; Charrel <i>et al.</i>, 2005; Charrel <i>et al.</i>, 2007; Santos <i>et al.</i>, 2007; Sanbonmatsu-Gámez <i>et al.</i>, 2009). Imported cases have also been reported in non-endemic countries such as Germany (Imirzalioglu <i>et al.</i>, 2006). Some vector species of the sand fly fevers have been identified, such as <i>P. papatasi</i> in Italy, <i>P. perniciosus</i> in Spain, Portugal and southern France, and <i>P. major</i> s.l in Turkey (Konstantinou <i>et al.</i>, 2007; Dedet, 2008; Ergunay <i>et al.</i>, 2012).

transovarial and transtadial transmission, both sexes are known to include viruses that can be efficiently detected. One approach is to identify virus sequences in blood-fed female sand flies and retain the males for morphologic identification and barcoding.

Conclusion

Unlike surveillance for mosquitoes or ticks, sampling for phlebotomine sand flies is much less practised in Europe. There may be many reasons for this, but undoubtedly the restricted range of sand flies to southern Europe has so far

negated the need for surveillance in parts of central and northern Europe. However, with current climatic changes, and the evidence presented elsewhere on changes in sand fly distribution in Europe (Medlock *et al.*, 2014), countries in previously non-endemic areas are becoming concerned about the prospect of a change in distribution both in latitude and altitude. Much of the expertise in sand fly sampling and surveillance in Europe exists with entomologists neighbouring the Mediterranean. This paper aims to share that expertise so that sand fly surveillance activities can be expanded to new locations in Europe, to further improve our understanding of the distribution of our native fauna, but also to better inform

public and veterinary health risks assessments on sand fly-borne disease.

References

- Abonnenc, E. (1972) Les phlébotomes de la région Éthiopienne (Diptera, Psychodidae). *Mém ORSTOM* 55, 1–290.
- Alcover, M.M., Gramiccia, M., Di Muccio, T., Ballart, C., Castillejo, S., Picado, A., Portús, M. & Gállego, M. (2012) Application of molecular techniques in the study of natural infection of *Leishmania infantum* vectors and utility of sandfly blood meal digestion for epidemiological surveys of leishmaniasis. *Parasitology Research* 111(2), 515–523.
- Alexander, B. (2000) Sampling methods for phlebotomine sand flies. *Medical and Veterinary Entomology* 14, 109–122.
- Alkan, C., Bichaud, L., de Lamballerie, X., Alten, B., Gould, E.A. & Charrel, R.N. (2013) Sandfly-borne phleboviruses of Eurasia and Africa: epidemiology, genetic diversity, geographic range, control measures. *Antiviral Research* 100(1), 54–74.
- Anez, N. & Tang, Y. (1997) Comparison of three methods for age grading of female Neotropical phlebotomine sand flies. *Medical and Veterinary Entomology* 11, 3–7.
- Artemiev, M.M. (1980) A revision of sand flies of subgenus *Adlerius* (diptera, Phlebotominae, *Phlebotomus*). *Zoologicheskii Zhurnal* 59, 1177–1192 (in Russian).
- Badakhshan, M., Sadraei, J. & Moin-Vaziri, V. (2011) Morphometric and morphological variation between two different populations of *Phlebotomus major* s.l. from endemic and non-endemic foci of visceral leishmaniasis in Iran. *Journal of Vector Ecology* 36(1), 153–158.
- Bañuls, A.L., Hide, M. & Prugnolle, F. (2007) *Leishmania* and the leishmaniasis: a parasite genetic update and advances in taxonomy, epidemiology and pathogenicity in humans. *Advances in Parasitology* 64, 1–109.
- Bañuls, A.L., Bastien, P., Pomares, C., Arevalo, J., Fisa, R. & Hide, M. (2011) Clinical pleomorphism in human leishmaniasis, with special mention of asymptomatic infection. *Clinical Microbiology and Infection* 17(10), 1451–61.
- Bowden, J. & Morris, M.G. (1975) The influence of moonlight on catches of insects in light traps in Africa. The effective radius of a mercury-vapour light trap and the analysis of catches using effective radius. *Bulletin of Entomological Research* 65, 303–348.
- Branco, S., Alves-Pires, C., Maia, C., Cortes, S., Cristovão, J.M., Gonçalves, L., Campino, L. & Afonso, M.O. (2013) Entomological and ecological studies in a new potential zoonotic leishmaniasis focus in Torres Novas municipality, Central Region, Portugal. *Acta Tropica* 125(3), 339–348.
- Charrel, R.N., Gallian, P., Navarro-Mari, M., Nicoletti, L., Papa, A. & Sánchez-Seco, M.P. (2005) Emergence of Toscana virus in Europe. *Emerging Infectious Diseases* 11(11), 1657–1663.
- Charrel, R.N., Izri, A., Temmam, S., Delaunay, P., Toga, I. & Dumon, H. (2007) Cocirculation of 2 genotypes of Toscana virus, southeastern France. *Emerging Infectious Diseases* 13(3), 465–468.
- Charrel, R.N., Moureau, G., Temmam, S., Izri, A., Marty, P., Parola, P., da Rosa, A.T., Tesh, R.B. & de Lamballerie, X. (2009) Massilia virus, a novel Phlebovirus (Bunyaviridae) isolated from sandflies in the Mediterranean. *Vector Borne and Zoonotic Disease* 9(5), 519–530.
- Dedet, J.P. (2008) The control of diseases transmitted by phlebotomine sandflies to humans, ever achievable? 3rd CVBD Symposium, 16–19 April 2008, Germany, proceeding 16–21.
- Detinova, T.S. (1962) *Age-Grouping Methods in Diptera of Medical Importance with Special Reference to Some Vectors of Malaria*. WHO Monograph Series, 47, Geneva, World Health Organization.
- Di Nicuolo, G., Pagliano, P., Battisti, S., Starace, M., Mininni, V., Attanasio, V. & Faella, F.S. (2005) Toscana virus central nervous system infections in southern Italy. *Journal of Clinical Microbiology* 43, 6186–6188.
- Dionisio, D., Valassina, M., Cuifolini, M.G., Vivarelli, A., Esperti, F. & Cusi, M.G. (2001) Encephalitis without meningitis due to sandfly fever virus serotype Toscana. *Clinical Infectious Disease* 32(8), 1241–1243.
- Disney, R.H. (1996) A trap for Phlebotomine sandflies attracted to rats. *Bulletin of Entomological Research* 56(3), 445–451.
- Dvorak, V., Halada, P., Hlavackova, K., Dokianakis, E., Antoniou, M. & Volf, P. (2014) Identification of phlebotomine sand flies (Diptera: Psychodidae) by matrix-assisted laser desorption/ionization time of flight mass spectrometry. *Parasites and Vectors* 7, 21.
- Echevarria, J.M., de Ory, F., Guisasaola, M.E., Sanchez-Seco, M. P., Tenorio, A. & Lozano, A. (2003) Acute meningitis due to Toscana virus infection among patients from both the Spanish Mediterranean region and the region of Madrid. *Journal Clinical Virology* 26(1), 79–84.
- Ergunay, K., Erisoz Kasap, O., Kocak Tufan, Z., Turan, M., Ozkul, A. & Alten, B. (2012) Molecular Evidence Indicates That Phlebotomus major sensu lato (Diptera: Psychodidae) is the vector species of the recently-identified sandfly fever Sicilian virus variant: Sandfly Fever Turkey Virus. *Vector-Borne Zoonotic Diseases* 12(8), 690–698.
- Erisoz Kasap, O., Belen, A., Kaynas, S., Simsek, F.M., Biler, L., Ata, N. & Alten, B. (2009) Activity patterns of sand fly species (Diptera: Psychodidae) and comparative performance of different traps in an endemic CL focus in Cukurova Plain, Southern Anatolia. *Acta Veterinaria Brno* 78(2), 327–335.
- Erisoz Kasap, O., Votypka, J. & Alten, B. (2013) The Distribution of the *Phlebotomus major* (Diptera: Psychodidae) complex in Turkey. *Acta Tropica* 127(3), 204–211.
- Espinola, H.N., Gonzalez, T.D., & Tonn, R.J. (1983) A useful trap for collecting sand flies (Diptera: Psychodidae, Phlebotominae). *Bulletin of the Society of Vector Ecology* 8, 65–66.
- Faiman, R., Cuño, R. & Warburg, A. (2009) Comparative efficacy of three suction traps for collecting Phlebotomine sand flies (Diptera: Psychodidae) in open habitats. *Journal of Vector Ecology* 34, 114–118.
- Feliciangeli, M.D. (2004) Natural breeding places of phlebotomine sandflies. *Medical and Veterinary Entomology* 18(1), 71–80.
- Gómez-Saladín, E., Doud, C.W. & Maroli, M. (2005) Short report: surveillance of *Leishmania* sp. among sand flies in Sicily (Italy) using a fluorogenic real-time polymerase chain reaction. *American Journal of Tropical Medicine and Hygiene* 72(2), 138–141.
- Gressitt, J.L. & Gressitt, M.K. (1962) An improved malaise trap. *Pacific Insects* 4, 87–90.
- Gutiérrez, M.A.C., Vivero, R.J., Vélez, I.D., Porter, C.H. & Uribe, S. (2014) DNA barcoding for the identification of sand fly species (Diptera, Psychodidae, Phlebotominae) in Colombia. *PLoS One* 9(1), e85496.
- Hanson, W.J. (1961) The breeding places of *Phlebotomus* in Panama. *Annals of the Entomological Society of America* 54, 317–322.
- Imirzalioglu, C., Schaller, M. & Bretzel, R.G. (2006) Sandfly fever Naples virus (serotype Toscana) infection with meningeal

- involvement after a vacation in Italy. *Deutsche Medizinische Wochenschrift* 131, 2838–2840.
- Jiménez, M., González, E., Iriso, A., Marco, E., Alegret, A., Fúster, F. & Molina, R. (2013) Detection of *Leishmania infantum* and identification of blood meals in *Phlebotomus perniciosus* from a focus of human leishmaniasis in Madrid, Spain. *Parasitology Research* 112(7), 2453–2459.
- Khalid, N., Elnaïem, D., Aboud, M., Al Rabba, F. & Tripet, F. (2010) Morphometric and molecular differentiation of *Phlebotomus* (Phlebotomus) sandflies. *Medical and Veterinary Entomology* 24(4), 352–360.
- Killick-Kendrick, R. (1999) The biology and control of phlebotomine sand flies. *Clinics in Dermatology* 17, 279–289.
- Killick-Kendrick, R., Wilkes, T.J., Alexander, J., Bray, R.S., Rioux, J.-A. & Bailly, M. (1985) The distance attraction of CDC light traps to phlebotominae sand flies. *Annales De Parasitologie Humaine et Comparee* 60, 763–767.
- Kline, D., Hogsette, J.A. & Müller, G. (2011) Comparison of various configurations of CDC-type traps for the collection of *Phlebotomus papatasi* Scopoli in southern Israel. *Journal of Vector Ecology* 36(Supplement 1), 212–218.
- Konstantinou, G.N., Papa, A. & Antoniadis, A. (2007) Sandfly-fever outbreak in Cyprus: are phleboviruses still a health problem? *Travel Medicine and Infectious Disease* 5(4), 239–242.
- Kuiper, G.P. (1938) The magnitude of the sun, the stellar temperature scale and bolometric corrections. *Astrophysical Journal* 88, 429–471.
- Lane, R.P. (1993) Sand flies (Phlebotominae). in Lane, R.P. & Crosskey, R.W. (eds) *Medical Insects and Arachnids*. London, Chapman and Hall, Ch. 4, 78–119.
- Latrofa, M.S., Dantes-Torres, F., Weigl, S., Tarallo, V.D., Parisi, A., Traversa, D. & Otranto, D. (2011) Multilocus molecular and phylogenetic analysis of phlebotomine sand flies (Diptera: Psychodidae) from southern Italy. *Acta Tropica* 119 (2–3), 91–98.
- Latrofa, M.S., Annoscia, G., Dantes-Torres, F., Traversa, D. & Otranto, D. (2012) Towards a rapid molecular identification of the common phlebotomine sand flies in the Mediterranean region. *Veterinary Parasitology* 184(2–4), 267–270.
- Lewis, D.J. (1982) A taxonomic review of the genus *Phlebotomus* (Diptera: Psychodidae). *Bulletin of the British Museum (Natural History), Entomology Series* 52, 1–35.
- Maia, C., Dionísio, L., Afonso, M.O., Neto, L., Cristóvão, J.M. & Campino, L. (2013) Leishmania infection and host-blood feeding preferences of phlebotomine sandflies and canine leishmaniasis in an endemic European area, the Algarve Region in Portugal. *Memorias do Instituto Oswaldo Cruz* 108(4), 481–487.
- Maroli, M., Feliciangeli, M.D. & Arias, J. (1997) *Metodos de captura, conservacion y montaje de los flebotomos (Diptera: Psychodidae)*. Documento OPS/HCP/HCT/95/97. Washington, DC, Organizacion Panamericana de la Salud.
- Maroli, M., Feliciangeli, M.D., Bichaud, L., Charrel, R.N. & Gradoni, L. (2013) Phlebotomine sandflies and the spreading of leishmaniasis and other diseases of public health concern. *Medical and Veterinary Entomology* 27(2), 123–147.
- Marquardt, W.C., Black, W.C., Freier, J., Hagedorn, H.H., Hemingway, J., Higgs, S., James, A.A., Kondratieff, B. & Moore, C.G. (2005) *Biology of Disease Vectors*. 2nd edn. Elsevier Academic Press, China, 785 p.
- Martin, F.R., McCreddie, J.W. & Colbo, M.H. (1994) Effect of trap site, time of day, and meteorological factors on abundance of host-seeking mammalophilic Black Flies (Diptera: Simuliidae). *The Canadian Entomologist* 126, 283–289.
- Martín-Sánchez, J., Gállego, M., Barón, S., Castillejo, S. & Morillas-Marquez, F. (2006) Pool screen PCR for estimating the prevalence of *Leishmania infantum* infection in sandflies (Diptera: Nematocera, Phlebotomidae). *Transactions of the Royal Society of Tropical Medicine and Hygiene* 100(6), 527–532.
- McCombie-Young, T.C., Richmond, A.E. & Brendish, G.R. (1926) Sand flies and sand fly fever in the Peshawar district. *Indian Journal of Medical Research* 13, 961–1021.
- Medlock, J.M., Hansford, K.M., Van Bortel, W., Zeller, H. & Alten, B. (2014) A summary of the evidence for the change in European distribution of phlebotomine sand flies (Diptera: Psychodidae) of public health importance. *Journal of Vector Ecology* 39(1), 72–77.
- Moncaz, A., Faiman, R., Kirstein, O. & Warburg, A. (2012) Breeding Sites of *Phlebotomus sergenti*, the Sand Fly. Vector of Cutaneous Leishmaniasis in the Judean Desert. *PLoS Neglected Tropical Diseases* 6(7), e1725.
- Montoya-Lerma, J. & Lane, R.P. (1996) Factors affecting host preference of *Lutzomyia evansi* (Diptera: Psychodidae) a vector of visceral leishmaniasis in Colombia. *Bulletin of Entomological Research* 86, 43–50.
- Moore, C.G. & Gage, K.L. (1996) Collecting methods for vector surveillance. pp. 471–491. in Beatty, B.J. & Marquardt, W.C. (ed) *The Biology of Disease Vectors*. Niwot, CO, University Press of Colorado.
- Niang, A.A., Hervy, J.-P., Depaquit, J., Bousset, P., Davidson, I., Geoffroy, B., Leger, N., Trouillet, J., Killick-Kendrick, R., Killick-Kendrick, M., Rioux, J.-A. & Brunches, J. (2004) Sand flies of the Afrotropical region. CD-ROM. IRD/IFAN, IRD Editions.
- Papa, A., Konstantinou, G., Pavlidou, V. & Antoniadis, A. (2006) Sandfly fever virus outbreak in Cyprus. *Clinical Microbiology and Infection* 12(2), 192–194.
- Pesson, B., Ready, J.S., Benabdennbi, I., Martín-Sánchez, I., Esseghir, S., Cadi-Soussi, M., Morillas-Marquez, F. & Ready, P.D. (2004) Sandflies of the *Phlebotomus perniciosus* complex: mitochondrial introgression and a new sibling species of *P. longicuspis* in the Moroccan Rif. *Medical and Veterinary Entomology* 18(1), 25–37.
- Rossi, E., Bongiorno, G., Ciolli, E., Di Muccio, T., Scalone, A., Gramiccia, M., Gradoni, L. & Maroli, M. (2008) Seasonal phenology, host-blood feeding preferences and natural *Leishmania* infection of *Phlebotomus perniciosus* (Diptera, Psychodidae) in a high-endemic focus of canine leishmaniasis in Rome province, Italy. *Acta Tropica* 105(2), 158–165.
- Rutledge, L.C. & Mooser, H.L. (1972) Biology of immature sand flies (Diptera: Psychodidae) at the bases of trees in Panama. *Environmental Entomology* 1, 300–309.
- Sanbonmatsu-Gámez, S., Pérez-Ruiz, M., Palop-Borrás, B. & Navarro-Marí, J.M. (2009) Unusual manifestation of Toscana virus infection, Spain. *Emerging Infectious Diseases* 15(2), 347–348.
- Santos-De Marco, T., De Mello Gaia, M.C. & Peçanha Brazil, R. (2002) Influence of the lunar cycle on the activity of phlebotomine sand flies (Diptera: Psychodidae). *Journal of the American Medical Association* 18(2), 114–118.
- Santos, L., Simões, J., Costa, R., Martins, S. & Lecour, H. (2007) Toscana virus meningitis in Portugal, 2002–2005. *Euro-surveillance* 12(6), 125–127.
- Schönian, G., Kuhls, K. & Mauricio, I.L. (2011) Molecular approaches for a better understanding of the epidemiology and population genetics of *Leishmania*. *Parasitology* 138(4), 405–425.

- Service, M.W. (1993) *Mosquito Ecology: Field Sampling Methods*. London, Chapman and Hall. p. 482.
- Southwood, T.R.E. & Henderson, P.A. (2000) *Ecological Methods*. 3rd edn. Blackwell Science, p. 575.
- Thatcher, V.E. (1968) Arboreal breeding sites of phlebotomine sand flies in Panama. *Annals of the Entomological Society of America* 61, 1141–1143.
- Theodor, O. (1958) Psychodidae-Phlebotominae. pp. 1–55. in Lindner, E. (ed) *Die Fliegen der Palaerktischen Region*. Stuttgart, Germany, E. Schweizerbart'sche.
- Tiwary, P., Kumar, D., Rai, M. & Sundar, S. (2012) PCR-RFLP based method for molecular differentiation of sand fly species *Phlebotomus argentipes*, *Phlebotomus papatasi*, and *Sergentomyia babu* found in India. *Journal of Medical Entomology* 49(6), 1515–1518.
- Townes, H. (1962) Design for a Malaise trap. *Proceedings of the Entomological Society of Washington* 64, 253–262.
- Valenta, D.T., Tang, Y. & Anez, N. (1995) A new method to determine the distance at which phlebotomine sand flies are attracted to light under field conditions. Proceedings of the 2nd International Symposium on Phlebotomine Sand Flies (ISOPS II) Merida, Venezuela. *Boletin de la Direccion de Malariologia y Saneamiento Ambiental* 35(Supplementary 1), 353–358.
- Verani, P., Ciufolini, M.G., Caciolli, S., Renzi, A., Nicoletti, L., Sabatinelli, G., Bartolozzi, D., Volpi, G., Amaducci, L., Coluzzi, M., Paci, P. and Balducci, M., et al. (1988) Ecology of viruses isolated from sand flies in Italy and characterized of a new Phlebovirus (Arabia virus). *American Journal of Tropical Medicine and Hygiene* 38(2), 433–439.
- Veronesi, E., Pilani, E., Carrieri, R. & Bellini, R. (2007) Trapping sandflies (Diptera: Psychodidae) in the Emilia-Romagna region of Northern Italy. *Journal of Vector Ecology* 32, 313–318.
- Volf, P. & Volfova, V. (2011) Establishment and maintenance of sand fly colonies. *Journal of Vector Ecology* 36(Supplement 1), S1–S9.
- WHO (1975) *Manual on Practical Entomology in Malaria, Part II*. Geneva, WHO Offset Publication, 191 p.
- WHO (1979) *Studies on Leishmaniasis Vectors/Reservoirs and Their Control in the World, General Review and Inventory*. WHO/VBC/79.749, Geneva, 88 p.
- WHO (1980) *Studies on Leishmaniasis Vectors/Reservoirs and Their Control in the World, Part III: Middle East*. WHO/VBC/80.776, Geneva, 78 p.
- Yaghoobi-Ershadi, M.R. & Javadian, E. (1996) Seasonal variation of *Leishmania major* infection rates in sand flies from rodent burrows in Isfahan Province, Iran. *Medical and Veterinary Entomology* 10, 181–184.

I.7. Caractérisation des populations de phlébotomes

I.7.1. Les outils moléculaires

Les méthodes moléculaires sont devenues de plus en plus incontournables pour comprendre la biologie des organismes. Elles sont utilisées, notamment, pour répondre à un grand nombre de questions écologiques. Les marqueurs génétiques tels que les allozymes, les microsatellites ou les séquences d'ADN mitochondrial et nucléaire ont montré leur intérêt pour les études de génétique des populations ou encore de phylogénie pour un grand nombre d'organismes [51].

Concernant les phlébotomes, classiquement les outils moléculaires utilisés sont : les isoenzymes, les hydrocarbures cuticulaires, les RFLP, l'amplification aléatoire d'ADN polymorphe (RAPD), les marqueurs mitochondriaux (Cytochrome b, NADH) et plus rarement les marqueurs microsatellites [52]. Cependant, très peu d'études portent sur *P. ariasi*, espèce principale dans notre région d'étude [3, 53-55] et un seul papier concerne l'analyse de marqueurs microsatellites [56]. Ces derniers marqueurs présentent de multiples avantages pour les études de génétique des populations: ils sont co-dominants, généralement neutres, avec un taux de mutations élevé, très polymorphes, abondants et bien répartis dans le génome. Ils peuvent permettre d'étudier les populations à différentes échelles : pays, régions ou même en sympatrie.

I.7.2. Les outils phénotypiques

L'analyse des formes est utilisée fréquemment dans de nombreux types d'études biologiques. En effet, certains processus biologiques ou écologiques peuvent entraîner l'apparition de phénotypes différents entre populations ou entre les individus au sein même d'une population. Par exemple, la maladie, les blessures, mais aussi le développement embryonnaire, l'adaptation aux conditions géographiques locales, ou encore la diversification évolutive à long terme. Des différences morphologiques entre des individus ou des populations peuvent être le signe de réponses différentes aux mêmes pressions évolutives (ou des différences dans les pressions évolutives elles-mêmes).

La morphométrie géométrique est une approche statistique qui permet l'étude et l'analyse de la forme d'objet et/ou d'organe de manière objective et chiffrée. Il existe deux approches différentes : l'étude par contour (relativement peu utilisée) ou par points de repère (« landmarks »). La morphométrie géométrique nous permet de savoir si, par exemple, différentes variables (environnementales ou géographiques) provoquent un effet sur la forme. Il est possible de répondre à cette question en déterminant, par un calcul de probabilités, si l'association entre les variables n'est pas due au hasard.

Le remplacement de la distance utilisée dans la morphométrie traditionnelle par les coordonnées de points de repère ne nous oblige pas à abandonner les méthodes classiques d'analyse statistique des données morphométriques, telles que l'analyse en composantes principales (PCA) et l'analyse canonique des variables aléatoires (CVA). Ce sont des méthodes utilisées fréquemment pour explorer la structure des données. Leurs résultats fournissent des diagrammes de dispersion des spécimens qui décrivent la variation entre les individus ou la différenciation entre les groupes. Ces modèles donnent également souvent des indices sur les causes de variation ou de différenciation ; informations qui sont renforcées par les graphiques montrant les dimensions le long desquelles la plupart des spécimens varie ou la plupart des groupes diffère.

La morphométrie géométrique permet donc de mettre en évidence des transformations morphologiques s'opérant au sein ou entre des populations subissant des pressions environnementales, biologiques ou climatiques variées [57]. Cette approche a d'ailleurs été largement utilisée pour mettre en évidence des caractéristiques différentielles au sein de populations en sympatrie ou entre des populations en allopatrie. Par exemple, une étude a démontré que certains traits morphologiques variaient entre des populations locales de *P. papatasi* [58]. En effet, sur les 36 caractères testés dans cette étude, 17 d'entre eux se sont révélés de qualité (principalement au niveau des pattes (tibia et fémur) et sur les ailes) pour mettre en évidence des différences entre ces populations.

I.7.3. Structure des populations de *P. ariasi*

Malgré une aire de répartition importante, il existe peu d'études sur la structure génétique de *P. ariasi*. Une étude sur la phylogéographie de cette espèce réalisée à partir des données du cytochrome b a montré une importante diversité intraspécifique ainsi que l'existence de deux lignées mitochondriales, une limitée à l'Algérie et l'autre très dispersée dans les pays du pourtour méditerranéen. La lignée franco-marocaine est caractérisée par une importante diversité d'haplotypes montrant une structuration géographique [3]. Deux marqueurs microsatellites mis au point par Aransay et al. [59] pour *P. perniciosus* ont pu être utilisés pour *P. ariasi* avec d'autres marqueurs (cytochrome b et facteur d'élongation EF-1 α) afin d'étudier la colonisation du sud-ouest de la France par cette espèce. Elle serait originaire d'Afrique du Nord avec un passage des Pyrénées et une expansion démographique probablement survenue pendant les plus chaudes périodes interglaciaires de l'Holocène [56, 60].

Kamhawi et al. [53] ont mis en évidence par chromatographie gaz-liquide des hydrocarbures cuticulaires deux populations (une sylvatique et une péri-urbaine) de *P. ariasi* dans le Sud de la France. Cette signature chimique est sous contrôle génétique, mais il peut exister des variations qualitatives et quantitatives de ce signal épicuticulaire en fonction de l'âge, du sexe et des conditions environnementales [61, 62]. La différence existante au niveau des hydrocarbures cuticulaires suggère l'absence de flux de gènes entre ces deux populations [53]. Cependant, la structure génétique des populations de *P. ariasi* n'a, jusqu'à présent, jamais été réalisée au niveau d'une région ou bien même d'un foyer de transmission. La faible capacité de dispersion des phlébotomes, connus pour être de mauvais voiliers [63], suggère l'existence de populations très structurées, même sur de petites aires géographiques.

II. Matériels et Méthodes

II.1. Présentation de la zone d'étude

II.1.1. Caractéristiques générales

La zone d'étude se situe dans le Sud de la France, sur le massif de l'Oiselette entre les deux vallées de l'Hérault (Ganges) et de l'Arre (Le Vigan). L'échantillonnage a été effectué le long d'un transect de 14 km qui relie les villes Saint-Julien-de-la-Nef et le Vigan, en passant par Roquedur-le-haut à 603 mètres d'altitude (Figure 10). Vingt-et-une stations ont été sélectionnées le long de ce transect (Tableau 1). Les analyses génétiques ont été effectuées sur les individus capturés en août 2011 à l'aide de pièges lumineux. Les individus issus d'une capture dans une station extérieure au transect (Lacan, ST21) ont été utilisés comme groupe externe (Tableau 1). Pour chaque station, les coordonnées géographiques et l'altitude (table 1) ont été collectées à l'aide d'un GPS de poche (Magellan Triton 2000). Les résultats ont été transférés dans le logiciel SIG ArcGIS v9.3 pour produire une carte (Figure 10). Des données descriptives de chacune des stations ont aussi été relevées (orientations des murs, versants, micro-habitats, hôtes présents visibles (données non décrites dans ce travail)).

Tableau 1 : Descriptif des stations échantillonnées (PL : Pièges lumineux ; PC : Pièges collants)

Station	Altitude	Groupe versant/ altitude	Coordonnées		Altitude (m)	Biotope	Type de piège
			Nord	Est			
ST01*	A1	S1	43,96548	3,686828	175	Localité	PL + PC
ST02*	A2	S1	43,96663	3,685075	228	Localité	PC
ST03*	A2	S1	43,96733	3,683149	244	Localité	PL + PC
ST04*	A3	S2	43,97462	3,675871	321	Localité / Clapier	PL + PC
ST05*	A3	S2	43,97595	3,677038	322	Rural	PC
ST06	A3	S2	43,97687	3,677551	341	Chenil	PL + PC
ST07*	A3	S2	43,97632	3,675457	354	Localité	PL + PC
ST08‡	A4	S3	43,97683	3,669611	443	Localité	PC
ST09‡	A5	S3	43,9746	3,659549	586	Poulailler	PL
ST10	A5	S3	43,97416	3,657616	606	Poulailler	PL
ST11*	A5	S3	43,97317	3,657214	603	Localité	PL + PC
ST12*	A5	S3	43,97144	3,655944	573	Rural	PC
ST13*	A5	S3	43,97321	3,650088	539	Rural	PL + PC
ST14*	A4	S4	43,97235	3,637441	417	Rural	PC
ST15	A3	S4	43,97495	3,635153	397	Rural	PL + PC
ST16*	A3	S4	43,97765	3,634949	362	Rural	PC
ST17*	A3	S5	43,97961	3,627845	343	Rural	PL + PC
ST18*	A2	S5	43,98478	3,623463	282	Rural	PL + PC
ST19	A2	S5	43,98409	3,616661	255	Localité	PL + PC
ST20*	A2	S5	43,98392	3,614822	245	Localité / Bergerie	PL + PC
ST21	A5	S6	43,95889	3,662474	500	Localité	PL

* Stations en commun avec Rioux et al. [1]

‡ Stations non incluses dans les analyses spatiotemporelles



Figure 12 : Carte de la zone d'étude. (●) indiquent les sites d'échantillonnage

Le choix du transect au sein du massif de l'Oiselette pour cette étude a été guidé par le fait qu'une étude similaire avait été effectuée entre mai et octobre 1977 par Rioux et al. [1]. Ainsi, le long de ce même transect sur les 22 stations étudiées en 1977, 14 stations ont été ré-échantillonnées au cours de notre étude et sont donc communes aux deux études (Tableau 1). Nous avons donc pu comparer sur ces 14 stations la distribution des espèces de phlébotomes et leurs proportions relatives en fonction de l'altitude et du versant entre 1977, 2011, 2012 et 2013. Cette région est connue comme étant une zone endémique pour la leishmaniose humaine et canine causée par *L. infantum* [34]. Cette affection a d'ailleurs été signalée chez plusieurs chiens au cours de l'étude (données non publiées).

De nombreux animaux sont présents dans cette zone suggérant un nombre important d'hôtes potentiels pour les phlébotomes. D'une part, des animaux domestiques : poulets, moutons, canards, oies, chevaux, lapins, chats et chiens dans les stations anthropisées. D'autre part des animaux sauvages dans les stations plus rurales : renards, rongeurs, lézards, oiseaux, etc.

La zone d'étude est partagée en deux versants ayant une orientation Sud-est et Nord-Ouest (Figure 11). Les stations ont été regroupées par altitude : groupe A1 : stations entre 100 et 200 m, groupe A2 : stations entre 200 et 300 m, groupe A3 : stations entre 300 et 400 m, groupe A4 : stations entre 400 et 500 m et groupe A5 : stations > 500 m (Table 1).

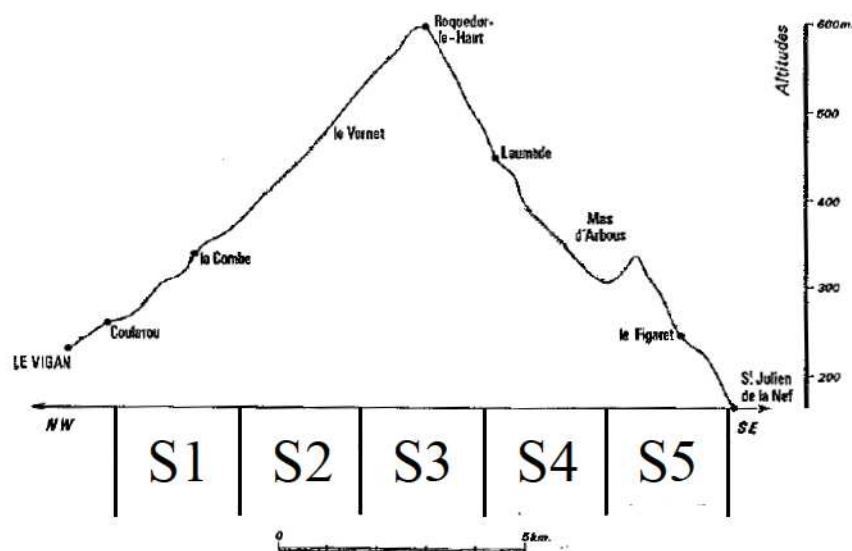


Figure 13 : Coupe transversale de la zone d'étude. NW : Nord-Ouest ; SE : Sud-Est ; S1 : Vallée de l'Arre versant nord, stations entre 200 et 300 m, S2 : mi-pente versant nord, stations entre 300 et 500 m, S3 : sommet versant nord, stations entre 500 et 600 m, S4 : mi-pente versant sud, stations entre 300 et 500 m, S5 : Vallée de l'Hérault versant sud, stations entre 300 et 100 m. Figure adaptée de Rioux et al. [1]

Enfin, nous avons regroupé les stations par groupe d'altitudes et par versant selon l'ordre adopté par Rioux et al. [1] : groupe S1 : Vallée de l'Arre versant nord, stations entre 200 et 300 m, groupe S2 : mi-pente versant nord, stations entre 300 et 500 m, groupe S3 : sommet versant nord, stations entre 500 et 600 m, groupe S4 : mi-pente versant sud, stations entre 300 et 500 m et enfin groupe S5 : Vallée de l'Hérault versant sud, stations entre 300 et 100 m (Tableau 1 et Figure 11).

II.1.2. Contexte climatique

Le Sud de la France subit les influences de la Méditerranée avec des hivers doux et des étés très chauds. En effet, la région du Vigan est soumise à un climat méditerranéen sub-humide [64] et présente des températures moyennes variant entre 5°C et 9°C en hiver et entre 19°C et 23°C en été avec une moyenne des précipitations annuelles comprise entre 1300 et 1500 mm.

II.1.3. Végétation

Le transect est défini par une succession de milieux ruraux et semi-ruraux le long d'une route départementale. La végétation est caractéristique des zones de « Garrigue » avec la présence entre autre de chênes verts, d'oliviers, de salsepareilles et de thyms. Le paysage est composé principalement de chênes et de pins dans les régions les plus sauvages mais présente aussi des zones anthropisées (villages, cultures, élevages). Les stations choisies présentent des écosystèmes diversifiés avec des sites dans un environnement « semi-rural » (murs le long des routes), ou anthropisés (villages, maisons isolées) avec présence ou non d'animaux (bergeries, élevages d'oies, clapiers, chenils).

II.1.4. Données démographiques

Notre zone d'étude se situe sur 3 communes, Le Vigan, Roquedur et Saint-Julien-de-la-Nef (3925, 217 et 123 habitants en 2012, respectivement). Il est important de noter que l'environnement a été relativement transformé depuis la dernière étude réalisée dans cette zone [1]. Selon l'Institut National de la Statistique et des Etudes Economique (INSEE), la population humaine a significativement augmenté au fil des années ainsi que le nombre d'habitations (Tableau 2). Une diminution du nombre d'exploitations agricoles est aussi observée (Tableau 2). Ces changements environnementaux et socio-économiques peuvent entraîner des changements importants sur les populations de phlébotomes dues par exemple à la destruction des gîtes larvaires de phlébotomes engendrée par la construction d'habitation, ou à une diminution des hôtes disponibles due à la réduction du nombre d'élevages.

Tableau 2 : Données démographiques de (1) Saint-Julien-de-la-Nef, (2) Roquedur-le-Haut et (3) Le Vigan (Institut National de la Statistique et des Etudes Economiques).

Année	Population			Nombre d'habitations			Nombre d'exploitations agricoles		
	1	2	3	1	2	3	1	2	3
1982	99	116	4517	/	/	/	/	/	/
1988	/	/	/	/	/	/	11	11	42
1990	140	133	4523	95	105	2557	/	/	/
1999	119	192	4448	94	112	2645	/	/	/
2000	/	/	/	/	/	/	8	6	12
2009	124	218	3959	126	130	2629	/	/	/

II.2. Enquête entomologique

II.2.1. Techniques de capture et conservation

De 2011 à 2013, des captures mensuelles ont été réalisées entre Mai et Novembre à l'aide de pièges lumineux (PL) et de pièges collants (PC) (Voir chapitre 1 - Techniques de captures). La combinaison de ces deux méthodes de piégeage permet de maximiser la possibilité de capturer les différentes espèces présentes [22]. 19 stations ont été échantillonnées en 2011 et une station (ST08) a été rajoutée en 2012 et 2013. Suite à la destruction d'un élevage d'oie, la station (ST09) a été déplacée dans un poulailler (Tableau 1). Afin d'assurer l'uniformité des données et la comparaison entre les 3 ans, ces trois stations ont été exclues des analyses spatio-temporelles. Les PL ont été posés dans 14 stations, entre 18h00 et 8h00, pendant 2 nuits consécutives une fois par mois. En 3 ans d'échantillonnage, 329 captures sur 649 nuits ont été réalisées. Les PC, quant à eux, ont été placés dans 18 stations et récupérés après 2 nuits. Ils ont été disposés dans différents biotopes : à l'intérieur et autour des habitations humaines, à proximité des animaux ou de la végétation et dans les barbacanes (fente verticale présente dans les murs de soutènement pour faciliter l'écoulement des eaux). Durant l'étude, 12196 PC ont été déposés, soit en moyenne 230 pièges par station. Les informations concernant les stations sont synthétisées dans le Tableau 1. Une fois les captures réalisées, les spécimens étaient individuellement transférés dans des tubes Eppendorf de 1,5 ml préalablement remplis d'alcool à 96% puis étiquetés.

Au cours de l'étude réalisée entre mai et octobre 1977, 4927 PC ont été disposés dans les 22 stations et récupérés tous les 15 jours [1].

II.2.2. Identification

Pour procéder à l'identification des individus ainsi qu'aux analyses moléculaires ou de morphométrie géométrique, chaque phlébotome a été déposé sur une lame. Avant le montage, la tête, les génitalia et les ailes ont été retirées à l'aide de fines aiguilles stériles à usage unique. Le thorax et le reste de l'abdomen ont été placés à sec dans des plaques 96 puits (MN Tubes strips, Macherey-Nagel, Hoerdt, France) à -20°C pour les études moléculaires. Les ailes ont été individuellement et manuellement transférées dans des tubes Eppendorf de 1,5 ml étiquetés et préalablement remplis d'alcool à 70%. La tête et les génitalia sont ensuite éclaircies dans une solution de Marc-André à chaud directement sur la lame puis montées dans de la gomme au chloral entre lame et lamelle.

L'identification des spécimens est réalisée individuellement sur la morphologie du pharynx, du cybarium et des génitalia pour les mâles ou des spermathèques pour les femelles à l'aide des clés d'Abonnenc [17], Lewis [65] et Killick-Kendrick et al. [66].

II.2.3. Relevé des données climatiques

Les relevés climatiques ont débuté en juin 2011 et ont été poursuivis jusqu'en octobre 2013. Durant la première année, 13 enregistreurs (iButton hygrocron, DS 1923) ont été placés au niveau de 13 stations à proximité des PL ou PC, et 7 autres ont été rajoutés en 2012 dans les stations manquantes. Ils ont été programmés pour mesurer la température et l'humidité relative toutes les 6 heures (à 6h, 12h, 18h et 24h). Durant ces 3 ans, certains enregistreurs ont été perdus ou détruits : station 1 (2012), station 10 et 15 (2013).

II.2.4. Analyses des données

Des tests de Kruskal-Wallis suivis de tests post-hoc effectués en utilisant des tests de Mann-Whitney avec correction de Bonferroni ont été réalisés pour tester les différences de température et d'humidité relative par mois. Selon la même procédure, au sein de chaque mois, les différences de température et d'humidité relative ont été testées en fonction du versant, de l'altitude et de la station (Voir chapitre 3 - Analyses des données climatiques). Les analyses ont été réalisées sous le logiciel R, version 3.1.1 [67].

La densité de phlébotomes par piège est définie comme étant le nombre de phlébotomes capturés par m² pour les PC et en nombre de phlébotomes capturés par piège par nuit pour les PL. Afin d'analyser l'impact de différents facteurs liés à l'environnement sur la présence et l'abondance des phlébotomes, deux types d'analyses ont été effectués.

Tout d'abord, l'exploration des données a été réalisée selon la méthodologie définie par Zuur et al. [68]. Pour étudier l'influence des variables environnementales sur la présence des différentes espèces de phlébotomes, un modèle linéaire généralisé (GLM) avec distribution binomiale a été utilisé. Pour cette analyse, les informations provenant des deux types de pièges ont pu être utilisées car il s'agissait d'une analyse présence/absence. Les variables explicatives utilisées étaient l'altitude, le versant, l'orientation des murs, la température moyenne et l'humidité relative moyenne. Dans un second temps, les effets des différentes variables sur l'abondance des espèces de phlébotomes ont été étudiés en utilisant un modèle additif généralisé (GAM) afin de détecter les relations non-linéaires. Pour cette analyse, seules les données des PC ont été utilisées. En effet, celles-ci sont plus représentatives de l'ensemble de la zone d'étude (plus grand nombre de réplicats et plus grande répartition spatiale). De plus, les densités des phlébotomes obtenues en PL *versus* PC ne peuvent pas être comparées entre elles. Étant donné que les données présentaient une sur-dispersion (variance observée des données supérieure à la variance théorique issue du modèle utilisé), un modèle additif généralisé avec une distribution binomiale négative et un lien logarithmique a été appliqué [69]. Les variables explicatives utilisées étaient l'altitude, le versant, l'orientation des murs, la température maximale / minimale et l'humidité relative maximale / minimale. En raison de la colinéarité (corrélation significative entre des variables) élevée entre certaines des variables explicatives (température maximale / minimale, humidité relative maximale / minimale), les modèles ont été ajustés séparément pour chaque variable. La sélection du modèle a été réalisée en utilisant les critères d'information d'Akaike (AIC) de chaque modèle univarié à l'aide de la commande « model.sel » du package MuMin [67]. La validation du modèle a été basée sur la méthode de Zuur et al. [70]. Toutes les analyses statistiques et graphiques ont été effectuées sous R, version 3.1.1 [67] à l'aide entre autres des packages lattice, mass, mgcv, et MuMin. Ces analyses ont été effectuées uniquement sur les deux espèces les plus représentées dans notre zone d'étude : *P. ariasi* et *S. minuta*. Le faible nombre de spécimens de *P. perniciosus* et *P. mascittii* collectés au cours de notre étude ne nous a pas permis de faire des analyses statistiques.

II.3. Etude de morphométrie géométrique

Dans le cadre de cette thèse, 374 spécimens de *P. ariasi* ont été utilisés, 186 mâles et 188 femelles, provenant de 16 stations. Le nombre d'individus sur les trois ans étant très important, l'analyse de morphométrie géométrique a été réalisée uniquement sur les spécimens de 2011. Seules les ailes droites ont été traitées dans cette étude. En effet, Rohlf et al. [71] conseillent d'utiliser pour les organes ou membres pairs, uniquement le côté droit ou gauche afin d'éviter un biais dû aux possibles phénomènes d'asymétrie entre les organes.

II.3.1. Préparation des ailes

La technique de préparation des ailes de phlébotomes est décrite dans : « **Prudhomme, J., Gunay, F., Rahola, N., Ouanaimi, F., Guernaoui, S., Boumezzough, A., Bañuls, A-L., Sereno, D., Alten, B., 2012. Wing size and shape variation of Phlebotomus papatasi (Diptera: Psychodidae) populations from the south and north slopes of the Atlas Mountains in Morocco. J Vector Ecol. 37(1): 137-147.** »

II.3.1.1. *Résumé*

Comme mentionné précédemment (voir chapitre 1 - Les outils phénotypiques), les analyses de morphométrie géométrique sont susceptibles d'apporter des informations précieuses aux études visant à déterminer la structure des populations. Les variations de taille et de forme sont intéressantes pour mieux connaître une espèce car elles sont généralement corrélées à sa biologie. Des variations de forme et de taille d'ailes ont déjà été observées dans des populations de phlébotomes provenant de différentes régions géographiques formant des populations différenciées au niveau intraspécifique [72-74]. Dans ce contexte, les principaux objectifs de cette étude étaient de déterminer s'il existait des différences phénotypiques au sein de l'espèce *P. papatasi* entre deux zones géographiques et climatologiques distinctes du Maroc et d'évaluer l'importance de la chaîne de montagnes de l'Atlas comme barrière entre les populations du nord et du sud. Une étude comparative de morphométrie géométrique sur différentes populations de *P. papatasi* provenant de foyers endémiques et non endémiques de LC au Maroc a donc été réalisée. Un total de 32 ailes (10 pour le Nord et 22 pour le Sud) a été utilisé provenant de 167 individus échantillonnés dans 10 sites de captures. Cette étude s'inscrivait dans un vaste projet s'intéressant à la biodiversité de *P. papatasi*, vecteur de *L. major* au Maroc.

Afin de visualiser les nervures alaires, ainsi que les différents points de repère indispensables pour l'étude de morphométrie, les ailes doivent être colorées. Dans cette publication, la technique de coloration a été améliorée à partir du procédé développé par Belen et al. [58] permettant une diminution du temps de préparation alaire de 45 minutes à moins de 10 minutes.

Tout d'abord, les ailes sont plongées dans du bleu de méthylène pendant 6 min. Puis, elles sont placées dans une succession de bains de quelques secondes : éthanol (75%), eau distillée et de nouveau dans de l'éthanol (75%). Pour permettre la fixation de la coloration, elles sont ensuite placées dans du xylène pendant 2 minutes. Toutes les ailes sont montées entre lame et lamelle dans de l'Euparal. Le montage est effectué à l'aide d'une loupe binoculaire. Les lames sont photographiées, numérisées et archivées en utilisant un microscope de dissection Leica Z16 APOA à zoom stéréoscopique avec un système de caméra DFC 425.

Les résultats de cette étude indiquent qu'il existe des différences significatives entre la taille et la forme des ailes des populations de *P. papatasi* du nord et du sud de l'Atlas. Elles pourraient être associées à un ensemble de facteurs biologiques et / ou environnementaux tels que l'altitude, les hôtes présents, l'écologie de l'espèce et les conditions climatiques.

Les méthodes de coloration mises au point dans ce travail ont ensuite été utilisées pour l'étude de morphométrie géométrique réalisée dans cette thèse.

II.3.1.2. Article 3: Wing size and shape variation of Phlebotomus papatasi (Diptera: Psychodidae) populations from the south and north slopes of the Atlas Mountains in Morocco.

Wing size and shape variation of *Phlebotomus papatasi* (Diptera: Psychodidae) populations from the south and north slopes of the Atlas Mountains in Morocco

Jorian Prudhomme¹, Filiz Gunay², Nil Rahola¹, Fouad Ouanaïmi³, Souad Guernaoui³,
Ali Boumezzough³, Anne-Laure Bañuls¹, Denis Sereno¹, and Bulent Alten^{1,2✉}

¹UMR MIVEGEC (IRD 224-CNRS 5290-Universités Montpellier 1 et 2), Montpellier, F-34394, France

²Hacettepe University, Faculty of Science, Department of Biology, Ecology Section, ESRL Laboratories, 06800 Beytepe, Ankara, Turkey, kaynas@hacettepe.edu.tr

³Université Cadi Ayyad, Faculté des Sciences Semlalia, Equipe Ecologie Animale et Environnement-LabL2E (URAC 32), BP 2390-40080 Marrakech-Maroc

Received 8 September 2011; Accepted 25 January 2012

ABSTRACT: The wing shape and size morphology of populations of the medically important phlebotomine sand fly, *Phlebotomus papatasi*, were examined in two endemic (south of the Atlas Mountains) and nonendemic (north of the Atlas Mountains) foci of cutaneous leishmaniasis by using geometric morphometrics in Morocco. Although it is present in all of Morocco, *P. papatasi* is the main vector of *Leishmania major* in only southern part of the Atlas Mountains. There are four major mountain ranges that serve as geographical barriers for species distribution in the study area and at least four gaps were recognized among these barriers. We found statistically significant differences in wing shape morphology between southern and northern populations. Analysis clearly recognized two main groups of populations on both sides of the mountains. The graphical depiction of Principal Component Analysis (PCA) and Canonical Variates Analysis (CVA) confirmed our morphometric study suggesting that the difference in wing morphology between the populations indicates that the population of *P. papatasi* shows phenotypic plasticity in the study area. According to centroid size analyses, which were used as measures of wing size differences among different sites, the north population of *P. papatasi* had relatively larger wings than the south population. *Journal of Vector Ecology* 37 (1): 137-147. 2012.

Keyword Index: *Phlebotomus papatasi*, geographic variation, geometric morphometrics, Morocco.

INTRODUCTION

Leishmaniasis is a major vector-borne disease endemic in Morocco, with two clinical types that exist in the country. Visceral leishmaniasis (VL), caused by *Leishmania infantum* and transmitted by species of the subgenus *Larrousius*, is widespread in the country and is more frequent in the northern part. Cutaneous leishmaniasis (CL) is widely distributed in Morocco and caused by either *Leishmania tropica* or *L. major*. CL due to *L. tropica* is widespread in the semi-arid regions with *Phlebotomus (Paraphlebotomus) sergenti* Parrot as the vector. CL due to *Leishmania major* is localized in the south, particularly in the per-arid area and southern part of the Atlas Mountain ranges. This CL has been known since 1914 (Foley and Vialatte 1914) and is caused by *L. major* zymodeme MON-25 (Rioux et al. 1986) transmitted by *Phlebotomus (Phlebotomus) papatasi* Scopoli (Guernaoui et al. 2006). Leishmaniasis is still a growing public health problem in Morocco. According to the Moroccan Ministry of Public Health, 359 cases of CL caused by *L. major*, 1,669 cases of CL by *L. tropica*, and 116 cases of VL by *L. infantum* were reported in 2001. In 2006, 2,240 new cases were reported.

Previous studies in Morocco (Rioux et al. 1984, 1997, Rioux 2001, Rispaïl et al. 2002) identified 17 species belonging to two genera, *Phlebotomus* and *Sergentomyia*,

and showed that the distribution of sand flies was related largely to the bioclimate (Guernaoui et al. 2006). There are five different bio-climatic zones in the country as Saharan, arid, semi-arid, sub-humid, and humid (Rioux et al. 1984). *P. papatasi*, the proven vector of *L. major*, is one of the dominant and widespread species in Morocco. In fact, *P. papatasi* is the dominant species in the arid plain in the southern part of the Atlas Mountains (Rioux et al. 1984). The high occurrence of *P. papatasi* in the lowland and its low frequency in the mountains could be explained by its preference for arid and peri-arid areas (Guernaoui et al. 2006). Moreover its density increases with increased aridity (Croset et al. 1974). Along with bioclimatic zones, altitude also acts on *P. papatasi* distribution by the habitats, relief, and by the climatic gradient on present in Morocco. According to Guernaoui et al. (2006) *P. papatasi* shows a negative correlation with the altitude. The density of this species is very important at lower altitudes between 400 and 600 m, and lower at the other altitudes. It is absent from 1,200 m altitude. Though *P. papatasi* populations exist throughout Moroccan territory, Gaud (1947) clearly showed that the biggest part of the population is distributed in the southern part of the Atlas ranges with numerous subpopulations. Rioux and de La Rocque (2003) confirmed this result. Rioux and de La Rocque (2003) also demonstrated that the main reservoir of *L. major*, *Meriones shawi*, is also present

in the whole country; however CL caused by *L. major* is only transmitted by the southern populations of *P. papatasi*. The questions are now whether there are any differences in terms of phenotypic or genetic features between southern and northern populations of *P. papatasi* and if so, whether phenotypic differences by themselves could be effective in transmitting *L. major* by only the southern populations of *P. papatasi*.

Size and shape variation are important in understanding ecological relationships because they generally correlate with the biology of many species. There has been considerable interest in the determinants of body size and how size varies through time and space. Geographic variation in body size is particularly controversial because both the patterns and their underlying mechanisms are matters of contention (Chown and Klok 2003). Similar to size, biological shape is one of the most conspicuous aspects of an organism's phenotype and provides a link between the genotype and the environment (Ricklefs and Miles 1994). In sand flies, significant morphometric wing shape variations were found in populations from different geographic regions, forming clusters or latitudinal clines in previous studies (Kasap and Alten 2006, Aytekin et al. 2007, Aytekin et al. 2009).

The main goals of this study were to utilize geometric morphometric approaches, which have the highest statistical power among alternatives (Rohlf 2000), to understand the possible phenotypic differences of *P. papatasi* populations between two geographically and climatologically distinct areas and to determine the importance of the Atlas Mountains as a barrier between southern and northern populations. It was hypothesized here that the body size of *P. papatasi* might change with bioclimatic differences between both sides of Atlas Mountains.

MATERIALS AND METHODS

The study area

Field studies were conducted from July to September 2010, in seven locations on southern, and in three locations on northern sides of the Atlas Mountains, Morocco. Table 1 shows information about altitudes, geographical references, bioclimatic and vegetation zones, eco regions and climatic conditions of the sampling stations located in the north (N) and south (S) portions of the Atlas Mountains, shown in Figure 1.

Morocco is subject to the different influences of the Mediterranean, the Atlantic Ocean and the Sahara. However, the Atlas, an imposing mountain range separating the northwest and southeast portions of the country, plays an important role in the climate of the area. The climate of Morocco lies between the Mediterranean climatic belt and the Saharan subtropical desert, with high mountain ranges introducing an altitudinal climatic gradient that overlies the latitudinal gradient. The southern slopes of the Atlas Mountains, which divert the moisture-laden Atlantic winds, have a rigorous pre-Saharan climate (desertic), while the northern slopes are relatively cool and well-watered (temperate). There are five different bioclimatic

zones: humid, subhumid, arid, semiarid, and saharian that create vegetation zones and eco-regions (Table 1) across the wetlands, rivers, desert, and mountains (UNESCO-FAO 1963, Rioux et al. 1984).

Sand fly collection and identification

Phlebotomus papatasi adults were collected using CDC miniature light traps (John W. Hock Co. FL, U.S.A.) during summer (July-September) in 2010. On each trapping night, one to three light traps were placed in each of the sampling localities. Light traps were placed 1 to 1.5 m above ground, and they were operated between 18:00 and 06:00 around/inside animal barns and houses used as main sampling stations. In addition, the light traps were also set near rodent burrows, poultry houses, and/or livestock. At the end of the sampling period, specimens from the traps were transferred into 1.5 ml Eppendorf tubes one by one with 96% ethanol.

Prior to mounting, heads, genitalia, and wings of the sand flies were removed. The heads and genitalia were cleared in KOH 19% for 2 h in Marc-Andre solution (chloral hydrate/acetic acid), then dehydrated and placed in creosote (beech wood oil) for a night. They were mounted in Euparal (Abonnenc 1972). The identification was made by examining the morphology of male genitalia, female spermathecae, and pharynxes using the standard keys of Perfiliev (1968), Abonnenc (1972), Artemiev (1991), and Killick-Kendrick et al. (1991).

Morphometric analysis

The number of specimens used in the geometric morphometrics analysis is shown in Table 1. All the specimens were screened under a light microscope for the presence of endoparasites to prevent possible variations which might affect the morphometric data (Mayr and Ashlock 1991). The wings were cleaned of scales and using the method of Belen et al. (2004), colored for viewing the veins with the modification of keeping the wings in methylene blue for 6 min at room temperature. They were then placed in 75% alcohol for a few seconds, after which they were transferred to cups containing distilled water for washing. The wings were again put in 75% alcohol for a few seconds and finally soaked in xylene for 2 min for fixation. As last step, all wings were mounted in Euparal on labelled slides. Slides were photographed using a Leica Z16 APOA stereoscopic zoom dissection microscope with DFC 425 digital camera system, digitized, and archived. All specimens were scored by a single experimenter. Photographs were first entered into tps-UTIL1.28 (Rohlf 2007).

A total of 32 specimens was used for the geometric morphometrics analysis (10 for the north and 22 for the south) (Table 1). Rohlf and Slice (1990) advised that for the paired organs, the one on the right side or left side should be used consistently to avoid possible negative allometric effects. Left and right wings for both southern and northern specimens were compared by using centroid sizes of the wings. No statistically significant difference was found between the right and left wings from southern (Wilcoxon-Mann-Whitney, $p=1$) and northern specimens

Table 1. Site description and sampling size of the stations in the study area.

Coordinates	Bioclimatic zones	Vegetation zone	Ecoregions	Annual Mean Precip. (mm)	Annual Mean Temp. (°C)	No. collected	No. used
34°55'N, 2°41' W	semi-arid	thermomediterranean	sclerophyll forests	400-500	21	11	2
28°45'N, 8°54'W	arid	inframediterranean	macaronessian tree	100-200	23	18	3
33° 45'N, 4° 47'W	sub-humid	thermomediterranean	sclerophyll forests	600-700	15.5	12	1
31°33'N, 4°15'W	saharian	maroc saharien	arboreous and steppe	100-200	23.5	7	1
31°13'N, 6°08' W	arid	thermo-mesomediterranean	sclerophyll forests, arboreous, presteppe and steppe	200-300	15.5	24	1
31°22'N, 5°59'W	semi-arid	supramediterranean	high altitude Atlasique steppe	300-400	15.5	15	1
31° 55'N, 4° 24'W	arid and saharian	maroc saharien and thermomediterranean	arboreous and steppe	100-200	18.5	24	13
29° 58'N, 9° 24'W	semi-arid	inframediterranean	macaronessian tree	400-500	15.5	10	2
34° 16'N, 4°22'W	sub-humid	supra-mesomediterranean	humid and sclerophyll forests	700-900	18.5	15	1
31°21' N, 8°4'W	sub-humid	supra-mountain mediterranean	sclerophyll forests	400-600	15.5	31	7

(Wilcoxon-Mann-Whitney, $p=-0.8833$). In this study, the right wings from females were only used in the size and shape analyses. Sixteen landmarks were chosen to be used according to Belen et al. (2004). The data were collected in the form of 2-D coordinates of the 16 landmarks. Landmarks were used for the analysis following the method of Rolf and Slice (1990) (Figure 2). The landmarks are the intersections of wing veins with the wing margin and intersections of cross veins with major veins. All wings were digitized twice in order to reduce the measurement error (z) (Arnqvist and Mårtensson 1998). Scaled, translated, and rotated landmark configurations were made using the GPA Procrustes superimposition method (Bookstein 1991, Rohlf 1993, 1999, Alibert et al. 2001). The coordinates were analyzed using tps-RELW 1.34 (Rohlf 2007) to calculate the eigen values for each principal warp. The variability in wing shape was assessed using the scores obtained for each individual on the first two relative warps by the realization of a principal component analysis (PCA). The relative warps correspond to the principal components and define a shape space in which individuals are replaced (Alibert et al. 2001). To better visualize the shape variation, we added only the deformations on the positive and negative extremes of the first two PCAs on the graph. A Canonical Variates Analysis (CVA) was also performed on landmark data by IMP CVAGEN60 (Zelditch et al. 2004) for comparing the populations collected from the south and the north, using group membership information and Barlett's test to examine differences among groups. "CVAGEN60" is the preferred version among CVAGEN programs when the sample size is smaller than the number of landmarks used. We used a number of PC axes equal to one less than the specimen count instead of all PC axes, while no variance is lost and difficulties related to matrix inversions in the CVA

are eliminated (Zelditch et al. 2004). The size morphometry of the sand flies were investigated using the centroid sizes of the wings as an estimator with the nonparametric Wilcoxon-Mann-Whitney test. Centroid size is the square root of the sum of squared distances of a set of landmarks from their centroid, i.e., the square root of the sum of the variances of the landmarks about that centroid in x- and y- directions (Bookstein 1991).

The coordinates of the landmarks obtained from tps-DIG were manipulated in Morphue (Slice 1998) to make comparisons among different populations by means of spline plots. The results were exaggerated twice for a better visualization. Twenty *P. papatasi* specimens from east of Anatolia, Turkey were included in the analysis as an out-group.

RESULTS

Insect wings are the most appropriate structures for geometric morphometric studies (Dvorak et al. 2006). When a PCA was conducted on 16 wing landmarks, the first two PCs summarized 60.67% of the total variances, respectively (PC1; 47.18% and PC2; 13.49%). The first PC suggests some differences in the relative positions of the landmarks regarding the apical part of the wing (Figures 3, 4). Main deformations centered on the medial of the wing on landmarks 1, 9 and 10-12 (Figure 4). The distribution of individuals along the two PCs is shown in Figure 5. PCA shows some overlap among individuals from all *P. papatasi* specimens examined in both sides of the Atlas Mountains. However, specimens of *P. papatasi* from the north tended to cluster together on the positive axis of PC1 suggesting a higher phenotypic distance relative to specimens from the south. When the shape differences of populations

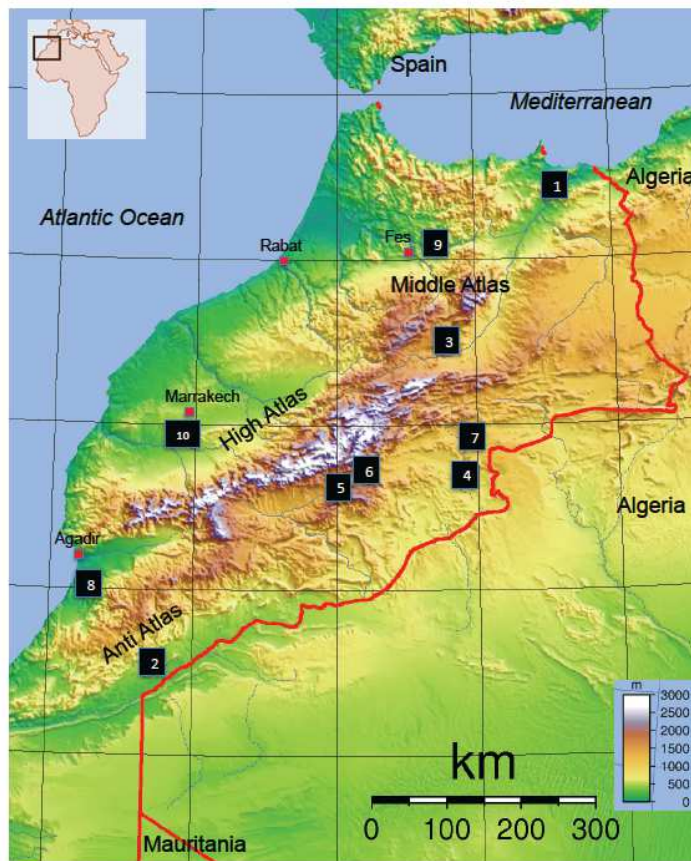


Figure 1. Map of the study area showing the main sampling stations, the main geographical barriers and altitude. 1. Sidi Athmane; 2. Tigmi Jdid; 3. Ait Brahim; 4. Es Sfalate; 5. Mirna; 6. Boumaine; 7. Errachidia; 8. Tafat; 9. Dar Bachir; 10. Akhlij (see Table 1 for geographical references and properties).

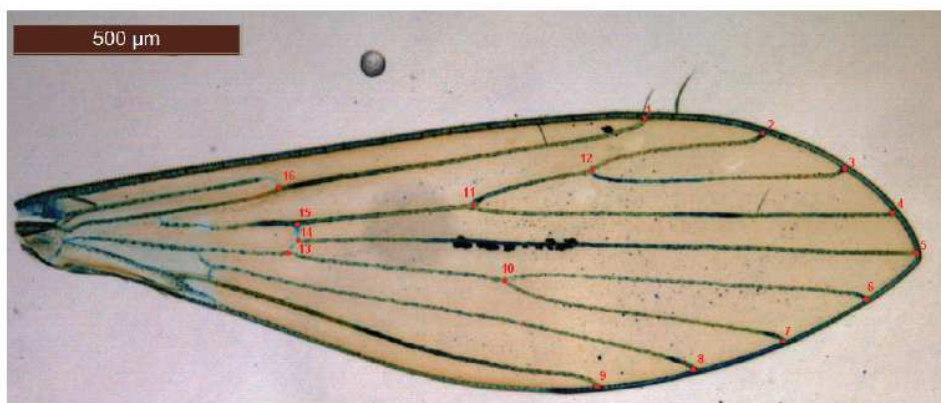


Figure 2. Location of the 16 wing landmarks used in the morphometric analysis of *Phlebotomus papatasi*.

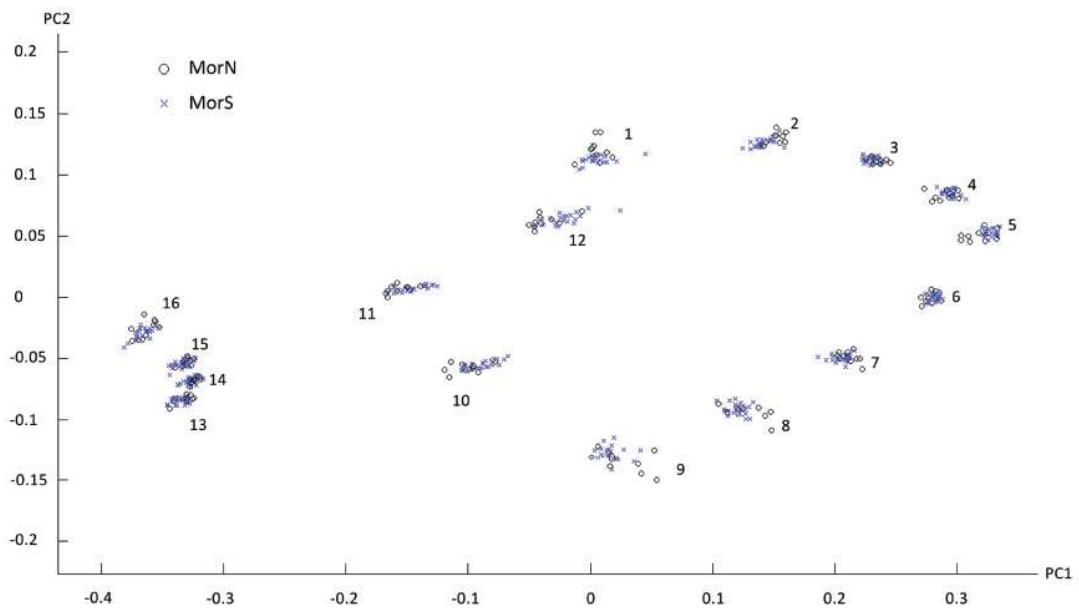


Figure 3. Output of consensus configuration by the GPA Procrustes superimposition method locating the 16 landmarks for each individual wing of the two populations. x: south of Atlas Mountains, o: north of Atlas Mountains.

were analyzed, CVA also showed some overlap in local populations of Morocco. There were distinct CVs in which Axis 1 Lambda= 0.6748, $\chi^2= 11.4075$, $df=2$, $P=0.00333342$. On the other hand, the cluster analyses conducted clearly recognized two main groups of populations that Morocco (Mor) formed a distinct group from Turkey (Tur) for the wings measured along the CV2 (Axis 1 Lambda= 0.2508, $\chi^2=66.3828$ $df=6$ $P=2.25076e-012$; Axis 2 Lambda= 0.7132 $\chi^2=16.2222$ $df=2$ $P=0.000300181$). In the first group (Morocco), it appeared that North Morocco (MorN) and South Morocco (MorS) populations of *P. papatasi* were different from each other and from the Turkish population along the first two axes in terms of phenotypic features (Figure 6).

Centroid sizes were used as measures of overall wing size differences among populations. The size differences among the populations were significant (Wilcoxon-Mann-Whitney Test: $p=0.0004583$) with the specimens from the north of the Atlas and Turkey as an out-group displaying considerably larger wings (Figure 7).

DISCUSSION

This is the first comparative morphometric study of different populations of *P. papatasi* occurring in endemic and non-endemic foci of CL in Morocco. The results for size and shape presented here indicate that there are some significant differences among populations. This may be associated with a set of biological and/or environmental factors such as altitude, host population, ecological, and climatic effects.

Body size is correlated with a large number of ecological, physiological, and genetic variables. A complete understanding of the effect of bio-geographical factors

requires information on the genetic and environmental components of body size (Kuclu et al. 2011). For instance, it is generally accepted that the body size of mosquitoes is ultimately a result of larval habitat quality (Schneider et al. 2004, 2007, Jirakanjanakit et al. 2007) and larval competition (Gimnig et al. 2002). Because the properties of larval habitats and crowding conditions were not recorded, we cannot exclude the influence of their variation on the observed size changes. Therefore, the studies conducted here are based only on size and shape differences, and we cannot predict how much of the geographic variation in body size is a result of genetic differences and how much is due to phenotypic plasticity of individuals growing in different environments. Geometric morphometrics provides us with a powerful tool to describe and quantify subtle shape and size variations. However, it is obvious that environmental effects are not the only contributions to the formation of phenotypes. Accordingly, genetic effects on body size and shape should be considered in future studies.

Phlebotomus papatasi is widely distributed around the Mediterranean basin, North Africa, throughout the Middle East, and eastward across the entire Indian subcontinent. Depaquit et al. (2008) demonstrated that genetic variability in *P. papatasi* contrasts with that observed within other species having a wide distribution like *P. (Paraphlebotomus) sergenti* in the Old World or *Lutzomyia (Lutzomyia) longipalpis* in the New World. The authors hypothesized that all populations (26) of *P. papatasi* over its distribution area (18 countries) have similar vectorial capacities. However, its distribution is highly disjunctive within that range, depending on locally occurring environmental factors, precipitation, temperature, physical barriers, altitude, latitude, habitat availability, and the distribution and abundance of vertebrate hosts (Ghosh et al. 1999). If we

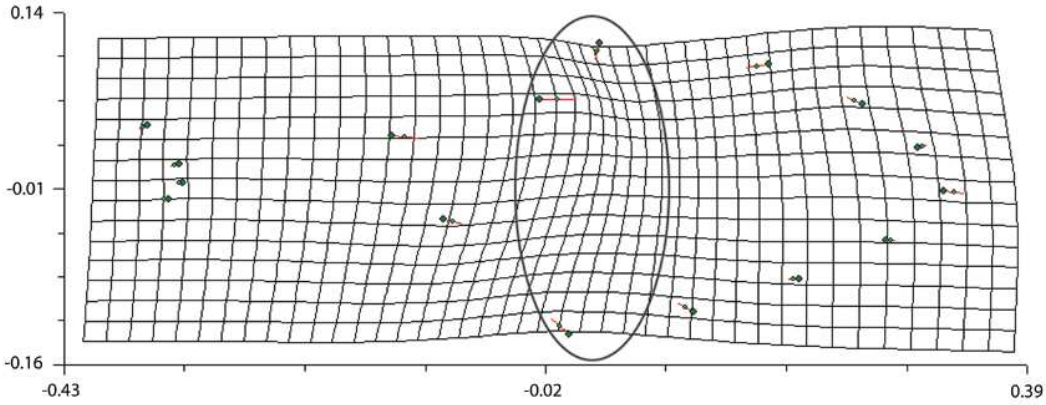


Figure 4. Thin-plate spline deformation grid showing the differences between the mean of the *P. papatasi* wings from the south and north parts of the Atlas Mountains (individuals magnified 2x; vectors are also shown).

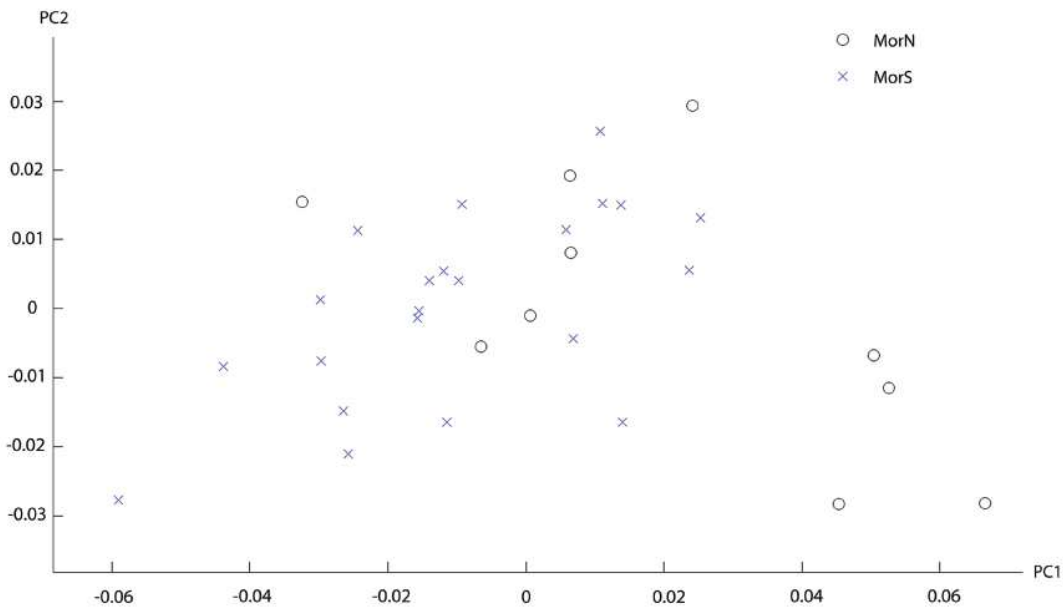


Figure 5. Distribution of the individuals of *P. papatasi* along the first two PCs. Principal component analysis of tangent space coordinates derived from GPA of the original coordinates that was conducted for the 16 landmarks digitized from the wings. Horizontal axis, PC1; vertical axis PC2. Signs indicate each individual. x: south of Atlas Mountains, o: north of Atlas Mountains.

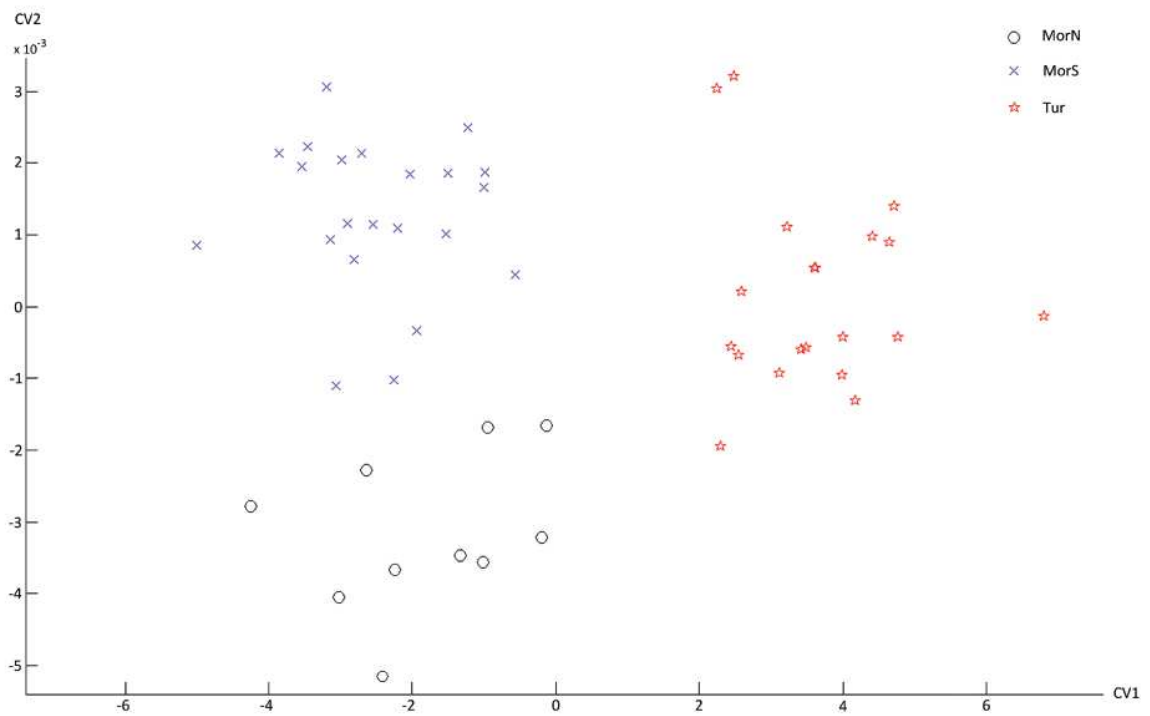


Figure 6. Distribution of *P. papatasi* individuals along the first two CVs. Canonical Variates Analysis of tangent space coordinates derived from GPA of the coordinates that was conducted for the 16 landmarks digitized from the wings. Horizontal axis, CV1, Vertical axis, CV2. Signs indicate each individual. x : south of Atlas Mountains, o : north of Atlas Mountains, * : individuals from Turkey (out-group).

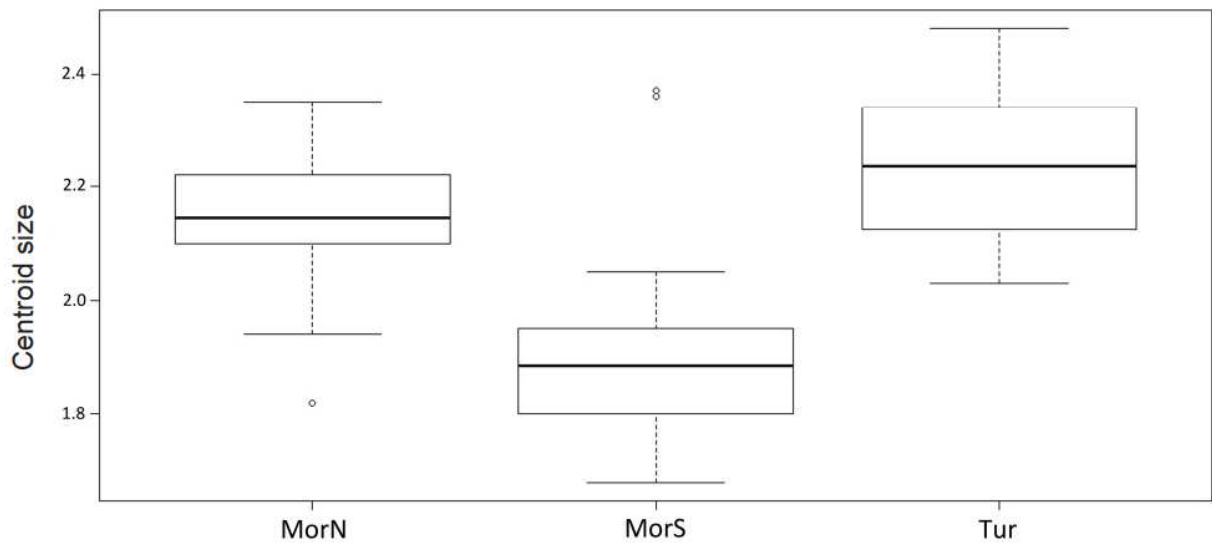


Figure 7. Mean, standard deviation, and error of centroid wing sizes of each population in *P. papatasi*. MorS: South of Atlas Mountains, MorN: North of Atlas Mountains, Tur: Turkey.

take into account the wide-range distribution of this species and also any kind of flying insects, full isolation should not be expected at first. On the other hand, Boussaa et al. (2008) compared four Moroccan populations with other Mediterranean basin populations from Spain, Cyprus, and Syria using isoenzymatic analysis, and the authors clearly revealed the genetic distance between populations of western Mediterranean countries (Morocco and Spain) and eastern countries according to nine polymorphic and three monomorphic enzymes. However, sand flies are poor fliers and the individuals rarely disperse more than several hundred meters, so it may be assumed that they may exist as local populations. Thus, genetic differences may be expected not only between geographical ecophenotypes from widely separated origins but also between local populations of *P. papatasi*. In Iran, genetic differentiation was detected between populations of this species sampled from different biotypes (Parvizi et al. 2003). Previous studies in Turkey clearly showed significant genetic and morphological differences between low altitudes and high altitudes and also between urban and rural populations of *P. papatasi*. (Volf et al. 2002, Alten et al. 2003). Dvorak et al. (2006) carried out a study to understand possible intraspecific variability of *P. sergenti* populations between Israel and southeast Anatolia by geometric morphometrics and RAPD analysis. The authors found statistically significant shape differences between the two populations and this result was fully supported by RAPD analysis to explain the genetic difference.

Guernaoui et al. (2010) demonstrated the phenotypic variation in the genitalia of male *P. papatasi* between the populations from central (north side of Atlas) and south (south side of Atlas Mountains) Morocco. They suggested that these malformations in male genitalia are associated with some specific environmental factors. The results for size and shape presented here indicate that there are differences among populations located in southern and northern parts of the Atlas Mountains in spite of a low number of examined specimens. The differences among the populations primarily occurred in the size and in the shape, respectively. This may also be associated with factors such as ecological and climatic effects. Similarly, four *P. papatasi* populations from southeast Anatolia were found to be significantly different according to altitude, and the differences among the populations primarily occurred in the wing size and shape (Belen et al. 2004). In our study, when whole size differences of the landmark locations were analyzed by centroid size analysis, two groups of populations on both sides of Atlas Mountains were recognized.

We obtained similar results when the wing shape differences of populations were analyzed. Similar to size, wing shape might be influenced by many factors and micro-environmental variation of some climatic parameters such as temperature and/or humidity. Kasap and Alten (2005, 2006) for *P. papatasi* and Aytekin et al. (2009) for *An. superpictus* demonstrated remarkable effect of temperature and humidity under laboratory conditions. Variations in wing shape can be related to flight performance and

nonlinear patterns in shape might also arise from selection by environmental variables such as relative humidity or saturation deficit of atmosphere showing nonlinear changes with altitude (Hoffman and Shirriffs 2002). We also identified the considerable deformation of wings between the populations (Figure 4). Thin-plate spline deformation analyses showed that this deformation on the medial part of the wings was mostly due to the relative effect of the southern populations. The medial of the wing of flying insects plays the most important role in flying capability (Johansson et al. 2009). If we consider this information, determining the deformation on the wings of especially the south population may explain why the biggest part of the population is located south of Atlas Mountain.

Temperature is one of the main factors preventing the spread of both visceral and cutaneous leishmaniasis. This ecological factor varies with altitude according to the thermal altitudinal gradient and latitude. The possible relationship between the leishmaniasis transmission and altitude may be closely related to many factors such as the temperature suitable for the evolution of *Leishmania* in sand flies (Rioux et al. 1985). Aytekin et al. (2007) found that even though there are three major mountain ranges that may serve as geographical barriers for sand fly species distribution and four main gaps were recognized in southern Anatolia, Turkey, there are no significant differences in wing morphology in *P. papatasi* specimens. It means that this species may disperse in large areas, vertically and horizontally. But in same study, the researchers also confirmed that there was a negative correlation among *Leishmania* transmission based on species, geographical barriers and altitude. In Turkey, *P. papatasi* can transmit HCL (human cutaneous leishmaniasis) vectored by *L. tropica* in the arid lowland of the hyper endemic area in southeast Anatolia. Guernaoui et al. (2006) proposed that altitude has an influence upon the spatial distribution and density of the sand fly species. The authors showed the distribution of *P. papatasi* populations between the northern and the southern parts of the Atlas Mountains has been limited by altitude (1000 m). The occurrence of *P. papatasi* in the lowlands and its low frequency in the mountains could be explained by its preferences for the arid and peri-arid areas. Moreover, its density increases with the aridity (Croset et al. 1974). Cross and Hyams (1996) predicted that raising the ambient temperature by 1-5° C through global warming would result in the expansion of the geographical distribution of *P. papatasi* in southwest Asia and extend the transmission season of *L. major*. In addition, this species is active throughout the year in the urban area of Marrakech, Morocco (Boussaa et al. 2005). This suggests the necessity of preventing the risk of extension of *L. major* foci from southern Morocco as predicted by Rioux et al. (1997).

In temperate climates, phlebotomine populations display marked seasonal variations in abundance, reaching high densities only during the summer months. The high levels of phenotypic diversity suggest that *P. papatasi* populations are able to maintain large effective population sizes in spite of the marked seasonality imposed by the

cold winter temperatures. A similar scenario is also met by Afrotropical vector populations in dry savanna/sahelian regions. In *An. arabiensis*, the strong seasonal fluctuations in abundance do not seem to affect the overall genetic diversity and current effective population size in dry areas of Sudan and Senegal where rains last for less than five months (Taylor et al. 1993, Simard et al. 2000).

Phlebotomus papatasi is active throughout the year in Morocco, absent occasionally in some samples because of meteorological disturbances. These results suggest that this vector does not diapause in Morocco (Baussaa et al. 2005). Two density peaks were identified by the authors in June and November. Between these two peaks, the density presented significant micro-variations because of the effect of the wind. On the other hand, the significant role of temperature on *P. papatasi* distribution was demonstrated by Singh (1999) and Kasap and Alten (2005). According to these studies, this species is less active at lower temperatures. Moreover, Srinivasan et al. (1993) showed a significant negative correlation between *P. papatasi* density and rainfall. Together with phenotypic correlations with arid and humid bioclimate, these results may explain why *L. major* transmission by *P. papatasi* can be easier in the south of the Atlas Mountain compared to the north.

This study is one component of a large-scale multi-level work that has been planned on population genetics and bio-ecologic studies in the same areas. The levels of phenotypic variation in our samples are more likely to reflect the local environmental pressures to which these populations are subjected. However, possible correlation between phenotypic differentiation and genetic and/or geographic distances should be considered with larger sample sizes in future studies.

Acknowledgments

We are grateful to IRD and CNRS for financial support. BA is financed by Infectiopole Sud, France. This study was partially funded by EU grant GOCE-2003-010284 EDENext and is cataloged by the EDENext Steering Committee as EDENext004 (<http://www.edenext.eu>). The contents of this publication are the sole responsibility of the authors and don't necessarily reflect the views of the European Commission. We especially thank Dr. Henry R. Rupp for English corrections and his contributions.

REFERENCES CITED

- Abonnenc, E. 1972. Les phlébotomes de la région éthiopienne (Diptera, Psychodidae). *Mém. ORSTOM*. 55: 289.
- Alibert, P., B. Moureau, J.L. Dommergues, and B. David. 2001. Differentiation at a microgeographical scale within two species of ground beetle, *Carabus auronitens* and *C. nemoralis* (Coleoptera, Carabidae): A geometrical morphometric approach. *Zool. Scripta*. 30: 299-316.
- Alten, B., S. Caglar, S. Kaynas, and F. Simsek. 2003. Evaluation of protective efficacy of K-OTAB impregnated bed nets for cutaneous leishmaniasis control in southeast Anatolia, Turkey. *J. Vector Ecol.* 28: 53-64.
- Arnqvist, G. and T. Mårtensson. 1998. Measurement error in geometric morphometrics: Empirical strategies to assess and reduce its impact on measures of shape. *Acta Zool. Acad. Sci. Hungary*. 44: 73-96.
- Artemiev, M.M. 1991. A classification of the subfamily Phlebotominae. *Parasitologia* 33: 69-77.
- Aytekin, A.M., B. Alten, S.S. Caglar, Y. Ozbel, S. Kaynas, F.M. Simsek, O. Erisoz Kasap, and A. Belen. 2007. Phenotypic variation among local populations of phlebotomine sand flies (Diptera: Culicidae) in southern Turkey. *J. Vector Ecol.* 32: 226-234.
- Aytekin, S., A.M. Aytekin, and B. Alten. 2009. Effect of different larval rearing temperatures on the productivity (R_0) and morphology of the malaria vector *Anopheles superpictus* Grassi (Diptera: Culicidae) using geometric morphometrics. *J. Vector Ecol.* 34: 32-42.
- Belen, A., B. Alten, and A.M. Aytekin. 2004. Altitudinal variation in morphometric and molecular characteristics of *Phlebotomus papatasi* populations. *Med. Vet. Entomol.* 18: 343-350.
- Bookstein, F.L. 1991. *Morphometric Tools for Landmark Data*. Cambridge University Press. 435 pp.
- Boussaa, S., S. Guernaoui, B. Pesson, A. Boumezzough. 2005. Seasonal fluctuations of phlebotomine sand fly populations (Diptera: Psychodidae) in the urban area of Marakech, Morocco. *Acta Trop.* 95: 86-91.
- Boussaa, S., S. Perrotey, A. Boumezzough, R. Harrak, S. Hilali, and B. Pesson, 2008. Isoenzymatic characterization of *Phlebotomus papatasi* (Diptera: Psychodidae) of the Marrakech area, Morocco. *J. Med. Entomol.* 45: 370-374.
- Chown, S.L. and C. Jaco Klok. 2003. Altitudinal body size clines: latitudinal effects associated with changing seasonality. *Ecography* 26: 445-455.
- Croset, H., J.A. Rioux, N. Leger, R. Houin, M. Cadi Soussi, N. Benmansour, and M. Maistre. 1974. Les méthodes d'échantillonnage des populations de phlébotomes en région Méditerranéenne. In: J.A. Rioux (ed.), *Ecologie des leishmanioses*. CNRS, Paris, pp. 139-151.
- Cross, E.R. and K.C. Hyams. 1996. The potential effect of global arming on the geographic and seasonal distribution of *Phlebotomus papatasi* in Southwest Asia. *Environ. Hlth. Perspect.* 104: 724-727.
- Depaquit, J., E. Lienard, A. Verzeaux-Griffon, H. Ferté, A. Bounamous, J.C. Gantier, H.A. Hanafi, R.L. Jacobson, M. Maroli, V. Moin-Vaziri, F. Müller, Y. Ozbel, M. Svobodova, P. Volf, N. Léger. 2008. Molecular homogeneity in diverse geographical populations of *Phlebotomus papatasi* (Diptera, Psychodidae) inferred from ND4 mtDNA and ITS2 rDNA Epidemiological consequences. *Infect. Genet. Evol.* 8: 159-170.
- Dvorak, V., A.M. Aytekin, B. Alten, S. Skarupova, J. Votýpka, and P. Volf. 2006. A comparison of the intraspecific variability of *Phlebotomus sergenti* Parrot, 1917 (Diptera: Psychodidae). *J. Vector Ecol.* 31: 229-238.

- Foley, H. and C. Vialatte. 1914. Existence dans le Sud marocain du bouton d'orient à l'état endémique. *Bull. Soc. Path. Exot.* 7: 114-115.
- Gaud, J. 1947. Phlébotomes du Maroc. *Bull. Soc. Sci. Natur. Du Maroc.* 27: 207-212.
- Ghosh, K.N., J.M. Mukhopadhyay, H.Guzman, R.B. Tesh, and L.E. Munstermann. 1999. Interspecific hybridization and genetic variability of *Phlebotomus* sandflies. *Med. Vet. Entomol.* 13: 78-88.
- Gimnig, J.E., M. Ombok, S. Otieno, M.G. Kaufman, J.M. Vulule, and E.D. Walker. 2002. Density-dependent development of *Anopheles gambiae* (Diptera: Culicidae) larvae in artificial habitats. *J. Med. Entomol.* 39: 162-172.
- Guernaoui, S., A. Boumezzough, and A. Laamrani. 2006. Altitudinal structuring of sand flies (Diptera:Psychodidae) in the High-Atlas mountains (Morocco) and its relation to the risk of leishmaniasis transmission. *Acta Trop.* 97: 246-351.
- Guernaoui, S., K. Ramaoui, N. Rahola, C. Barnabe, D. Sereno, and A. Boumezzough. 2010. Malformations of the genitalia in male *Phlebotomus papatasi* (Scopoli) (Diptera: Psychodidae). *J. Vector Ecol.* 35: 13-19.
- Hoffmann, A.A. and J. Shirriffs. 2002. Geographic variation for wing shape in *Drosophila serrata*. *Evolution.* 56: 1068-1073.
- Jirakanjanakit, N., S. Leemingsawat, S. Thongrunkiat, C. Apiwathnasorn, S. Singhaniyom, C. Bellec, and J.P. Dujardin. 2007. Influence of larval density or food variation on the geometry of the wing of *Aedes (Stegomyia) aegypti*. *Trop. Med. Intl. Hlth.* 12: 1354-1360.
- Johansson, F., M. Söderquist and F. Bokma. 2009. Insect wing shape evolution: independent effects of migratory and mate guarding flight on dragonfly wings. *Biol. J. Linn. Soc.* 97: 362-372.
- Kasap, O. and B. Alten. 2005. Laboratory estimation of degree-day developmental requirements of *Phlebotomus papatasi* (Diptera: Psychodidae). *J. Vector Ecol.* 30: 328-333.
- Kasap, O. and B. Alten, 2006. Comparative demography of the sand fly *Phlebotomus papatasi* (Diptera:Psychodidae) at constant temperature. *J. Vector Ecol.* 31: 378-385.
- Killick-Kendrick, R., Y. Tang, M. Killick-Kendrick, D.K. Sang, M.K. Sirdar, L. Ke, R.W. Ashford, J. Schorscher, and R.H. Johnson. 1991. The identification of female sandflies of the subgenus *Larrousius* by the morphology of the spermathecal ducts. *Parasitologia* 33: 335-347.
- Kuclu, O., A. Aldemir, and B. Demirci. 2011. Altitudinal variation in the morphometric characteristics of *Aedes vexans* Meigen from northeastern Turkey. *J. Vector Ecol.* 36: 30-41.
- Mayr, E. and P.D. Ashlock. 1991. *Principles of Systematic Zoology*. McGraw-Hill, New York.
- Parvizi, P., M. Benlarbi, and P.D. Ready. 2003. Mitochondrial and Wolbachia markers for the sandfly *Phlebotomus papatasi*: little population differentiation between peridomestic sites and gerbil burrows in Isfahan province, Iran. *Med. Vet. Entomol.* 17: 351-362.
- Perfiliew, P.P. 1968. *Fauna of USSR Diptera, Phlebotomidae (sandflies)*. *Fauna of the USSR.* 3(2) Israel Program for Scientific Translations, Jerusalem, Israel, 195 pp.
- Ricklefs, R.E. and D.B. Miles. 1994. Ecological and evolutionary inferences from morphology: an ecological perspective. In: P.C. Wainwright and S.M. Reilly (eds). *Ecological Morphology*. University of Chicago Press. 13-41.
- Rioux, J.A., P. Rispaill, G. Lanotte, and J. Lepart. 1984. Relations phlébotomes-bioclimats en écologie des leishmanioses. Corollaires épidémiologiques. L'exemple du Maroc. *Bull. Soc. Bot. Fr.* 13:549-557.
- Rioux, J.A., J.P. Aboulker, G. Lanotte, R. Killick-Kendrick, and Martni-Dumas. 1985. Ecologie des leishmanioses dans le sud de la France. 21. Influence de la température sur le développement de *Leishmania infantum* Nicolle, 1908 chez dans *Phlebotomus ariasi*. *Ann. Parasitol. Hum. Comp.* 60: 221-229.
- Rioux, J.A., E. Guilvard, J. Dereure, G. Lanotte, M. Denial, F. Pratlong, E. Serres, and A. Belmonte. 1986. Infection naturelle de *Phlebotomus papatasi* (Scopoli, 1786) par *Leishmania major* Mon-25. A propos de 28 souches isolées dans un foyer du sud Marocain. In: *Leishmania*. Taxinomie et Phylogénèse. Applications écoépidémiologiques. (Coll. Int. CNRS/INSERM, 1984), IMEEE, Montpellier, France, pp. 471-480.
- Rioux, J.A., O. Akalay, J. Perieres, J. Dereure, J. Mahjour, H. N. Le Houerou, N. Leger, P. Desjeux, M. Gallego, A. Saddiki, A. Barkia, and H. Nachi. 1997. L'évaluation écoépidémiologique du "risque leishmanien" au Sahara atlantique marocain. Intérêt heuristique de la relation "Phlébotomes-bioclimats". *Ecol. Méditerran.* 23: 73-92.
- Rioux, J.A. 2001. Trente ans de coopération franco-marocaine sur les leishmanioses: Dépistage et analyse des foyers. Facteurs de risque. Changements climatiques et dynamique nosogéographique. *Bull. Ass. Anc. Elèves. Inst. Past.* 168: 90-101.
- Rioux, J.A. and S. de La Rocque. 2003. Climats, leishmanioses et trypanosomiases. Changements climatiques, maladies infectieuses et allergiques. *Ann. Inst. Pasteur* 16: 41-62.
- Rispail, P., J. Dereure, and D. Jarry. 2002. Risk zones of human leishmaniasis in the western Mediterranean basin. Correlations between vectors sand flies, bioclimatology and phytosociology. *Mem. Inst. Oswaldo Cruz* 97: 477-483.
- Rohlf, F.J. and D. Slice. 1990. Extensions of the procrustes method for the optimal superimposition of landmarks. *Syst. Zool.* 39: 40-59.
- Rohlf, F.J. 1993. Relative warp analysis and an example of its application to mosquito wings. In: L.F. Marcus, E. Bello, and A. Garcia-Valdecasas (eds.). *Contributions to Morphometrics*. Madrid, Nacional de Ciencias Naturales. 264 pp.
- Rohlf, F.J. 1999. Shape statistics: Procrustes superimpositions and tangent spaces. *J. Class.* 16: 197-223.

- Rohlf, F.J. 2000. Statistical power comparisons among alternative morphometric methods. *Am. J. Phys. Anthropol.* 111: 463-478.
- Rohlf, F.J. 2007. *TpsUTIL*. Department of Ecology and Evolution, State University of New York at Stony Brook.
- Schneider, J.R., A.C. Morrison, H. Astete, T.W. Scott, and M.L. Wilson. 2004. Adult size and distribution of *Aedes aegypti* (Diptera: Culicidae) associated with larval habitats in Iquitos, Peru. *J. Med. Entomol.* 41: 634-642.
- Schneider, J.R., A. Mori, J. Romero-Severson, D.D. Chadee, and D.W. Severson. 2007. Investigations of dengue-2 susceptibility and body size among *Aedes aegypti* populations. *Med. Vet. Entomol.* 4: 370-376.
- Simard, F., T. Lehmann, J. Lemasson, M. Diatta, and D. Fontenille. 2000. Persistence of *Anopheles arabiensis* during the severe dry season conditions in Senegal: an indirect approach using microsatellite loci. *Insect Mol. Biol.* 9: 467-479.
- Singh, K.V. 1999. Studies on the role of climatological factors in the distribution of phlebotomine sand flies (Diptera: Psychodidae) in semi-arid areas of Rajasthan, India. *J. Arid Environ.* 42: 43-48.
- Slice, D.E. 1998. *Morpheus*. Software for morphometric research. Revision 01-30-98. Department of Ecology and Evolution, State University of New York, Stony Brook, NY.
- Srinivasan, R., K.N. Panicker, and V. Dhanda. 1993. Population dynamics of *Phlebotomus papatasi* (Diptera: Phlebotomidae) in Pondicherry, India. *Acta Trop.* 54: 125-130.
- Taylor, C.E., Y.T. Toure, M. Coluzzi, V. Petrarca. 1993. Effective population size and persistence of *Anopheles arabiensis* during the dry season in West Africa. *Med. Vet. Entomol.* 7: 351-357.
- UNESCO-FAO. 1963. *Bioclimatic of the Mediterranean Zone. Explanatory Notes*. Published in 1963 by the United Nations Educational, Scientific and Cultural Organization Place de Fontenoy, Paris-7e and the Food and Agriculture Organization of the United Nations, Viale delle Terme di Caracalla, Rome Printed by Firmin-Didot, Mesnil-sur-l'Estrée (Eure). 60 pp.
- Volz, P., Y. Ozbel, F. Akkafa, M. Svobodova, J. Votypka, and K.P. Chang. 2002. Sand flies (Diptera: Phlebotominae) in Sanliurfa, Turkey: relationship of *Phlebotomus sergenti* with the epidemic of anthroponotic cutaneous leishmaniasis. *J. Med. Entomol.* 39: 12-15.
- Zelditch M.L., H.D. Swiderski, H.D. Sheets, and W.L. Fink. 2004. *Geometric Morphometrics for Biologists*. Elsevier Academic Press. London. 443 pp.

II.3.2. Analyses des données

Les images de chaque aile ont été enregistrées sous le format adéquat à l'aide du logiciel tpsUtil version 1.60 [75] puis les coordonnées cartésiennes de 16 points de repère, choisis d'après la méthodologie de Belen et al. [58] ont été numérisées à l'aide du logiciel tpsDIG2 version 2.18 [76]. Ces points de repère correspondent à l'intersection des nervures alaires avec le bord de l'aile et les intersections des nervures alaires entre elles (Figure 12). Toutes les ailes ont été numérisées deux fois afin de réduire l'erreur de mesure (z) [77]. La taille moyenne (« centroid size ») des ailes de chaque individu a été calculée après élimination des différences de position, d'échelle et d'orientation. Cette taille moyenne correspond à la racine carrée de la somme des carrés des distances de l'ensemble des points de repère par rapport à leur point moyen central, c'est-à-dire la racine carrée de la somme des variances des points de repère par rapport à leur point moyen central, en abscisse et en ordonnée [74]. La comparaison des « centroid sizes » a été effectuée sous le logiciel R, version 3.1.1 [67] à l'aide d'un test non paramétrique de Wilcoxon-Mann-Whitney ou d'un test de Kruskal Wallis, suivis d'un test post-hoc de Mann-Whitney avec correction de Bonferroni, pour mettre en évidence une différence, si elle existe, au niveau de la taille des ailes chez nos individus.

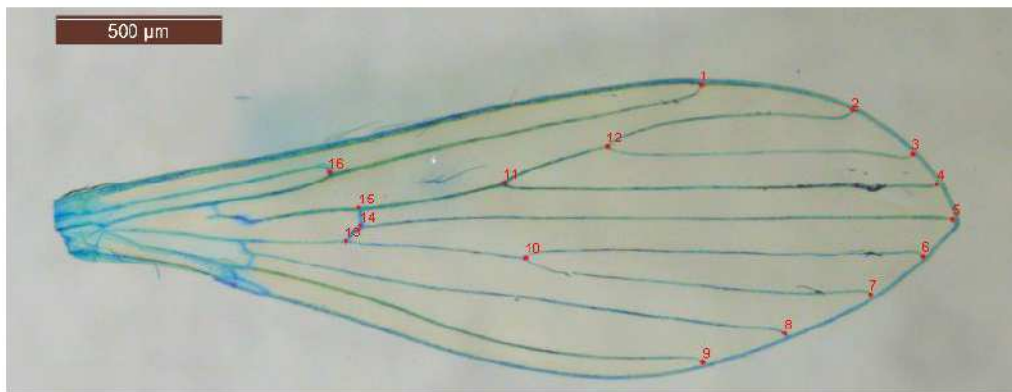


Figure 14 : Localisation des 16 points de repère sur une aile de *Phlebotomus ariasi*.

Ensuite, les différences de position, d'échelle et d'orientation de ces points de repère dans le plan cartésien doivent être retirées afin de ne laisser que les différences de forme (Figure 13). Les analyses morphométriques et les sorties graphiques ont été réalisées à l'aide du logiciel CLIC [78] en utilisant principalement les modules TET, MOG et PAD. Les points de repère ou « landmarks » ont subi la méthode de superimposition des procrustes dans le but de produire des variables de formes (partial warps) [79-82]. Les composantes principales (relative warps) ont pu ensuite être utilisées afin de comparer les populations de *P. ariasi*. Afin d'évaluer le degré de similitude entre les populations, les distances de Mahalanobis entre populations ont été calculées en utilisant le module PAD du logiciel CLIC [78]. Un test de permutation non paramétrique avec 1000 permutations a ensuite été réalisé afin de tester le seuil de significativité de ces distances. L'allométrie (variation de forme associée à la variation de taille) a aussi pu être estimée. Pour faciliter les différentes analyses, nous avons organisé les ailes suivant les paramètres que nous voulions tester : sexe, mois, versant, altitude et station.

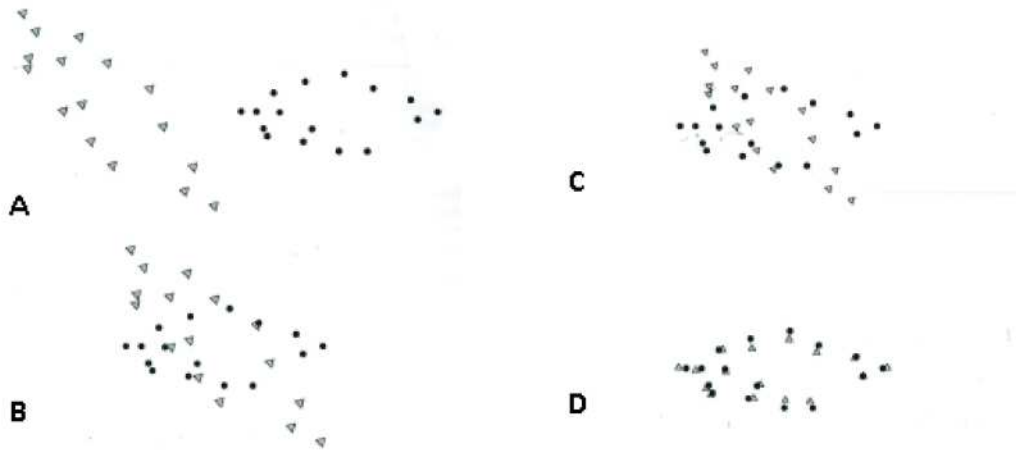


Figure 15 : Schéma de l'élimination des variations entre deux ailes (◄ points de repère sur l'aile 1 ; ● points de repère sur l'aile 2). (A) configuration d'origine de deux ailes ; (B) Elimination des différences de position ; (C) Elimination des différences d'échelle ; (D) Elimination des différences d'orientation. Ces trois ajustements permettent de mettre en avant uniquement les variations de forme. Figure provenant de Zelditch et al. [57].

II.4. Caractérisation génétique

Tout d'abord, des marqueurs microsatellites ont été mis au point et validés sur un petit nombre d'échantillons (Voir chapitre 6 - Développement des marqueurs microsatellites). Ensuite, 11 de ces marqueurs, les plus polymorphes, ont été utilisés afin de réaliser la caractérisation génétique des populations de *P. ariasi* au sein de notre zone d'étude. (Voir chapitre 6 - Ecosystème et structure génétique des populations de *P. ariasi*). Le nombre d'individus étant très important, l'analyse génétique a été réalisée uniquement sur les spécimens de 2011.

II.4.1. Extraction d'ADN

L'extraction d'ADN a été effectuée à l'aide de deux méthodes : une automatisée et une manuelle. En effet, les ADN des spécimens collectés sur le transect ont été extraits de manière automatisée en raison de leur très grand nombre, alors que les quelques dizaines de spécimens provenant d'autres pays ont été extraits manuellement. Les deux méthodes d'extraction ont donné des qualités de résultats similaires pour tous les loci microsatellites étudiés au cours de ce travail.

L'extraction automatisée a été réalisée sur la plateforme de l'UMR 190 Virus Emergents, Université Aix-Marseille-IRD-EHESP selon deux méthodes, la première à l'aide du protocole Chelex [83] et la seconde selon le protocole décrit dans Zapata et al. [84]. Le thorax, l'abdomen et les pattes ont été broyés individuellement à l'aide d'un Mixer Mill MM 300 (Qiagen, Venlo, Netherlands) avec une bille de tungstène de 3 mm de diamètre tournant à une fréquence de 30 cycles par secondes pendant 3 minutes dans 200 µL de milieu minimum essentiel d'Eagle (EMEM). Ensuite, 140 µL de ce broyat ont été utilisés pour la purification de l'ADN, soit par ajout d'une suspension de résine de Chelex [83], soit à l'aide d'une station de travail (Eppendorf epMotion 5075) et d'un kit d'extraction Macherey-Nagel NucleoSpin 96 Virus kits. Ce kit (ADN-ARN) permettra de tester ultérieurement la présence de phlébovirus par RT PCR.

L'extraction manuelle a été réalisée selon la méthode CTAB, à partir d'une version modifiée du protocole proposé par Edwards [85]. Le phlébotome entier a été broyé à l'aide

d'un piston pellet dans 200 μ L de tampon d'extraction contenant du Tris-HCl 1M pH 8,0, 0,5 M d'EDTA, 1,4 M de NaCl et 2% de cetyltriméthylammonium bromure (CTAB). Le mélange a ensuite été incubé à 65°C pendant 5 minutes. La solution a été centrifugée à 12000 rotations par minute (rpm) pendant 5 minutes après l'ajout de 200 μ L de chloroforme. Le surnageant a été prélevé et ajouté dans 200 μ L d'isopropanol, puis centrifugé pendant 15 minutes à 15000 rpm. Le culot a ensuite été rincé à l'aide de 200 μ L d'éthanol à 70%, centrifugé 5 minutes à 12000 rpm, séché 5 minutes au speed-vac (DNA Mini Centrifugal Evaporator, Heto Holten, Allerød, Danemark), re-suspendu dans 30 μ L d'eau et enfin incubé toute la nuit à température ambiante.

Pour les deux techniques d'extraction, l'ADN extrait a ensuite été placé à -20°C pour les études moléculaires

II.4.2. Développement des marqueurs microsatellites

Une banque de marqueurs microsatellites a été réalisée à partir de spécimens de *P. ariasi* mâles capturés dans notre zone d'étude. Cette banque a ensuite été enrichie par des motifs microsatellites (AG, AC, AAC, AGG, ACG, AAG, ACAT et ATCT). Puis les loci microsatellites ont été clonés, isolés puis séquencés par pyroséquençage haut débit 454 GS-FLX Titanium (GS FLX®, RocheDiagnostics®, Bâle, Suisse) pour validation. Une centaine de paires d'amorces a été identifiée et 35 ont été validées sur la base de la détection, sans ambiguïté, d'amplicons polymorphes à l'aide d'une température d'hybridation fixe et d'un protocole de génotypage sur gel de polyacrylamide. Ces étapes ont été effectuées par une entreprise privée (Genoscreen, Institut Pasteur de Lille, France) selon la méthodologie décrite dans Malausa et al. [86].

Sur les 35 couples d'amorces proposés, 14 ont été sélectionnés pour la poursuite de l'étude sur la base des critères suivants : taux de GC, position des microsatellites dans la séquence, structure du motif répété, résultats des PCR et électrophorégrammes clairs ne présentant qu'un ou deux pics. Les informations relatives aux 14 marqueurs microsatellites sont synthétisées dans le tableau 6.

II.4.3. Amplification d'ADN et génotypage

Ces marqueurs ont d'abord été testés sur 43 *P. ariasi* provenant de différentes localités (France (Roquedur et Nice), Portugal, Espagne, Turquie et Chypre) et sur 7 autres espèces (voir chapitre 6 - Développement des marqueurs microsatellites). L'ADN de chaque spécimen a été génotypé sur 16 loci microsatellites, les 14 sélectionnés ci-dessus et deux marqueurs microsatellites AAm20 et AAm24 développés par Aransay et al. [59] (Tableau 6). Ensuite, 1692 individus, capturés en 2011, ont été génotypés pour les 11 loci les plus polymorphes : aria1, aria2, aria3, aria4, aria5, aria10, aria11, aria12, aria13, aria14 and aria15 afin de réaliser la caractérisation génétique des populations de *P. ariasi* dans notre zone d'étude.

Les ADN extraits ont été amplifiés selon les conditions décrites ci-après. Chaque mélange réactionnel de 25 μ L était composé de 1 pmol d'amorces sens et antisens, l'amorce sens étant marquée avec un fluorochrome FAM, ATT0565 ou HEX (Tableau 6), 5 ng d'ADN, 6 pmol de dNTP, 2,5 μ L de tampon 10X et 0,25 μ L de Taq polymérase (Roche Diagnostics®, 5 UI / μ L).

Les amplifications ont été effectuées dans un thermocycleur (Mastercycler, Eppendorf) selon les conditions suivantes : une étape de dénaturation initiale à 95°C pendant 10 min ; 40

cycles à 95°C pendant 30 s, 30 s à la température d'hybridation de chaque locus (Tableau 6) et 72°C pendant 1 min ; et enfin une étape d'extension finale à 72°C pendant 10 min.

Tableau 3 : Descriptif des 16 marqueurs microsatellites utilisés pour *Phlebotomus ariasi* (Th : température d'hybridation ; Gp : groupe de multiplexage).

Nom	Séquence des amorces (5'-3')	Numéro GeneBank	Motif répété	Th	Fluoro-chromes	Gp	Taille (pb)
aria1	5'-TCCGCTCTTATCATCAATCG-3'	KJ526156	(TC) ₁₋₈	55	FAM	1	194-208
	5'-AGTGCCTCTCATCCGCTAGT-3'						
aria2	5'-CCTGCCAGAGTTTAATTATTTG-3'	KJ526157	(AG) ₂₋₆	55	FAM	1	105-113
	5'-AGTTTCCTCGAAAATTGCCA-3'						
aria3	5'-ATTCCGCTTCACCACCAAC-3'	KJ526158	(TC) ₆₋₁₂	52	FAM	2	124-136
	5'-ATCCCTTAATTTTCCGTGG-3'						
aria4	5'-ACTTTGTGAACTCGTGAGGC-3'	KJ526159	(CT) ₆₋₉	51	FAM	3	114-120
	5'-CGTAGCAGGAGTAAGAATTTATG-3'						
aria5	5'-GGACGATGACGCCAAGTATC-3'	KJ526160	(TC) ₅₋₇	60	FAM	4	160-164
	5'-AATCGTGAGTGAGGCCGAAAC-3'						
aria7	5'-GCCCGATAGTGTAAATGGGAA-3'	KJ526161	(TC) ₇₋₁₀	55	ATT0565	1	155-161
	5'-ACGGATGTCTTCGCTCTCAG-3'						
aria8*	5'-CAAAGACGGACACTTCCACA-3'	KJ526162	(AG) ₇₋₁₁	55	ATT0565	3	124-132
	5'-CCGATAAATGGTGGACTTGG-3'						
aria9	5'-AAAATGCGTGTCACTGTGGT-3'	KJ526163	(GA) ₆₋₉	55	ATT0565	2	163-169
	5'-TTCAGCCGGTAATTGGTGTA-3'						
aria10	5'-AGTAAACCCTTCAAGTGCGC-3'	KJ526164	(CA) ₉₋₁₂	59	ATT0565	4	205-211
	5'-CCAGAAGCCCTTTTCACACC-3'						
aria11	5'-GTGCTGGGAGGACAAGAATC-3'	KJ526165	(TC) ₅₋₈	55	HEX	1	93-101
	5'-CACCAAGTGCAGGAGTGAGA-3'						
aria12	5'-TTCCACCTACACATGCCTCA-3'	KJ526166	(TC) ₅₋₉	55	HEX	3	80-88
	5'-GCTCTTAGATGTGGGCGAAA-3'						
aria13	5'-GACCGGTTGTGATTAATGTGC-3'	KJ526167	(AG) ₄₋₉	55	HEX	2	103-113
	5'-CGATGATCGCCATTACCTT-3'						
aria14	5'-AGTTATAGGCAACTGGGCGG-3'	KJ526168	(GA) ₉₋₁₈	51	HEX	3	192-210
	5'-TTTAGCCCAAACACTACGCACA-3'						
aria15	5'-TTAGGGTATAACCACAGCCGC-3'	KJ526169	(CT) ₄₋₉	55	HEX	4	119-129
	5'-TTGTCAGGCTGCCGATCT-3'						
AAm20	5'-ACAAGCGAGTCATAGAGTCCG-3'	AJ303377	(GAG) ₄₋₅	57	FAM	5	183-185
	5'-CTGGTGGAGGTGAGTTGAG-3'						
AAm24	5'-CTATTCGCCCACTTGG-3'	AJ303378	(GCA) ₆	52	HEX	5	170
	5'-TCAATCGACATTCGGACAGGC-3'						

Le génotypage a été effectué à l'aide d'un séquenceur automatisé ABI Prism 3130 Genetic Analyser (Applied Biosystems®) avec 1 µL de produit PCR ajouté dans un mélange standard de 0,5 µL de marqueur de taille GENESCAN 500LIZ (Applied Biosystems®) et de 12,5 µL de formamide (Hi-Di) (Applied Biosystems®). Les profils ont été lus et analysés à l'aide du logiciel GeneMapper 4.0 (Applied Biosystems®). Le multiplexage durant le génotypage a été réalisé par l'association de séquences microsatellites de tailles différentes

et/ou marquées par des fluorochromes de différentes couleurs. Le multiplexage durant la PCR n'a pas été testé.

II.4.4. Analyses des données

Afin d'étudier la structure génétique des populations de *P. ariasi*, nous avons analysé les données à différentes échelles: stations, mois de capture, versants et groupes d'altitude. Les stations ont été subdivisées par micro-habitats quand cela était possible (ex : mur, maison, etc.). De même, nous avons utilisé les groupes définis précédemment pour le versant et l'altitude (Voir ci-dessus - Caractéristiques générales).

Pour chaque groupe d'individus, le nombre moyen d'allèles (N_a), la richesse allélique moyenne (R_s), l'hétérozygotie attendue non biaisé moyenne (H_e), l'hétérozygotie moyenne observée (H_o), la diversité intra-population moyenne (H_s) et le coefficient de consanguinité (F_{IS}) ont été calculés à l'aide de FSTAT 2.9.3 [87] et de GENETIX 4.04 [88]. L'écart à l'équilibre de Hardy-Weinberg ainsi que le déséquilibre de liaison ont été mesurés à l'aide de GENEPOP version 4.2.2 [89] et FSTAT 2.9.3, respectivement.

La présence d'allèles nuls a été testée avec MICRO-CHECKER 2.2.3 [25]. Les loci, pour lesquels nous avons confirmé la présence d'allèles nuls, n'ont pas été utilisés dans les analyses de génétique des populations.

Les tests d'isolement par la distance ont été effectués en utilisant GENEPOP [89]. La corrélation entre les distances géographiques et génétiques a été analysée par un test de Mantel (10^4 permutations).

II.5. Détection des leishmanies par qPCR

La recherche de leishmanies a été effectuée sur 918 ADN de phlébotomes femelles capturées en 2011, à l'aide d'une qPCR selon le protocole de Ceccarelli et al. [90]. Les amorces définies dans cet article sont : MLF: 5'-CGTTCTGCGAAAACCGAAA-3' (amorce sens) et MLR: 5'-CGGCCCTATTTTACACCAACC-3' (amorce anti-sens)

Elles amplifient une région conservée de 111 pb d'ADN kinétoplastique de *Leishmania* (numéro genebank : Z35272). Cette technique permet de détecter *L. major*, *L. tropica*, *L. amazonensis*, *L. braziliensis*, *L. infantum*, *L. chagasi*, *L. lainsoni*, *L. guyanensis* et *L. tarentolae* avec une sensibilité de 10 fg d'ADN (équivalent à 1 parasite) (testée sur une gamme d'ADN de *L. major*) (C. Cassan, communication personnelle, 10 Juillet 2015). Tous les individus ont été amplifiés selon un protocole précis. Chaque mélange réactionnel de 6 μ L était composé de 1 μ L d'ADN, 3 μ L de LightCycler 480 SYBR Green I master mix (Roche) et 0,05 μ L de chaque amorce. Les amplifications ont été effectuées dans un thermocycleur qPCR LC480 (Roche) sous les conditions suivantes : une pré-incubation à 94°C pendant 10 min, suivie de 40 cycles à 94°C pendant 30 s, 60°C pendant 20 s et 72°C pendant 20 s. A la fin de chaque élongation une détection de fluorescence est réalisée. La réaction a tout d'abord été testée en duplicata sur des pools de 8 individus. N'ayant obtenu aucun résultat positif, nous avons continué les amplifications individuellement afin de vérifier que les résultats négatifs n'étaient pas dus à un effet de dilution.

III. Analyses des données climatiques

La transmission des *Leishmania* peut être influencée par les changements environnementaux et climatiques soit directement (effet de la température sur le développement du parasite chez le vecteur) soit indirectement (changement dans le comportement et l'aire de distribution du vecteur) [4]. De plus, il est maintenant bien démontré que la température et l'humidité relative jouent un rôle majeur dans la distribution spatiale et la dynamique saisonnière des populations de vecteurs [91-93].

Dans ce contexte, le but de ce chapitre est de décrire la région d'étude d'un point de vue climatique et d'analyser la variabilité de la température et de l'humidité relative le long du transect de 14 km, considérant les mois et les années de collecte et certains paramètres environnementaux (versant, altitude, station) (voir chapitre 2 - Caractéristiques générales).

Nous avons débuté les relevés climatiques en juin 2011 et ceux-ci se sont poursuivis jusqu'en octobre 2013. Durant la première année (juin 2011), 13 enregistreurs de données de températures et d'humidité relative (iButton hygrocron, DS 1923) ont été placés dans 13 stations à proximité des PL ou PC, et 7 autres ont été rajoutés en février 2012 dans les stations qui en étaient dépourvues. Ces collecteurs ont été programmés pour mesurer et enregistrer la température et l'humidité relative toutes les 6 heures (à 6h, 12h, 18h et 24h). Durant ces 3 ans, certains enregistreurs ont malheureusement été perdus ou détruits : station 1 (2012), station 10 et 15 (2013). Les données récoltées pour chaque station de 2011 à 2013 sont synthétisées dans la figure 14.

Selon les données analysées durant les périodes de capture, la température moyenne était de 21,3°C, 20,4°C et 19,9°C, et l'humidité relative moyenne de 60,1%, 70,6% et 69,4% pour 2011, 2012 et 2013, respectivement. La température maximale / minimale était de 46,1°C / 2,6°C, 46,6°C / 5,6°C et 47,6°C / 4,6°C, tandis que l'humidité relative maximum / minimum était de 100% / 13,8%, 100% / 20,9% et 100% / 25,7% pour 2011, 2012 et 2013 respectivement. Aucune différence significative pour la température et l'humidité relative n'a été observée entre ces 3 ans durant la saison d'activité des phlébotomes (test de Kruskal-Wallis, p -value = 0,9535 et p -value = 0,7413, respectivement).

Quand les données climatiques (température et humidité relative) sont regroupées par mois, une différence significative de température et d'humidité relative est observée (p -value < 0,05, pour les deux variables). Le test post-hoc effectué a montré des différences significatives entre chaque mois (p -value < 0,05, pour tous les mois) (Figure 15). Comme attendu, la température et l'humidité relative sont donc significativement différentes entre chaque mois.

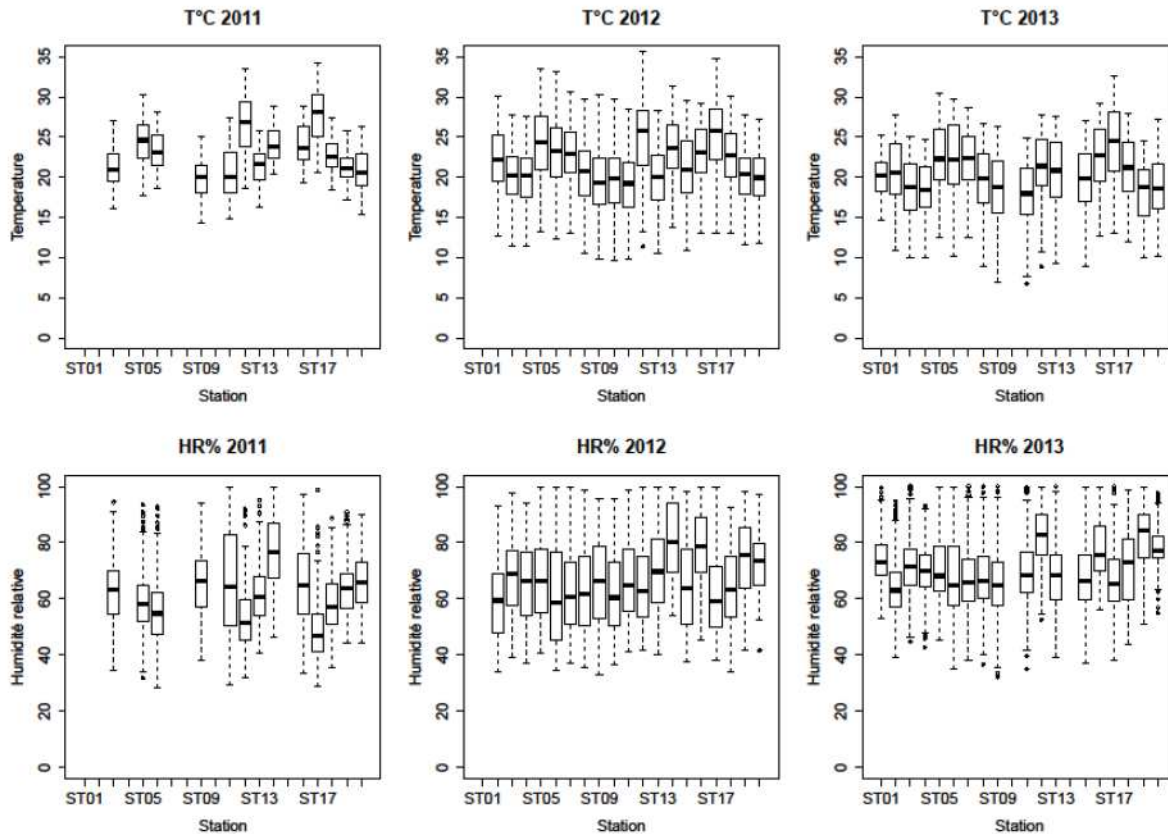


Figure 16 : Boxplot de la température (°C) et de l'humidité relative (HR%) par station et par année. Les valeurs manquantes représentent les enregistreurs perdus. Le boxplot (ou boîte à moustache) représente une distribution des valeurs avec la médiane (trait épais), une boîte s'étendant du quartile 0,25 au quartile 0,75 et un intervalle de confiance de 95% pour la médiane.

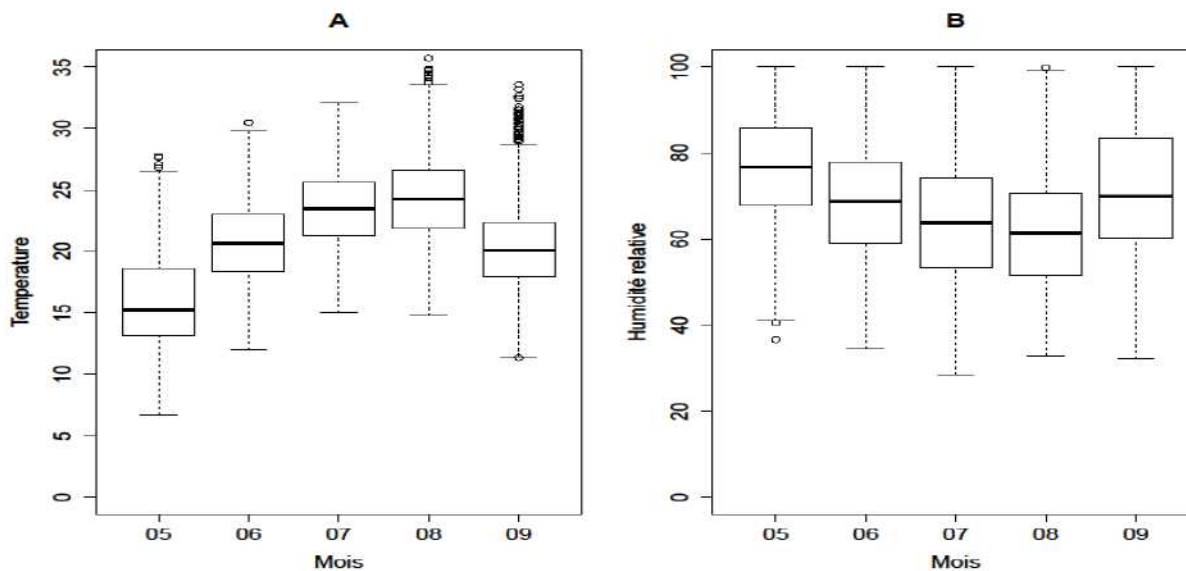


Figure 17 : Boxplot montrant les variabilités de température (A) et d'humidité relative (B) par mois. Le boxplot (ou boîte à moustache) représente une distribution des valeurs avec la médiane (trait épais), une boîte s'étendant du quartile 0,25 au quartile 0,75 et un intervalle de confiance de 95% pour la médiane.

Au sein de chaque mois, les différences de température et d'humidité relative ont été testées en fonction du versant, de l'altitude et de la station. Nous obtenons les résultats suivants :

- Une différence significative de la température par versant pour les mois de juin, août et septembre (test de Wilcoxon-Mann-Whitney, p -value < 0,05 pour chacun de ces mois) ainsi que de l'humidité relative pour chaque mois (test de Wilcoxon-Mann-Whitney, p -value < 0,05, pour mai, juin, juillet, août et septembre).
- Une différence significative de la température par altitude (p -value < 0,05, pour chaque mois) ainsi que pour l'humidité relative pour les mois de juin, juillet, août et septembre (p -value < 0,05, pour chacun de ces mois). Les résultats des tests post-hoc sont synthétisés dans le tableau 4.
- Et enfin, une différence significative de température et d'humidité relative par station (p -value < 0,05, pour chaque mois et chaque variable). Les résultats des tests post-hoc sont synthétisés dans l'annexe 1.

Pour les analyses de présence / abondance des plébotomes, ces données ont ensuite été regroupées par période de 3 jours correspondant aux dates de capture sur le terrain.

Tableau 4 : Significativité des tests post-hoc (tests de Mann-Whitney avec correction de Bonferroni) réalisés sur la température et l'humidité relative par altitude et par mois (*: significatif, NS: Non Significatif).

Mois	Altitude	Température				Humidité relative			
		A	B	C	D	A	B	C	D
Mai	B	NA							
	C	NA	*						
	D	NA	NS	NS					
	E	NA	NS	*	*				
Juin	B	NS				NS			
	C	*	*			NS	*		
	D	*	NS	NS		NS	NS	NS	
	E	NS	NS	*	*	NS	NS	NS	NS
Juillet	B	NS				*			
	C	*	*			*	*		
	D	*	*	*		NS	*	*	
	E	NS	NS	*	*	*	*	NS	*
Août	B	*				*			
	C	*	*			*	*		
	D	*	*	NS		*	NS	*	
	E	*	NS	*	*	*	*	NS	NS
Septembre	B	*				NS			
	C	*	*			NS	NS		
	D	*	*	NS		NS	*	*	
	E	*	NS	*	*	NS	NS	NS	*

Aucune information n'étant disponible pour l'année 1977, nous n'avons pas pu comparer les données climatiques de nos trois années d'étude avec celles de la période de collecte effectuée par Rioux et al. [1].

En conclusion, ces analyses nous ont permis premièrement de comparer les données climatiques entre nos trois années d'études et deuxièmement d'obtenir des détails précis sur la variabilité de température et d'humidité relative par mois, versant, altitude et station dans notre zone d'étude.

Il est important de noter que nous n'avons obtenu aucune différence significative entre nos trois années de collecte. Ces résultats soulignent que les variations dans l'organisation des populations de phlébotomes entre les 3 années, si elles existent, ne pourront pas être expliquées par les facteurs climatiques. Cependant, comme attendu les différences de température et d'humidité relative sont très significatives entre chaque mois. Des différences également importantes sont observées entre les deux versants de notre zone d'étude pour la plupart des mois, mis à part le mois de juillet. Il existe plus de différences climatiques (température et humidité relative) entre les groupes d'altitude durant le mois d'août et entre les stations durant les mois d'août et septembre (par ordre décroissant septembre, août, juillet, juin et mai pour la température et août, septembre, juillet, mai et juin pour l'humidité relative). En effet, en mai il existe peu de différences climatiques entre les stations, elles sont beaucoup plus différenciées en juillet, comme par exemple pour la station 14 (Annexe 1).

L'hypothèse de travail est que ces fluctuations climatiques ont un impact sur les populations de phlébotomes en créant des microclimats propres au développement de ces populations de manière localisée, comme l'avait suspecté Rioux et al. [1]. Dans le prochain chapitre, nous explorons l'influence de ces différences sur la présence, l'abondance, la densité et l'organisation des populations de phlébotomes d'un point de vue spatio-temporel le long du transect de notre région d'étude (Voir chapitre 4 - Impact de l'écosystème sur l'organisation spatio-temporelle des populations de phlébotomes).

IV. Impact de l'écosystème sur l'organisation spatio-temporelle des populations de phlébotomes

Cette étude est détaillée dans l'article en révision dans le journal « *Parasites & Vectors* » : « **Prudhomme J., Rahola N., Toty C., Cassan C., Roiz D., Vergnes B., Thierry M., Rioux J-A, Alten B.*, Sereno D.*, Bañuls A-L*.** (submitted). *Ecology and spatiotemporal dynamics of sandflies in the Mediterranean Languedoc region (Roquedur area, Gard, France).* »

IV.1. Résumé

La région des Cévennes est sous l'influence d'un climat méditerranéen et présente des conditions topographiques et climatiques favorisant la présence de phlébotomes. Dans cette région, les phlébotomes sont présents de mai à octobre avec un pic d'abondance atteint en juillet-août lorsque les températures moyennes sont au maximum.

Les variables climatiques, sont des facteurs importants pouvant fortement influencer les populations de phlébotomes et la transmission des leishmanioses. Ces facteurs joueraient un rôle important non seulement au moment de la capture des adultes mais aussi, et surtout, à la ponte et durant le développement larvaire. Rioux et al. [42] ont souligné une augmentation de 2°C entre 1946 et 2004 dans la station météorologique de Montpellier-Fréjorgues, située à environ 60 km de notre zone d'étude. Cette étude montre l'importance du suivi des populations de vecteurs, de la surveillance des changements climatiques et des évolutions de l'environnement socio-culturels pour comprendre l'évolution de ces populations. De plus, l'expansion actuelle de l'aire de distribution des leishmanioses en France souligne la nécessité d'accroître les connaissances sur ces insectes.

Dans ce contexte, l'objectif de ce travail était d'étudier la dynamique spatio-temporelle des populations de phlébotomes et de déterminer les variables environnementales qui influencent cette dynamique (température, humidité relative, altitude, etc.). Pour ce faire, nous avons réalisé une enquête entomologique sur 3 ans (2011-2013) dans notre zone d'étude. La distribution des espèces aux niveaux spatial et temporel, leur densité et abondance ont ensuite été comparées avec les données collectées en 1977, les données climatiques (température, humidité relative) et environnementales (altitude, versant, orientation des murs).

Durant cette étude, 4 espèces de phlébotomes ont été recensées : *Phlebotomus ariasi*, *Sergentomyia minuta* (les deux espèces prédominantes), *P. perniciosus* et *P. mascittii*. Leurs activités saisonnières s'étendaient de mai à octobre avec des pics en juillet-août quand la température moyenne est optimale. Nous avons montré que la présence et l'abondance des phlébotomes sont fortement influencées par l'altitude. En dehors de l'altitude, la température et l'humidité jouent aussi un rôle majeur. Une température élevée et une humidité relative basse sont les conditions idéales de capture. L'orientation de la zone d'étude (le versant) et l'orientation des murs ont eux aussi un impact significatif sur la présence / abondance des phlébotomes. Cette étude a permis de mettre en évidence des différences interspécifiques et les effets non-linéaires des variables climatiques sur l'abondance des phlébotomes. De plus, bien que l'environnement ait considérablement changé en 30 ans, aucune différence

significative dans la distribution spatiale et la dynamique saisonnière des espèces de phlébotomes et leurs proportions relatives n'a été constatée. Ces résultats démontrent la stabilité des populations de phlébotomes au court du temps et suggèrent une grande capacité d'adaptation de ces insectes, à court et long terme, dans des écosystèmes changeants.

IV.2. Article 4 : Ecology and spatiotemporal dynamics of sandflies in the Mediterranean Languedoc region (Roquedur area, Gard, France).

1 **Ecology and spatiotemporal dynamics of sandflies in the**
2 **Mediterranean Languedoc region (Roquedur area, Gard, France)**

3
4
5
6
7
8
9
10
11 Jorian PRUDHOMME^{1§} [jorian.prudhomme@hotmail.fr], Nil RAHOLA¹ [nil.rahola@ird.fr], Céline TOTY¹
12 [celine.toty@ird.fr], Cécile CASSAN¹ [cecile.cassan@ird.fr], David ROIZ¹ [davidroiz@gmail.com], Baptiste
13 VERGNES¹ [baptiste.vergnes@ird.fr], Magali THIERRY¹ [magalithierry@wanadoo.fr], Jean-Antoine RIOUX²
14 [j.a.rioux@wanadoo.fr], Bulent ALTEN^{3*} [kaynas@hacettepe.edu.tr], Denis SERENO^{4*} [denis.sereno@ird.fr]
15 and Anne-Laure BAÑULS^{1*} [anne-laure.banuls@ird.fr].
16
17
18
19
20

21
22
23 1. UMR MIVEGEC (IRD 224 - CNRS 5290 – Université Montpellier), Centre IRD, F34394 Montpellier,
24 France.
25

26
27 2. Faculté de Médecine, Université Montpellier, 2 rue Ecole de médecine, 34000 Montpellier, France
28

29 3. Hacettepe University, Faculty of Science, Department of Biology, Ecology Section, ESRL Laboratories, 0680
30 Beytepe, Ankara, Turkey. 4. UMR INTERTRYP (IRD - CIRAD 177), Centre IRD, F34394 Montpellier, France.
31
32

33
34
35 17 * These authors contributed equally to the work
36

37 18 § Correspondence: Jorian Prudhomme; E-mail: jorian.prudhomme@hotmail.fr
38

39 19 IRD (Institut de Recherche pour le Développement)
40

41 20 911 Avenue Agropolis, 34394 Montpellier
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

21 Abstract

22 **Background:** Phlebotomine sandflies are hematophagous insects widely present in Western
23 Mediterranean countries and known for their role as *Leishmania* vectors. During the last ten
24 years, the risk of leishmaniasis re-emergence has increased in France. However, sandfly
25 biology and ecology in the South of France remain poorly known because the last detailed
26 study on their spatiotemporal dynamics was performed over 30 years ago. The aim of the
27 present study was to update our knowledge on sandfly ecology by determining their
28 spatiotemporal dynamics and by investigating the relationship between
29 environmental/climatic factors and the presence and abundance of sandflies in the South of
30 France.

31 **Methods:** An entomological survey was carried out during three years (2011-2013) along a
32 14 kilometer-long transect. The findings were compared with the data collected along the
33 same transect in 1977. Data loggers were placed in each station and programmed to record
34 temperature and relative humidity every six hours between April 2011 and November 2014.
35 Several environmental factors (such as altitude, slope and wall orientation) were characterized
36 at each station.

37 **Results:** Four sandfly species were collected: *Phlebotomus ariasi* and *Sergentomyia minuta*,
38 which were predominant, *P. perniciosus* and *P. mascittii*. Sandfly activity within the studied
39 area started in May and ended in October with peaks in July-August at the optimum average
40 temperature. We found a positive effect of altitude and temperature and a negative effect of
41 relative humidity on *P. ariasi* and *S. minuta* presence. We detected interspecific differences
42 and non-linear effects of these climatic variables on sandfly abundance. Although the
43 environment has considerably changed in 30 years, no significant difference in sandfly
44 dynamics and species diversity was found by comparing the 1977 and 2011-2013 data.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45 **Conclusion:** Our study shows that this area maintains a rich sandfly fauna with high *P. ariasi*
46 population density during the active season. This represents a risk for *Leishmania*
47 transmission. The analysis revealed that the presence and abundance of *P. ariasi* and *S.*
48 *minuta* were differently correlated with the environmental and climatic factors. Comparison
49 with the data collected in 1977 highlighted the sandfly population stability, suggesting that
50 they can adapt, in the short and long term, to changing ecosystems.

51

52 **Keywords:** *Sandflies, South of France, Ecology, Spatiotemporal dynamics*

53 Background

54 Phlebotomine sandflies (Diptera, Psychodidae) are hematophagous insects generally
55 active at dusk and during the night. They are abundant in peri-urban and rural environments,
56 often close to human and domestic animal populations. Although it is thought that sandflies
57 do not fly far away from their breeding sites, one species (*P. ariasi*) can move further than 1
58 km [1]. Sandflies are present in temperate regions during the summer and in tropical regions
59 throughout the year [2]. They are widely distributed in Western Mediterranean countries [3-
60 5]. In France, five sandfly species have been described so far: *Phlebotomus ariasi* Tonnoir,
61 1921, *P. perniciosus* Newstead, 1911, *P. mascittii* Grassi, 1908, *P. sergenti* Parrot, 1917, *P.*
62 *papatasi* (Scopoli, 1786) (Corsica) and *Sergentomyia minuta* (Rondani, 1843).

63 *Phlebotomus* species are known for their role as vectors of medically important
64 pathogens, such as the parasitic protozoa of the genus *Leishmania* (the causative agents of
65 leishmaniasis) [6], the *Bartonella bacilliformis* bacterium [6] and arthropod-borne viruses
66 (Phlebovirus) recognized as human pathogens (Toscana virus, Naples virus and Sicilian
67 virus)[7, 8]. In the Languedoc region, *P. ariasi* is the main proven vector of *Leishmania*
68 *infantum*, while *P. perniciosus* is the second one [9, 10].

69 During the last 10 years, the risk of emergence or re-emergence of leishmaniasis and
70 *Phlebovirus* has considerably increased in France [11, 12]. On the other hand, the last detailed
71 study on the spatiotemporal dynamics of sandflies in the South of France was carried more
72 than 30 years ago [10]. Climatic variables, especially temperature, are important factors for
73 the distribution of pathogens and their vectors. These relationships must be dissected in the
74 context of the current climate change. For instance, at the meteorological station of
75 Montpellier-Fréjorgues, located about 60 km from our study area, an increase of 1.895°C (\pm
76 0.068) in the mean annual temperature between 1946 and 2004 was observed [13]. This study
77 highlights the importance of climate change and the effect of environmental modifications on

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

78 the dynamics and changes in sandfly populations. Furthermore, the current increase of
79 leishmaniasis distribution in France underlines the necessity to improve our knowledge about
80 the ecology of these insect vectors. Therefore, the objective of this work was to investigate the
81 ecology and spatiotemporal dynamics of sandfly populations and especially of the two main
82 species encountered in the study area: *P. ariasi*, the main vector of *L. infantum*, and *S. minuta*,
83 not involved in *L. infantum* transmission. First, we assessed the effect of climatic and
84 environmental factors on the dynamics of these two species that are characterized by different
85 ecology and life history traits. We then compared our results with those of the study by Rioux
86 et al. [10] to identify modifications in sandfly seasonal dynamics and abundance after 30
87 years.

88

89 Methods

90 **Study area**

91 The field study was performed in the South of France, on the “massif de l’Oiselette”
92 upland between the “Hérault” (Ganges, Hérault) and “Arre” (Le Vigan, Gard) valleys along a
93 14 km transect that links the villages of Saint-Julien-de-la-Nef and le Vigan, including
94 Roquedur-le-Haut (at 601m above sea level) (Fig. 1). This transect is characterized by a
95 succession of rural and semi-rural environments along a road. The weather conditions are
96 those typical of the Mediterranean sub-humid climate [14] and the area is characterized by the
97 presence of “Garrigue” vegetation, such as *Quercus ilex* and *Quercus pubescens*.

98 The “massif de l’Oiselette” area was chosen because a first spatiotemporal study on
99 sandfly was conducted by Rioux et al. [10] in the same geographical region between May and
100 October 1977. Twenty sampling stations were selected (Table 1) of which 14 were in
101 common with the previous study. This allowed comparing the data on sandfly densities
102 collected at different altitudes on the two opposite sides of the massif in 1977, 2011, 2012 and

103 2013. Moreover, human and canine leishmaniasis caused by *L. infantum* is endemic in this
104 area [15], and during the current study, several cases of canine leishmaniasis were observed
105 (unpublished data).

106 A variety of domestic animals that are potential sandfly hosts (chickens, sheep, ducks,
107 geese, horses, rabbits, cats and dogs) are present in this area. Some sampling stations were
108 located in rural areas because various wild animals could also be sandfly hosts [16].

109

110 **Sandfly collection and identification**

111 Sandflies were collected using CDC miniature light traps (LT) (John W. Hock Co. FL,
112 U.S.A.) and sticky traps (ST) (20 x 20 cm sheets of white paper soaked with castor oil)[17]
113 each month, between May and November, in 2011, 2012 and 2013. Nineteen stations were
114 sampled in 2011 and an additional one (station 8) was added in 2012 and 2013 (Table 1).

115 In 14 sampling sites (Table 1), one or two LT were set up (inside and/or outside houses,
116 animal barns, etc.) and were operated between 18:00 pm and 08:00 am for two nights. Over
117 three years, a total of 329 LT were set up during 649 nights of trapping. In 18 stations (Table
118 1), a total of 12,196 ST (mean: 230 ST per station) were placed in various biotopes, inside and
119 around human dwellings and animal housing, close to the vegetation and inside crevices in the
120 walls. The ST were collected after two nights.

121 Captured specimens were transferred individually into 1.5 ml Eppendorf tubes with
122 90% ethanol and labeled. Prior to mounting, the sandfly head, genitalia and wings were
123 removed. Wings and body were stored separately for future geometric morphometric and
124 genetic analysis, respectively. The head and genitalia were cleared in Marc-André solution
125 (chloral hydrate/acetic acid) and mounted in chloral gum [2]. Specimen identification was
126 individually verified based on the morphology of the pharynxes and/or the male genitalia or

127 female spermathecae, as described by Abonnenc [2], Lewis [3] and Killick-Kendrick et al.
128 [18].

129 In 1977, 4927 ST, changed every 15 days, were placed in 22 stations between May and
130 October [10]. The environment of the studied area has considerably changed in 30 years.
131 According to the data from the French “Institut National de la Statistique et des Etudes
132 Economique” (INSEE), the human population and the number of houses in this area have
133 significantly increased over the years, whereas the numbers of farms has decreased (Table
134 2). To compare the current and the 1977 ST data, sampling stations were grouped, based on
135 the station altitude (from 100 m to 606 m) and the slope, in five different groups (Table 1),
136 according to the classification used by Rioux et al. [10], 1) Hérault valley (100-300m), 2)
137 Mid-slope/South side (300-400m), 3) Summit (500-600m), 4) Mid-slope/North side (300-
138 500m) and 5) Arre valley (200-300m).

140 **Collection of climatic data**

141 To investigate whether variations in local temperature and relative humidity could affect
142 sandfly presence or abundance, 20 temperature/humidity data loggers (iButton hygchron,
143 DS 1923) were placed at each station in areas close to the traps. They were programmed to
144 record temperature (°C) and relative humidity (RH%) every six hours from April 2011 to
145 November 2014. Analyses were performed using the data recorded during each trapping
146 period (three days). The mean/minimum/maximum temperature and the
147 mean/minimum/maximum relative humidity were then computed. At each location, a
148 handheld GPS (Magellan Triton 2000) was used to record the coordinates. Results were
149 transferred to the ArcGIS v9.3 GIS software to produce a map (Fig. 1). Data were collected
150 on the habitat, wall orientation, slope (summit and South or North side) and site
151 characteristics. No climatic data were available for 1977.

152

1
2 **153 Data analysis**
3

4
5 **154** Analyses were performed separately for ST and LT to determine the relation between
6
7 **155** the presence/abundance of sandflies and different parameters (such as altitude, slope, wall
8
9 **156** orientation, mean/minimum/maximum temperature and mean/minimum/maximum relative
10
11 **157** humidity). Sandfly density was calculated as the number of sandflies per m² for ST and as the
12
13 **158** number of sandflies by trap per night for LT. Data exploration was based on the protocol
14
15 **159** described by Zuur et al. [19].
16
17

18
19 **160** The effects of the environmental and climatic variables on the presence of the different
20
21 **161** sandfly species were analyzed using the Generalized Linear Model (GLM) with binomial
22
23 **162** distribution. The effects of the environmental and climatic variables on the abundance of the
24
25 **163** various sandfly species were analyzed using Generalized Additive Models (GAMs) to detect
26
27 **164** non-linear relationships. As the data showed a significant over-dispersion, the model was
28
29 **165** fitted using a negative binomial error distribution and a logarithm link [20]. The explanatory
30
31 **166** variables were: altitude, slope, wall orientation, mean/minimum/maximum temperature and
32
33 **167** mean/minimum/maximum relative humidity. Model selection was performed using the
34
35 **168** Akaike information criterion for each variable and a forward stepwise model selection
36
37 **169** procedure [21]. Validation was performed based on Zuur et al. [22]. Statistical analyses were
38
39 **170** performed using the R statistical package, version 3.1.2 [23], with the packages mgcv, mass,
40
41 **171** gam, lattice and MuMin among others.
42
43
44
45
46
47

48
49 **172** These analyses were only performed on *P. ariasi* and on *S. minuta*. It was not relevant
50
51 **173** to include *P. perniciosus* and *P. mascittii* due to the low number of collected specimens.
52

53
54 **174** The temperature effects on the estimates of inter-annual average sandfly abundance
55
56 **175** (2011-2013) for *P. ariasi* and *S. minuta* were analyzed using GAMs with negative binomial
57
58 **176** distribution, as described above. We assumed that a three-year survey was long enough to
59
60
61
62
63
64
65

177 bring informative results about the relationships between annual climatic variables and annual
178 sandfly abundance (*P. ariasi* and *S. minuta*).

179

180 Results

181 **Sandfly fauna**

182 Overall, 15,488 specimens (7949 males and 7539 females) belonging to four sandfly
183 species were collected in the 20 stations over three years (Table 3). As expected, *P. ariasi*
184 (93.23%) was the predominant species in the study area, whereas *S. minuta* (6.18%), *P.*
185 *perniciosus* (0.48%) and *P. mascittii* (0.11%) were less abundant.

186 During the three years, the distribution of each sandfly species in the different stations
187 did not change significantly (Kruskal-Wallis test, p -value = 0.5706, p -value = 0.131, p -value
188 = 0.815, p -value = 0.7715 for *P. ariasi*, *P. perniciosus*, *P. mascittii* and *S. minuta* captured by
189 ST: and p -value = 0.6487, p -value = 0.6411, p -value = 0.2282, p -value = 0.9989 for *P. ariasi*,
190 *P. perniciosus*, *P. mascittii* and *S. minuta* captured by LT, respectively). *P. ariasi* and *P.*
191 *perniciosus* were present in almost all sampling sites, while *S. minuta* was more abundant in
192 rural biotopes (especially, stations 5, 12, 13, 15 and 17).

193

194 **Seasonal dynamics**

195 Overall, *P. ariasi* was the predominant species during the active season. *S. minuta* was
196 also continuously present during the season, although it was less abundant compared with *P.*
197 *ariasi*. Conversely, *P. perniciosus* and *P. mascittii* were rare throughout the studied period
198 (Fig.2).

199 There was a difference in terms of density peaks between trap types. The mean density
200 (based on ST captures) for all sandfly species was highest in July during the first two years.
201 However, in 2013, *P. ariasi* and *S. minuta* were slightly more abundant in August, *P.*

202 *perniciosus* in June and *P. mascittii* in July (Fig. 2A and B). Based on the LT capture data, *P.*
203 *ariasi* and *P. perniciosus* showed a peak of density in July, while *P. mascittii* was more
204 abundant in August (Fig. 2C and D). Nevertheless, the monthly density of the four considered
205 species did not significantly change during the three years of study (Kruskal-Wallis test, *p*-
206 value = 0.9362, *p*-value = 0.8196, *p*-value = 0.8049, *p*-value = 0.9674 for *P. ariasi*, *P.*
207 *perniciosus*, *P. mascittii* and *S. minuta* captured by ST; and *p*-value = 0.9511, *p*-value =
208 0.9957, *p*-value = 0.3204, *p*-value = 0.7226 for *P. ariasi*, *P. perniciosus*, *P. mascittii* and *S.*
209 *minuta* captured with LT, respectively).

211 Altitude distribution

212 *P. ariasi* and *S. minuta* were found at each altitude interval, with a peak of abundance at
213 mid slope (300-400m) for *P. ariasi* and at the summit (500-600m) for *S. minuta* (Table 4). A
214 significant positive relationship was found between the presence (based on LT and ST
215 captures) of *P. ariasi* (*p*-value < 0.001) and *S. minuta* (*p*-value < 0.001) and the altitude (Figs.
216 3A and 3E). The abundance of these species, evaluated by ST captures, was also positively
217 correlated with altitude (*p*-value < 0.0001 for both species). *P. ariasi* abundance increased
218 until it reached a plateau between 400 m and 500 m and after 500 m started to decrease (Fig.
219 4A). *S. minuta* abundance reached a plateau at 500 m (Fig. 5A).

221 Effect of climatic conditions

222 Based on the analysis of the data collected by the temperature/humidity data loggers, no
223 significant difference in temperature and relative humidity was observed between years
224 (Kruskal-Wallis test, *p*-value = 0.9535 for temperature and *p*-value = 0.7413 for relative
225 humidity) (Table 5). We found a positive relationship between the presence (LT and ST) of *P.*
226 *ariasi* and *S. minuta* and the mean temperature (*p*-value < 0.0001, for the two species) (Figs.

227 3B and 3F) and a significant negative relationship between the presence of *P. ariasi* and *S.*
228 *minuta* and the relative humidity (p -value < 0.0001 , for both species) (Figs. 3C and 3G).

229 The abundance of *P. ariasi*, based on the ST captures, tended to be negatively correlated
230 with the maximum relative humidity during the day (p -value = 0.0533) (Fig. 4C). On the
231 other hand, *S. minuta* abundance was negatively correlated with the minimum relative
232 humidity during the night and the maximum relative humidity during the day (p -value $<$
233 0.0001 for minimum and p -value = 0.0172 for maximum relative humidity) (Figs. 5B and
234 5C).

235 We observed a positive relationship between the abundance (ST captures) of these two
236 species and temperature. *P. ariasi* abundance increased with the temperature until 35°C and
237 above this temperature, it started to decrease (p -value < 0.0001 and p -value < 0.001 for
238 minimum (night) and maximum (day) temperature, respectively) (Fig. 4D). *S. minuta*
239 abundance also increased with the temperature (p -value = 0.0292 and p -value = 0.00304 for
240 minimum (night) and maximum (day) temperature respectively) (Figs. 5D and 5E).

241

242 **Effect of slope and wall orientation**

243 In 1980, Rioux *et al.* suspected an effect of the slope to explain the altitude distribution
244 of sandfly populations along this transect. For *P. ariasi* and *S. minuta*, no significant
245 correlation between their presence (ST and LT captures) and slope or wall orientation was
246 found (p -value = 0.23124 and p -value = 0.0947 for slopes, p -value = 0.145668 and p -value =
247 0.7153 for wall orientation for *P. ariasi* and *S. minuta*, respectively) (Figs. 3D and 3H).

248 However, a significant correlation between abundance (based on ST captures) of *P.*
249 *ariasi* and wall orientation was found (p -value = 0.018) as well as a significant correlation
250 between *S. minuta* and slope (p -value = 0.0158) and wall orientation (p -value < 0.001) (Figs.
251 4F, 5F and 5G).

252

253 **Inter-annual relationship of temperature and *P. ariasi* abundance**

254 The abundance of *P. ariasi* was related to the annual mean/minimum/maximum
255 temperature (p -value < 0.0001 for all three) (Figs. 6A, B and C). *P. ariasi* abundance
256 increased with the annual mean temperature until 20°C and above this temperature it
257 decreased. *S. minuta* abundance also increased with the annual mean/minimum/maximum
258 temperature, but without any limiting temperature (p -value = 0.00146, p -value = 0.0551 and
259 p -value = 0.004, respectively) (Figs. 6D, E and F).

260

261 **Comparison with the data collected in 1977**

262 In 1977, 5043 individuals (4215 males and 828 females) were captured by Rioux *et al.*
263 (1980). Their abundance was comparable to that of the current study: *P. ariasi* (86.36%), *S.*
264 *minuta* (13.27%), *P. perniciosus* (0.26%) and *P. mascittii* (0.12%) (Kruskal-Wallis test, p -
265 value = 0.9899).

266 When we compared the data from the 14 stations in common between the two studies
267 (Table 1), the same peaks of density for *P. ariasi* (at mid slope) and *S. minuta* (at the top)
268 were observed (Table 6).

269 As no climatic record was available for 1977, we could not compare these data.

270

271 **Discussion**

272 For this study, two different trap types were used (LT and ST) because they provide
273 complementary information on the sandfly host-seeking and resting populations. LT
274 preferentially target highly phototropic species [2], and allow capturing large numbers of
275 samples and measuring the relative changes in abundance over time and space [24].
276 Moreover, LT are useful to determine the seasonal activity because they capture active

277 sandflies. On the other hand, ST are not expensive, can be easily produced in large numbers
278 and are a method of capture by interception rather than attraction. Therefore, they provide
279 data on resting individuals [9]. Finally, some sandfly species, such as *S. minuta* in Southern
280 France, are not attracted by light traps.

281 In the present study, three *Phlebotomus* and one *Sergentomyia* species were identified
282 in the Roquedur area, among which *P. ariasi* and *P. perniciosus*, the two major known vectors
283 of leishmaniasis in France. Their spatiotemporal distribution (month/station) did not
284 significantly change during the study period. Similar to previous studies [10, 13], *P. ariasi*
285 was the predominant species (93.23%) and *S. minuta* was the second most abundant one in the
286 study area (6.18%). *P. perniciosus*, which accounted for only 0.48% of captures in the study
287 area, is fairly abundant in the Provence region [25]. In agreement with previous data [26], *P.*
288 *mascittii* represented a very small proportion of the total sandfly captures (0.11%). This low
289 density may be due to a specific ecological niche for this species (such as caves or tunnels)
290 [10, 27, 28].

291 Sandfly activity in the region started in May and ended in October, with a peak in
292 July-August when the average temperature was optimal for sandflies (between 20 and 30°C).
293 *P. ariasi* and *P. perniciosus* were abundant in anthropic biotopes. Conversely, *S. minuta* was
294 more frequently captured in “semi-rural” stations. This difference could be possibly explained
295 by their trophic preferences: mammals and avian species (especially domestic species such as
296 dog, sheep and chicken) for *P. ariasi* and *P. perniciosus*, and reptiles for *S. minuta* [2, 29].

297 Sandfly presence/abundance was significantly influenced by altitude. Nevertheless, *P.*
298 *ariasi* was collected at almost all altitudes with a peak of abundance in mid-slope areas. Its
299 highest densities were recorded between 400 and 500 m of altitude, although this species can
300 be found >800 m above sea level and also in subalpine and mountain climates [9, 30]. *S.*
301 *minuta* was present at all altitudes with the highest total number at the summit (500-600 m).

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

302 Similar results were also reported by Rioux et al. [10]. Although altitude is not an ecological
303 factor by itself, sandfly distribution is influenced by environmental biotic and abiotic features
304 related to altitude, such as temperature [31-33]. However, based on the data collected with
305 LT, *P. ariasi* and *P. perniciosus* abundances seem to be also strongly influenced by the host
306 abundance and availability, regardless of the altitude. For example, in the poultry farm located
307 in station 10 (606m), where many hosts were available, more than one thousand individuals
308 were captured with a LT in one night. In contrast, *S. minuta* prefers wild habitats with the
309 highest abundance in “semi-rural” stations situated at the summit.

310 Besides altitude, sandfly presence and abundance were significantly influenced by
311 temperature and relative humidity. Specifically, *P. ariasi* abundance increased with the
312 temperature until 35°C and decreased with the increasing of relative humidity. Similar
313 findings were reported for *P. ariasi* in Spain [30]. This species can be found in a variety of
314 habitats and usually prefers relatively high temperatures with moderate relative humidity.
315 According to the literature, the lowest temperature of activity for *P. ariasi* is around 15 °C
316 [34], its optimal nocturnal temperature ranges between 19 and 21 °C [9] and the maximum
317 temperature is around 30°C [35]. This is in agreement with our findings: lowest temperature
318 of activity around 10°C, optimal nocturnal temperature between 20 and 25°C and maximum
319 temperature around 35°C. In comparison with *P. ariasi*, *S. minuta* shows a higher tolerance to
320 higher temperatures and lower relative humidity. In our study, the lowest temperature for this
321 species was around 20°C, its abundance increased with the temperature and decreased rapidly
322 with a relative humidity higher than 60%. No maximum temperature was detected. Moreover,
323 our findings indicate that the effect of temperature on sandfly abundance is non-linear, and
324 therefore, simplistic approaches about the influence of climate change on sandfly populations
325 must be avoided. Indeed, higher temperatures may have a negative effect on sandfly
326 populations, as described for other vectors, such as mosquitoes [36].

327 Previous studies suspected that slope and wall orientation may have a significant
1
2 328 impact on the presence/abundance of sandflies [10, 37, 38]. In our study, *P. ariasi* and *S.*
3
4 329 *minuta* were more abundant in South-oriented slopes and South-oriented walls. These two
5
6 330 features, which are related to temperature and relative humidity, can be also used as good
7
8
9 331 predictors of sandfly abundance.

11 332 The comparison with the data from the study carried out in the same area in 1977 [10]
12
13 333 allowed investigating the changes in the sandfly population during the last 30 years. The
14
15 334 environment has considerably changed in 30 years (Table 2). The consequences of habitat
16
17 335 transformation by urbanization, housing improvement and reduction of host abundance could
18
19 336 also have affected the sandfly population. Moreover, Rioux et al. [13] reported that the annual
20
21 337 mean temperature has increased over the years. Therefore we expected a change in sandfly
22
23 338 abundance and distribution. However, data comparison did not highlight any significant
24
25 339 difference in the relative abundance and spatiotemporal distribution of the different species
26
27 340 between studies (2011 to 2013 *versus* 1977), despite the different methodologies: ST were left
28
29 341 for 15 days in the study by Rioux et al. [10] and only for two nights in this study. This
30
31 342 suggests that the sandfly population in this area is stable and, therefore, can adapt to
32
33 343 environmental changes. Because of the methodological differences, the densities of sandflies
34
35 344 by m² could not be compared.

36 345 According to Ashford et al. [34], in Mediterranean regions, the temperature of
37
38 346 leishmaniasis foci are generally between 20°C and 30°C in July and between 5°C and 10°C in
39
40 347 January, the optimal temperatures for *P. ariasi* and *P. perniciosus*, the two main leishmaniasis
41
42 348 vectors in the South of France. However, no information about *Leishmania* transmission
43
44 349 during the last 30 years is available. The increase of temperature and the impact of socio-
45
46 350 ecological changes in this region could have modified *Leishmania* transmission.
47
48 351 Environmental, population and individual factors have to be considered together to
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

352 understand epidemiology and transmission ecology of parasites [39]. Rioux et al. [40]
353 determined that in France, an area with a mean sandfly density (based on ST capture) above
354 20 specimens/m² is an area with high leishmaniasis transmission. Our study area (*P. ariasi*
355 maximum density: 40.11, 25.75 and 50.43 specimens/m² in 2011, 2012 and 2013,
356 respectively) can be considered as a zone at risk of leishmaniasis transmission.

358 Conclusions

359 This study provides useful information about the environmental and climatic factors,
360 such as altitude, temperature, relative humidity, slopes and wall orientation, that can affect the
361 presence and abundance of *P. ariasi* and *S. minuta*. Our findings indicate that all these factors
362 must be taken into account in a species-specific analysis of sandflies to evaluate the risk of
363 *Leishmania* transmission. Although the environment has been considerably transformed in our
364 study area in 30 years, sandfly abundance has not significantly changed between 1977 and
365 2011-2013, highlighting the sandfly capacity to adapt, in the short and long term, to the
366 ecosystem modifications.

367 Our findings show that the study area, which is located in the Cévennes range of
368 mountains with favorable topographic and climatic conditions for sandflies, has a rich sandfly
369 population and harbors important vector species, such as *P. ariasi*. The high *P. ariasi*
370 population density throughout the active season represents a risk of *L. infantum* transmission.
371 Moreover, in the context of climate change, it is important to study the optimal conditions for
372 vector species, the non-linear effects of temperature and to collect information on changes of
373 these insect populations. Careful monitoring of environmental variables and their effect on
374 vector biology is a key issue for the implementation of control strategies.

376 Competing interests

377 The authors declare that they have no competing interests.

378

379 Authors' contributions

380 JP, NR, CT, CC, DR, BV, JAR, BA, ALB and DS wrote the manuscript. JP, NR, CT, CC,

381 BV, JAR, BA, ALB and DS carried out the field work. JP, NR, CT, CC, MT and DS carried

382 out the dissections and identifications. JP and DR performed the statistical analysis. BA, DS

383 and ALB designed the study. All authors read and approved the final manuscript.

384

385 Acknowledgments

386 This study was funded by the EU grant FP7-261504 EDENext and is catalogued by the

387 EDENext Steering Committee as EDENextXXX (<http://www.edenext.eu>). The contents of

388 this publication are the sole responsibility of the authors and do not necessarily reflect the

389 views of the European Commission. We are particularly grateful to Mr. Lacoste, IRD (Institut

390 de Recherche pour le Développement) and CNRS for financial support. We also thank

391 Çağışan Karacaoğlu for making the map and Elisabetta Andermacher for assistance in

392 preparing and editing the manuscript.

393

394 References

- 1
2 395 1. Rioux J, Killick-Kendrick R, Leaney A, Turner D, Bailly M, Young C: **Ecologie des Leishmanioses dans le sud de la France. 12. Dispersion horizontale de *Phlebotomus ariasi* Tonnoir, 1921. Experiences preliminaires. *Annales de parasitologie* 1979, 54(6):673-682.**
- 3 396
- 4 397
- 5 398 2. Abonnenc E: **Les phlébotomes de la région éthiopienne (Diptera, Psychodidae).** 1972:1-290.
- 6 399
- 7 399 3. Lewis D: **A taxonomic review of the genus *Phlebotomus* (Diptera: Psychodidae).** *Entomology series* 1982, 45(2):121-209.
- 8 400
- 9 401 4. Franco F, Morillas-Marquez F, Barón S, Morales-Yuste M, Gálvez R, Díaz-Sáez V, Pesson B, Alves-Pires C, Depaquit J, Molina R *et al*: **Genetic structure of *Phlebotomus (Larrousius) ariasi* populations, the vector of *Leishmania infantum* in the western Mediterranean: Epidemiological implications.** *International Journal for Parasitology* 2010, 40(11):1335-1346.
- 10 402
- 11 403
- 12 404
- 13 404 5. Ready P: **Leishmaniasis emergence in Europe.** *Euro surveillance : bulletin Européen sur les maladies transmissibles = European communicable disease bulletin* 2010, 15(10):19505.
- 14 405
- 15 406
- 16 407 6. Dolmatova AV, Demina NA: **Les phlébotomes (Phlebotominae) et les maladies qu'ils transmettent.** 1966, 18:1-169.
- 17 408
- 18 409 7. Bichaud L, Souris M, Mary C, Ninove L, Thirion L, Piarroux R, Piarroux R, Lamballerie X, Charrel R: **Epidemiologic Relationship between Toscana Virus Infection and *Leishmania infantum* Due to Common Exposure to *Phlebotomus perniciosus* Sandfly Vector.** *PLoS Neglected Tropical Diseases* 2011, 5(9):e1328.
- 19 410
- 20 410
- 21 411
- 22 412
- 23 413 8. Bichaud L, Izri A, Lamballerie X, Moureau G, Charrel R: **First detection of Toscana virus in Corsica, France.** *Clinical Microbiology and Infection* 2014, 20(2):O101-O104.
- 24 414
- 25 414 9. Rioux J, Golvan Y, Croset H, Tour S, Hovin R, Abonnenc E, Petitdidier M, Vollhardt Y, Dedet JP, Albaret JL *et al*: **Epidémiologie des leishmanioses dans le Sud de la France.** *Monographie de l'Institut National de la Santé et de la Recherche Médicale* 1969, 37:1-228.
- 26 415
- 27 416
- 28 417
- 29 418 10. Rioux J, Killick-Kendrick R, Perieres J, Turner D, Lanotte G: **Ecologie des Leishmanioses dans le Sud de la France. 13. Les sites de "flanc de coteau", biotopes de transmission privilégiés de la Leishmaniose viscérale en Cévennes.** *Annales de parasitologie* 1980, 55(4):445-453.
- 30 419
- 31 419
- 32 420
- 33 421 11. Dedet J-P: **Les leishmanioses en France métropolitaine.** *BEH Hors-série* 2010:9-12.
- 34 422
- 35 423 12. Depaquit J, Gr M, Grandadam M, adam JP, Fouque F, Andry P-E, Peyrefitte C: **Arthropod-borne viruses transmitted by Phlebotomine sandflies in Europe: a review.** *Euro surveillance : bulletin Européen sur les maladies transmissibles = European communicable disease bulletin* 2010, 15(10):19507.
- 36 424
- 37 424
- 38 425
- 39 426 13. Rioux J, Carron S, Dereure J, Perieres J, Zeraia L, Franquet E, Babinot M, Gallego M, Prudhomme J: **Ecology of leishmaniasis in the South of France. 22. Reliability and representativeness of 12 *Phlebotomus ariasi*, *P. perniciosus* and *Sergentomyia minuta* (Diptera: Psychodidae) sampling stations in Vallespir (eastern French Pyrenees region).** *Parasite (Paris, France)* 2013, 20:34.
- 40 427
- 41 428
- 42 429
- 43 429
- 44 430 14. Daget P: **Le bioclimat Méditerranéen: Analyse des formes climatiques par le système d'Emberger.** *Vegetation* 1977, 34(2):87-103.
- 45 431
- 46 432
- 47 433 15. Lachaud L, Dedet J, Marty P, Faraut F, Buffet P, Gangneux J, Ravel C, Bastien P: **Surveillance of leishmaniasis in France, 1999 to 2012.** *Euro surveillance : bulletin Européen sur les maladies transmissibles = European communicable disease bulletin* 2013, 18(29):20534.
- 48 434
- 49 435
- 50 435
- 51 436 16. Guy M, Killick-Kendrick R, Gill G, Rioux J, Bray R: **Ecology of leishmaniasis in the south of France. 19. Determination of the hosts of *Phlebotomus ariasi* Tonnoir, 1921 in the Cévennes by bloodmeal analyses.** *Annales de parasitologie humaine et comparée* 1984, 59(5):449-458.
- 52 437
- 53 438
- 54 439
- 55 440 17. Alexander B: **Sampling methods for phlebotomine sandflies.** *Medical and Veterinary Entomology* 2000, 14(2):109-122.
- 56 441
- 57 441
- 58 442 18. Killick-Kendrick R, Tang Y, Killick-Kendrick M, Sang D, Sirdar M, Ke L, Ashford R, Schorscher J, Johnson R: **The identification of female sandflies of the subgenus *Larrousius* by the morphology of the spermathecal ducts.** *Parassitologia* 1991, 33:335-347.
- 59 443
- 60 444

- 445 19. Zuur AF, Ieno EN, Elphick CS: **A protocol for data exploration to avoid common statistical**
 1 446 **problems.** *Methods in Ecology and Evolution* 2010, 1:3-14.
- 2 447 20. Hoef J, Boveng P: **Quasi-Poisson vs. negative binomial regression: how should we model**
 3 448 **overdispersed count data?** *Ecology* 2007, 88(11):2766-2772.
- 4 449 21. Crawley MJ: **Statistical computing: an introduction to data analysis using S-Plus:** John Wiley
 5 450 **and Sons %@ 9780471560401; 2002.**
- 6 451 22. Zuur AF, Saveliev AA, Ieno EN: **A beginner's guide to generalised additive mixed models**
 7 452 **with R:** Highland Statistics Limited; 2014.
- 8 453 23. Team RC: **R: A language and environment for statistical computing.** R Foundation for
 9 454 **Statistical Computing, Vienna, Austria, 2012.** ISBN 3-900051-07-0; 2014.
- 10 455 24. Alten B, Ozbel Y, Ergunay K, Kasap O, Cull B, Antoniou M, Velo E, Prudhomme J, Molina R,
 11 456 **Bañuls A et al: Sampling strategies for phlebotomine sand flies (Diptera: Psychodidae) in**
 12 457 **Europe.** *Bulletin of entomological research* 2015:1-15.
- 13 458 25. Peyrefitte CN, Gr M, adam JP, Bessaud M, Andry P-E, Fouque F, Caro V, Diancourt L,
 14 459 **Schuffenecker I, Pagès F et al: Diversity of *Phlebotomus perniciosus* in Provence,**
 15 460 **Southeastern France: Detection of Two Putative New Phlebovirus Sequences.** *Vector-Borne*
 16 461 **and Zoonotic Diseases 2013, 13(9):630-636.**
- 17 462 26. Naucke T, Lorentz S, Rauchenwald F, Aspöck H: ***Phlebotomus (Transphlebotomus) mascittii***
 18 463 **Grassi, 1908, in Carinthia: first record of the occurrence of sandflies in Austria (Diptera:**
 19 464 **Psychodidae: Phlebotominae).** *Parasitology Research* 2011, 109(4):1161-1164.
- 20 465 27. Schmitt C: **Untersuchungen zur Biologie und Verbreitung von *Phlebotomus***
 21 466 **(*Transphlebotomus*) *mascittii*, Grassi 1908 (Diptera: Psychodidae) in Deutschland.**
 22 467 ***Diplomarbeit Inst f Med Parasitol* 2002:1-91.**
- 23 468 28. Naucke T, Menn B, Massberg D, Lorentz S: **Winter activity of *Phlebotomus***
 24 469 **(*Transphlebotomus*) *mascittii*, Grassi 1908 (Diptera: Psychodidae) on the island of Corsica.**
 25 470 ***Parasitology Research* 2008, 103(2):477-479.**
- 26 471 29. Campino L, Cortes S, Dionísio L, Neto L, Afonso MO, Maia C: **The first detection of**
 27 472 ***Leishmania major* in naturally infected *Sergentomyia minuta* in Portugal.** *Memórias do*
 28 473 ***Instituto Oswaldo Cruz* 2013, 108(4):516-518.**
- 29 474 30. Ballart C, Guerrero I, Castells X, Barón S, Castillejo S, Alcover M, Portús M, Gállego M:
 30 475 **Importance of individual analysis of environmental and climatic factors affecting the**
 31 476 **density of *Leishmania* vectors living in the same geographical area: the example of**
 32 477 ***Phlebotomus ariasi* and *P. perniciosus* in northeast Spain.** *Geospatial health* 2014, 8(2):389-
 33 478 403.
- 34 479 31. Guernaoui S, Boumezzough A, Laamrani AE: **Altitudinal structuring of sand flies (Diptera:**
 35 480 **Psychodidae) in the High-Atlas mountains (Morocco) and its relation to the risk of**
 36 481 **leishmaniasis transmission.** *Acta Tropica* 2006, 97(3):346-351.
- 37 482 32. Ozbel Y, Sanjoba C, Alten B, Asada M, Depaquit J, Matsumoto Y, Demir S, Siyambalagoda R,
 38 483 **Rajapakse R, Matsumoto Y: Distribution and ecological aspects of sand fly (Diptera:**
 39 484 **Psychodidae) species in Sri Lanka.** *Journal of Vector Ecology* 2011, 36:S77-S86.
- 40 485 33. Neteler M, Roiz D, Rocchini D, Castellani C, Rizzoli A: **Terra and Aqua satellites track tiger**
 41 486 **mosquito invasion: modelling the potential distribution of *Aedes albopictus* in north-**
 42 487 **eastern Italy.** *International Journal of Health Geographics* 2011, 10(1):49.
- 43 488 34. Ashford R, Bettini S, Peters W, Killick-Kendrick R: **Ecology and epidemiology: Old World. *The***
 44 489 ***leishmaniasis in biology and medicine Volume I Biology and epidemiology* 1987:365-424.**
- 45 490 35. Rioux J, Aboukler JP, Lanotte G, Killick-Kendrick R, Martini-Dumas A: **Ecologie des**
 46 491 **Leishmanioses dans le sud de la France. 21 - Influence de la température sur le**
 47 492 **developpement de *Leishmania infantum* Nicolle, 1908 chez *Phlebotomus ariasi* Tonnoir,**
 48 493 **1921. Etude expérimentale.** *Annales de parasitologie humaine et comparée* 1985, 60(3):221-
 49 494 229.

495 36. Mordecai E, Paaijmans K, Johnson L, Balzer C, Ben-Horin T, Moor E, McNally A, Pawar S, Ryan
1 496 S, Smith T *et al*: **Optimal temperature for malaria transmission is dramatically lower than**
2 497 **previously predicted.** *Ecology Letters* 2013, **16**(1):22-30.

3 498 37. Ostfeld RS, Roy P, Haumaier W, Canter L, Keesing F, Rowton ED: **Sand fly (*Lutzomyia***
4 499 ***vexator*)(Diptera: Psychodidae) populations in upstate New York: abundance,**
5 500 **microhabitat, and phenology.** *Journal of medical entomology* 2004, **41**(4):774-778.

6 501 38. Belen A, Alten B: **Seasonal dynamics and altitudinal distributions of sand fly (Diptera:**
7 502 **Psychodidae) populations in a cutaneous leishmaniasis endemic area of the Cukurova**
8 503 **region of Turkey.** *Journal of Vector Ecology* 2011, **36**:S87-S94.

9 504 39. Giraudoux P, Raoul F, Pleydell D, Craig P: **Multidisciplinary studies, systems approaches and**
10 505 **parasite eco-epidemiology: something old, something new.** *Parasite (Paris, France)* 2008,
11 506 **15**(3):469-476.

12 507 40. Rioux J, Croset H, Lanotte G: **Ecologie d'un foyer Méditerranéen de leishmaniose viscérale.**
13 508 **Essai de modélisation.** *Colloques Internationaux du CNRS* 1977, **239**:295-305.

14 509
15 510
16
17
18
19
20

21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Table 1. Sampling stations in the study area (LT: CDC miniature light traps; ST: Sticky Traps)

Station number	Group**	Coordinates		Altitude (m)	Biotope	Traps
		North	East			
ST01*	1	43.96548	3.686828	175	hamlet	LT + ST
ST02*	1	43.96663	3.685075	228	hamlet	ST
ST03*	1	43.96733	3.683149	244	hamlet	LT + ST
ST04*	2	43.97462	3.675871	321	hamlet / hutch	LT + ST
ST05*	2	43.97595	3.677038	322	rural	ST
ST06	2	43.97687	3.677551	341	kennel	LT + ST
ST07*	2	43.97632	3.675457	354	hamlet	LT + ST
ST08	3	43.97683	3.669611	443	hamlet	ST
ST09	3	43.9746	3.659549	586	poultry farm	LT
ST10	3	43.97416	3.657616	606	poultry farm	LT
ST11*	3	43.97317	3.657214	603	hamlet	LT + ST
ST12*	3	43.97144	3.655944	573	rural	ST
ST13*	3	43.97321	3.650088	539	rural	LT + ST
ST14*	4	43.97235	3.637441	417	rural	ST
ST15	4	43.97495	3.635153	397	rural	LT + ST
ST16*	4	43.97765	3.634949	362	rural	ST
ST17*	5	43.97961	3.627845	343	rural	LT + ST
ST18*	5	43.98478	3.623463	282	rural	LT + ST
ST19	5	43.98409	3.616661	255	hamlet	LT + ST
ST20*	5	43.98392	3.614822	245	hamlet / sheep barn	LT + ST

* Stations present in the study by Rioux et al. [10].

** Groups, according to the classification by Rioux et al. [10]: 1, Hérault valley (100-300m); 2, Mid-slope/South side (300-400m); 3, Summit (500-600m); 4, Mid-slope/North side (300-500m); and 5, Arre valley (200-300m).

Table 2. Main demographic data for Saint-Julien-de-la-Nef, Roquedur-le-Haut and Le Vigan in 1982, 1988, 1990, 1999, 2000 and 2009 (Institut National de la Statistique et des Etudes Economiques). Only these years were available in the INSEE web archive.

Year	Population			Number of houses			Number of farms		
	Saint Julien de la Nef	Roquedur	Le Vigan	Saint Julien de la Nef	Roquedur	Le Vigan	Saint Julien de la Nef	Roquedur	Le Vigan
1982	99	116	4517	/	/	/	/	/	/
1988	/	/	/	/	/	/	11	11	42
1990	140	133	4523	95	105	2557	/	/	/
1999	119	192	4448	94	112	2645	/	/	/
2000	/	/	/	/	/	/	8	6	12
2009	124	218	3959	126	130	2629	/	/	/

Table 3. Number and relative abundance (%) of sandfly species in the study area during the three-year survey (sampling with light and sticky traps). The details of the captures by type of traps are summarized in Table 4.

Year	Species	Number ^a	Females	Males	Percentage (%) ^b
2011	<i>Phlebotomus ariasi</i>	2866	934	1932	90.52
	<i>Sergentomyia minuta</i>	258	109	149	8.15
	<i>Phlebotomus perniciosus</i>	39	10	29	1.23
	<i>Phlebotomus mascittii</i>	3	3	0	0.09
	Total	3166	1056	2110	100
2012	<i>P. ariasi</i>	4508	2830	1678	92.83
	<i>S. minuta</i>	320	116	204	6.59
	<i>P. perniciosus</i>	21	0	21	0.43
	<i>P. mascittii</i>	7	7	0	0.14
	Total	4856	2953	1903	100
2013	<i>P. ariasi</i>	7065	3361	3704	94.63
	<i>S. minuta</i>	379	162	217	5.08
	<i>P. perniciosus</i>	15	1	14	0.20
	<i>P. mascittii</i>	7	6	1	0.09
	Total	7466	3530	3936	100
Total		15488	7539	7949	100

^a Total number of specimens collected by CDC light traps and sticky paper in all sampling stations.

^b Relative abundance: $nx/N * 100$, nx: number of individuals belonging to species x, N: total number of sampled individuals.

Table 4. Density of sandfly species collected at the different altitude ranges during the three-year survey in Roquedur-le-Haut. Higher densities at mid slopes are observed.

Sticky Traps (total density/m²)					
Groups	Year	<i>Phlebotomus ariasi</i>	<i>Sergentomyia minuta</i>	<i>Phlebotomus perniciosus</i>	<i>Phlebotomus mascittii</i>
Hérault valley (100-300m)	2011	3.32	0.00	0.09	0.00
	2012	1.44	0.00	0.00	0.00
	2013	2.85	0.14	0.07	0.06
Mid-slope/South side (300-400m)	2011	40.11	1.24	0.92	0.17
	2012	15.95	2.10	0.58	0.05
	2013	24.04	3.20	0.09	0.06
Summit (500-600m)	2011	26.25	7.79	0.33	0.00
	2012	19.41	6.29	0.09	0.00
	2013	35.46	9.61	0.00	0.00
Mid-slope/North side (300-500m)	2011	38.84	0.90	0.00	0.00
	2012	25.75	1.69	0.00	0.00
	2013	50.43	1.04	0.15	0.00
Arre Valley (200-300m)	2011	9.46	8.26	0.47	0.00
	2012	3.12	1.72	0.00	0.00
	2013	5.44	1.07	0.00	0.00
Light Traps (total density/night/trap)					
Groups	Year	<i>P. ariasi</i>	<i>S. minuta</i>	<i>P. perniciosus</i>	<i>P. mascittii</i>
Hérault valley (100-300m)	2011	0.15	0.00	0.00	0.00
	2012	0.13	0.00	0.00	0.00
	2013	0.36	0.00	0.00	0.00
Mid-slope/South side (300-400m)	2011	2.36	0.00	0.00	0.00
	2012	2.50	0.00	0.01	0.01
	2013	6.93	0.00	0.00	0.03
Summit (500-600m)	2011	1.11	0.00	0.01	0.00
	2012	19.28	0.00	0.01	0.01
	2013	39.71	0.00	0.07	0.01
Mid-slope/North side (300-500m)	2011	0.08	0.00	0.00	0.00
	2012	0.04	0.24	0.00	0.00
	2013	0.28	0.02	0.00	0.00
Arre Valley (200-300m)	2011	3.82	0.00	0.07	0.00
	2012	0.94	0.00	0.00	0.01
	2013	4.81	0.00	0.03	0.00

Table 5. Average/maximum/minimum temperature (°C) and relative humidity (%) during the capture period (May to September) for 2011, 2012 and 2013.

	Temperature (°C)			Relative humidity (%)		
	Average	Maximum	Minimum	Average	Maximum	Minimum
2011	21.3	46.1	2.6	60.1	100	13.8
2012	20.4	46.6	5.6	70.6	100	20.9
2013	19.9	47.6	4.6	69.4	100	25.7

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Table 6. Total density per m² of sandfly species collected in the 14 stations in common with the study by Rioux et al. [10] at the different altitude ranges in the studied area. Higher densities at mid slopes are observed.

Sticky Traps Density/m ² [Percentage (%) ^a]	Year	<i>Phlebotomus ariasi</i>	<i>Sergentomyia minuta</i>	<i>Phlebotomus perniciosus</i>	<i>Phlebotomus mascittii</i>
Hérault valley (100-300m)	1977	5.12 [96.6%]	0.00 [0.0%]	0.18 [3.4%]	0.00 [0.0%]
	2011	3.32 [97.4%]	0.00 [0.0%]	0.09 [2.6%]	0.00 [0.0%]
	2012	1.44 [100%]	0.00 [0.0%]	0.00 [0.0%]	0.00 [0.0%]
	2013	2.85 [91.3%]	0.14 [4.5%]	0.07 [2.2%]	0.06 [1.9%]
Mid-slope/South side (300-400m)	1977	29.84 [86.2%]	4.18 [12.1%]	0.36 [1.0%]	0.22 [0.6%]
	2011	38.50 [94.9%]	1.24 [3.1%]	0.78 [1.9%]	0.04 [0.1%]
	2012	13.93 [84.3%]	2.10 [12.7%]	0.45 [2.7%]	0.05 [0.3%]
	2013	23.29 [87.4%]	3.20 [12.0%]	0.09 [0.3%]	0.06 [0.2%]
Summit (500-600m)	1977	27.59 [85.6%]	4.66 [14.4%]	0.00 [0.0%]	0.00 [0.0%]
	2011	26.25 [76.4%]	7.79 [22.7%]	0.33 [1.0%]	0.00 [0.0%]
	2012	17.11 [72.8%]	6.29 [26.8%]	0.09 [0.4%]	0.00 [0.0%]
	2013	32.70 [77.6%]	9.42 [22.4%]	0.00 [0.0%]	0.00 [0.0%]
Mid-slope/North side (300-500m)	1977	63.65 [94.7%]	3.54 [5.3%]	0.00 [0.0%]	0.00 [0.0%]
	2011	34.52 [99.1%]	0.30 [0.9%]	0.00 [0.0%]	0.00 [0.0%]
	2012	24.29 [98.3%]	0.43 [1.7%]	0.00 [0.0%]	0.00 [0.0%]
	2013	44.14 [99.8%]	0.00 [0.0%]	0.08 [0.2%]	0.00 [0.0%]
Arre valley (200-300m)	1977	19.18 [92.3%]	1.40 [6.7%]	0.00 [0.0%]	0.20 [1.0%]
	2011	7.16 [45.7%]	8.26 [52.7%]	0.25 [1.6%]	0.00 [0.0%]
	2012	2.99 [63.5%]	1.72 [36.5%]	0.00 [0.0%]	0.00 [0.0%]
	2013	5.21 [83.0%]	1.07 [17.0%]	0.00 [0.0%]	0.00 [0.0%]

^aRelative abundance: $n_x/N * 100$, n_x : number of individuals belonging to species x, N: total number of sampled individuals.

Figure legends

Figure 1. Map of the study area. Red circles indicate the sampling stations.

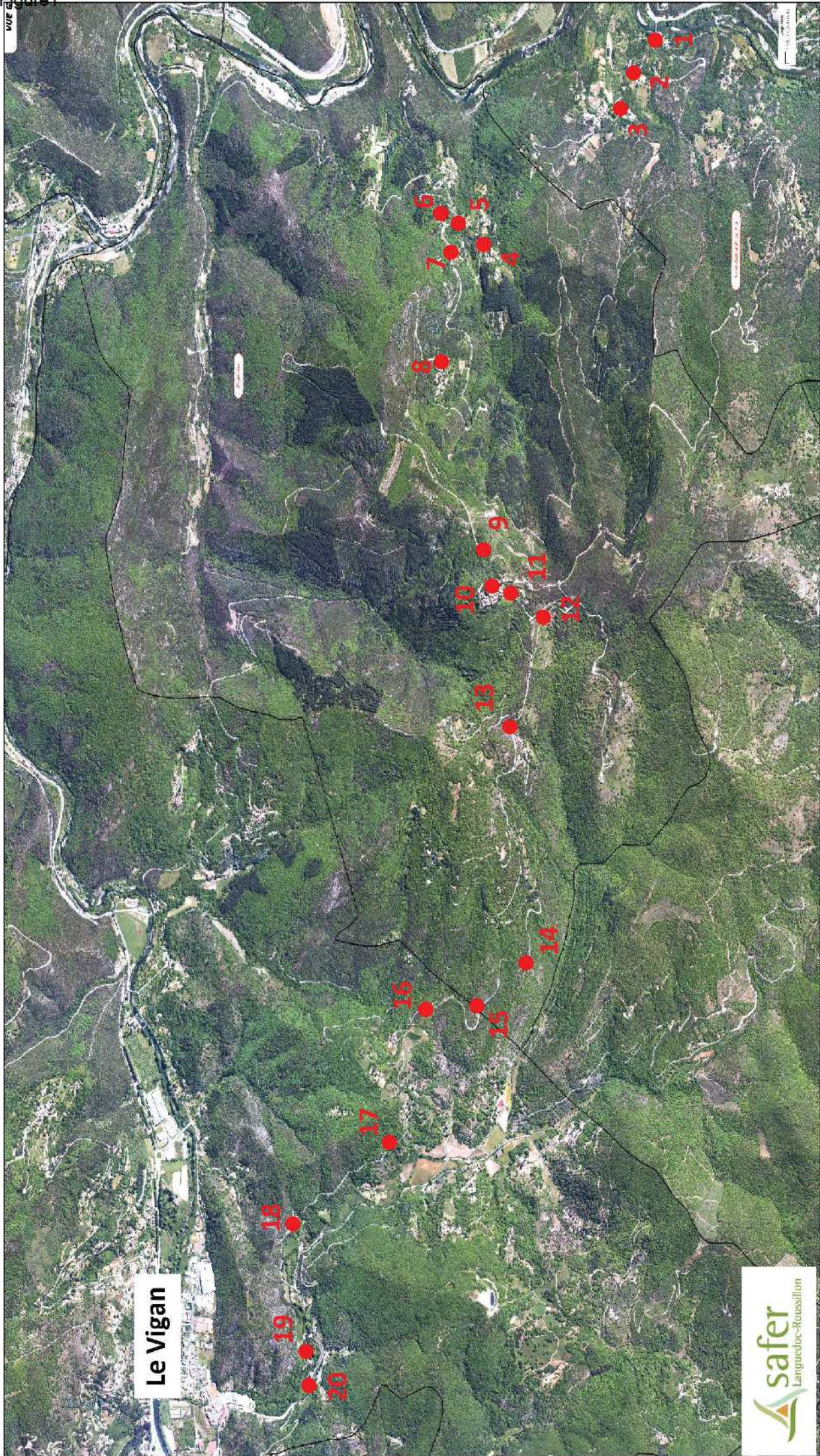
Figure 2. Seasonal dynamics of the sandfly species captured by sticky traps (A and B) and light traps (C and D) in the study area. Red line: *Phlebotomus ariasi*, blue line: *Sergentomyia minuta*, orange line: *P. perniciosus*, green line: *P. mascittii* and black line: maximum peak of density.

Figure 3. Boxplot showing the number of times *Phlebotomus ariasi* (upper histograms) or *Sergentomyia minuta* sandflies were found (presence) or not (absence) in ST and LT traps according to the altitude (A and E), relative humidity (B and F), temperature (C and G) and slope features (D and H).

Figure 4. *Phlebotomus ariasi* density (m^2) relative to altitude (A), minimum relative humidity (B), maximum relative humidity (C), minimum temperature (D), maximum temperature (E) and trap orientation (in degree) with N = North, E = East, S = South and W = West (G). Black dots: field data, lines: selected Generalized Additive Models. Temperature and relative humidity were calculated during the monthly captures by separating the temperature/humidity data collected during the day and during the night.

Figure 5. *Sergentomyia minuta* density (m^2) relative to altitude (A), maximum relative humidity (B), minimum relative humidity (C), minimum temperature (D), maximum temperature (E), slope (F) and trap orientation (in degree) with N = North, E = East, S = South and W = West (G). Black dots: field data, lines: selected Generalized Additive Models. Temperature and relative humidity were calculated during monthly captures by separating the temperature/humidity data collected during the day and during the night. Only the graphics for significant results are shown.

Figure 6. Relationship (Generalized Additive Models) between climatic variables and sandfly density (mean of the three years). *Phlebotomus ariasi* density (m^2) and annual mean temperature (A), annual minimum temperature (B) and annual maximum temperature (C). *Sergentomyia minuta* density (m^2) and annual mean temperature (C), annual minimum temperature (D) and annual maximum temperature (E). In comparison with *P. ariasi*, *S. minuta* shows higher tolerance to higher temperature.



Le Vigan

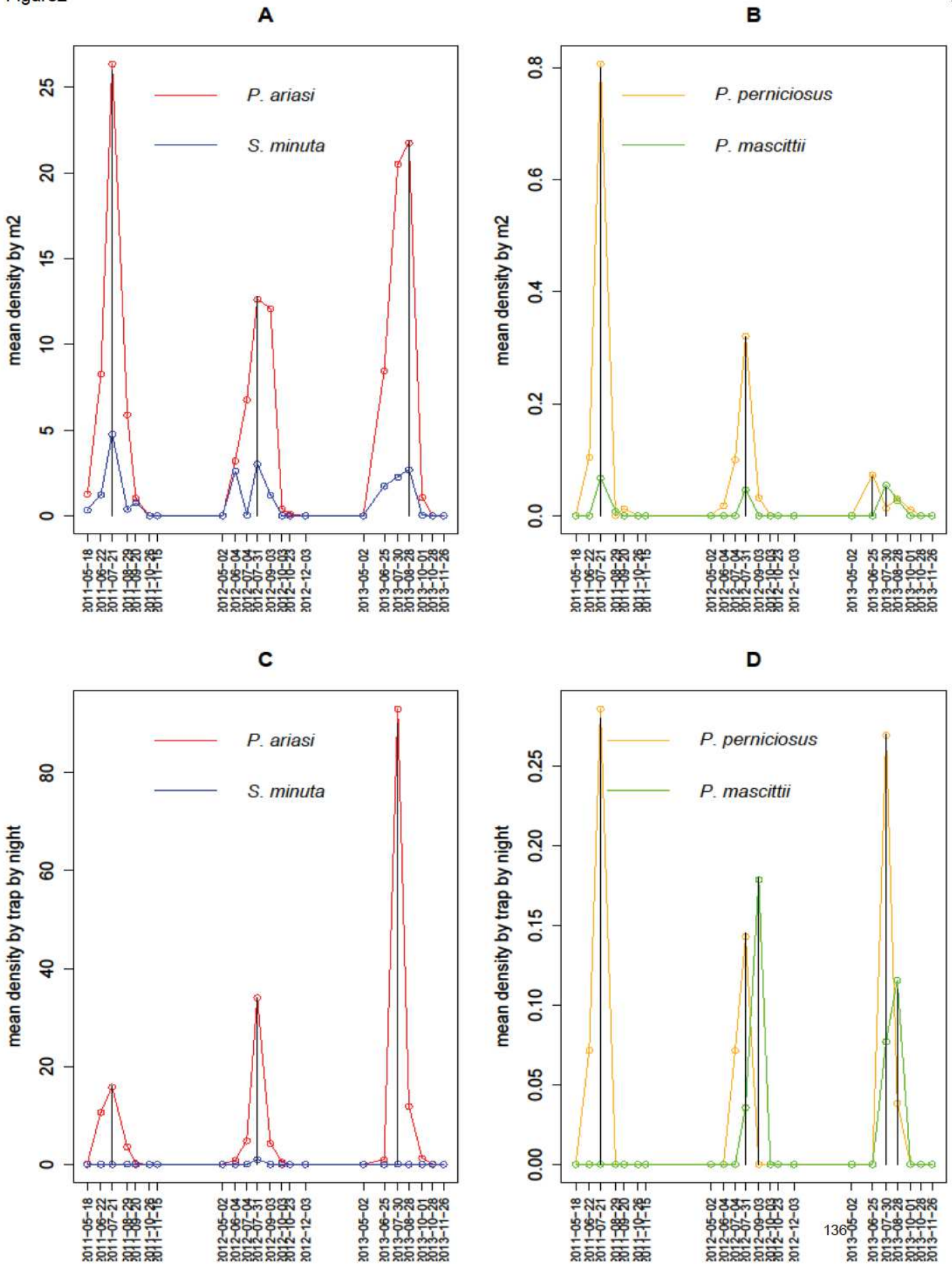


● Stations



0 500 1.000 2.000 3.000 Meters

Figure2



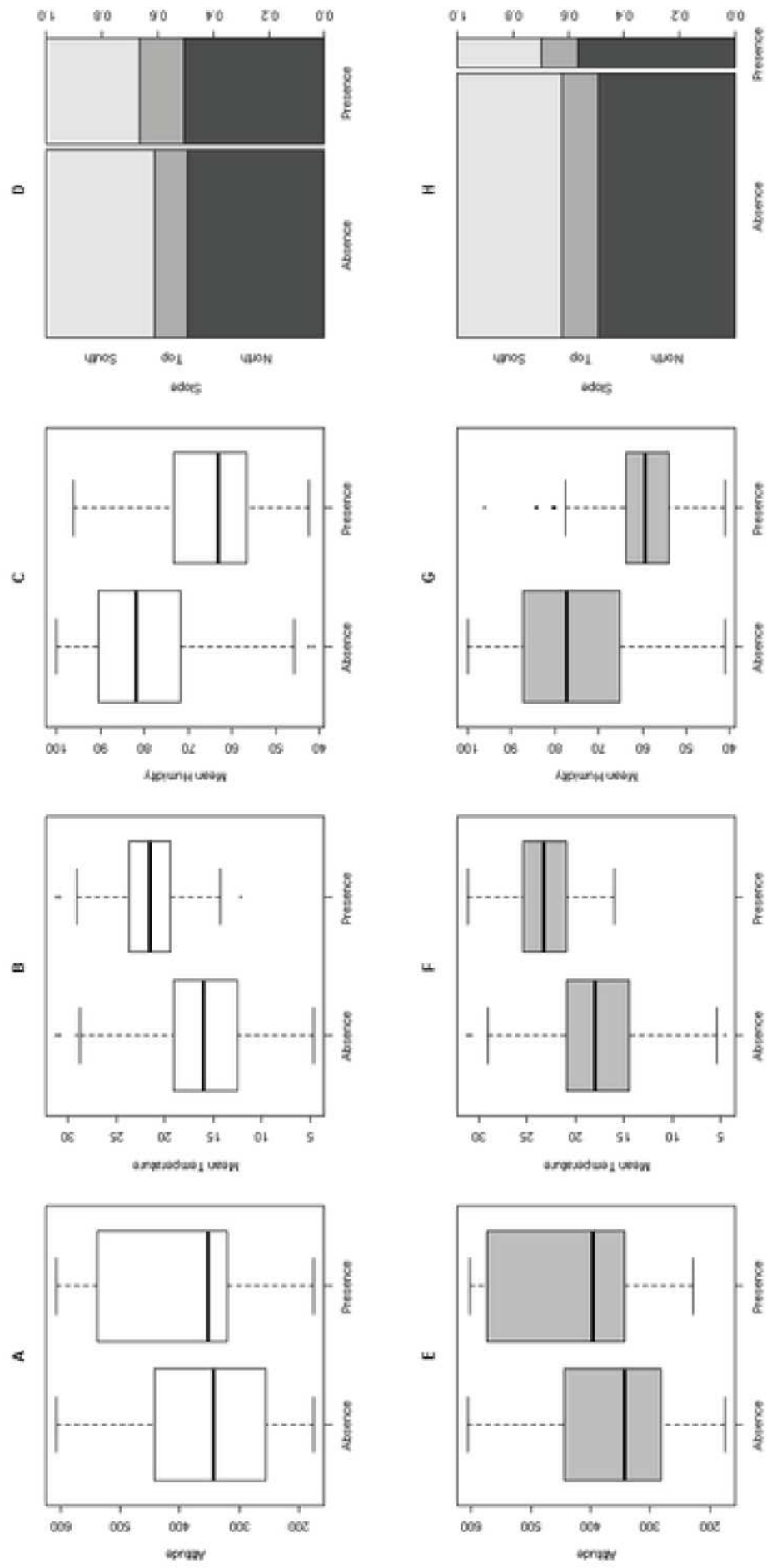


Figure 4

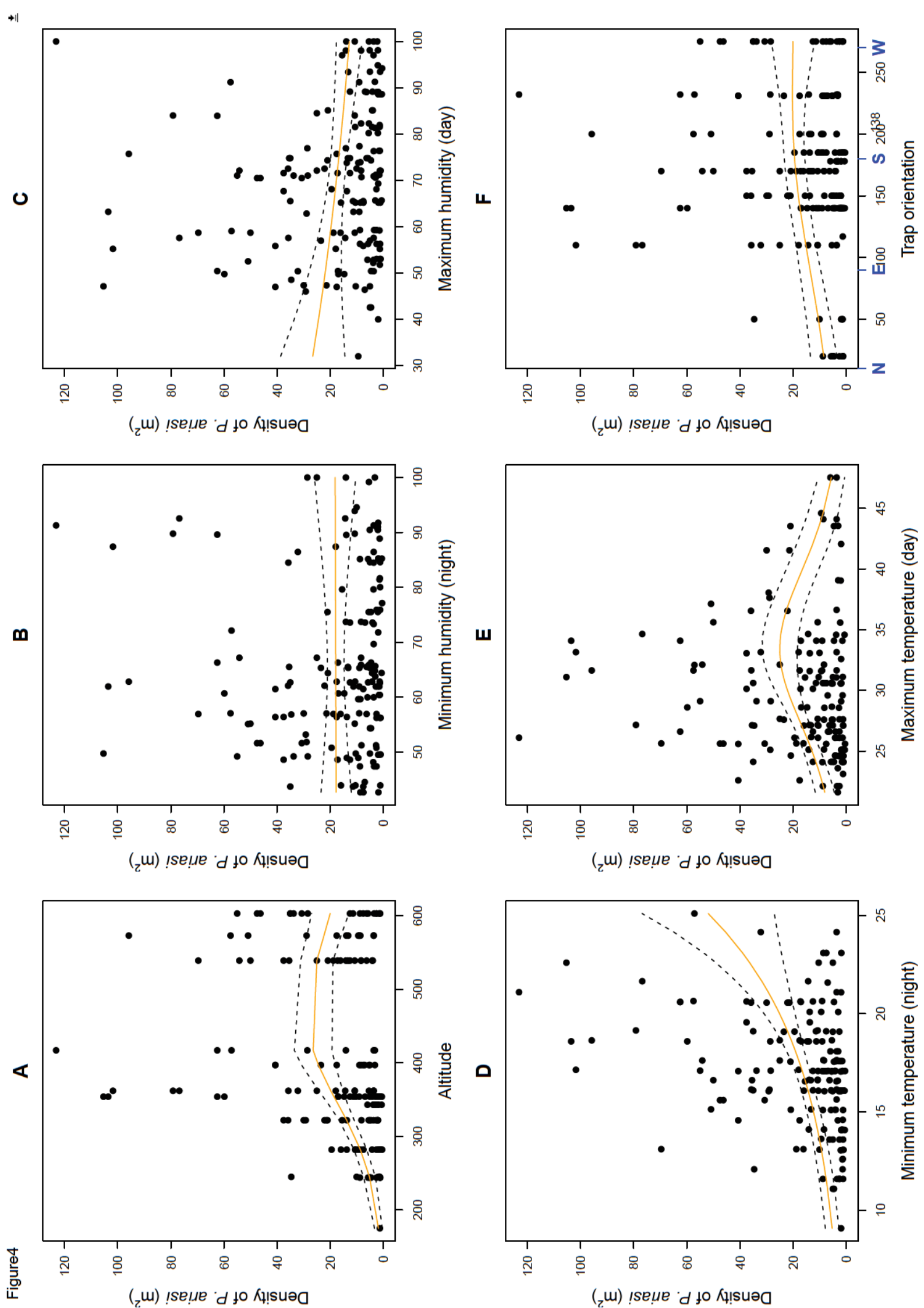


Figure5

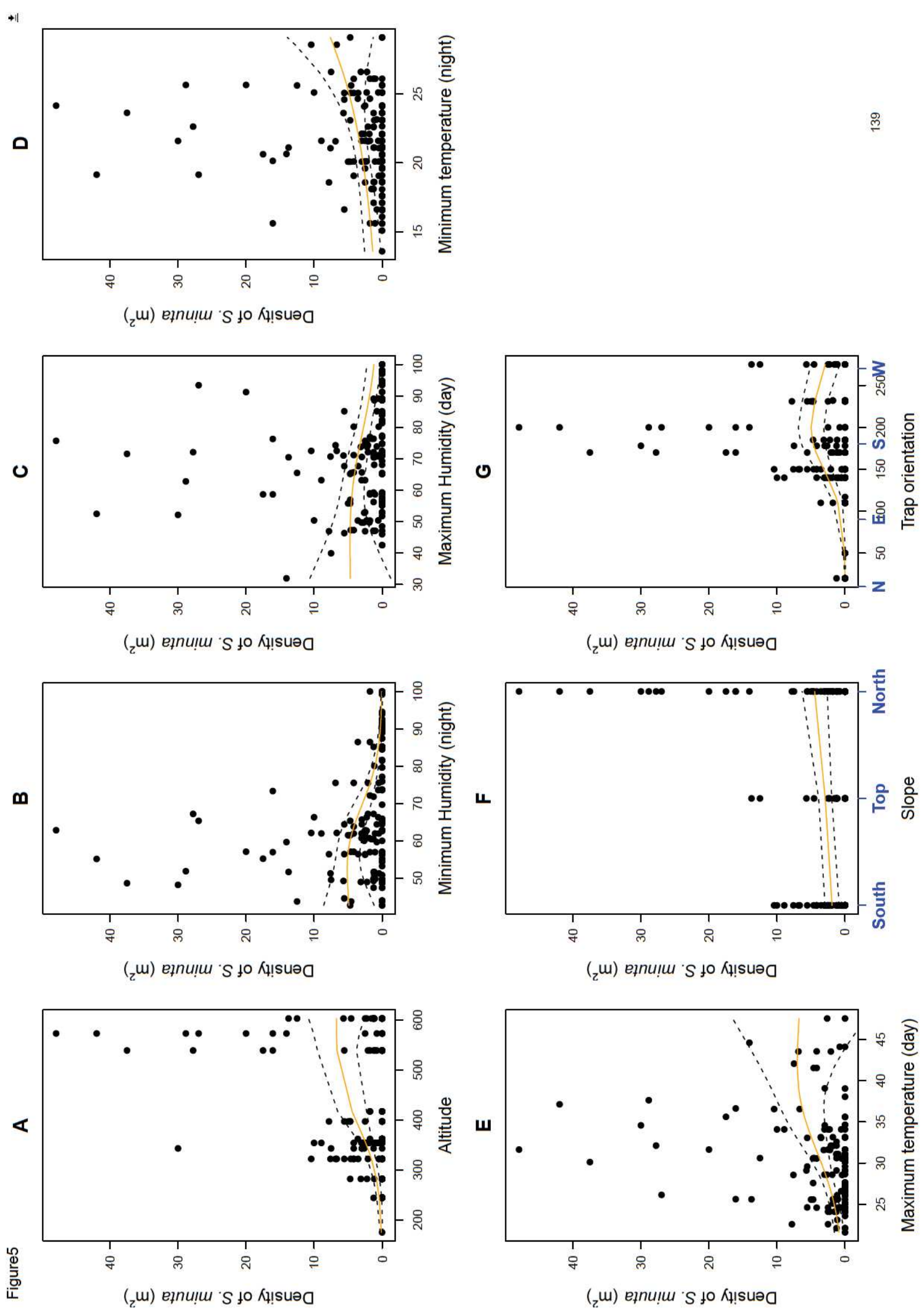
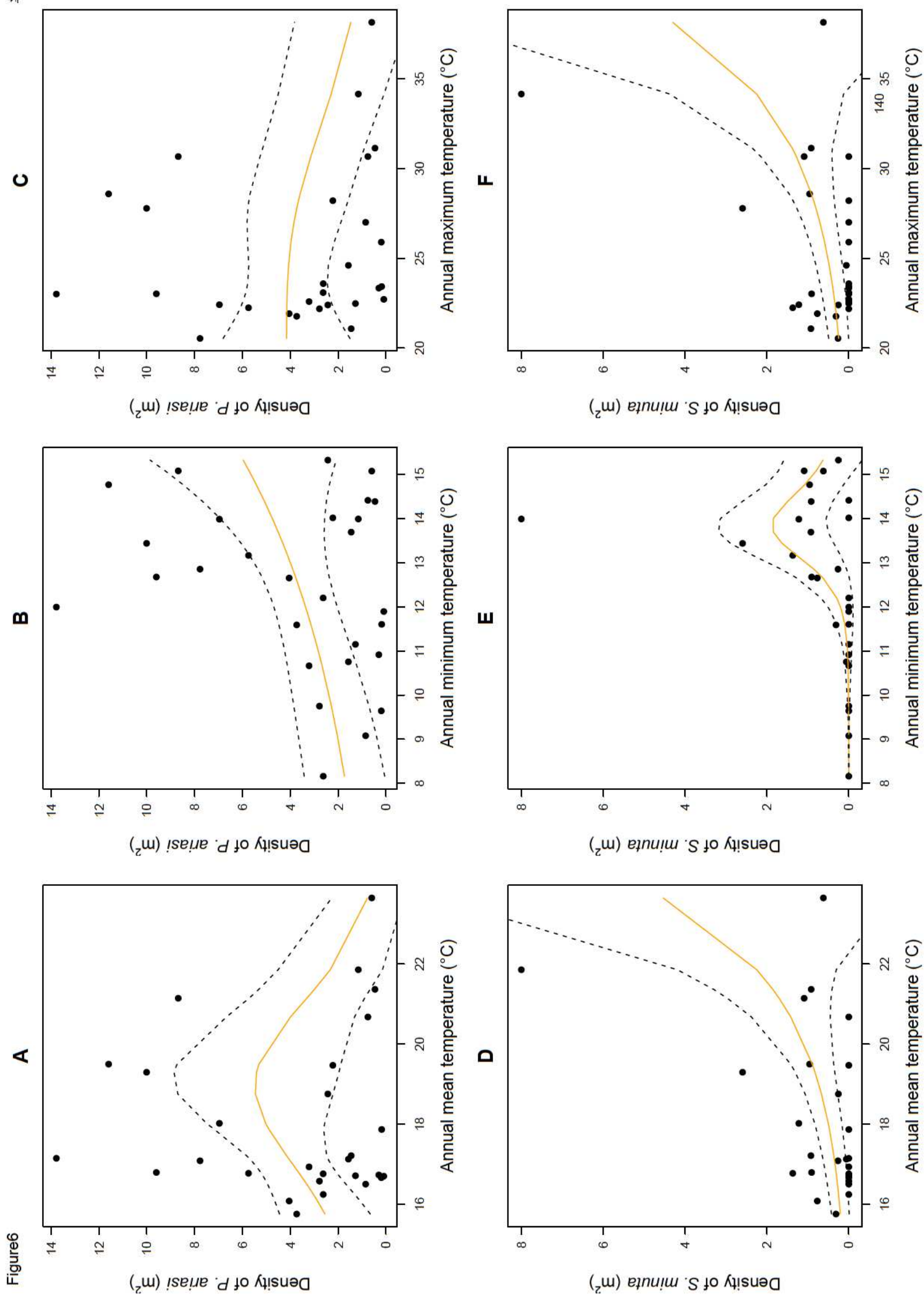


Figure 6



V. Plasticité phénotypique et capacité d'adaptation de *P. ariasi*

La structuration phénotypique des populations de *P. ariasi* dans notre zone d'étude est décrite dans : « *Prudhomme J., Cassan C., Hide M., Toty C., Rahola N., Vergnes B., Dujardin J-P., Alten B., Sereno D.*; Bañuls A-L*. Phenotypic variation of Phlebotomus ariasi in the region of Roquedur (Gard, France). En cours de rédaction.* »

V.1. Echantillons

Pour cette analyse, 374 *P. ariasi*, capturés en 2011, ont été utilisés, 186 mâles et 188 femelles, provenant de 16 stations de capture (Tableau 5).

Tableau 5: Nombre d'ailes de *P. ariasi* utilisées par station pour l'étude de morphométrie géométrique (SE: Sud-Est; NO: Nord-Ouest; Groupe d'altitude: A1 (100-200m), A2 (200-300m), A3 (300-400m), A4 (400-500m), A5 (>500); ♂: male; ♀: femelle).

Station	Versant	Groupe d'altitude	Nombre d'ailes par mois											
			Mai		Juin		Juillet		Août		Septembre		Total	
			♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
ST03	SE	A2	0	0	4	4	2	6	0	0	0	0	6	10
ST04	SE	A3	0	0	1	14	6	0	0	0	0	0	7	14
ST05	SE	A3	0	0	8	0	0	0	0	0	0	0	8	0
ST06	SE	A3	0	0	6	14	4	16	8	0	0	1	18	31
ST09	SE	A5	0	0	9	9	2	5	0	0	0	0	11	14
ST10	SE	A5	0	0	0	0	0	2	0	1	0	0	0	3
ST11	NO	A5	1	0	9	23	3	19	5	2	3	0	21	44
ST12	NO	A5	4	0	6	3	0	0	0	0	0	0	10	3
ST13	NO	A5	0	0	7	0	0	1	1	1	4	1	12	3
ST14	NO	A4	3	0	12	0	2	0	0	0	2	0	19	0
ST15	NO	A3	0	0	5	0	2	0	1	0	0	0	8	0
ST16	NO	A3	1	0	12	0	0	0	0	0	0	0	13	0
ST17	NO	A3	0	0	0	2	0	4	0	0	0	0	0	6
ST18	NO	A2	0	0	8	4	5	9	2	0	1	0	16	13
ST19	NO	A2	0	0	0	4	8	4	0	0	0	0	8	8
ST20	NO	A2	0	0	27	32	2	7	0	0	0	0	29	39
Total			9	0	114	109	36	73	17	4	10	2	186	188

V.2. Résumé

P. ariasi, est l'espèce prédominante de notre zone d'étude et le vecteur prouvé de *L. infantum* dans le Sud de la France (Voir chapitre 4 - Impact de l'écosystème sur l'organisation spatio-temporelle des populations de phlébotomes). Cependant, la biologie et l'écologie de cette espèce restent mal connues. De plus, jusqu'à présent, aucune étude n'a utilisé les méthodes de morphométrie géométrique pour étudier les variations de forme et de taille des ailes de *P. ariasi* en France. Les principaux objectifs de cette étude étaient de mettre en évidence, si elles existent, les variations phénotypiques de *P. ariasi* dans notre région d'étude et de déterminer les facteurs qui influencent ces phénotypes.

Pour ce faire, nous avons réalisé des captures, entre mai et octobre 2011, le long d'un transect de 14 km dans la région de Roquedur (Gard, France). Sur les 20 stations

échantillonnées, les paramètres environnementaux (tels que l'altitude et le versant) ont été recueillis. Après identification de l'espèce, l'analyse phénotypique des ailes de *P. ariasi* a été effectuée à l'aide d'une approche de morphométrie géométrique.

Les analyses ont montré dans un premier temps, qu'il existait des différences importantes de forme et de taille entre les ailes des mâles et celles des femelles. Ce résultat peut s'expliquer par un dimorphisme sexuel, phénomène observable chez beaucoup d'espèces d'insectes. Dans un second temps, nous avons pu observer des variations phénotypiques entre les mois de juin et juillet pour les deux sexes. Il ne nous a pas été possible de tester les autres mois (mai, août et septembre) en raison du faible nombre d'échantillons. Enfin, des différences de taille et de forme par groupe d'altitudes pour les mâles et les femelles en juin ont été mises en évidence ainsi que la présence d'une structuration spatiale en juin pour les femelles par versant et station. Ces résultats soulignent l'existence de pressions environnementales et climatiques variables selon les paramètres testés (altitude, versant, station). Cependant, la présence d'un polymorphisme plus prononcé chez les femelles suggère que les pressions de sélection agissant sur les deux sexes sont différentes.

En conclusion, les variations phénotypiques observées chez *P. ariasi* reflètent les pressions environnementales auxquelles ces populations sont soumises. Nos résultats soulignent l'existence d'une plasticité phénotypique et d'une importante capacité d'adaptation de ces insectes, même à un niveau sympatrique.

V.3. Article 5 : Phenotypic variation of *Phlebotomus ariasi* in the region of Roquedur (Gard, France).

Phenotypic variation of *Phlebotomus ariasi* in the region of Roquedur (Gard, France)

Jorian PRUDHOMME^{1§} [jorian.prudhomme@hotmail.fr], Cécile CASSAN¹ [cecile.cassan@ird.fr], Mallorie HIDE¹ [mallorie.hide@ird.fr], Céline TOTY¹ [celine.toty@ird.fr], Nil RAHOLA¹ [nil.rahola@ird.fr], Baptiste VERGNES¹ [baptiste.vergnes@ird.fr], Jean-Pierre DUJARDIN² [dujardinbe@gmail.com], Bulent ALTEN^{3*} [kaynas@hacettepe.edu.tr], Denis SERENO^{2*} [denis.sereno@ird.fr] and Anne-Laure BANULS^{1*} [anne-laure.banuls@ird.fr].

1. UMR MIVEGEC (IRD 224 - CNRS 5290 - Université de Montpellier), 911 avenue Agropolis, F34394 Montpellier, France.

2. UMR INTERTRYP (IRD - CIRAD 177), Centre IRD, F34394 Montpellier, France.

3. Hacettepe University, Faculty of Science, Department of Biology, Ecology Section, ESRL Laboratories, 0680 Beytepe, Ankara, Turkey.

* These authors contributed equally to the work

§ Correspondence: Jorian Prudhomme; E-mail: jorian.prudhomme@hotmail.fr

I.R.D (Institut de Recherche pour le Développement)

911 Avenue Agropolis, 34394 Montpellier

Abstract

1 *Phlebotomus ariasi* Tonnoir, 1921, is the predominant sandfly species in Cevennes region
2 and a proven vector of *Leishmania infantum* in the South of France. Even if this species is
3 widely present in Western Mediterranean countries and is of medical importance, its biology
4 and ecology remain poorly know. Furthermore, until now no study used geometric
5 morphometrics to explore the phenotypic variation of *P. ariasi* in France. The main goals of
6 this work are to investigate the phenotypic variation of *P. ariasi* in a region of Montpellier
7 which is characterized by climatic and environmental fluctuations, and to determine which
8 spatiotemporal factors influence the sandfly phenotypes. Sandflies were captured along a 14
9 kilometer-long transect in 2011 from May to October and several environmental data (such as
10 altitude, slope) were collected for each station. Morphological analysis of *P. ariasi* wings
11 were performed by a geometric morphometric approaches.

12 We evidenced morphological variations among local populations of *P. ariasi*. First, a
13 sexual dimorphism was observed and shape and size variations between months (in particular
14 June and July) for both gender. During June, we highlighted differences in wing phenotypes
15 according to altitude for both sexes and by slope and station for female.

16 The phenotypic variations, observed in *P. ariasi* along the transect under study, point out
17 that these populations are subjected to environmental pressures. Nevertheless, it seems that
18 sandflies are more sensitive to extrinsic factors in June and July, suggesting a phenotypic
19 plasticity and a structuring in microgeographic populations.

20

21 *Keywords: Sandflies, South of France, Geometric morphometry, Phlebotomus ariasi,*
22 *Phenotypic plasticity*

23 Introduction

24 *Phlebotomus ariasi* Tonnoir, 1921, is the predominant sandfly species in the Cevennes
25 region [1] and one of the two proven vector, with *Phlebotomus perniciosus* Newstead, 1911,
26 of leishmaniasis caused by *Leishmania infantum* in the South of France [2]. The resting sites
27 of *P. ariasi* are found in houses, animal sheds, caves and holes in walls (“barbacanes”), near
28 roads or in villages. This hematophagous insect is active during night and dusk, present
29 during summer in temperate regions, abundant in peri-urban and rural environments and often
30 close to human and domestic animal populations [3]. This species is widely present in
31 Western Mediterranean countries [4-6].

32 Currently, biology and ecology of *P. ariasi* remain poorly know. During the past 10
33 years, the risk of emergence or re-emergence of this disease has increased in France [7]. The
34 current extension of the leishmaniasis distribution underlines the need to increase knowledge
35 on this vector. However, only few studies have been performed to understand the population
36 structure of this species such as analysis of cuticular hydrocarbons [8], isoenzymes [9],
37 random amplified polymorphic DNA [10], sequences of mitochondrial cytochrome b gene [5]
38 and microsatellite markers [11]. But until now, no study using geometric morphometrics
39 approaches have been done to investigate phenotypic variations of *P. ariasi* in France.
40 Significant morphometric wing shape variations were found in sandfly populations
41 originating from various geographic regions, forming clusters or latitudinal clines in other
42 species such as *P. papatasi* [12, 13], *P. sergenti* [14, 15] or *Lutzomyia longipalpis* [16]. These
43 studies highlighted the existence of local phenotypic variations linked to environmental
44 factors.

45 The main goals of this work were to investigate phenotypic variations of *P. ariasi*
46 population collected in the region of Montpellier which is characterized by climatic and

47 environmental fluctuations, and to determine which spatiotemporal factors (month, station,
48 altitude or slope) influence the sandfly phenotypes.

49

50 Materials and methods

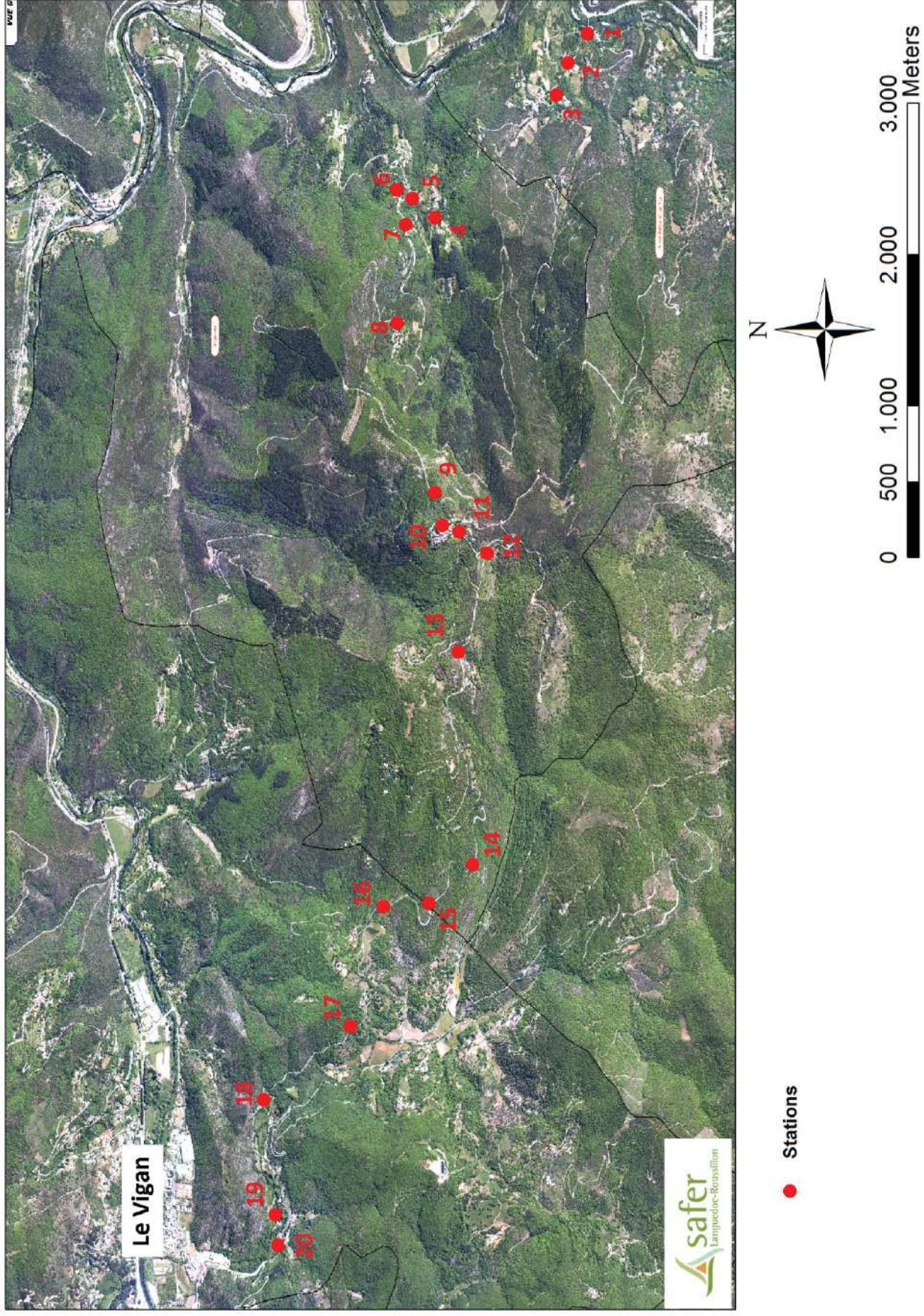
51 **The study area**

52 The field study was performed in the South of France, on the hill “le massif de
53 l’Oiselette” located between two valleys: “Hérault” (Ganges, Hérault) and “Arre” (Le Vigan,
54 Gard). Sandflies were collected along a 14 km transect between Saint Julien de la Nef and le
55 Vigan, including Roquedur-le-haut (at 601m above sea level) (Figure 1). 20 stations were
56 selected along this transect (Table 1 and Figure 1). This region is subject to the Mediterranean
57 sub-humid climat [17] and characterized by the presence of “Garrigue” species such as
58 *Quercus ilex* and *Q. pubescens*.

59 This geographical area is known to be an endemic area for human and canine
60 leishmaniasis caused by *L. infantum* [1]. Various domestic animals that are potential hosts for
61 sandflies, were present throughout the transect such as chicken, sheep, ducks, geese, horses,
62 rabbits, cats and dogs. Furthermore, some stations were located in semi-rural areas and thus
63 various wild animals could be also hosts for sandflies [18].

64 To test the effect of environmental factors on the sandfly wing size and shape, the
65 stations were organized according to the slopes and altitudes. Therefore, stations were
66 grouped into two slope groups: Southeast (SE) and Northwest (NW), and five altitude groups:
67 A1 (200-300m), A2 (300-400m), A3 (400-500m), A4 (>500) (Table 2).

Figure 1. Map of the study area showing the main sites sampled. Red circles indicate the sampling localities.



68 **Sandfly collection and identification**

69 Sandfly collections were performed monthly between May and November 2011 with
70 CDC miniature light traps (John W. Hock Co. FL, U.S.A.) and flight interception traps (20 x
71 20 cm white paper cover with castor oil) [19]. Along this transect, 20 stations were selected.
72 In 14 sampling sites (Table 1), one or two light traps were set up for two nights (inside and/or
73 outside of houses, animal barns, etc.), operating between 18:00 pm and 08:00 am. A total of
74 105 light traps were set during 210 nights of trapping. In 17 stations (Table 1), a total of 3589
75 sticky traps were used and a mean of 189 sticky traps by station were placed in various
76 biotopes, inside and around human dwelling and animal housing, close to the vegetation and
77 crevices in the walls. The sticky traps were settled up in the sampling sites for 2 consecutive
78 days.

79 At the end of the sampling period, specimens from the traps were transferred
80 individually into 1.5 ml Eppendorf tubes with 96% ethanol and labeled accordingly. Prior to
81 mounting, heads, genitalia and wings of the sandflies were removed. The heads and genitalia
82 were cleared in Marc-André solution (chloral hydrate / acetic acid) and mounted in chloral
83 gum [3]. Specimen identification was individually verified based on the morphology of the
84 pharynges and / or the male genitalia or female spermathecae, using the keys of Abonnenc
85 [3], Lewis [6] and Killick-Kendrick et al. [20]. From the identifications, we selected the wings
86 of the *P. ariasi* specimens, which are in majority in our sample.

Table 1. Sampling stations in the study area (CDC: CDC miniature light traps; ST: Sticky Traps)

Station number	Coordinates		Altitude (m)	Biotope	Traps
	North	East			
ST01	43.96548	3.686828	175	Hamlet	CDC + ST
ST02	43.96663	3.685075	228	Hamlet	ST
ST03	43.96733	3.683149	244	Hamlet	CDC + ST
ST04	43.97462	3.675871	321	Hamlet / Hutch	CDC + ST
ST05	43.97595	3.677038	322	Rural	ST
ST06	43.97687	3.677551	341	Kennel	CDC + ST
ST07	43.97632	3.675457	354	Hamlet	CDC + ST
ST08	43.97683	3.669611	443	Hamlet	ST
ST09	43.9746	3.659549	586	Poultry	CDC
ST10	43.97416	3.657616	606	Poultry	CDC
ST11	43.97317	3.657214	603	Hamlet	CDC + ST
ST12	43.97144	3.655944	573	Rural	ST
ST13	43.97321	3.650088	539	Rural	CDC + ST
ST14	43.97235	3.637441	417	Rural	ST
ST15	43.97495	3.635153	397	Rural	CDC + ST
ST16	43.97765	3.634949	362	Rural	ST
ST17	43.97961	3.627845	343	Rural	CDC + ST
ST18	43.98478	3.623463	282	Rural	CDC + ST
ST19	43.98409	3.616661	255	Hamlet	CDC + ST
ST20	43.98392	3.614822	245	Hamlet / Sheep barn	CDC + ST

Table 2. Number of *Phlebotomus ariasi* wings used by station for the geometric morphometric analysis (SE: South-East; NW: North-West; Altitude groups: 1 (100-200m), 2 (200-300m), 3 (300-400m), 4 (400-500m), 5 (>500); ♂: male; ♀: female).

Station	Slope	Altitude groups	Wings number by month											
			May		June		July		August		September		Total	
			♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
ST03	SE	A2	0	0	4	4	2	6	0	0	0	0	6	10
ST04	SE	A3	0	0	1	14	6	0	0	0	0	0	7	14
ST05	SE	A3	0	0	8	0	0	0	0	0	0	0	8	0
ST06	SE	A3	0	0	6	14	4	16	8	0	0	1	18	31
ST09	SE	A5	0	0	9	9	2	5	0	0	0	0	11	14
ST10	SE	A5	0	0	0	0	0	2	0	1	0	0	0	3
ST11	NW	A5	1	0	9	23	3	19	5	2	3	0	21	44
ST12	NW	A5	4	0	6	3	0	0	0	0	0	0	10	3
ST13	NW	A5	0	0	7	0	0	1	1	1	4	1	12	3
ST14	NW	A4	3	0	12	0	2	0	0	0	2	0	19	0
ST15	NW	A3	0	0	5	0	2	0	1	0	0	0	8	0
ST16	NW	A3	1	0	12	0	0	0	0	0	0	0	13	0
ST17	NW	A3	0	0	0	2	0	4	0	0	0	0	0	6
ST18	NW	A2	0	0	8	4	5	9	2	0	1	0	16	13
ST19	NW	A2	0	0	0	4	8	4	0	0	0	0	8	8
ST20	NW	A2	0	0	27	32	2	7	0	0	0	0	29	39
Total			9	0	114	109	36	73	17	4	10	2	186	188

87 **Wing preparation**

88 A total of 374 specimens of *P. ariasi* were used for the geometric morphometrics
89 analysis (186 males and 188 females) (Table 2). Rohlf et al. [21] suggest using only one side
90 for the pair of organs or limbs to avoid a bias because of possible asymmetry between the two
91 sides. In this study, only the right wings were used. Wings removed from each specimen (see
92 above) were mounted in Euparal on labeled slides. Before mounting, the wings were stained
93 using the method previously described in Prudhomme et al. [13]. Slides were photographed
94 using a Leica Z16 APOA stereoscopic zoom dissection microscope with DFC 425 digital
95 camera system, digitized, and archived.

96

97 **Morphometric analysis**

98 Pictures were first entered into tps-Util 1.60 [22]. Then, 16 landmarks were used for the
99 analysis following the method of Rohlf et al. [21] with tpsDIG2 2.18 software [23] (Figure 2).
100 Landmarks are located at the intersections of wing veins with the wing margin and at the
101 intersections of cross veins with major veins (Figure 2). The morphometric analyses as well as
102 graphical outputs were performed using various modules of the CLIC package [24]. The sizes
103 of the wings were analyzed using the centroid sizes of these wings as an estimator using a
104 nonparametric Wilcoxon-Mann-Whithney test or Kruskal Wallis test followed by a post-hoc
105 test using Mann-Whitney tests with Bonferroni correction, using the R statistical package,
106 version 3.1.2 [25]. Centroid size is the square root of the sum of squared distances of a set of
107 landmarks from their centroid, i.e. the square root of the sum of the variances of the
108 landmarks about that centroid in x- and y- directions [26]. The landmark configurations were
109 scaled, translated, and rotated against the consensus configuration by the GLS Procrustes
110 superimposition method [26-29] in order to produce shape variables (partial warps). The
111 principal components (relative warps) [26] were used to compare population samples. To

112 assess the degree of similarity between populations, pairwise Mahalanobis distances between
113 populations were calculated using PAD software and tested by nonparametric permutation
114 tests with 1000 iterations each.

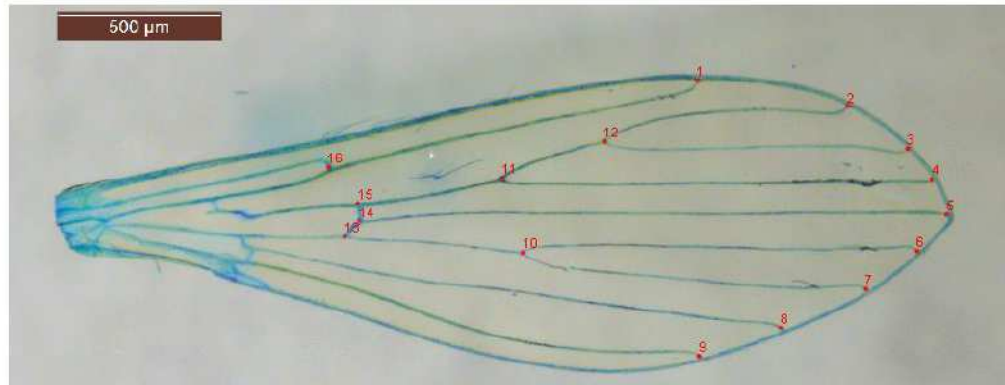


Figure 2. Location of the 16 wing landmarks used in the morphometric analysis of *Phlebotomus ariasi*.

Results

Sexual dimorphism

116 The wing shape showed statistically significant differences between males and females.
117 It appeared that females and males of *P. ariasi* were different from each other along the first
118 axis in terms of phenotypic features (Figures 3 and 4). Mahalanobis distances were
119 significantly different between both sexes (Adjusted p -values < 0.0001). Moreover, simple
120 reclassification scores to the sexes were equal to 96% for males and 93% for females,
121 supporting the observed differentiation between the two groups. Centroid sizes were used as a
122 measure of the overall wing size differences among populations. The size differences linked
123 to the gender were significant (Wilcoxon-Mann-Whitney Test: p -value < 0.0001), with the
124 females displaying larger wings than males (Figure 5). The contribution of size to wing shape
125 differentiation was 72% (Figure 6).

126 Since a phenotypic difference between males and females was observed, we decided to
127 perform the analysis considering the month of capture separately for each sex.

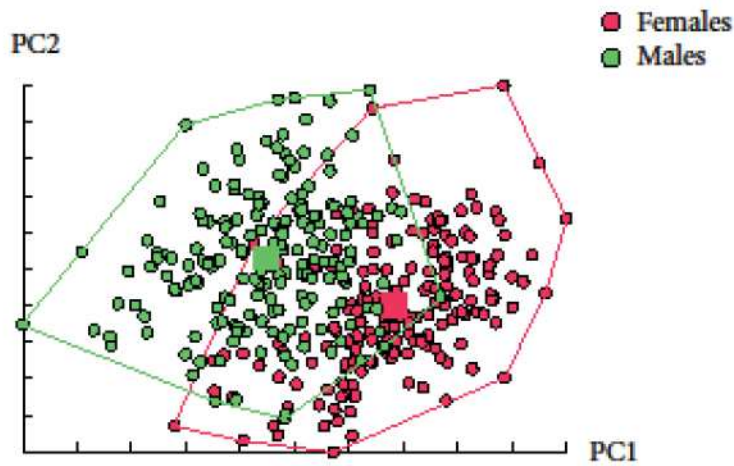


Figure 3. Distribution of the individuals of *Phlebotomus ariasi* along the first two principal components by gender. Horizontal axis: principal component 1; Vertical axis: principal component 2. Signs indicate each individual. Pink: Females; Green: males.

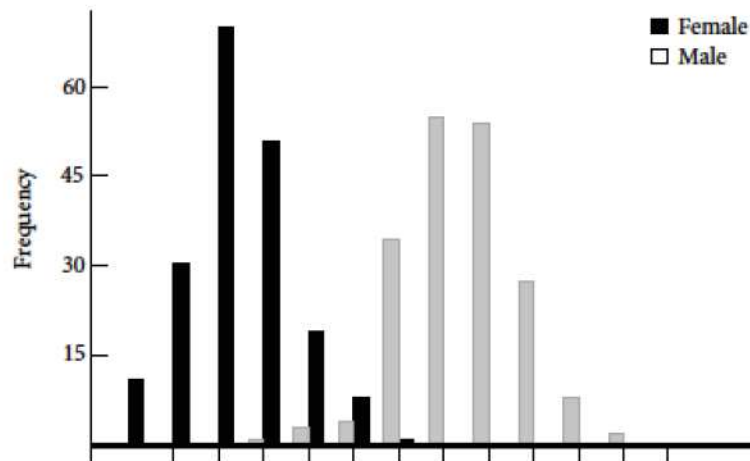


Figure 4. Canonical variate frequencies generated in the discriminant analysis by gender. Black bars: females; Gray bars: males.

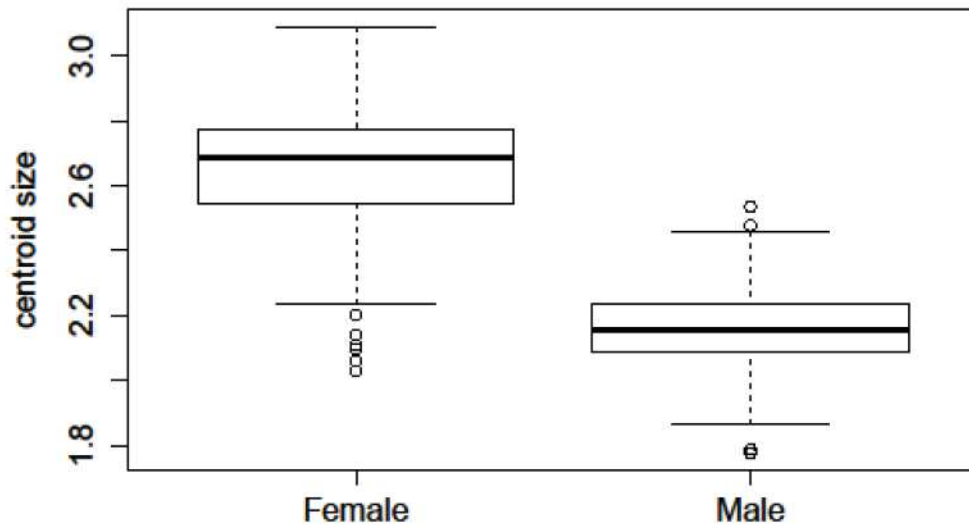


Figure 5. Mean, standard deviation, and error of centroid wing sizes for each sex.

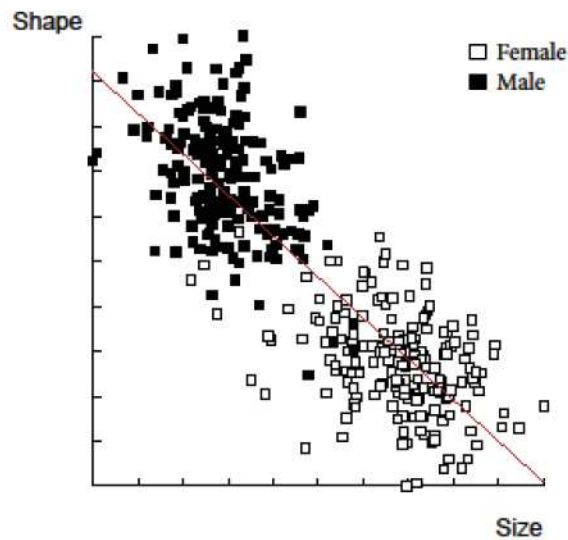


Figure 6. Regression of first discriminant factor on centroid size. Vertical axis: first discriminant factor, representing 100% of the total discrimination; Horizontal axis: centroid size of the wing. White squares: females; Black squares: males. Regression line is shown.

128 **Differentiation by month**

129 Mahalanobis distances to study wing shape were significantly different between the
 130 months; between June-July for females (Adjusted p -values < 0.0001) and between June-July
 131 and July-August for males (Adjusted p -values < 0.05 after Bonferonni correction; eight

132 components, 85.53% of total shape variance) (Figures 7 and 8). For females, it was not
133 possible to test shape differentiation for the other months due to the small number of
134 specimens (Table 2). Indeed, for the discriminant analyses, the sampling size is limiting
135 because in the case of small populations, the low number of principal components does not
136 allow to explain the variance of the dataset. Moreover, simple reclassification scores to the
137 month groups were on average equal to 73.6% for males (58-88%) and 81 for females (80-
138 82%), supporting the differentiation observed between samples.

139 Concerning wing size, for females, a Kruskal Wallis test revealed a significant effect of
140 month on the centroid size ($\chi^2 = 11.6239$, p -value = 0.02038). A post-hoc test using Mann-
141 Whitney tests with Bonferroni correction showed significant differences between June and
142 July (p -value = 0.03741), between June and September (p -value = 0.02588) and between July
143 and September (p -value = 0.03678) (Figure 9A). For males, no size difference between
144 months was ascertained ($\chi^2 = 8.5591$, p -value = 0.07312) (Figure 9B). The contribution of
145 size to wing shape differentiation was 0% for females and for males 43% and 2%
146 (Discriminant factor 1 and 2, respectively) (Figure 10).

147 These results showed a strong phenotypic structuring between months in particular
148 between June and July for both sexes. Since a phenotypic difference between these two last
149 months was observed, the following analyses performed to determine the possible effects of
150 slope, altitude and station were realized for females and males in June and July separately. It
151 was not possible to test for the other months due to a too small number of specimens (Table
152 2).

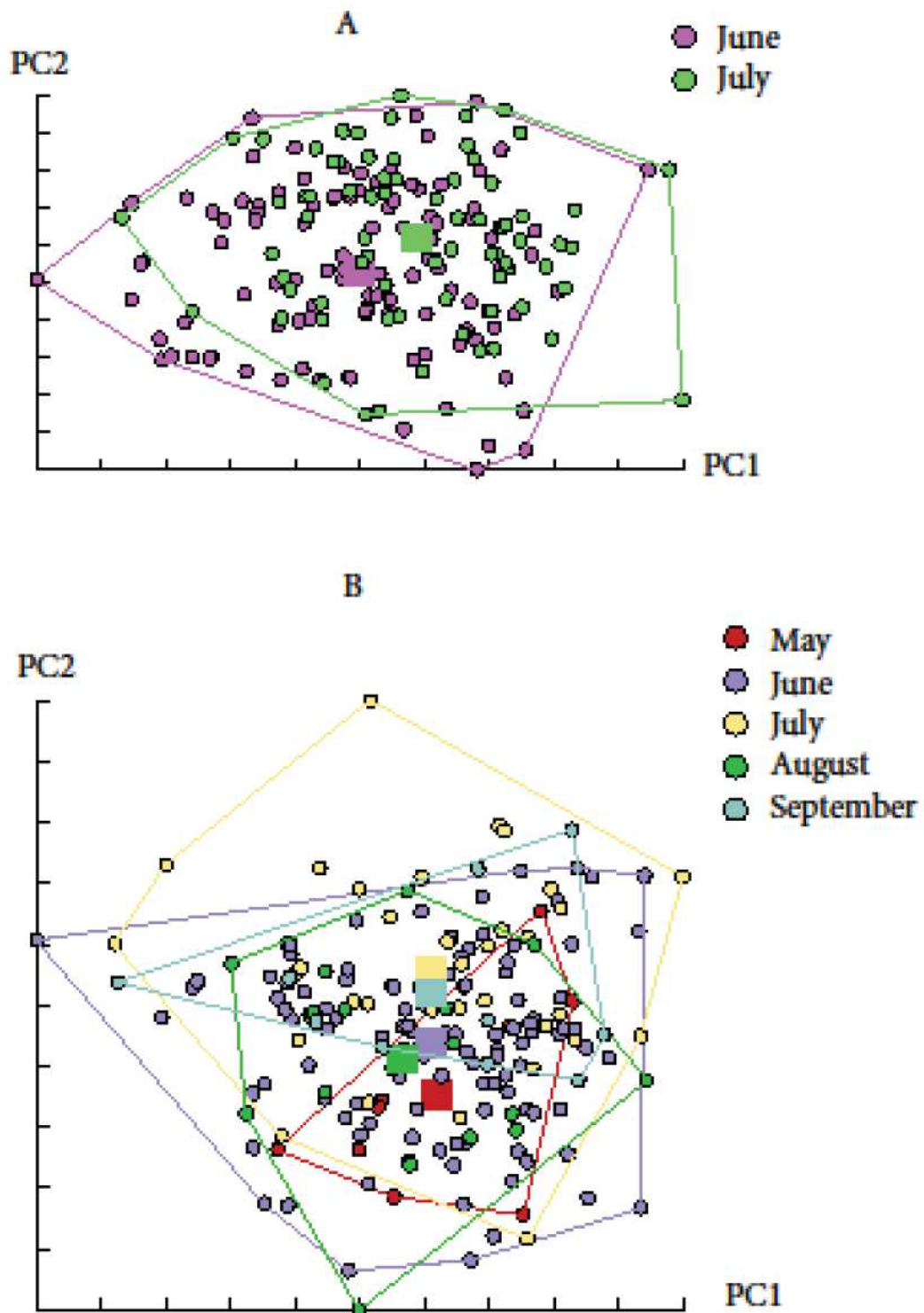


Figure 7. Distribution of the individuals of *Phlebotomus ariasi* along the first two principal components for females (A) and males (B) by month. Horizontal axis: principal component 1; Vertical axis: principal component 2. Signs indicate each individual.

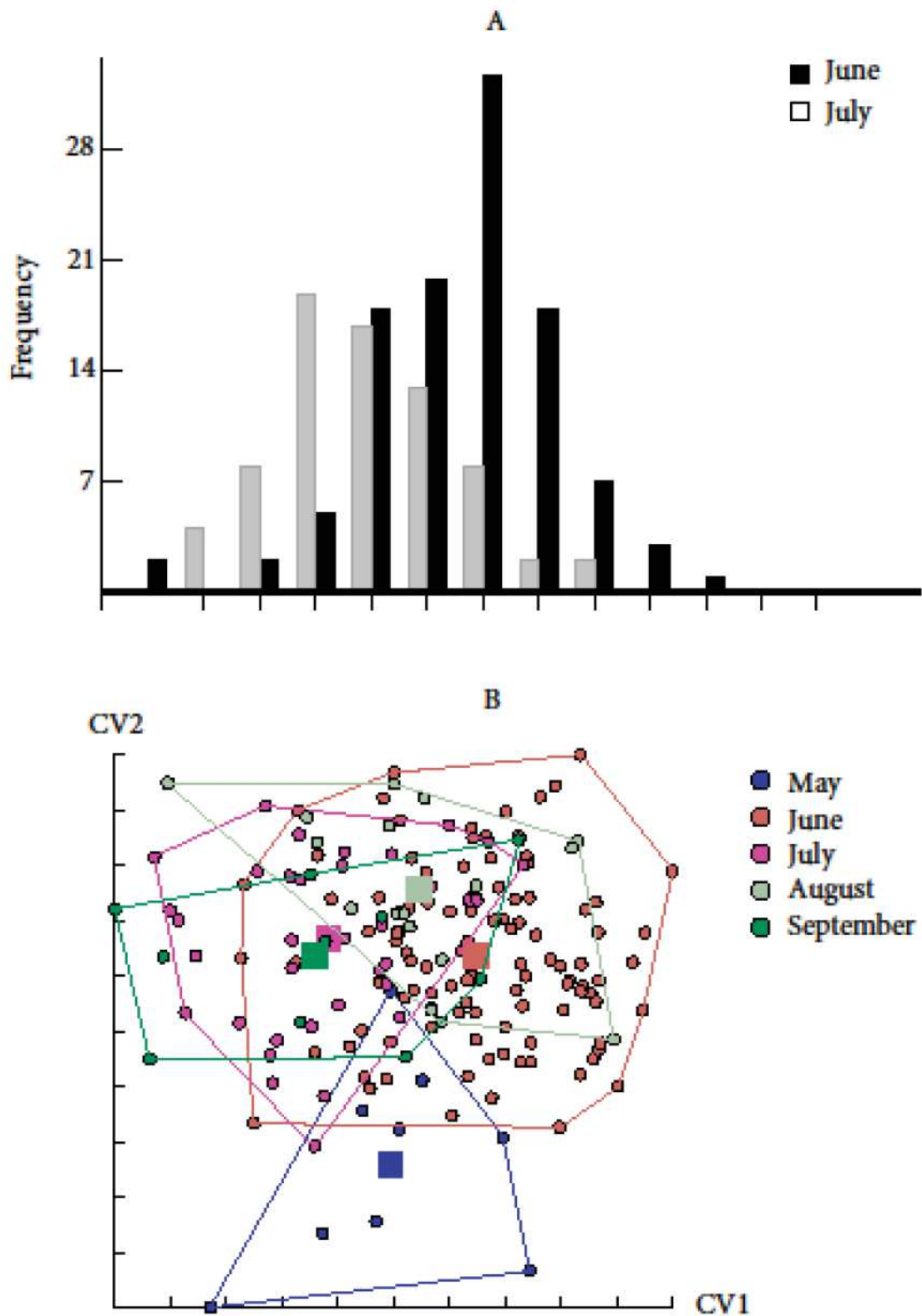


Figure 8. Canonical variate frequencies generated in the discriminant analysis for females (A) and distribution of the individuals along the first two canonical variates for males (B). Horizontal axis (B): canonical variate 1; Vertical axis (B): canonical variate 2. Signs indicate each individual.

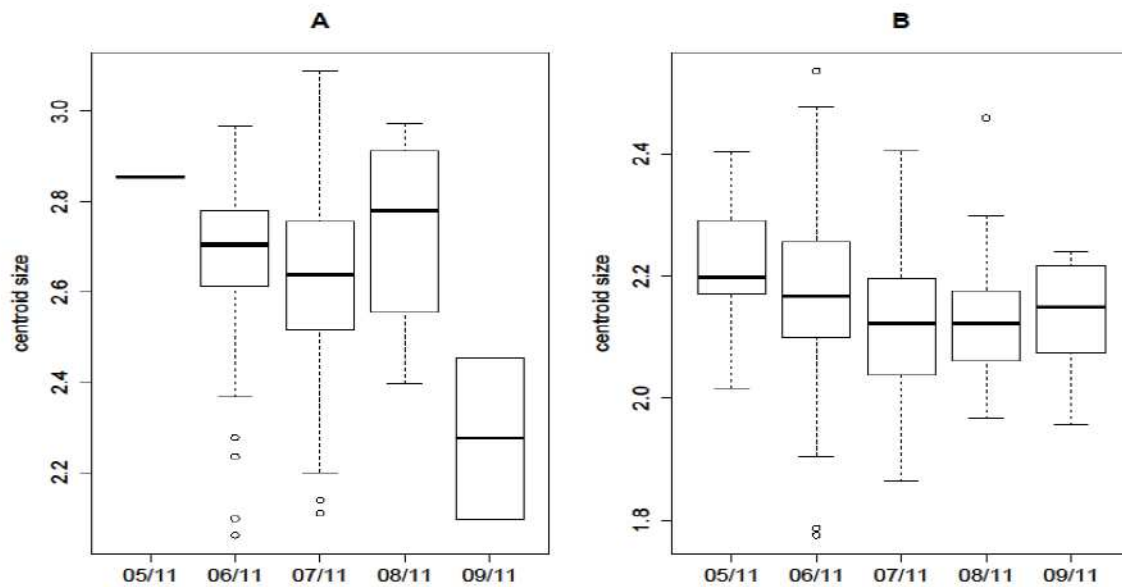


Figure 9. Mean, standard deviation, and error of centroid wing sizes by month for females (A) and males (B).

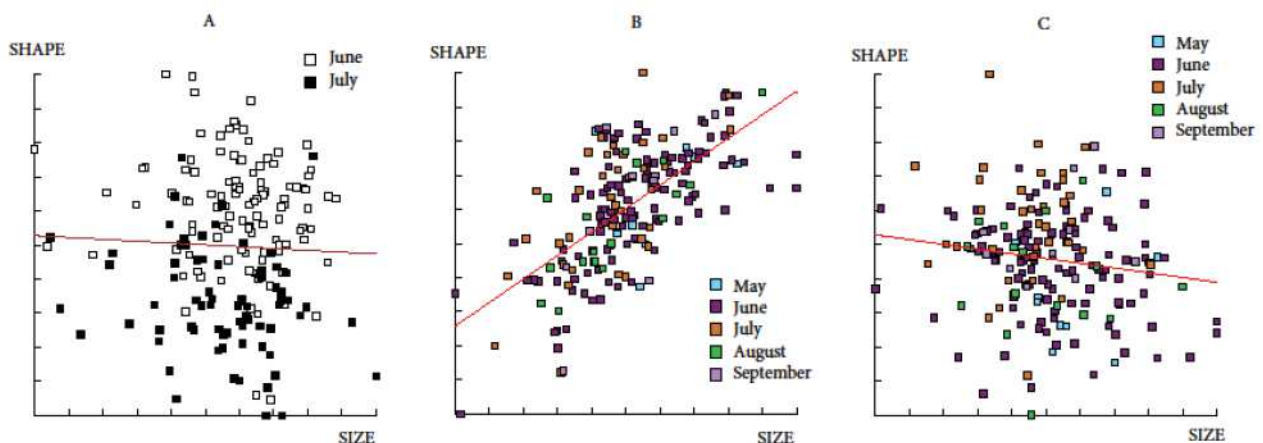


Figure 10. Regression of first discriminant factor on centroid size for females (A) and of the two first for males. Horizontal axis: centroid size of the wing; Vertical axis (A): discriminant factor 1, representing 100% of the total discrimination; Vertical axis (B and C): discriminant factor 1 and 2, representing 24% and 17% of the total discrimination. Regression line is shown.

Differentiation by slope

153 As detailed above to determine the possible effect of slope on the *P. ariasi* phenotype,
 154 we performed analysis on wings considering each sex and the two months, June and July,
 155 separately (see samples in table 2).

156 Mahalanobis distances to study wing shape were significantly different between the
157 slopes (Adjusted p -value < 0.01) only for females in June (Figures 11 and 12). Moreover,
158 simple reclassification scores to the slope groups were on average equal to 95% (80-90%),
159 supporting the observed differentiation between samples. However, no significant difference
160 was observed between the slopes for the females in July (Adjusted p -value > 0.05) and for the
161 males whatever the month (Adjusted p -values > 0.05 ; 13 components, 96.5% of total shape
162 variance) (data not shown).

163 Furthermore, the Kruskal Wallis test to study wing size revealed also no significant
164 effect of slopes in June and July ($\chi^2 = 1.0064$, p -value = 0.3158; $\chi^2 = 0.4636$, p -value =
165 0.4959, respectively) for females and also for males ($\chi^2 = 0.4915$, p -value = 0.4833; $\chi^2 =$
166 0.6771, p -value = 0.4106) (data not shown).

167 The contribution of size to wing shape differentiation was 0% and 32% for males in
168 June and July, respectively, and 0% for females for both months (Figure 13).

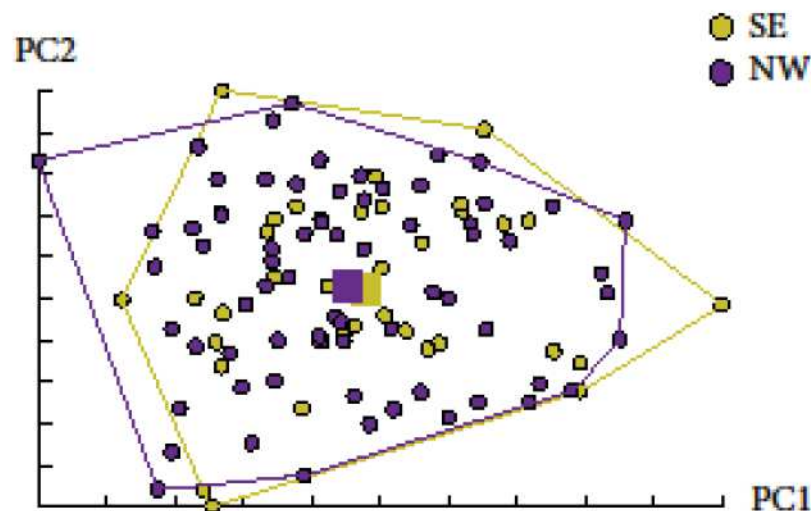


Figure 11. Distribution of the individuals of *Phlebotomus ariasi* females from June along the first two principal components. Horizontal axis: principal component 1; Vertical axis: principal component 2. Signs indicate each individual. Yellow: South-East slope (SE), purple: North-West slope (NW).

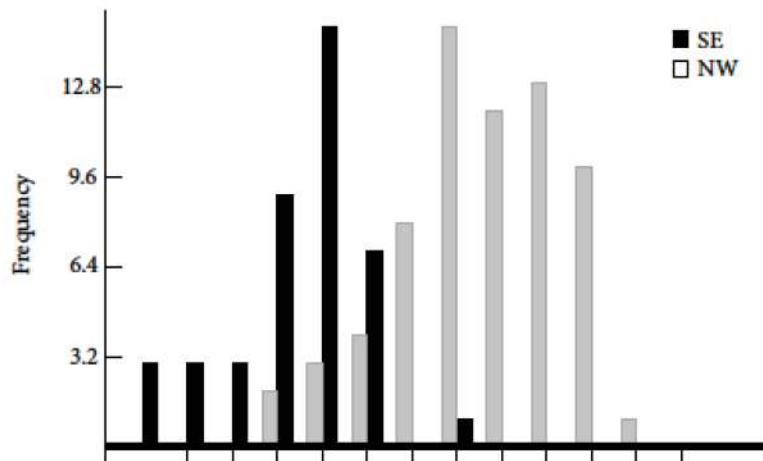


Figure 12. Canonical variate frequencies generated in the discriminant analysis for females in June by slope. Black bars: South-East slope (SE); Gray bars: North-West slope (NW).

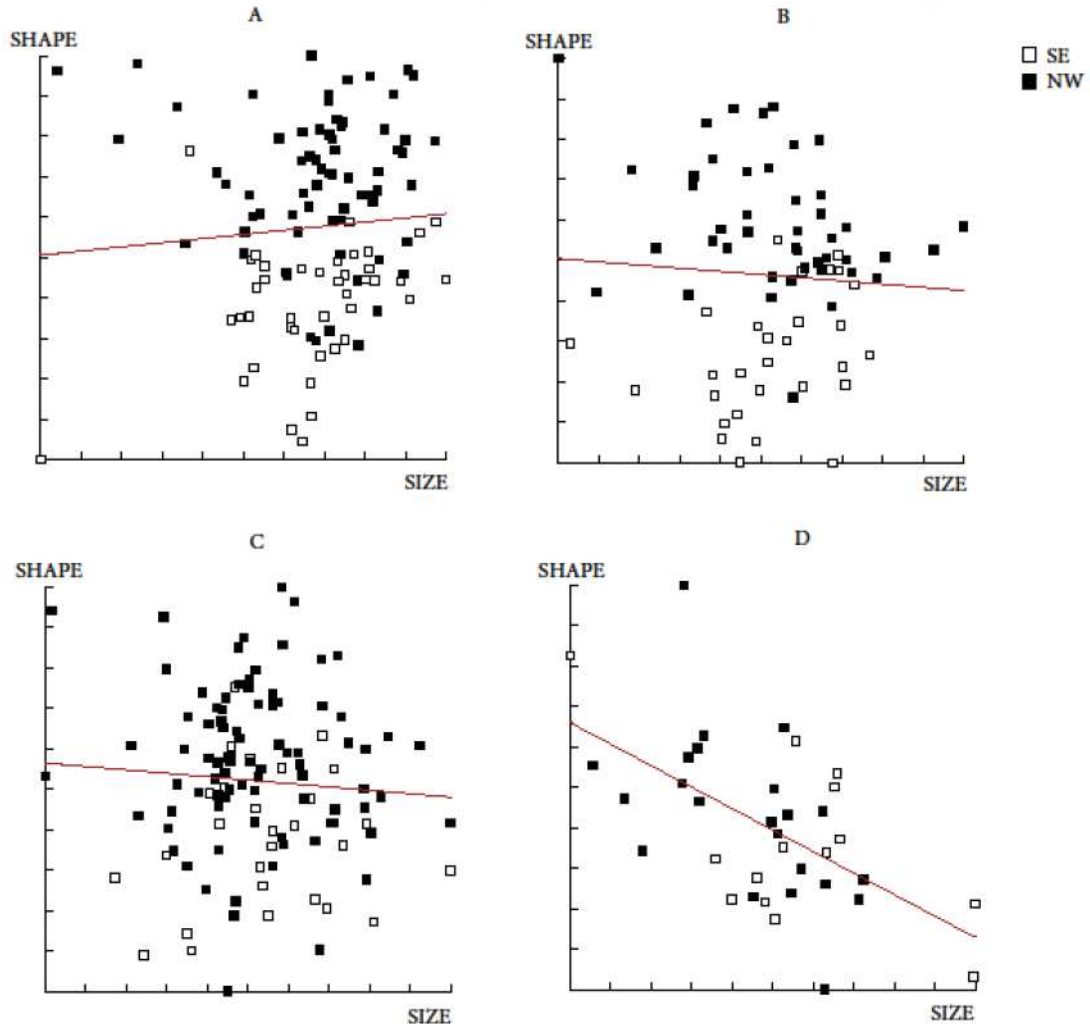


Figure 13. Regression of first discriminant factor on centroid size in June and July for females (A and B) and for males (C and D). Horizontal axis: centroid size of the wing; Vertical axis: first discriminant factor, representing 100% of the total discrimination. Regression line is shown. SE: South-East slope; NW: North-West slope.

169 **Differentiation by altitude**

170 The effect of altitude on *P. ariasi* phenotype was tested for males and females in June
171 and July separately (see the sample in Table 2). In June, Mahalanobis distances used to study
172 wing shape were significantly different between the altitude groups A1 and A2 for females
173 (Adjusted p -value < 0.01667) and between group A3 and all the other groups for males
174 (Adjusted p -value < 0.00833 , 11 components, 93.2% of total shape variance) (Figures 14 and
175 15). Moreover, simple reclassification scores to the altitude groups were on average equal to
176 73% (65-80%) for females and 53% (43-75%) for males, supporting the observed
177 differentiation between samples. However, in July, no significant difference was observed
178 between the altitude groups for females and males (Adjusted p -value > 0.01667 , 19
179 components, 99.2% of total shape variance and Adjusted p -value > 0.01667 , 4 components,
180 71.3% of total shape variance, respectively) (data not shown). To realize the analyses with
181 males in July, the altitude group 4 had to be removed because of a low number of specimens
182 ($n=2$).

183 Concerning wing size, in June, a significant effect of altitude on centroid size was
184 observed for females ($\chi^2 = 7.0282$, p -value = 0.02977), the post-hoc test showed significant
185 differences between groups A1 (200-300m) and A2 (300-400m) (p -value = 0.022). For males,
186 the difference was strongly significant between groups A1 (200-300m) and A3 (400-500m)
187 ($\chi^2 = 12.4331$, p -value = 0.006038) with a p -value = 0.0076 for the post-hoc test (Figure 16).
188 Conversely, no significant effect of altitude were found on the centroid sizes in July for both
189 sexes ($\chi^2 = 4.9144$, p -value = 0.08567; $\chi^2 = 0.4848$, p -value = 0.9222, for females and males
190 respectively) (data not shown).

191 The contribution of size to wing shape differentiation was 7% and 0% for females and
192 48% and 1% for males in June (Figure 17) and 37% and 14% for females and 36% and 0% for
193 males in July (data not shown).

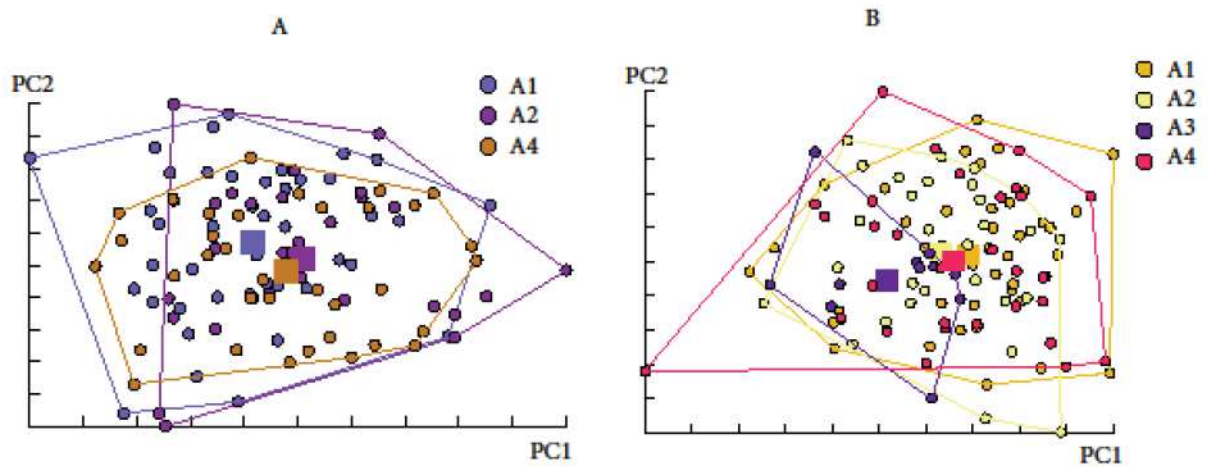


Figure 14. Distribution of the individuals of *Phlebotomus ariasi* females (A) and males (B) by altitude groups in June along the first two principal components. Horizontal axis: principal component 1; vertical axis: principal component 2. Signs indicate each individual. Altitude groups: A1 (200-300m), A2 (300-400m), A3 (400-500m), A4 (>500)

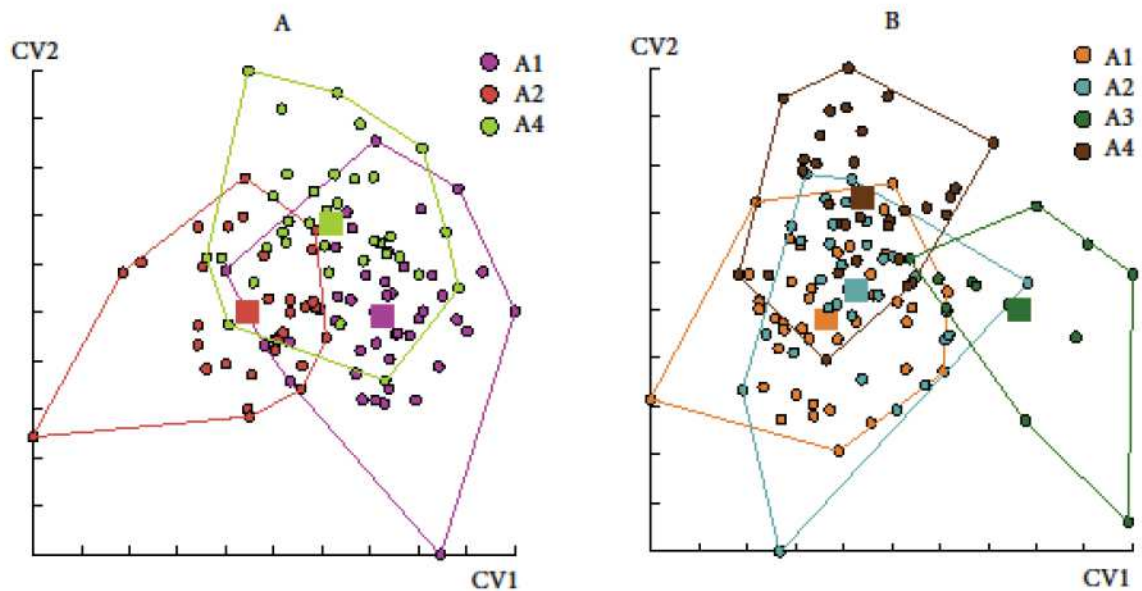


Figure 15. Distribution of the individuals along the first two canonical variates for females (A) and males (B) by altitude groups in June. Horizontal axis: canonical variate 1; Vertical axis: canonical variate 2. Signs indicate each individual. Altitude groups: A1 (200-300m), A2 (300-400m), A3 (400-500m), A4 (>500)

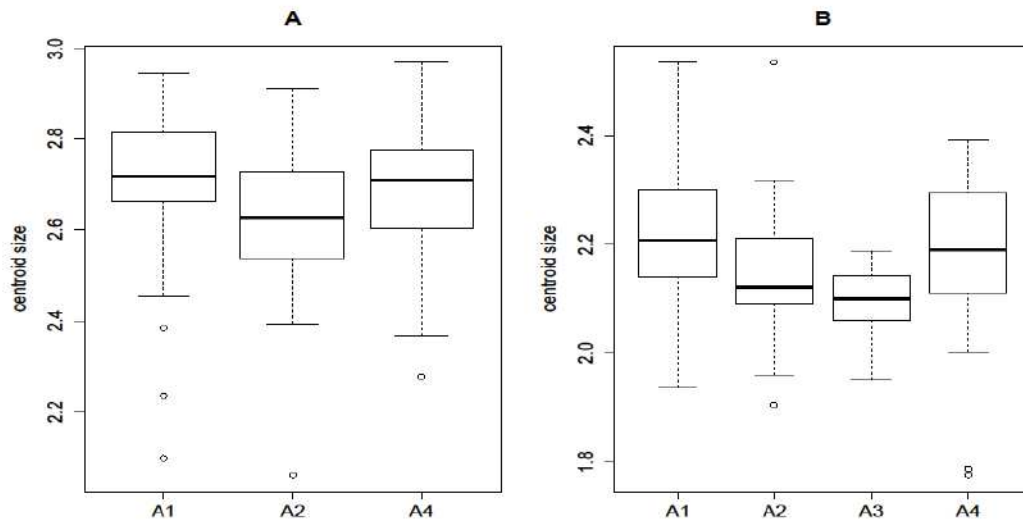


Figure 16. Mean, standard deviation, and error of centroid wing sizes by altitude groups for females (A) and males (B) in June. Altitude groups: A1 (200-300m), A2 (300-400m), A3 (400-500m), A4 (>500)

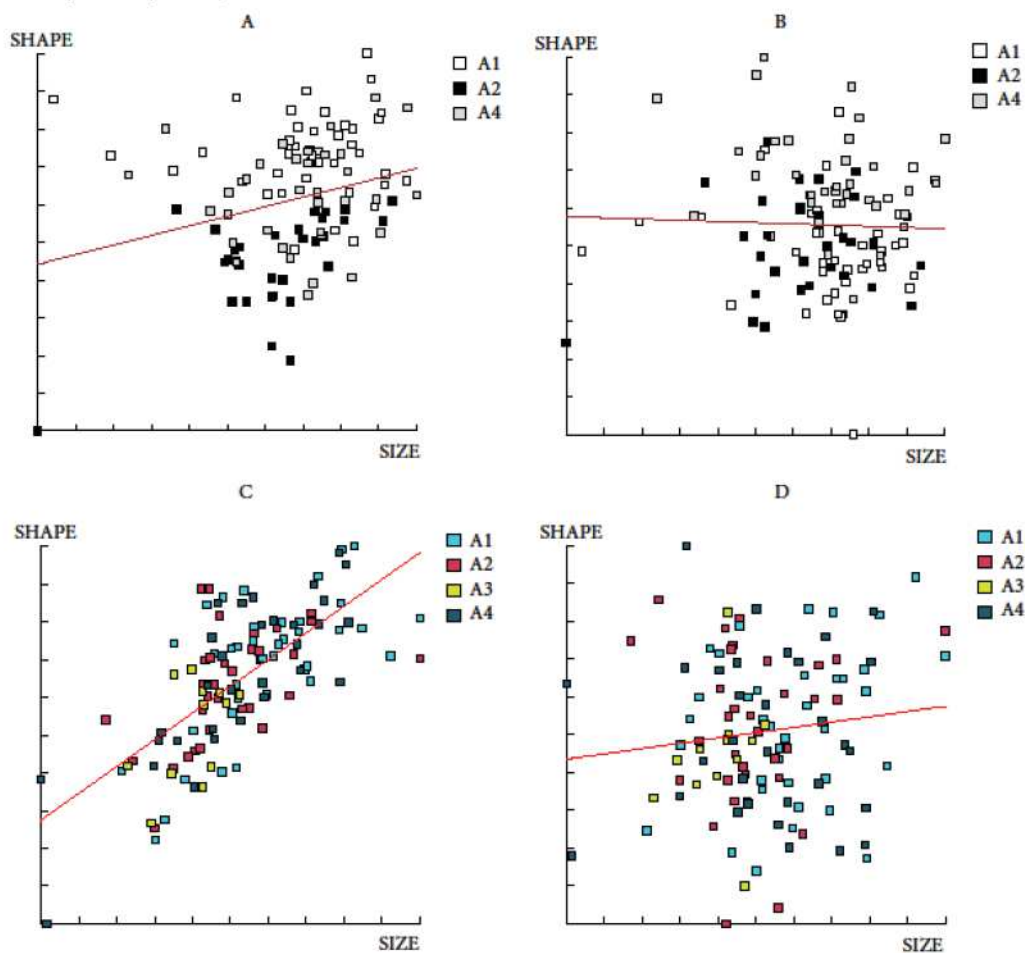


Figure 17. Regression of first discriminant factor on centroid size by altitude groups in June for females (A and B) and for males (C and D). Horizontal axis: centroid size of the wing. Vertical axis: discriminant factor 1 (A and C) or discriminant factor 2 (B and D). Regression line is shown. Altitude groups: A1 (200-300m), A2 (300-400m), A3 (400-500m), A4 (>500)

194 **Phenotypic differentiation by station**

195 In order to observe a possible effect of stations on wing phenotype, several analyses
 196 were realized by sex in June and July. Due to low number of individuals in certain stations,
 197 we performed a serial of discriminant analyses first considering all the stations and then
 198 excluding stations with a low number of individuals in order to increase the number of
 199 principal components and thus the variance explained (Table 3).

Table 3. Number of *Phlebotomus ariasi* wings used by station for the geometric morphometric analysis (Station: Number of individuals by station included in the analysis; NC: Number of components included in the analysis; V: percentage of shape variance explained by the components included in the analysis).

	Females June		Females July			Males June			Males July			
NC	3	8	3	4	5	3	4	5	2	3	4	
V (%)	59.29	87.67	55.32	64.88	70.04	54.91	63.67	71.24	54.65	67.69	75.66	
Station	ST03	4	/	6	6	6	4	/	/	/	/	/
	ST04	14	14	/	/	/	/	/	/	6	6	6
	ST05	/	/	/	/	/	8	8	8	4	4	/
	ST06	14	14	16	16	16	6	6	6	/	/	/
	ST09	9	9	5	5	/	9	9	9	/	/	/
	ST10	/	/	/	/	/	/	/	/	/	/	/
	ST11	23	23	19	19	19	9	9	9	3	/	/
	ST12	/	/	/	/	/	6	6	6	/	/	/
	ST13	/	/	/	/	/	7	7	7	/	/	/
	ST14	/	/	/	/	/	12	12	12	/	/	/
	ST15	/	/	/	/	/	5	5	/	/	/	/
	ST16	/	/	/	/	/	12	12	12	/	/	/
	ST17	/	/	4	/	/	/	/	/	/	/	/
	ST18	4	/	9	9	9	8	8	8	5	5	5
	ST19	4	/	4	/	/	/	/	/	8	8	8
	ST20	32	32	7	7	7	27	27	27	/	/	/

200 For females in June, Mahalonobis distances used to study wing shape were not
 201 significantly different between stations for the first analysis (data not shown) but significant
 202 between ST19 and ST20 for the second one (Table 4 and Figures 18 and 19). In July, even if

203 CVAs showed some grouping by station (Figure 20), Mahalanobis distances were not
 204 significantly different between stations for all analyses (Table 4). For males, no significant
 205 difference was observed between stations for June and July (data not shown). The results of
 206 the shape analyses and the contribution of size to wing shape differentiation are synthesized in
 207 Table 4.

Table 4. Synthesis of the Mahalanobis distances results (NC: Number of components included in the analysis; V: percentage of shape variance explained by the components included in the analysis; *P*-values: *P*-value after Bonferonni correction (* significant); Allometry: percentage of size contribution to wing shape differentiation for the first component (1) and the second one (2)).

Analysis	NC	V (%)	Adjusted <i>P</i> -values	Allometry	
				1	2
Females in June	3	59.29	$P > 0.00179$	26%	1%
	8	87.67	$P < 0.00500$ *	25%	22%
Females in July	3	55.32	$P > 0.00179$	38%	13%
	4	64.88	$P > 0.00333$	41%	15%
	5	70.04	$P > 0.00500$	33%	23%
Males in June	3	54.91	$P > 0.00076$	48%	1%
	4	63.67	$P > 0.00091$	49%	0%
	5	71.24	$P > 0.00111$	48%	1%
Males in July	2	54.65	$P > 0.00500$	36%	0%
	3	67.69	$P > 0.00833$	35%	1%
	4	75.66	$P > 0.01667$	26%	8%

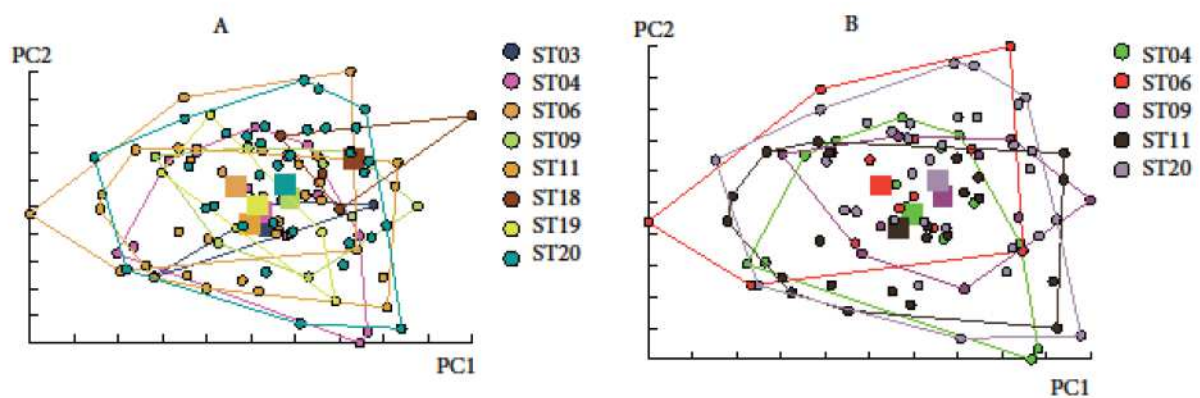


Figure 18. Distribution of the individuals of *Phlebotomus ariasi* females by station in June with 3 (A) and 8 components (B) along the first two principal components. Horizontal axis: principal component 1; Vertical axis: principal component 2. Signs indicate each individual.

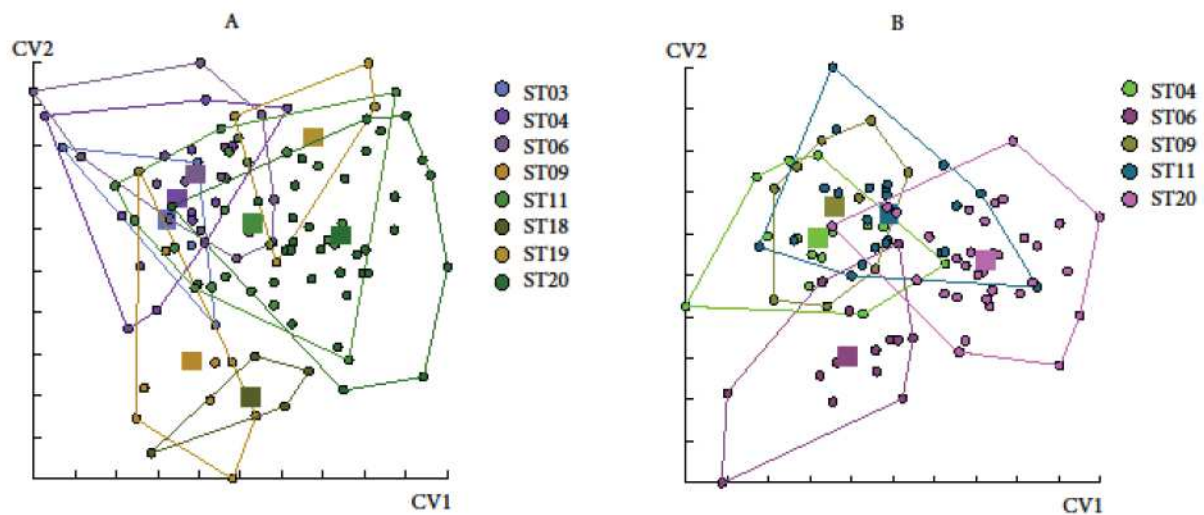


Figure 19. Distribution of the individuals along the first two canonical variates for females by station in June with 3 (A) and 8 components (B). Horizontal axis: canonical variate 1; Vertical axis: canonical variate 2. Signs indicate each individual.

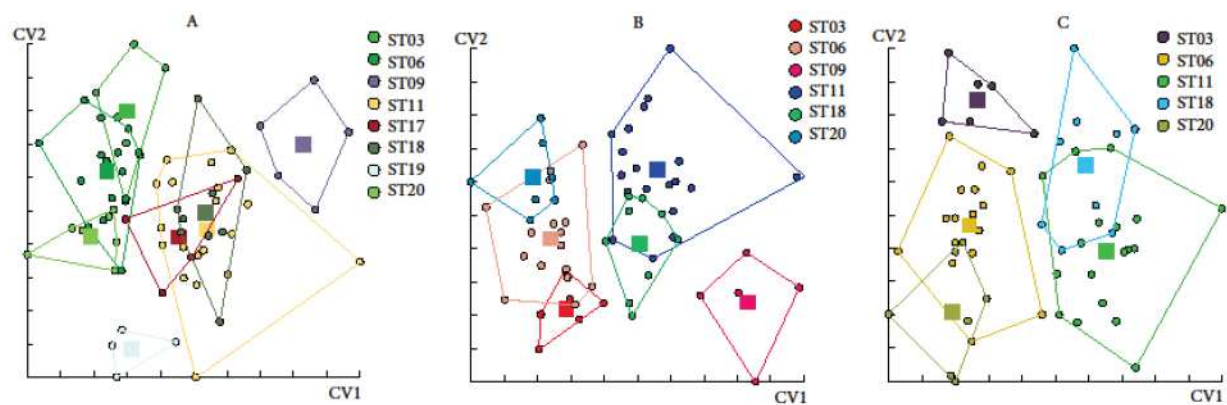


Figure 20. Distribution of the individuals along the first two canonical variates for females by station in July with 3 (A), 4 (B) and 5 components (C). Horizontal axis: canonical variate 1; Vertical axis: canonical variate 2. Signs indicate each individual.

208 Kruskal Wallis tests used to study wing size revealed no significant effect of station on
 209 centroid size in June ($\chi^2 = 12.4043$, p -value = 0.1915; $\chi^2 = 18.3754$, p -value = 0.07327) and
 210 in July ($\chi^2 = 12.5229$, p -value = 0.1294; $\chi^2 = 11.6$, p -value = 0.2368) for females and males,
 211 respectively.

212 Discussion

213 **Sexual dimorphism**

214 Our results showed significant differences in wing shape and size between males and
215 females; females having larger wings. Sexual dimorphism is widely observed in many insects
216 such as *Aedes* mosquitoes [30], *Anopheles*, *Culex* and *Ochlerotatus* mosquitoes [31],
217 *Drosophila melanogaster* [32], and *D. subobscura* [33]. Thin-plate spline deformation
218 analyses showed that the deformation was mostly present on the medial part of the wings. The
219 medial of the wings of flying insects is known to play an essential role in flying capability
220 [34]. Because of blood meal necessity, the female sandflies might need a greater flying
221 capacity compared to males. This could explain the wing differences between them. A
222 previous study based on a mark-release-recapture method showed that *P. ariasi* females
223 present a dispersion ability of more than 1km [35]. It would be interesting to measure the
224 dispersion capacity of males in order to test this hypothesis.

225

226 **Wing phenotype differentiation by month**

227 We observed a difference of wing shape between June and July within both sexes and a
228 difference of size only for females (smaller in July). It was not possible to make any
229 conclusion for the other months (May, August and September) due to the low number of
230 individuals captured. The variations observed between June and July may be due to variation
231 in environmental conditions. The individuals captured in June and July have certainly
232 emerged at the end of May and June, respectively. Major temperature and relative humidity
233 variation were observed between these two months. Moreover, the temperature variation
234 between night and day is greater in May than in June or July. It is known that the climatic
235 parameters influence the entry into diapause and the exit and also the larvae development of
236 insects [36]. *P. ariasi*, as most of northern insect species, presents a period of diapause (at the

237 4th instar larvae stage) in order to survive over the winter and postpone the reproduction until
238 favorable conditions [37]. The presence of climatic variations between months, at the end
239 and/or at the beginning of the sandfly season may be responsible of differences in the
240 development of the larvae before and/or after the diapause [38]. The conditions for the
241 beginning and end of diapause are still not well known [36]. This study highlights wing
242 differences between months but these results should be considered with larger sample sizes
243 and other species.

244

245 **Environmental factors and phenotypic variation in June**

246 In our study, we found shape and size differences by altitude for males and females in
247 June. These results are more likely to reflect differential environmental and climatic pressures
248 according to group of altitudes. Previous studies highlighted the importance of altitude for the
249 biology of sandflies since biotic and abiotic characteristics are highly correlated with
250 altitudinal gradients [12, 39]. Particularly, the size of adults is largely influenced by
251 temperature, with larger adults generated at low temperatures and smaller adults at high
252 temperatures [12]. It is known that larvae development is influenced by temperature
253 conditions [12, 40]; a high temperature will produce a rapid development and thus small
254 individuals. Assuming that wing size reflects the size of specimens, centroid size should
255 predict larger individuals at higher altitudes [41]. However, the size differences observed by
256 altitude groups did not show larger wings at higher altitudes. This correlation may not be
257 observed in this study because of too small altitudinal differences between sampling sites.

258 We also found a significant spatial differentiation in June for females by slope and
259 station. The absence of spatial differentiation observed in males, conversely to females could
260 be a consequence of stronger sex-specific selection pressures such as the host availability for
261 blood meal in the sampled stations. The strong sexual dimorphism observed also support the

262 existence of sex-specific selection pressures (see above). Nevertheless, the reasons of
263 differential shape and size between sexes have not been well explored [42].

264

265 Conclusion

266 In conclusion, this study indicates that phenotypical variations exist among local
267 populations of *P. ariasi*, providing four main results: sexual dimorphism, shape and size
268 variation between months (June and July in particular) and different wing phenotypes by
269 altitude for both sexes and by slope and stations for females.

270 The levels of phenotypic variation observed in *P. ariasi* populations are more likely to
271 reflect the local environmental pressures to which these populations are subjected. These data
272 underline the plasticity and the capacity of adaptation of these insects even at a sympatric
273 level.

274

275 Acknowledgments

276 This study was funded by EU grant FP7-261504 EDENext and is catalogued by the
277 EDENext Steering Committee as EDENextXXX (<http://www.edenext.eu>). The contents of
278 this publication are the sole responsibility of the authors and do not necessarily reflect the
279 views of the European Commission. We are particularly grateful to the EDENext PhDB
280 group, Professor Rioux for the interesting and useful discussions and the habitants from the
281 study area for their kindness and availability. We also thank Mr. Lacoste (Inkerman
282 foundation), IRD (Institut de Recherche pour le Développement) and CNRS for financial
283 support.

- 285 1. Rioux JA, Killick-Kendrick R, Perieres J, Turner D, Lanotte G: **Ecologie des Leishmanioses dans**
286 **le Sud de la France. 13. Les sites de "flanc de coteau", biotopes de transmission privilégiés**
287 **de la Leishmaniose viscérale en Cévennes.** *Annales de parasitologie* 1980, 55(4):445-453.
- 288 2. Rioux JA, Carron S, Dereure J, Perieres J, Zeraia L, Franquet E, Babinot M, Gallego M,
289 Prudhomme J: **Ecology of leishmaniasis in the South of France. 22. Reliability and**
290 **representativeness of 12 *Phlebotomus ariasi*, *P. perniciosus* and *Sergentomyia minuta***
291 **(Diptera: Psychodidae) sampling stations in Vallespir (eastern French Pyrenees region).**
292 *Parasite (Paris, France)* 2013, 20:34.
- 293 3. Abonnenc E: **Les phlébotomes de la région éthiopienne (Diptera, Psychodidae).** 1972:1-290.
- 294 4. Ready P: **Leishmaniasis emergence in Europe.** *Euro surveillance : bulletin Européen sur les*
295 *maladies transmissibles = European communicable disease bulletin* 2010, 15(10):19505.
- 296 5. Franco F, Morillas-Marquez F, Barón S, Morales-Yuste M, Gálvez R, Díaz-Sáez V, Pesson B,
297 Alves-Pires C, Depaquit J, Molina R *et al*: **Genetic structure of *Phlebotomus (Larrousius)***
298 ***ariasi* populations, the vector of *Leishmania infantum* in the western Mediterranean:**
299 **Epidemiological implications.** *International Journal for Parasitology* 2010, 40(11):1335-1346.
- 300 6. Lewis D: **A taxonomic review of the genus *Phlebotomus* (Diptera: Psychodidae).** *Bulletin of*
301 *the British Museum (Natural History)* 1982, 45(2):121-209.
- 302 7. Dedet J-P: **Les leishmanioses en France métropolitaine.** *BEH Hors-série* 2010:9-12.
- 303 8. Kamhawi S, Molyneux D, Killick-Kendrick R, Milligan P, Phillips A, Wilkes T, Killick-Kendrick M:
304 **Two populations of *Phlebotomus ariasi* in the Cévennes focus of leishmaniasis in the south**
305 **of France revealed by analysis of cuticular hydrocarbons.** *Medical and Veterinary*
306 *Entomology* 1987, 1(1):97-102.
- 307 9. Pesson B, Wallon M, Floer M, Kristensen A: **[Isoenzymatic studies of Mediterranean**
308 **populations of sandflies of the subgenus *Larrousius*].** *Parassitologia* 1991, 33:471-476.
- 309 10. Martin-Sanchez J, Gramiccia M, Pesson B, Morillas-Marquez F: **Genetic polymorphism in**
310 **sympatric species of the genus *Phlebotomus*, with special reference to *Phlebotomus***
311 ***perniciosus* and *Phlebotomus longicuspis* (Diptera, Phlebotomidae).** *Parasite (Paris, France)*
312 2000, 7(4):247-254.
- 313 11. Mahamdallie S, Pesson B, Ready P: **Multiple genetic divergences and population expansions**
314 **of a Mediterranean sandfly, *Phlebotomus ariasi*, in Europe during the Pleistocene glacial**
315 **cycles.** *Heredity* 2010, 106(5):714-726.
- 316 12. Aytekin A, Alten B, Caglar SS, Ozbel Y, Kaynas S, Simsek F, Kasap O, Belen A: **Phenotypic**
317 **variation among local populations of phlebotomine sand flies (Diptera: Psychodidae) in**
318 **southern Turkey.** *Journal of vector ecology : journal of the Society for Vector Ecology* 2007,
319 32(2):226-234.
- 320 13. Prudhomme J, Gunay F, Rahola N, Ouanaïmi F, Guernaoui S, Boumezzough A, Bañuls A,
321 Sereno D, Alten B: **Wing size and shape variation of *Phlebotomus papatasi* (Diptera:**
322 **Psychodidae) populations from the south and north slopes of the Atlas Mountains in**
323 **Morocco.** *Journal of vector ecology : journal of the Society for Vector Ecology* 2012,
324 37(1):137-147.
- 325 14. Dvorak V, Aytekin A, Alten B, Skarupova S, Votycka J, Volf P: **A comparison of the**
326 **intraspecific variability of *Phlebotomus sergenti* Parrot, 1917 (Diptera: Psychodidae).**
327 *Journal of vector ecology : journal of the Society for Vector Ecology* 2006, 31(2):229-238.
- 328 15. Dvorak V, Votycka J, Aytekin A, Alten B, Volf P: **Intraspecific variability of natural**
329 **populations of *Phlebotomus sergenti*, the main vector of *Leishmania tropica*.** *Journal of*
330 *vector ecology : journal of the Society for Vector Ecology* 2011, 36 Suppl 1:S49-57.
- 331 16. Riva J, Pont F, Ali V, Matias A, Mollinedo S, Dujardin JP: **Wing geometry as a tool for studying**
332 **the *Lutzomyia longipalpis* (Diptera: Psychodidae) complex.** *Memórias do Instituto Oswaldo*
333 *Cruz* 2001, 96(8):1089-1094.

- 334 17. Daget P: **Le bioclimat Méditerranéen: Analyse des formes climatiques par le système**
335 **d'Emberger.** *Vegetation* 1977, **34(2):87-103.**
- 336 18. Guy M, Killick-Kendrick R, Gill G, Rioux JA, Bray RS: **Ecology of leishmaniasis in the south of**
337 **France. 19. Determination of the hosts of *Phlebotomus ariasi* Tonnoir, 1921 in the**
338 **Cévennes by bloodmeal analyses.** *Annales de parasitologie humaine et comparée* 1984,
339 **59(5):449-458.**
- 340 19. Alten B, Ozbel Y, Ergunay K, Kasap O, Cull B, Antoniou M, Velo E, Prudhomme J, Molina R,
341 **Bañuls A et al: Sampling strategies for phlebotomine sand flies (Diptera: Psychodidae) in**
342 **Europe.** *Bulletin of entomological research* 2015:1-15.
- 343 20. Killick-Kendrick R, Tang Y, Killick-Kendrick M, Sang D, Sirdar M, Ke L, Ashford R, Schorscher J,
344 **Johnson R: The identification of female sandflies of the subgenus *Larrousius* by the**
345 **morphology of the spermathecal ducts.** *Parassitologia* 1991, **33:335-347.**
- 346 21. Rohlf FJ, Slice D: **Extensions of the Procrustes method for the optimal superimposition of**
347 **landmarks.** *Systematic Biology* 1990, **39(1):40-59.**
- 348 22. Rohlf FJ: **tpsUtil, file utility program. version 1.60. . Department of Ecology and Evolution,**
349 **State University of New York at Stony Brook** 2015.
- 350 23. Rohlf FJ: **tpsDIG2 version 2.18. . Stony Brook, NY: Department of Ecology and Evolution, State**
351 **University of New York** 2015.
- 352 24. **MoMe-CLIC. Morphometrics in Medical Entomology – Collection of Landmark for**
353 **Identification and Characterization.** [<http://mome-clic.com/>]
- 354 25. Team RC: **R: A language and environment for statistical computing.** R Foundation for
355 **Statistical Computing, Vienna, Austria, 2012.** ISBN 3-900051-07-0; 2014.
- 356 26. Bookstein FL: **Morphometric tools for landmark data: Cambridge univ. press Cambridge etc;**
357 **1991.**
- 358 27. Rohlf F: **Shape statistics: Procrustes superimpositions and tangent spaces.** *Journal of*
359 **Classification** 1999, **16:197-223.**
- 360 28. Rohlf F: **Relative warp analysis and an example of its application to mosquito.** *Contributions*
361 **to morphometrics** 1993, **8:131.**
- 362 29. Alibert P, Moureau B, Dommergues J-L, David B: **Differentiation at a microgeographical scale**
363 **within two species of ground beetle, *Carabus auronitens* and *C. nemoralis* (Coleoptera,**
364 **Carabidae): a geometrical approach.** *Zoologica Scripta* 2001, **30(4):299-311.**
- 365 30. Wormington JD, Juliano SA: **Sexually dimorphic body size and development time plasticity**
366 **in *Aedes mosquitoes* (Diptera: Culicidae).** *Evolutionary Ecology Research* 2014, **16:223-234.**
- 367 31. Virginio F, Vidal P, Suesdek L: **Wing sexual dimorphism of pathogen-vector culicids.**
368 ***Parasites & Vectors* 2015, 8(1).**
- 369 32. Bitner-Mathá B, Klaczko L: **Plasticity of *Drosophila melanogaster* wing morphology: effects**
370 **of sex, temperature and density.** *Genetica* 1999, **105(2):203-210.**
- 371 33. Gilchrist GW, Huey RB: **Plastic and genetic variation in wing loading as a function of**
372 **temperature within and among parallel clines in *Drosophila subobscura*.** *Integrative and*
373 ***Comparative Biology* 2004, 44:461-470.**
- 374 34. Johansson F, Söderquist M, Bokma F: **Insect wing shape evolution: independent effects of**
375 **migratory and mate guarding flight on dragonfly wings.** *Biological Journal of the Linnean*
376 ***Society* 2009, 97(2):362-372.**
- 377 35. Killick-Kendrick R, Rioux JA, Bailly M, Guy M, Wilkes T, Guy F, Davidson I, Knechtli R, Ward R,
378 **Guilvard E et al: Ecology of leishmaniasis in the south of France. 20. Dispersal of**
379 ***Phlebotomus ariasi* Tonnoir, 1921 as a factor in the spread of visceral leishmaniasis in the**
380 **Cévennes.** *Annales de parasitologie humaine et comparée* 1984, **59(6):555-572.**
- 381 36. Lawyer P, Young D: **Diapause and quiescence in *Lutzomyia diabolica* (Diptera: Psychodidae).**
382 ***Parassitologia* 1991, 33:353-360.**
- 383 37. Volf P, Volfova V: **Establishment and maintenance of sand fly colonies.** *Journal of vector*
384 **ecology : journal of the Society for Vector Ecology** 2011, **36 Suppl 1:S1-9.**

- 385 38. Ready P, Croset H: **Diapause and laboratory breeding of *Phlebotomus perniciosus* Newstead**
386 **and *Phlebotomus ariasi* Tonnoir (Diptera: Psychodidae) from southern France.** *Bulletin of*
387 *entomological research* 1980, **70**:511-623.
- 388 39. Belen A, Alten B, Aytakin A: **Altitudinal variation in morphometric and molecular**
389 **characteristics of *Phlebotomus papatasi* populations.** *Medical and Veterinary Entomology*
390 2004, **18**(4):343-350.
- 391 40. Aytakin S, Aytakin A, Alten B: **Effect of different larval rearing temperatures on the**
392 **productivity (R₀) and morphology of the malaria vector *Anopheles superpictus* Grassi**
393 **(Diptera: Culicidae) using geometric morphometrics.** *Journal of vector ecology : journal of*
394 *the Society for Vector Ecology* 2009, **34**(1):32-42.
- 395 41. Dujardin J-P, Pont F, Baylac M: **Geographical versus interspecific differentiation of sand flies**
396 **(Diptera: Psychodidae): a landmark data analysis.** *Bulletin of entomological research* 2003,
397 **93**(1):87-90.
- 398 42. Chown S, Gaston K: **Body size variation in insects: a macroecological perspective.** *Biological*
399 *reviews of the Cambridge Philosophical Society* 2010, **85**(1):139-169.

400

401

VI. Une structuration génétique en sous-populations micro-géographiques

VI.1. Développement des marqueurs microsatellites

Tout d'abord, des marqueurs microsatellites ont été mis au point pour *P. ariasi* et testés sur un petit nombre d'échantillons. La procédure est décrite dans : « **Prudhomme J., Toty C., Kasap OE., Rahola N., Vergnes B., Maia C., Campino L., Antoniou M., Jimenez M., Molina R., Cannet A., Alten B., Sereno D.*, Bañuls A-L.*, 2014. New microsatellite markers for multi-scale genetic studies on *Phlebotomus ariasi* Tonnoir, vector of *Leishmania infantum* in the Mediterranean area. *Acta Trop.* 142: 79-85. » [94]**

VI.1.1. Résumé

Le but de ce travail était de développer des marqueurs microsatellites polymorphes afin d'étudier la structure génétique des populations de *P. ariasi* à différentes échelles géographiques. 16 loci microsatellites ont été utilisés, 14 mis au point dans cette étude et 2 précédemment décrits [59]. Ces marqueurs ont été testés sur des populations de *P. ariasi* provenant de 3 localités : Roquedur-le-Haut (Gard, France), Nice (Alpes Maritimes, France) et Arrábida (Setúbal, Portugal). La transférabilité de ces amorces microsatellites a aussi été vérifiée sur sept autres espèces : *P. perniciosus* Newstead, 1911, *P. sergenti* s.l. Parrot (1917), *P. mascittii* Grassi (1908), *P. papatasi* (Scopoli, 1786), *P. Tobbi* Adler, Theodor et Lourie (1930), *P. perfiliewi* s.l Parrot (1930) et *Sergentomyia minuta* (Rondani, 1843).

Les résultats préliminaires de cette étude montrent une forte différenciation entre les échantillons de France et ceux du Portugal mais aucune entre les deux populations françaises. L'hypothèse d'une divergence plus récente entre les deux populations françaises en opposition à celle plus ancienne des populations françaises et portugaises est cohérente avec les données obtenues.

En définitive, cette étude montre l'utilité des marqueurs microsatellites pour les analyses de génétique des populations de *P. ariasi* à différentes échelles géographiques. De plus, de manière intéressante, certains loci se sont révélés adaptés pour la caractérisation génétique d'autres espèces.

Les marqueurs microsatellites mis au point au cours de cette étude ont ensuite été utilisés pour l'analyse de la structure génétique des populations de *P. ariasi* dans notre zone d'étude (Voir : VI.2. Ecosystème et structure génétique des populations de *P. ariasi*).

VI.1.2. Article 6 : New microsatellite markers for multi-scale genetic studies on *Phlebotomus ariasi* Tonnoir, vector of *Leishmania infantum* in the Mediterranean area.



New microsatellite markers for multi-scale genetic studies on *Phlebotomus ariasi* Tonnoir, vector of *Leishmania infantum* in the Mediterranean area



Jorian Prudhomme^{a,*}, Céline Toty^a, Ozge Erisoz Kasap^b, Nil Rahola^a, Baptiste Vergnes^a, Carla Maia^c, Lenea Campino^c, Maria Antoniou^d, Maribel Jimenez^e, Ricardo Molina^e, Arnaud Cannet^f, Bulent Alten^b, Denis Sereno^{a,1}, Anne-Laure Bañuls^{a,1}

^a UMR MIVEGEC (IRD 224 – CNRS 5290 – Universités Montpellier 1 et 2), 911 Avenue Agropolis, F34394 Montpellier, France

^b Hacettepe University, Faculty of Science, Department of Biology, Ecology Section, ESRL Laboratories, Beytepe, 0680 Ankara, Turkey

^c Unidade de Parasitologia Médica, Centro de Malária e outras Doenças Tropicais, Instituto de Higiene e Medicina Tropical (IHMT), Universidade Nova de Lisboa (UNL), Rua da Junqueira, 100, 1349-008 Lisboa, Portugal

^d Laboratory of Clinical Bacteriology, Parasitology, Zoonoses, and Geographical Medicine, Faculty of Medicine, University of Crete, Voutes, Heraklion, 71003 Crete, Greece

^e Unidad de Entomología Médica, Servicio de Parasitología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Ctra. Majadahonda-Pozuelo s/n, Majadahonda, 28220 Madrid, Spain

^f Inserm U1065, Centre Méditerranéen de Médecine Moléculaire, C3M, Université de Nice-Sophia Antipolis, 151, route St Antoine de Ginestière, BP 2 3194, 06204 Nice Cedex, France

ARTICLE INFO

Article history:

Received 24 June 2014

Received in revised form 21 October 2014

Accepted 26 October 2014

Available online 1 November 2014

Keywords:

Phlebotomus ariasi

Microsatellites

Population genetics

ABSTRACT

The population structure of *Phlebotomus ariasi*, a proven vector of *Leishmania infantum* in the Mediterranean area, is still poorly understood. Previously, only two microsatellite loci had been developed to study the population genetics of this species. Herein we use these loci and determined fourteen novel microsatellite loci, useful for the characterization of *P. ariasi* populations. These loci were tested on three populations of *P. ariasi*, two from France and one from Portugal. In addition, the usefulness of these markers was also evaluated on seven other sandfly species.

We show, that for *P. ariasi*, 15 of the 16 loci selected were polymorphic, with a mean of 4.25 alleles and an observed heterozygosity of 0.299. Within the *P. ariasi* population of France, 11 loci were polymorphic, with an average of 2.44 alleles and an observed heterozygosity of 0.2177. The fixation index was moderate among the French populations but high between French and Portuguese populations. In addition, eight loci were also found to be amplifiable in six other *Phlebotomus* species.

These results demonstrate the usefulness of this new set of microsatellite loci for population structure and molecular ecology studies of *P. ariasi* at various spatial scales, but also of other sandfly species.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

Phlebotomine sandflies are insect vectors that transmit Leishmaniasis, human and veterinary diseases caused by protozoa

belonging to the *Leishmania* genus. About 14 million people are infected worldwide by these parasites, with an estimated 1.3 million new cases and estimated 20,000 to 30,000 deaths that occur annually (Alvar et al., 2012). During the past 10 years, the disease has spread considerably in France. An increase in the incidence of cutaneous leishmaniasis cases associated with an extension of the disease endemic area is observed (Dedet, 2010). Climate change might affect distribution of this disease by the effect of environmental variation on the range and seasonal abundances of the vector species (Ready, 2010). Although this disease is extensively studied in Europe, the biology and ecology of sandflies is still poorly known.

Phlebotomus ariasi Tonnoir, 1921, is the predominant sandfly species in Cevennes region (Rioux et al., 1980) and one of the two proven vector, with *Phlebotomus perniciosus* Newstead, 1911, of

* Corresponding author. Tel.: +33 467 416 348; fax: +33 621 504 351.

E-mail addresses: jorian.prudhomme@hotmail.fr (J. Prudhomme), celine.toty@ird.fr (C. Toty), ozgeerisoz@yahoo.com (O.E. Kasap), nil.rahola@ird.fr (N. Rahola), baptiste.vergnes@ird.fr (B. Vergnes), CarlaMaia@ihmt.unl.pt (C. Maia), lcampino@ihmt.unl.pt (L. Campino), antoniou@med.uoc.gr (M. Antoniou), mjimenez@isciii.es (M. Jimenez), rmolina@isciii.es (R. Molina), arnaud.cannet@unice.fr (A. Cannet), kaynas@hacettepe.edu.tr (B. Alten), denis.sereno@ird.fr (D. Sereno), anne-laure.banuls@ird.fr (A.-L. Bañuls).

¹ These authors contributed equally to the work.

leishmaniasis caused by *Leishmania infantum* in the South of France (Rioux et al., 2013). *P. ariasi* are found resting in houses, animal sheds, caves and holes in walls (“barbacanes”), near roads or in villages. This species has a wide geographical area distribution, including many countries of the Western Mediterranean region such as Algeria, France, Italy, Morocco, Portugal, Spain and Tunisia (Ballart et al., 2012; Barón et al., 2011; Boudabous et al., 2009; Boussaa et al., 2010; Branco et al., 2013; Franco et al., 2010; Lewis, 1982; Ready, 2010; Rossi et al., 2007). However, the population structure of *P. ariasi* is currently not well known. Only few studies have been performed on this species such as analysis of cuticular hydrocarbons (Kamhawi et al., 1987), isoenzymes (Pesson et al., 1991), random amplified polymorphic DNA (Martín-Sánchez et al., 2000) and sequences of mitochondrial cytochrome b gene (Franco et al., 2010). Among available molecular markers for molecular ecology studies, microsatellite markers have proved to be useful tools to study population genetic structure (Selkoe and Toonen, 2006). These molecular markers allow working at various scales to compare populations among countries and between regions, but also within endemic areas. However, few studies using microsatellite loci have been carried out on Mediterranean sandflies (for example: *P. papatasi* (Hamarshah et al., 2009; Khalid et al., 2012), *P. perniciosus* (Aransay et al., 2003) and *P. ariasi* (Mahamdallie et al., 2011)). The previous population genetic study of *P. ariasi* (Mahamdallie et al., 2011), used two microsatellite loci developed in a prior study for *P. perniciosus* (Aransay et al., 2001) with other genetic markers (DNA sequences from mitochondrial cytochrome b and nuclear elongation factor-1a) to understand the population expansions of this species in Europe during the Pleistocene glacial cycles.

The aim of this work was to develop a suite of polymorphic microsatellite loci to study the *P. ariasi* population structure and molecular ecology at different geographic scale, including micropopulations.

Sixteen microsatellite loci were used to characterize *P. ariasi*, including the two previously developed by Aransay et al. (2001) for *P. perniciosus*. The 14 other loci were developed herein. In addition, the transferability of the microsatellite primers developed in this study was tested in 7 other sandfly species: *P. perniciosus*, *P. sergenti* s.l. Parrot (1917), *P. mascittii* Grassi (1908), *P. papatasi* (Scopoli, 1786), *P. tobbi* Adler, Theodor and Lourie (1930), *P. perfiliewi* s.l. Parrot (1930), and *Sergentomyia minuta* (Rondani, 1843). As the specimens could be intermediate morphotypes between sergenti and similis we prefer to use *P. sergenti* s.l. for the morphotypes processed. Based on the paper of Depaquit et al. (2013), we also prefer to use *P. perfiliewi* s.l. instead of using *P. perfiliewi* s.s., *P. transcaucasicus* s.s. or *P. galilaeus* s.s. as the species status of these taxa is still doubtful.

2. Material and methods

2.1. DNA extractions

Two DNA extraction methods were employed. To obtain the DNA of the French individuals, we used an automated technique using the Chelex method (Wang and Wang, 2012) because they were handled together with a large sample of several thousands sandflies. The entire sandflies were ground using a Mixer Mill MM300 (QIAgen, Venlo, Netherlands) with one 3-mm tungsten bead at a frequency of 30 cycles s^{-1} for 3 min in the presence of 200 μ L Eagle’s Minimal Essential Medium. A volume of 140 μ L of each sample was utilized for DNA purification using the Eppendorf epMotion 5075 working station and Macherey-Nagel NucleoSpin 96 kits. For all the other specimens, since they were not numerous, DNA was manually extracted using the CTAB method,

according to a modified version of the protocol proposed by Edwards (1998). The entire sandfly was mixed with 200 μ L of extraction buffer containing 1 M Tris–HCl, pH 8.0, 0.5 M EDTA, 1.4 M NaCl and 2% Cetyl trimethyl ammonium bromide (CTAB). The mixture was then incubated at 65 °C for 5 min. The solution was centrifuged at 12,000 rpm for 5 min after addition of chloroform (200 μ L). The supernatant was extracted and added to 200 μ L of isopropanol, followed by centrifugation at 15,000 rpm for 15 min. The pellet was washed with 200 μ L 70% ethanol, centrifuged at 12,000 rpm for 5 min, air dried, resuspended in 30 μ L water and incubated overnight at room temperature.

Both DNA extraction methods were deemed equally efficient and gave similar amplification results for all the microsatellite loci used during this study.

2.2. Isolation of microsatellite DNA loci and identification

Microsatellite markers were determined from *P. ariasi* males captured in Roquedur area (Gard, France). Microsatellites were isolated and cloned before being sequenced by capillary sequencing (3730XL[®], Applied BioSystems[®], Foster City, CA) and high throughput 454 GS-FLTitanium pyrosequencing (GS FLX[®], Roche Diagnostics[®], Bâle, Swiss). They were developed, validated and genotyped by fragment analysis on the capillary sequencer. These steps were performed by a private company (Genoscreen, Pasteur Institute of Lille, France) according to the methodology described in Malausa et al. (2011). One hundred primer pairs were designed, and a further 48 selected for further testing based on the detection of unambiguous polymorphic amplicons using fixed annealing temperature PCR and a polyacrylamide gel genotyping protocol. Of these, 14 were selected for further study on the basis of different criteria (percentage of GC content, position of microsatellites in the sequence, structure of repeated array, Polymerase chain reaction (PCR) results, clear electrophoregrams which only displayed one or two peaks).

2.3. DNA amplification and genotyping

All the individuals were amplified according to the following conditions. Each 25 μ L reaction mix was composed of 1 pmol of forward and reverse primers, the forward being labeled with a fluorochrome FAM, ATT0565 or HEX (Table 1), 5 ng of DNA template, 6 pmol of dNTP mix, 2.5 μ L of buffer 10X and 0.25 μ L of *Taq* polymerase (Roche Diagnostics[®], 5 UI/ μ L). Amplifications were carried out in a thermal cycler using the following conditions: an initial denaturation step at 95 °C for 10 min, 40 cycles at 95 °C for 30 s, annealing temperature of each locus (see Table 1) for 30 s at 72 °C for 1 min and a final extension step at 72 °C for 10 min.

Genotyping was performed on an ABI Prism 3130 Genetic Analyser (Applied Biosystems[®]) automated sequencer with 1 μ L of PCR product added into a standard loading mix: 0.5 μ L of an internal standard-size GENESCAN 500LIZ and 12.5 μ L of formamide (Hi-Di) (both Applied Biosystems[®]). Profiles were read and analyzed using GENEMAPPER 4.0 (Applied Biosystems, Foster City (CA)). The DNA of each sandfly was genotyped for all the 16 selected loci, including the previously designed AAm20 and AAm24 (see Table 1) (Aransay et al., 2001). Several trials showed that multiplexing loci amplification was possible for those PCR products characterized by different sizes (no overlapping zones), or by labeling with different fluorochromes.

2.4. Data analysis

Observed heterozygosity (H_o), mean genetic diversity (H_s), expected heterozygosity (H_e) and fixation index (F_{ST}) were computed using FSTAT, version 2.9.3.2 (Goudet, 1995).

Table 1
 Details of the 16 microsatellite markers developed for *P. arisi* species; name, primer sequences, GenBank Accession number to reference clone, structure of repeated array, thermocycling conditions (annealing temperature: Ta), fluorescent dye, groups for multiplexing, genetic variation (alleles number: Na), allele size range, observed heterozygosity (H_o), mean genetic diversity (H_s), expected heterozygosity (H_e), computed by FSTAT version 2.9.3.2 (Goudet, 1995) and proportion of polymorphic loci (P).

Name	Primer sequence (5'-3')	GeneBank accession no.	Repeat type	Ta (°C)	Labeling dye	Groups	Na	Allele size (bp)	H_o	H_s	H_e	P
aria1	5'-TCCGCTTATCATCAATCG-3' 5'-AGTGGCTTCATCCGCTAGT-3'	KJ526156	(TC)1-8	55	FAM	1	4	194–208	0.154	0.129	0.491	0.093
aria2	5'-CCTGCCAGAGTTAATTATTG-3' 5'-AGTTTCTCGAAAATTGCCA-3'	KJ526157	(AG)2-6	55	FAM	1	4	105–113	0.402	0.478	0.568	0.302
aria3	5'-ATTCGGCTTACCACCAAC-3' 5'-ATCCCTTAATTTCCGTGG-3'	KJ526158	(TC)6-12	52	FAM	2	6	124–136	0.435	0.506	0.717	0.558
aria4	5'-ACTTTGTGAACCTCTGAGGC-3' 5'-CGTAGCAGGAGTAAGAATTTAATG-3'	KJ526159	(CT)6-9	51	FAM	3	4	114–120	0.437	0.472	0.493	0.372
aria5	5'-GGACGATGACGCCAAGTATC-3' 5'-AATCGTCTGAGTCAGGCCAAGC-3'	KJ526160	(TC)5-7	60	FAM	4	3	160–164	0.351	0.459	0.457	0.395
aria7	5'-CCCCATAGTCTAATGGAA-3' 5'-AGGATGCTTCGCTCTCAG-3'	KJ526161	(TC)7-10	55	ATT0565	1	4	155–161	0.238	0.233	0.459	0.116
aria8*	5'-CAAGACGGACACTTCCACA-3' 5'-CCGATAAATGGTGGACTTGG-3'	KJ526162	(AG)7-11	55	ATT0565	3	4	124–132	0.095	0.242	0.335	0.046
aria9	5'-AAATGGGTGTCACCTGTGGT-3' 5'-TTCAGCCGTAATTTGGTGTGTA-3'	KJ526163	(GA)6-9	55	ATT0565	2	4	163–169	0.190	0.282	0.520	0.093
aria10	5'-AGTAAACCTTCAAGTGGCC-3' 5'-CCAGAGCCCTTTTCACACC-3'	KJ526164	(CA)9-12	59	ATT0565	4	3	205–211	0.427	0.399	0.437	0.279
aria11	5'-GTGCTGGGAGGACAAAGATC-3' 5'-CACCAAGTGCAGGAGTGAGA-3'	KJ526165	(TC)5-8	55	HEX	1	4	93–101	0.427	0.407	0.455	0.279
aria12	5'-TTCACCTACACATGCCCTCA-3' 5'-GCTCTTAGATGTGGCCGAAA-3'	KJ526166	(TC)5-9	55	HEX	3	2	80–88	0.011	0.012	0.012	0.023
aria13	5'-GACCGTGTGATTAATGTGC-3' 5'-CGATATCGCCATTTACTCT-3'	KJ526167	(AG)4-9	55	HEX	2	6	103–113	0.599	0.589	0.667	0.628
aria14	5'-AGTTATAGGCAACTGGCCGG-3' 5'-TTTAGCCCAACTACGCACA-3'	KJ526168	(GA)9-18	51	HEX	3	9	192–210	0.598	0.732	0.776	0.698
aria15	5'-TTAGGGTATACACAGCCGC-3' 5'-TTGTCAGGCTGCCGATCT-3'	KJ526169	(CT)4-9	55	HEX	4	5	119–129	0.319	0.406	0.422	0.302
Aam20*	5'-ACAGCGATCATAGATCCG-3' 5'-CTGGTGGAGGTGAGTTGAG-3'	AJ303377	(GAG)4-5	57	FAM	5	2	183–185	0.095	0.086	0.092	0.047
Aam24*	5'-CTATTCGCCGCCACTTGC-3' 5'-TCAATCGACATTCGGACAGGC-3'	AJ303378	(GCA)6	52	HEX	5	1	170	0.000	0.000	0.000	0

* Indicates significant deviations from Hardy–Weinberg equilibrium.

Indicates microsatellite loci developed in a prior study for *Phlebotomus perniciosus* (Aransay et al., 2001)

Table 2
Characterization of sandfly species populations used in this work.

Country and locality	Latitude	Longitude	Altitude (m)	Name of the providing institute	Species (number)
France, Roquedur (Gard)	N 43°58'23.41"	E 3°39'25.97"	603	IRD (Institut de Recherche pour le Développement)	<i>P. ariasi</i> (29) <i>P. perniciosus</i> (5) <i>P. mascittii</i> (3) <i>S. mnuta</i> (5)
France, Nice(Alpes Maritimes)	N 43°51'41.189"	E 7°18'25.579"	417	University of Nice Sophia Antipolis	<i>P. ariasi</i> (7)
Portugal, Arrábida (Setúbal)	N 38°28'36.70"	W 8°59'38.76"	330	Instituto de Higiene e Medicina Tropical	<i>P. ariasi</i> (7)
Spain	N 40°19'29.79"	W 3°47'46.87"	679	Instituto de Salud Carlos III	<i>P. perniciosus</i> (5)
Turkey, Imamoglu, Adana	N 37°18'2.11"	E 35°31'4.65"	215	Hacettepe University, Faculty of Science	<i>P. sergenti</i> (5) <i>P. tobbi</i> (6) <i>P. perfilewii</i> (6)
Cyprus	N 35°05'35.38"	E 33°26'00.42"	191	University of Crete	<i>P. papatasi</i> (6)

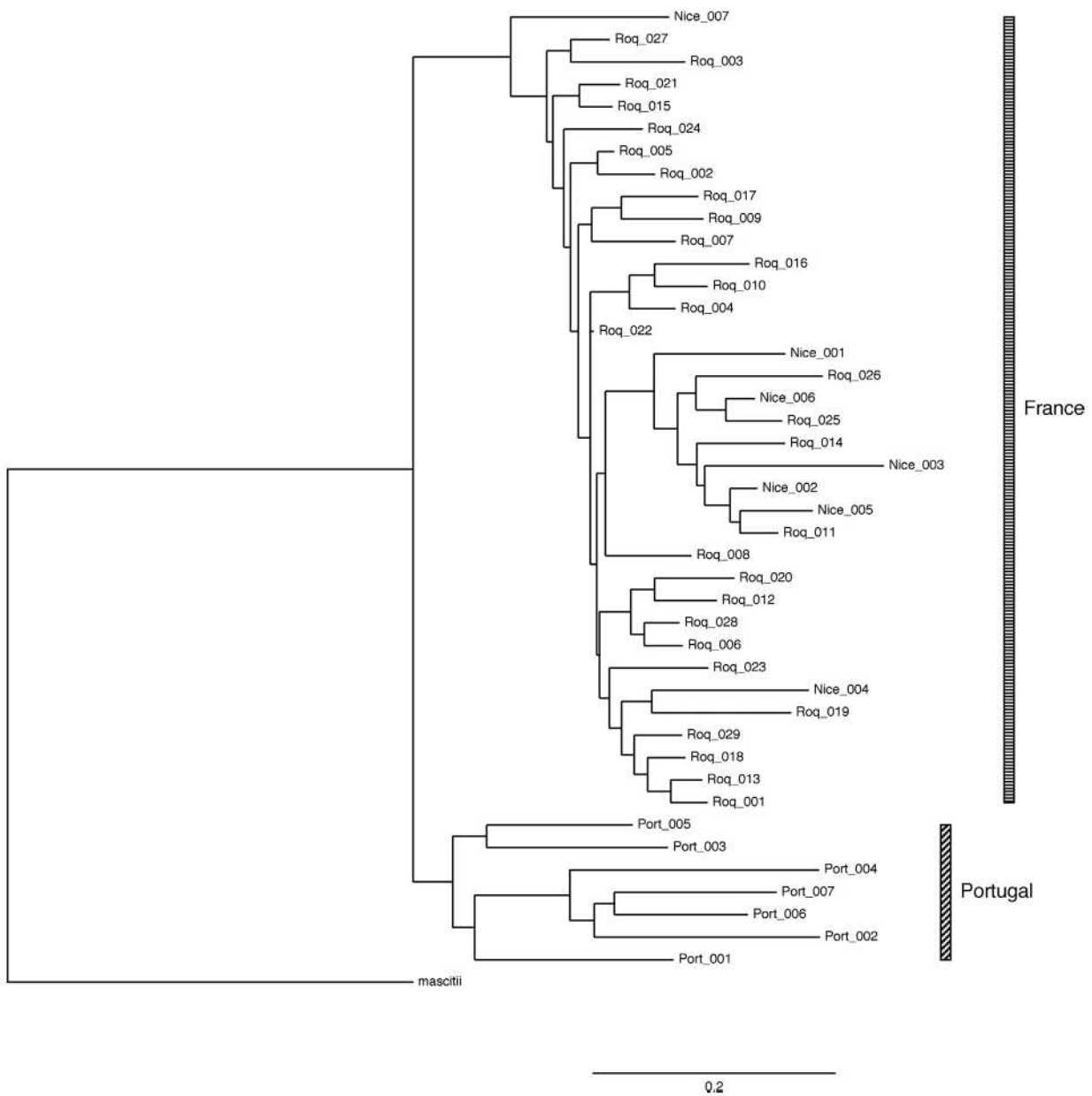


Fig. 1. Neighbor-joining phenogram based on pairwise genetic distances, studied by the Cavalli-Sforza chord measure (Dc), for the three *P. ariasi* populations (Roq = Roquedur, Nice = Nice and Port = Portugal). *P. mascittii* (*mascittii* = *P. mascittii*) was used as outgroup in tree construction.

Table 3

Individual estimations for the three populations of genetic variation (alleles number: Na); allele size range (dinucleotide repeat numbers), observed heterozygosity (H_o), mean genetic diversity (H_s), expected heterozygosity (H_e), inbreeding coefficient (F_{IS}), proportion of polymorphic loci (P) and structure of repeated array computed using GENETIX 4.05 (Belkhir et al., 2004).

Population	Locus	aria1	aria2	aria3	aria4	aria5	aria7	aria8	
Roquedur	Na	2	3	2	3	2	1	1	
	Allele size (bp)	206 (7)–208 (8)	109 (4)–113 (6)	126 (7)–128 (8)	114 (6)–118 (8)	162 (6)–164 (7)	157 (8)	126 (8)	
	H_e	0.0339	0.3347	0.4976	0.3549	0.4709	0	0	
	H_s	0.0345	0.3406	0.5064	0.3612	0.4791	0	0	
	H_o	0.0345	0.2069	0.4483	0.3103	0.4828	0	0	
	F_{IS}	0	0.397	0.117	0.143	–0.008	NA	NA	
	P	0.034	0.207	0.448	0.310	0.483	0	0	
Nice	Na	1	2	3	2	2	1	1	
	Allele size (bp)	206 (7)	111 (5)–113 (6)	126 (7)–130 (9)	116 (7)–118 (8)	162 (6)–164 (7)	157 (8)	126 (8)	
	H_e	0	0.4898	0.5612	0.4592	0.4592	0	0	
	H_s	0	0.5275	0.6044	0.4945	0.4945	0	0	
	H_o	0	0.2857	0.5714	0.4286	0.4286	0	0	
	F_{IS}	NA	0.478	0.059	0.143	0.143	NA	NA	
	P	0	0.286	0.571	0.428	0.429	0	0	
Portugal	Na	2	3	4	3	3	4	4	
	Allele size (bp)	194 (1)–204 (6)	105 (2)–111 (5)	124 (6)–136 (12)	114 (6)–120 (9)	160 (5)–164 (7)	155 (7)–161 (10)	124 (7)–132 (11)	
	H_e	0.3367	0.5204	0.3673	0.5204	0.3571	0.6633	0.6633	
	H_s	0.3626	0.5604	0.3956	0.5604	0.3846	0.7143	0.7143	
	H_o	0.4286	0.7143	0.2857	0.5714	0.1429	0.7143	0.2857	
	F_{IS}	–0.200	–0.304	0.294	–0.021	0.647	0.000	0.619	
	P	0.429	0.714	0.286	0.571	0.143	0.714	0.286	
Total	Repeat type	(TC)	(AG)	(TC)	(CT)	(TC)	(TC)	(AG)	
Population	aria9	aria10	aria11	aria12	aria13	aria14	aria15	AAM20	AAM24
Roquedur	1	2	2	2	5	8	3	1	1
	165 (7)	205 (9)–209 (11)	95 (6)–99 (7)	80 (5)–88 (9)	103 (4)–113 (9)	196 (11)–210 (18)	123 (6)–129 (9)	185 (5)	170 (6)
	0	0.1284	0.1284	0.0339	0.5832	0.7414	0.3948	0	0
	0	0.1307	0.1307	0.0345	0.5935	0.7544	0.4017	0	0
	0	0.1379	0.1379	0.0345	0.6552	0.7931	0.2414	0	0
	NA	–0.057	–0.057	0	–0.106	–0.052	0.403	NA	NA
	0	0.138	0.138	0.034	0.621	0.793	0.276	0	0
Nice	2	2	2	1	3	4	2	1	1
	165 (7)–167 (8)	205 (9)–209 (11)	95 (6)–99 (7)	88 (9)	103 (4)–109 (7)	192 (9)–204 (15)	123 (6)–125 (7)	185 (5)	170 (6)
	0.2449	0.4898	0.4898	0	0.6429	0.7041	0.2449	0	0
	0.2637	0.5275	0.5275	0	0.6923	0.7582	0.2637	0	0
	0	0.5714	0.5714	0	0.8571	0.7143	0	0	0
	1.000	–0.091	–0.091	NA	–0.263	0.063	1.000	NA	NA
	0	0.571	0.571	0	0.857	0.714	0	0	0
Portugal	4	3	4	1	3	3	3	2	1
	163 (6)–169 (9)	205 (9)–211 (12)	93 (5)–101 (8)	88 (9)	103 (4)–107 (6)	196 (11)–202 (14)	119 (4)–123 (6)	183 (4)–185 (5)	170 (6)
	0.5408	0.5204	0.5408	0	0.4490	0.6122	0.5000	0.2449	0
	0.5824	0.5604	0.5824	0	0.4835	0.6593	0.5385	0.2637	0
	0.5714	0.5714	0.5714	0	0.2857	0.2857	0.7143	0.2857	0
	0.020	–0.021	0.020	NA	0.429	0.586	–0.364	–0.091	NA
	0.571	0.286	0.714	0	0.286	0.286	0.714	0.286	0
Total	(GA)	(CA)	(TC)	(TC)	(AG)	(GA)	(CT)	(GAG)	(GCA)

Proportion of polymorphic loci (P) was also calculated. Deviations from Hardy–Weinberg equilibrium and linkage disequilibrium at each locus were measured using GENEPOP version 4.2.2 (Rousset, 2008). The individual estimations for the three populations of H_o , H_s , H_e and inbreeding coefficient (F_{IS}) were computed using GENETIX 4.05 (Belkhir et al., 1996–2004), together with P .

Genetic relatedness based on the proportion of shared alleles was studied by the modified Cavalli-Sforza chord measure (D_c) (Cavalli-Sforza and Edwards, 1967). These genetic distances were calculated using the POPULATIONS package 1.2.31 (Langella, in press, <http://bioinformatics.org/~tryphon/populations/>). The phenetic tree was constructed using neighbor-joining (NJ) clustering

Table 4

Cross-species transferability of eight microsatellite loci of the 16 microsatellite primers developed from our study in *P. ariasi*.

Locus	Phlebotomus mascittii (n=3)	Phlebotomus papatasi (n=6)	Phlebotomus perfiliewi s.l (n=6)	Phlebotomus perniciosus (n=10)	Phlebotomus sergenti (n=5)	Phlebotomus tobbi (n=6)	Sergentomyia minuta (n=5)
aria2	2	–	–	–	–	–	–
aria5	1	–	2	–	–	1	–
aria7	3	3	3	2	1	1	–
aria9	3	–	2	1	–	3	–
aria12	–	–	1	–	–	2	–
aria13	2	–	–	1	–	–	–
aria14	3	–	–	–	–	3	–
aria15	–	–	–	–	–	2	–

method (Saitou and Nei, 1987) and was edited with the computer program TreeDyn (Chevenet et al., 2006).

3. Results and discussion

3.1. Sampling

Eighty-four sandfly specimens from various countries were used: 43 *P. ariasi*, 36 from two regions of France (Roquedur, Gard ($n=29$); Nice, Alpes Maritimes ($n=7$)) and seven from Portugal (Arrábida, Setúbal), together with *P. perniciosus* (Spain, $n=5$; France, $n=5$), *P. sergenti* (Turkey, $n=5$), *P. mascittii* (France, $n=3$), *P. papatasi* (Cyprus, $n=6$), *P. tobbi* (Turkey, $n=6$), *P. perfliewi* s.l. (Turkey, $n=6$) and *S. minuta* (France, $n=5$) (Table 2).

Specimen identification was individually verified based on the morphology of the pharynx and/or the male genitalia or female spermathecae, using the keys of Theodor (1958), Abonnec (1972), Lewis (1982) and Killick-Kendrick et al. (1991).

3.2. Development of polymorphic microsatellite markers

A total of 48 microsatellite loci (available on request) containing repeat motifs were discovered, with an average sequence length of 140 bp (range 90–290). Of these, 14 were selected on the basis of different criteria (see Material and Methods). The details on selected loci are listed in Table 1. We tested the polymorphism of these loci for *P. ariasi* using 36 individuals from France and seven from Portugal. The 14 loci are complete microsatellite loci, consisting of dinucleotide motif. The sequences of the 16 loci reported in this paper are available in GenBank (accession numbers: KJ526156–KJ526169 (this study); AJ303377–AJ303378, Mahamdallie et al., 2011) (Table 1).

3.3. Genotyping and population genetics analysis

For the three populations of *P. ariasi* from Roquedur and Nice (France) and individuals from Portugal, all the loci, except AAm24, were polymorphic, with an average of 4.25 alleles per loci ($SD \pm 1.693$; range 2–9). Mean H_o , H_s and H_e were 0.299 ± 0.192 , 0.340 ± 0.210 and 0.431 ± 0.228 , respectively (Table 1). The proportion of polymorphic loci (P) varies from 9.3% to 69.8%. The fixation index (F_{ST}) revealed a moderate genetic differentiation between Roquedur and Nice ($F_{ST} = 0.1101$; P -value = 0.00033) but the F_{ST} was high and significant between Roquedur and Portugal ($F_{ST} = 0.4131$; P -value = 0.00033) and between Nice and Portugal ($F_{ST} = 0.3238$; P -value = 0.001). Deviation from Hardy–Weinberg equilibrium (H_{WE}) was only observed in locus aria8 for the total population and Linkage Disequilibrium (LD) was detected between loci aria10, aria11 and aria13. This linkage might be attributable to the physical proximity of these loci on the genome (leading to a non independent transmission of alleles of these different loci) or to admixture between different subpopulations, or to inadvertent sampling of a single isolated subpopulation.

The NJ phenogram confirms the high and significant F_{ST} values obtained between French samples (Roquedur and Nice) and Portugal (Fig. 1). In agreement with the low F_{ST} value, no structuring was observed between the two French populations of *P. ariasi* on the NJ phenogram (Fig. 1). These results are coherent with a more recent divergence between the two French populations than with the population from Portugal. The low bootstrap values obtained (less than 50%) indicate that the different *P. ariasi* populations share numerous alleles which can be explained by the recent divergence between these three populations.

Similar analyses performed within the population from Roquedur area, showed that 11 loci were polymorphic, with an average of 2.44 alleles ($SD \pm 1.825$; range 2–8). Mean H_o , H_s and H_e were

0.2177 ± 0.2546 , 0.2354 ± 0.2536 and 0.2314 ± 0.2492 , respectively (Table 3). P varies from 3.4% to 79.3%. This rate shows an important variation reflecting the different degree of polymorphism observed for each locus. Nevertheless, these results indicate that these microsatellite markers can also detect genetic diversity at a local scale. H_{WE} deviation and LD were also found in the Roquedur population. The causes (see above) of the observed Hardy Weinberg deviation and LD will be further determined by a population genetics study on a larger sympatric sample.

The inbreeding coefficient (F_{IS}) for Roquedur, Nice and Portugal was 0.077 (P -value = 0.0897), 0.151 (P -value = 0.1080) and 0.136 (P -value = 0.0412), respectively. A slight heterozygosity deficit was observed in the Portuguese population, which could be due to the small number of individuals tested ($n=7$). Nevertheless, these preliminary results suggest that this novel suite of 16 microsatellite loci is suitable for the study of *P. ariasi* population structure at different geographic scales.

3.4. Test of transferability

The same microsatellite loci can, on occasion, work well in other species—note the two microsatellites developed for *P. perniciosus* (Aransay et al., 2001) work well for population studies of *P. ariasi* (Mahamdallie et al., 2011; herein). To test the transferability of these 16 microsatellite loci to other local sandfly species, we tested these primers with seven other Mediterranean species: *P. mascittii*, *P. papatasi*, *P. perniciosus*, *P. perfliewi* s.l., *P. sergenti*, *P. tobbi*, and *S. minuta* (Table 4). Some eight loci were found to be transferable: *P. mascittii* (loci aria2, aria5, aria7, aria9, aria13 and aria14), *P. papatasi* (locus aria7), *P. perfliewi* s.l. (loci aria5, aria7, aria9 and aria12), *P. perniciosus* (loci aria7, aria9 and aria13), *P. sergenti* s.l. (locus aria7) and *P. tobbi* (loci aria5, aria7, aria9, aria12, aria14 and aria15) (Table 4).

For loci that were successfully amplified, the numbers of alleles per loci are given. No amplification was achieved in *S. minuta*.

None of these loci were amplified in *S. minuta*. The fact that these microsatellite loci can be amplified in various species indicates their usefulness for interspecific and intraspecific research within the genus *Phlebotomus*, especially for future genetic, phylogeographic or evolutionary studies.

4. Conclusions

It is known that microsatellite markers have multiple advantages: they are co-dominant, usually neutral, they have a high rate of mutations, they are highly polymorphic with a large number of alleles per locus and they are abundant and well distributed in the genome. These preliminary results prove the utility of this suite of microsatellite markers for population genetic studies of *P. ariasi* at different geographic scales. Some of them have been proven to be also suitable for genetic characterization of other sandfly species. In fine, the set of 15 (one was monomorphic in our study) polymorphic microsatellite markers developed in this work will allow us to study the sandfly population structure and molecular ecology at a local geographical scale. These studies will thus improve our knowledge regarding the biology and ecology of these insects of medical interest.

Acknowledgements

This study was funded by EU grant FP7-261504 EDENext and is catalogued by the EDENext Steering Committee as EDENext220 (<http://www.edenext.eu>). The contents of this publication are the sole responsibility of the authors and do not necessarily reflect the views of the European Commission.

We are particularly grateful to the EDENext PhDB group, Professor Rioux for the interesting and useful discussions and especially Yvonne Linton for English corrections and her contributions to this work.

We thank UMR D190 'Emergence des Pathologies Virales', Faculté de médecine-Timone, Marseille, for DNA extractions and the SeqGen platform (CeMEB Labex, France) for facilitating access to the ABI Prism 3130 Genetic Analyser.

We also thank IRD (Institut de Recherche pour le Développement) and CNRS for financial support. CM holds a FCT fellowship (SFRH/BPD/44082/2008).

References

- Abonnet, E., 1972. Les phlébotomes de la région Éthiopienne (Diptera, Psychodidae). *Mém. ORSTOM* 55, 1–290.
- Alvar, J., Vélez, I.D., Bern, C., Herrero, M., Desjeux, P., Cano, J., Jannin, J., den Boer, M., WHO Leishmaniasis Control Team, 2012. *Leishmaniasis worldwide and global estimates of its incidence*. *PLoS One* 7 (5), e35671.
- Aransay, A.M., Malarky, G., Ready, P.D., 2001. Isolation (with enrichment) and characterization of trinucleotide microsatellites from *P. perniciosus*, a vector of *L. infantum*. *Mol. Ecol. Notes* 1, 176–178.
- Aransay, A.M., Ready, P.D., Morillas-Márquez, F., 2003. Population differentiation of *Phlebotomus perniciosus* in Spain following postglacial dispersal. *Heredity* 90, 316–325.
- Ballart, C., Barón, S., Alcover, M.M., Portús, M., Gállego, M., 2012. Distribution of phlebotomine sand flies (Diptera: Psychodidae) in Andorra: first finding of *P. perniciosus* and wide distribution of *P. ariasi*. *Acta Trop.* 122 (1), 155–159.
- Barón, S.D., Morillas-Márquez, F., Morales-Yuste, M., Díaz-Sáez, V., Irigaray, C., Martín-Sánchez, J., 2011. Risk maps for the presence and absence of *Phlebotomus perniciosus* in an endemic area of leishmaniasis in southern Spain: implications for the control of the disease. *Parasitology* 138 (10), 1234–1244.
- Boudabous, R., Amor, S., Khayech, F., Marzouk, M., Bdira, S., Mezhoud, H., Azaïez, R., Sfar, M., Babba, H., 2009. The phlebotomine fauna (Diptera: Psychodidae) of the eastern coast of Tunisia. *J. Med. Entomol.* 46 (1), 1–8.
- Boussaa, S., Neffa, M., Pesson, B., Boumezzough, A., 2010. Phlebotomine sandflies (Diptera: Psychodidae) of southern Morocco: results of entomological surveys along the Marrakech-Ouarzazat and Marrakech-Azilal roads. *Ann. Trop. Med. Parasitol.* 104 (2), 163–170.
- Branco, S., Alves-Pires, C., Maia, C., Cortes, S., Cristovão, J.M., Gonçalves, L., Campino, L., Afonso, M.O., 2013. Entomological and ecological studies in a new potential zoonotic leishmaniasis focus in Torres Novas municipality, Central Region, Portugal. *Acta Trop.* 125 (3), 339–348.
- Belkhir, K., Borsa, P., Chikhi, L., Raufaste, N., Bonhomme, F., 1996–2004. GENETIX 4.05, logiciel sous Windows TM pour la génétique des populations. Laboratoire Génome, Populations, Interactions, CNRS UMR 5171, Université de Montpellier II, Montpellier (France). (<http://kimura.univ-montp2.fr/genetix/>).
- Cavalli-Sforza, L.L., Edwards, A.W.F., 1967. Phylogenetic analysis: models and estimation procedures. *Evolution* 32, 550–570.
- Chevenet, F., Brun, C., Bañuls, A.-L., Jacq, B., Christen, R., 2006. TreeDyn: towards dynamic graphics and annotations for analyses of trees. *BMC Bioinformatics* 12, 439.
- Dedet, J.-P., 2010. Les leishmanioses en France métropolitaine. *Bull. Epidémiol.* 38, 9–13.
- Depaquit, J., Bounamous, A., Akhouni, M., Augot, D., Sauvage, F., Dvorak, V., Chaibullinova, A., Pesson, B., Volf, P., Léger, N., 2013. A taxonomic study of *Phlebotomus* (Larrousius) *perfiliewi* s.l. *Infect. Genet. Evol.* 20, 500–508.
- Edwards, K.J., 1998. Miniprep procedures for the isolation of plant DNA. In: Karp, A., Isaac, P.G., Ingram, D.S. (Eds.), *Molecular Tools for Screening Biodiversity, Plants and Animals*. Chapman and Hall, New York, NY.
- Franco, F.A.L., Franco, O.A.L., Morillas-Márquez, F., Barón, S.D., Morales-Yuste, M., Gálvez, R., Díaz, V., Pesson, B., Alves-Pires, C., Depaquit, J., Molina, R., Afonso, O., Gállego, M., Guernaoui, S., Bounamous, A., Martín-Sánchez, J., 2010. Genetic structure of *Phlebotomus* (Larrousius) *ariasi* populations, the vector of *Leishmania infantum* in the western Mediterranean: epidemiological implications. *Int. J. Parasitol.* 40 (11), 1335–1346.
- Goudet, J., 1995. *fstat* version 1.2: a computer program to calculate F statistics. *J. Hered.* 86, 485–486.
- Hamarshah, O., Presber, W., Yaghoobi-Ershadi, M.R., Amro, A., Al-Jawabreh, A., Sawalha, S., Al-Lahem, A., Das, M.L., Guernaoui, S., Seridi, N., Dhiman, R.C., Hashiguchi, Y., Ghrab, J., Hassan, M., Schönian, G., 2009. Population structure and geographical subdivision of the *Leishmania* major vector *Phlebotomus papatasi* as revealed by microsatellite variation. *Med. Vet. Entomol.* 23 (1), 69–77.
- Kamhawi, S., Molyneux, D.H., Killick-Kendrick, R., Milligan, P.J., Phillips, A., Wilkes, T.J., Killick-Kendrick, M., 1987. Two populations of *Phlebotomus ariasi* in the Cévennes focus of leishmaniasis in the south of France revealed by analysis of cuticular hydrocarbons. *Med. Vet. Entomol.* 1 (1), 97–102.
- Khalid, N.M., Aboud, M.A., Alrabba, F.M., Elnaiem, D.E., Tripet, F., 2012. Evidence for genetic differentiation at the microgeographic scale in *Phlebotomus papatasi* populations from Sudan. *Parasit. Vectors* 5, 249.
- Killick-Kendrick, R., Tang, Y., Killick-Kendrick, M., Sang, D.K., Sirdar, M.K., Ke, L., Ashford, R.W., Schorscher, J., Johnson, R.H., 1991. The identification of female sandflies of the subgenus Larrousius by the morphology of the spermathecal ducts. *Parassitologia* 33, 337–347, Suppl.
- Langella, O. Populations 1.2.28: a population genetic software. 1999 CNRS UPR9034. [cited 2013 March 8]. Available from: <http://bioinformatics.org/~tryphon/populations/>
- Lewis, D.J., 1982. A taxonomic review of the genus *Phlebotomus* (Diptera:Psychodidae). *Bull. Br. Mus. Nat. Hist. Entomol.* 45, 121–209.
- Mahamdallie, S.S., Pesson, B., Ready, P.D., 2011. Multiple genetic divergences and population expansions of a Mediterranean sandfly, *Phlebotomus ariasi*, in Europe during the Pleistocene glacial cycles. *Heredity* 106, 1–13.
- Malaua, T., Gilles, A., Meglécz, E., Blanquart, H., Duthoy, S., Costedoat, C., Dubut, V., Pech, N., Castagnone-Sereno, P., Délye, C., Feau, N., Frey, P., Gauthier, P., Guillemaud, T., Hazard, L., Le Corre, V., Lung-Escarment, B., G. Malé, P.J., Ferreira, S., Martin, J.F., 2011. High-throughput microsatellite isolation through 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries. *Mol. Ecol. Resour.* 11, 638–644.
- Martin-Sánchez, J., Gramiccia, M., Pesson, B., Morillas-Márquez, F., 2000. Genetic polymorphism in sympatric species of the genus *Phlebotomus*, with special reference to *Phlebotomus perniciosus* and *Phlebotomus longicuspis* (Diptera, Phlebotomidae). *Parasite* 7, 247–254.
- Pesson, B., Wallon, M., Floer, M.T., Kristensen, A.R., 1991. Isoenzymatic studies of Mediterranean populations of sandflies of the subgenus Larrousius. *Parassitologia* 33, 471–476, Suppl.
- Ready, P.D., 2010. Leishmaniasis emergence in Europe. *Euro Surveill.* 15 (10), 1–11.
- Rioux, J., Carron, S., Dereure, J., Périères, J., Zeraïa, L., Franquet, E., Babinot, M., Gállego, M., Prudhomme, J., 2013. Ecology of leishmaniasis in the South of France. 22. Reliability and representativeness of 12 *Phlebotomus ariasi*, *P. perniciosus* and *Sergentomyia minuta* (Diptera: Psychodidae) sampling stations in Vallespir (eastern French Pyrenees region). *Parasite* 20 (34), 1–10.
- Rioux, J.A., Killick-Kendrick, R., Perieres, J., Turner, D.P., Lanotte, G., 1980. Ecologie des leishmanioses dans le Sud de la France. 13. Les sites de flanc de coteau, biotopes de transmission privilégiés de la Leishmaniose viscérale en Cévennes. *Ann. Parasit. Hum. Comp.* 55 (4), 445–453.
- Rossi, E., Rinaldi, L., Musella, V., Veneziano, V., Carbone, S., Gradoni, L., Cringoli, G., Maroli, M., 2007. Mapping the main *Leishmania phlebotomine* vector in the endemic focus of the Mt. Vesuvius in southern Italy. *Geospat. Health* 1 (2), 191–198.
- Rousset, F., 2008. GENEPOP'007: a complete re-implementation of the GENEPOP software for Windows and Linux. *Mol. Ecol. Resour.* 8 (1), 103–106.
- Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4, 406–425.
- Selkoe, K.A., Toonen, R.J., 2006. Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. *Ecol. Lett.* 9 (5), 615–629.
- Theodor, O., 1958. Psychodidae–phlebotominae. In: Lindner, E. (Ed.), *Die Fliegen der Palearktischen Region*. E. Schweizerbartsche Verlags, Stuttgart.
- Wang, Q., Wang, X., 2012. Comparison of methods for DNA extraction from a single chironomid for PCR analysis. *Pak. J. Zool.* 44 (2), 421–426.

VI.2. Ecosystème et structure génétique des populations de *P. ariasi*

Onze des marqueurs microsatellites les plus polymorphes décrits précédemment (Voir ci-dessus - Développement des marqueurs microsatellites) ont été utilisés afin de réaliser la caractérisation génétique de *P. ariasi* dans notre zone d'étude. Ils sont détaillés dans : « **Prudhomme J., Toty C., Cassan C., Rahola N., Vergnes B., Alten B., Sereno D. *, Bañuls A-L*.** *Advances in understanding the genetic population structure of Phlebotomus ariasi in the Mediterranean Languedoc region (Roquedur area, Gard, France).* *En cours de rédaction.* »

VI.2.1. Résumé

Dans le chapitre précédent, nous avons mis en évidence une structuration phénotypique des populations de *P. ariasi* dans notre zone d'étude liée aux facteurs environnementaux et reflétant une grande plasticité phénotypique des phlébotomes (voir chapitre 5 - Plasticité phénotypique et capacité d'adaptation de *P. ariasi*). Cependant, cette étude ne nous a pas renseignés sur l'organisation génétique des populations de *P. ariasi* qui, à ce jour, reste encore mal connue. Dans ce contexte, l'objectif de ce travail était d'étudier la structure génétique des populations de *P. ariasi* dans la région de Montpellier en utilisant les marqueurs microsatellites mis au point au cours de cette thèse [94].

Pour ce faire, nous avons réalisé des captures, entre mai et octobre 2011, le long d'un transect de 14 km dans la région de Roquedur (Gard, France). Sur les 20 stations échantillonnées, les paramètres environnementaux (tels que l'altitude et le versant) ont été déterminés. Après identification de l'espèce, l'analyse génétique de 1692 *P. ariasi* a été effectuée à l'aide des 11 marqueurs microsatellites les plus polymorphes mis au point précédemment.

Sur les 11 marqueurs microsatellites sélectionnés, deux loci présentaient des allèles nuls quelle que soit la subdivision et ont donc été éliminés pour les analyses de génétique des populations. Les 9 marqueurs microsatellites utilisés se sont révélés polymorphes à toutes les échelles spatiales (même micro-géographique) et les indices de diversité génétique étaient similaires quelle que soit l'échelle étudiée. L'estimation des indices de fixation (F_{ST}) a mis en évidence une faible mais significative différenciation génétique entre les stations d'échantillonnage et leurs sous-divisions (échelle micro-géographique). L'hypothèse de structuration micro-géographique était soutenue par les tests d'Hardy-Weinberg qui identifient les unités de reproduction à de petites échelles spatiales reliées entre elles par des événements de migration. Cette structuration particulière pourrait être la conséquence de la faible capacité de dispersion des phlébotomes. L'analyse détaillée (par mois et par sexe) au niveau micro-géographique a montré une dispersion plus prononcée pour les femelles en juin. Ces résultats peuvent être expliqués par des capacités de vol distinctes entre les sexes et une accessibilité aux ressources différentes selon les mois de collecte.

Il s'agit de la première étude de génétique des populations de *P. ariasi* réalisée à partir de données microsatellites dans le Sud de la France. La structure micro-géographique et temporelle des populations observée pour *P. ariasi* semble en accord avec le fonctionnement des foyers de transmission des *Leishmania*.

VI.2.2. Article 7 : Molecular advances in understanding the genetic population structure of *Phlebotomus ariasi* in the Mediterranean Languedoc region (Roquedur area, Gard, France).

Molecular advances in understanding the genetic population structure of *Phlebotomus ariasi* in the Mediterranean Languedoc region (Roquedur area, Gard, France).

Jorian PRUDHOMME^{1§} [jorian.prudhomme@hotmail.fr], Céline TOTY¹ [celine.toty@ird.fr], Cécile CASSAN¹ [cecile.cassan@ird.fr], Nil RAHOLA¹ [nil.rahola@ird.fr], Baptiste VERGNES¹ [baptiste.vergnes@ird.fr], Bulent ALTEN² [kaynas@hacettepe.edu.tr], Denis SERENO^{3*} [denis.sereno@ird.fr] and Anne-Laure BAÑULS^{1*} [anne-laure.banuls@ird.fr].

1. UMR MIVEGEC (IRD 224 - CNRS 5290 – Université de Montpellier), 911 avenue Agropolis, F34394 Montpellier, France.

2. Hacettepe University, Faculty of Science, Department of Biology, Ecology Section, ESRL Laboratories, 0680 Beytepe, Ankara, Turkey.

3. UMR INTERTRYP (IRD - CIRAD 177), Centre IRD, F34394 Montpellier, France.

* These authors contributed equally to the work

§ Correspondence: Jorian Prudhomme; E-mail: jorian.prudhomme@hotmail.fr

I.R.D (Institut de Recherche pour le Développement)

911 Avenue Agropolis, 34394 Montpellier

Abstract

1 The *Phlebotomus* species are known for their role as vectors of medically important
2 pathogens. In particular, *Phlebotomus ariasi* is the predominant sandfly species in Cevennes
3 region and one of proven vectors of *Leishmania infantum* in the South of France. This species
4 has a wide geographical area distribution but its population structure is currently not well
5 known. Therefore, the aim of this work was to study the molecular ecology and the
6 population structure of *P. ariasi* in the region of Montpellier using microsatellite markers.

7 Sandflies were captured along a 14 kilometer-long transect in 2011 from May to October
8 and several environmental data (such as altitude, slope) were collected for each station.
9 Genetic analysis of *P. ariasi* was performed using 11 microsatellite loci. To study the
10 organization of *P. ariasi* populations, we analyzed the data at different spatio-temporal
11 scales: microgeographical populations, stations, month of capture, slopes and groups of
12 altitude.

13 From the eleven selected microsatellite markers, two loci revealed null alleles whatever
14 the subdivisions and were thus removed for the population genetic analyses. The nine loci
15 used were polymorphic at all spatial scales even at microscale and the genetic diversity
16 indices remain similar whatever the subdivision under study. The fixation index revealed low
17 but significant genetic differentiation at low spatial scale, suggesting a microgeographic
18 population substructure in this species, possibly due to limited dispersal. The Hardy
19 Weinberg analyses supported that these microgeographic populations correspond to
20 independent reproductive units, even if migration events were detected between them.
21 Interestingly, we found a weaker spatial structuring for females than for males which might
22 be explained by a differential dispersal capability in flight according to sex. A higher
23 dispersion capacity for females compared to males could be due to the blood meal necessity.

24 This is the first study of population genetics studies on *P. ariasi* from the South of
25 France region using microsatellite markers. The structuring of sandflies in microgeographical
26 subpopulations seems to be in agreement with the organization of *Leishmania* transmission
27 foci.

28

29 *Keywords: Sandflies, South of France, Ecology, Population genetics, microgeographic*
30 *population, microsatellites*

31 Introduction

32 The *Phlebotomus* species are known for their role as vectors of medically important
33 pathogens, such as the kinetoplastida protozoan of the genus *Leishmania*, the causative agent
34 of leishmaniasis [1], *Bartonella bacilliformis* [1] and arthropod-borne viruses recognized as
35 human pathogens (Toscana virus, Naples virus and Sicilian virus) [2]. *Phlebotomus ariasi*
36 Tonnoir, 1921, is the predominant sandfly species in Cevennes region [3] and one of the two
37 proven vectors, with *Phlebotomus perniciosus* Newstead, 1911, of leishmaniasis caused by
38 *Leishmania infantum* in the South of France [4]. Sandflies are abundant in peri-urban and
39 rural environments and often close to human and domestic animal populations [4, 5]. In
40 particular, *P. ariasi* is found resting in houses, animal sheds, caves and holes in walls
41 (“barbacanes”), near roads or in villages. This species has a wide geographical area
42 distribution, including many countries of the Western Mediterranean region such as Algeria,
43 France, Italy, Morocco, Portugal, Spain and Tunisia [6-15]. This past 10 years, the risk of
44 emergence or re-emergence of leishmaniasis [16] and phlebovirus [17] has increased in
45 France probably linked to the ecology of the vectors. Nevertheless, the biology and ecology
46 of *P. ariasi* remain poorly known despite its important role as vector. As example, the
47 population structure of *P. ariasi* in *Leishmania* foci and the impact on transmission are
48 currently not well known. Only few studies have been performed on this species such as
49 analysis of cuticular hydrocarbons [18], isoenzymes [19], random amplified polymorphic
50 DNA [20], sequences of mitochondrial cytochrome b gene [11] and microsatellite markers.
51 Among available molecular markers for molecular ecology studies, microsatellite markers
52 have proved to be useful tools to study population genetic structure [21]. These molecular
53 markers allow working at various scales to compare populations among countries and
54 between regions, but also within endemic areas. A previous population genetic study of *P.*
55 *ariasi* [22] used two microsatellite loci, developed in a prior study for *P. perniciosus* [23],

56 with other genetic markers to understand the expansion of this species in Europe during the
57 Pleistocene glacial cycles. Recently, we previously described a set of 14 new microsatellite
58 loci for *P. ariasi*. The 11 most polymorphic loci of them were chosen and used for this study.

59 In this context, the aims of this work were to study the molecular ecology and the
60 population structure of *P. ariasi* in the region of Montpellier using a set of polymorphic
61 microsatellite loci already investigated in Prudhomme et al. [24].

62

63 Materials and methods

64 **The study area**

65 The field study was performed in the South of France, on the hill “le massif de
66 l’Oiselette” situated between the two valleys “Hérault” (Ganges, Hérault) and “Arre” (Le
67 Vigan, Gard). This work was performed throughout a 14 km transect from Saint Julien de la
68 Nef to le Vigan, including Roquedur-le-haut (at 601m above sea level) (Figure 1). This
69 region is subject to the Mediterranean sub-humid climat [25] and is characterized by the
70 presence of plant species typical of Garrigue habitat such as *Quercus ilex* and *Q. pubescens*.

71 This geographical area is known to be an endemic area for human and canine
72 leishmaniasis caused by *L. infantum* for a long time. This area is characterized by the
73 presence of different domestic animals, which are potential hosts for sandflies, such as
74 chicken, sheep, ducks, geese, horses, rabbits, cats, dogs and a wide variety of wild animals
75 such as foxes, rodents, lizards, birds, etc.

76

77 **Sandfly collection and identification**

78 Sandfly collections were performed monthly between May and November in 2011 with
79 CDC miniature light traps (John W. Hock Co. FL, U.S.A.) and flight interception traps (20 x
80 20 cm white paper cover with castor oil) [26]. 20 localities were sampled (Table 1 and Figure
81 1). In 14 sampling sites (Table 1), one or two light traps were set up for two nights (inside
82 and/or outside houses, animal barns, etc.). They were operated between 18:00 pm and 08:00
83 am. A total of 105 light traps were set during 210 nights of trapping. In 17 stations (Table 1),
84 a total of 3589 sticky traps were used and a mean of 189 sticky traps by station were placed
85 in various biotopes, inside and around human dwelling and animal housing, close to the
86 vegetation and crevices in the walls. The sticky traps were collected after 2 days. Specimens
87 from Lacan locality (ST21) situated outside transect were captured by CDC light traps in
88 August 2011 and were used as outgroup (Table 1). Because of the distance (1.5 km;
89 coordinates: 43.95889N, 3.662474E) from our transect, this station could not be indicated on
90 the map presented in Figure 1.

91 At the end of the sampling period, specimens from the traps were transferred
92 individually into 1.5 ml Eppendorf tubes with 96% ethanol and labeled accordingly. Prior to
93 mounting, heads, genitalia and wings of the sandflies were removed. The heads and genitalia
94 were cleared in Marc-André solution (chloral hydrate / acetic acid) and mounted in chloral
95 gum [27]. Specimen identification was individually performed based on the morphology of
96 the pharynges and / or the male genitalia or female spermathecae, using the keys of
97 Abonnenc [27], Lewis [6] and Killick-Kendrick et al. [28].

98

Figure 1. Map of the study area. Red circles indicate the sampling stations.

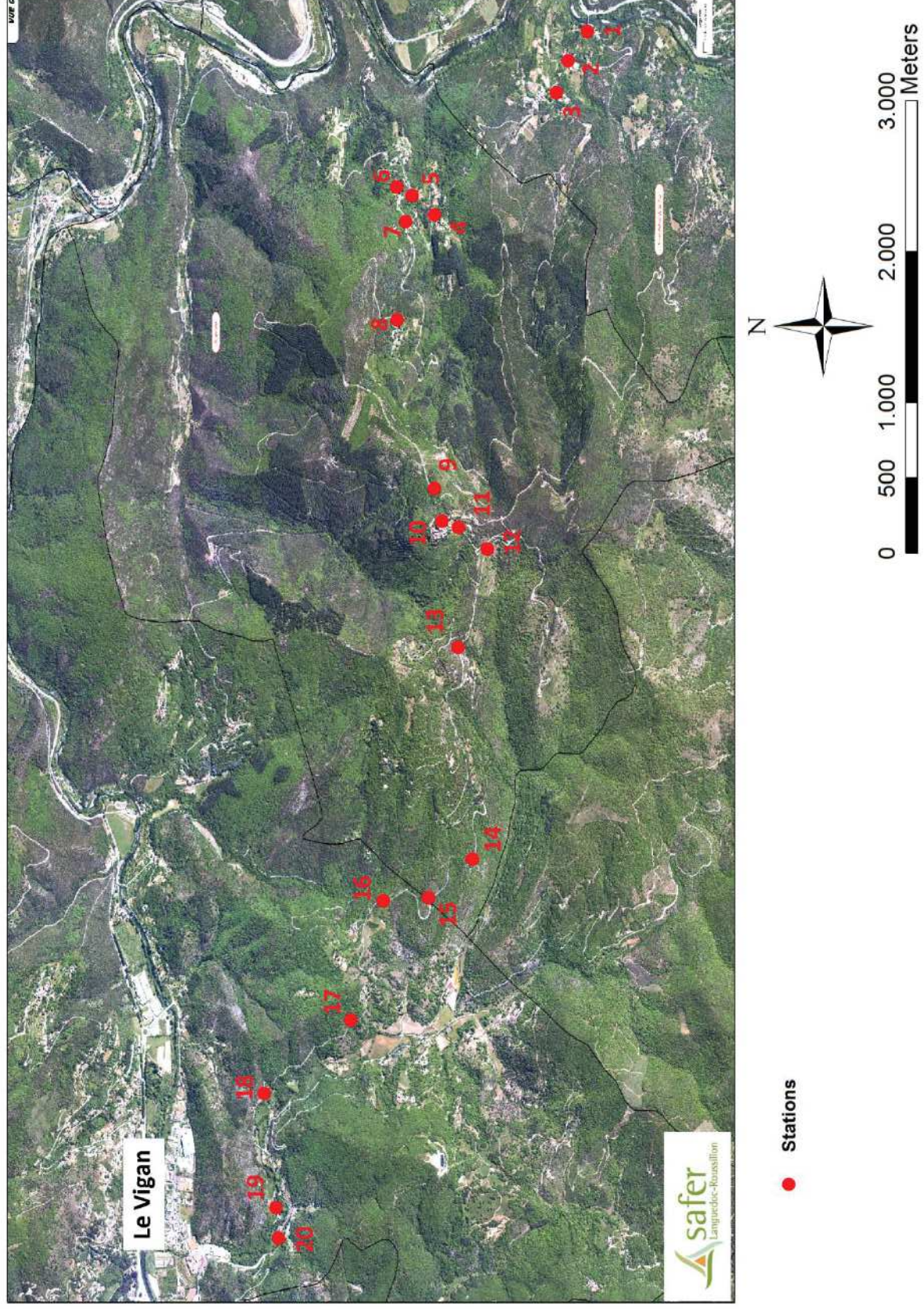


Table 1. Sampling stations in the study area. Description of the collection sites and number of sandflies captured and genotyped (SE: South-East; NW: North-West; Slope groups: S1 (Northern slope, 100-300m), S2 (Northern slope, 300 et 500 m), S3 (Northern slope, >500 m), S4 (Southern slope, 300 et 500 m), S5 (Southern slope, 300-100 m); Alt: altitude (m); Altitude groups: A1 (100-200m), A2 (200-300m), A3 (300-400m), A4 (400-500m), A5 (>500m); Ncap: Number of sandflies captured; Ngen: Number of genotyped sandflies; CDC: CDC miniature light traps; ST: Sticky Traps).

	Coordinates		Slope groups	Alt (m)	Altitude groups	Biotope	Ncap	Ngen	Traps	
	North	East								
ST01	43.96548	3.686828	S1	175	A1	Hamlet	0	0	CDC + ST	
ST02	43.96663	3.685075	S1	228	A2	Hamlet	2	0	ST	
ST03	43.96733	3.683149	S1	244	A2	Hamlet	58	55	CDC + ST	
ST04	43.97462	3.675871	S2	321	A3	Hamlet / Hutch	306	83	CDC + ST	
ST05	43.97595	3.677038	S2	322	A3	Rural	437	142	ST	
ST06	43.97687	3.677551	S2	341	A3	Kennel	117	114	CDC + ST	
ST07	43.97632	3.675457	S2	354	A3	Hamlet	258	131	CDC + ST	
ST08	43.97683	3.669611	S3	443	A4	Hamlet	0	0	ST	
ST09	43.97460	3.659549	S3	586	A5	Poultry farm	43	39	CDC	
ST10	43.97416	3.657616	S3	606	A5	Poultry farm	7	0	CDC	
ST11	43.97317	3.657214	S3	603	A5	Hamlet	301	221	CDC + ST	
ST12	43.97144	3.655944	S3	573	A5	Rural	134	97	ST	
ST13	43.97321	3.650088	S3	539	A5	Rural	198	114	CDC + ST	
ST14	43.97235	3.637441	S4	417	A4	Rural	198	132	ST	
ST15	43.97495	3.635153	S4	397	A3	Rural	73	60	CDC + ST	
ST16	43.97765	3.634949	S4	362	A3	Rural	130	118	ST	
ST17	43.97961	3.627845	S5	343	A3	Rural	26	23	CDC + ST	
ST18	43.98478	3.623463	S5	282	A2	Rural	202	150	CDC + ST	
ST19	43.98409	3.616661	S5	255	A2	Hamlet	71	70	CDC + ST	
ST20	43.98392	3.614822	S5	245	A2	Hamlet / Sheep barn	305	131	CDC + ST	
ST21	43.95889	3.662474	S6	500	A5	Hamlet	12	12	CDC	
	Total							2878	1692	

99 DNA extraction

100 DNA was extracted by two automated techniques according to the Chelex method
 101 [29] and the protocol described in Zapata et al. [30]. First, the entire sandflies were ground
 102 using a Mixer Mill MM300 (QIAGEN, Venlo, Netherlands) with one 3-mm tungsten bead at a
 103 frequency of 30 cycles s⁻¹ for 3 min in the presence of 200 µL Eagle's Minimal Essential
 104 Medium. A volume of 140 µL of each sample was then utilized for DNA purification by

105 adding a Chelex resin suspension [29] or by using the Eppendorf epMotion 5075 working
106 station and Macherey-Nagel NucleoSpin 96 kits.

107

108 **DNA amplification and genotyping**

109 Eleven of the most polymorphic loci out of the 16 microsatellite markers previously
110 described for *P. ariasi* [24], were selected (Table 2). All the individuals were amplified
111 according to the following conditions. Each 25 μ L reaction mix was composed of 1 pmol of
112 forward and reverse primers, the forward being labeled with a fluorochrome FAM, ATT0565
113 or HEX (Table 1), 5 ng of DNA template, 6 pmol of dNTP mix, 2.5 μ L of buffer 10X and
114 0.25 μ L of Taq polymerase (Roche Diagnostics®, 5 UI/ μ L). Amplifications were carried out
115 in a thermal cycler using the following conditions: an initial denaturation step at 95°C for 10
116 min, 40 cycles at 95°C for 30 s, annealing temperature of each locus (see Table 2) for 30 s at
117 72°C for 1 min and a final extension step at 72°C for 10 min. Genotyping was performed on
118 an ABI Prism 3130 Genetic Analyser (Applied Biosystems®) automated sequencer with 1 μ L
119 of PCR product added into a standard loading mix: 0.5 μ L of an internal standard-size
120 GENESCAN 500LIZ and 12.5 μ L of formamide (Hi-Di) (both Applied Biosystems®).
121 Profiles were read and analyzed using GENEMAPPER 4.0 (Applied Biosystems, Foster City
122 (CA)).

Table 2. Details of the 11 microsatellite markers selected; name, primer sequences, GenBank Accession number to reference clone, structure of repeated array, thermocycling conditions (annealing temperature: Ta), labeling dye, groups for multiplexing.

Name	Primer sequence (5'-3')	GeneBank Accession no.	Repeat type	Ta (°C)	Labelling dye	Groups
aria1	5'-TCCGCTCTTATCATCAATCG-3'	KJ526156	(TC)1-8	55	FAM	1
	5'-AGTGCGTCTCATCCGCTAGT-3'					
aria2	5'-CCTGCCAGAGTTAATTATTG-3'	KJ526157	(AG)2-6	55	FAM	1
	5'-AGTTTCCTCGAAAATTGCCA-3'					
aria3	5'-ATTCCGCTTCACCACCAAC-3'	KJ526158	(TC)6-12	52	FAM	2
	5'-ATCCCCTTAATTTCCGTGG-3'					
aria4	5'-ACTTTGTGAACTCGTGAGGC-3'	KJ526159	(CT)6-9	51	FAM	3
	5'-CGTAGCAGGAGTAAGAATTTATG-3'					
aria5	5'-GGACGATGACGCCAAGTATC-3'	KJ526160	(TC)5-7	60	FAM	4
	5'-AATCGTGAGTGAGGCGAAAC-3'					
aria10	5'-AGTAAACCCTCAAGTGC GC-3'	KJ526164	(CA)9-12	59	ATT0565	4
	5'-CCAGAAGCCCTTTTACACC-3'					
aria11	5'-GTGCTGGGAGGACAAGAATC-3'	KJ526165	(TC)5-8	55	HEX	1
	5'-CACCAAGTGCAGGAGTGAGA-3'					
aria12	5'-TTCCACCTACACATGCCTCA-3'	KJ526166	(TC)5-9	55	HEX	3
	5'-GCTCTTAGATGTGGGCGAAA-3'					
aria13	5'-GACCGGTTGTGATTAATGTGC-3'	KJ526167	(AG)4-9	55	HEX	2
	5'-CGATGATCGCCATTTACCTT-3'					
aria14	5'-AGTTATAGGCAACTGGGCGG-3'	KJ526168	(GA)9-18	51	HEX	3
	5'-TTTAGCCCAAACACTACGCACA-3'					
aria15	5'-TTAGGGTATACCACAGCCGC-3'	KJ526169	(CT)4-9	55	HEX	4
	5'-TTGTCAGGCTGCCGATCT-3'					

123 Data analysis

124 To study the organization of *P. ariasi* populations, we analyzed the data at different
125 scales: months of capture, slopes, groups of altitude, stations and microgeographic scale. To
126 work at the microgeographic scale, the stations were subdivided according to microhabitats
127 when it was possible (wall, house). To consider slopes and altitudes together, we created the
128 following groups: Six slope groups, group S1 (Northern slope, 100-300m), group S2
129 (Northern slope, 300 et 500 m), group S3 (Northern slope, >500 m), group S4 (Southern
130 slope, 300 et 500 m), group S5 (Southern slope, 300-100 m) and group S6 (Lacan station,

131 ST21) for the outgroup; and five altitude groups: group A1 (100-200m), group A2 (200-
132 300m), group A3 (300-400m), group A4 (400-500m), group A5 (>500) (Table 1).

133 The presence of null alleles was investigated with MICRO-CHECKER 2.2.3 [31]. For
134 each sampling group, mean number of alleles (N_a), mean allelic richness based on the
135 smallest sample size (R_s), mean unbiased expected heterozygosity (H_e), mean observed
136 heterozygosity (H_o), mean intra-population diversity (H_s) and F_{IS} were estimated with
137 FSTAT 2.9.3 [32] and GENETIX 4.04 [33]. Deviations from Hardy-Weinberg equilibrium
138 and linkage disequilibrium were measured using GENEPOP version 4.2.2 [34] and FSTAT
139 2.9.3, respectively. The number of migrant (N_m) were calculated for the microgeographic
140 population subdivisions using GENEPOP version 4.2.2.

141 Isolation by distance analyses were performed between individuals using GENEPOP
142 version 4.2.2. The correlation between geographical and genetic distance matrices were tested
143 with Mantel test (10^4 permutations).

144

145 Results

146 **Presence of null alleles**

147 According to MICROCHECKER, null alleles were detected in 5 microsatellite loci for
148 slope/altitude groups and altitude groups (aria1, aria2, aria10, aria14 and aria15) but in only 2
149 loci for station groups (aria2 and aria15). The results are detailed in Supplementary Table 1.
150 It was not possible to verify the presence of null alleles for the month groups since the
151 number of specimens exceeded 750 individuals which is the limit of the software. The
152 analysis detected more loci with probable null alleles in slope/altitude and altitude groups
153 than in station groups. Since for 3 loci (aria1, aria10, aria14), null alleles were not confirmed
154 at small scale (stations), these data suggested a Wahlund effect for these loci. Indeed, a
155 Wahlund effect generates heterozygote deficits that can be taken for null allele presence [35].

156 In this context, we removed only aria2 and aria15 and we performed all the following
157 analyses with 9 microsatellite markers.

158

159 **Variability**

160 Of 15228 data points (genotypes for 1692 individuals at 9 loci), there were 560 (3.68%)
161 missing values. For the global population, mean number of alleles (N_a) was 7.56; mean
162 allelic richness (R_s) was 7.43; mean unbiased expected heterozygosity (H_e) was 0.40; mean
163 observed heterozygosity (H_o) was 0.38 (Table 3). For the analyses by group see Tables 3 and
164 4, we can observe that the values of genetic diversity indices remained similar whatever the
165 subdivisions under study. For example, by groups (slope, altitude, month, station and
166 microgeographic subdivision), mean intrapopulation diversity (H_s) varied between 0.40 and
167 0.41.

168

169 **Hardy-Weinberg Equilibrium**

170 The Hardy Weinberg equilibrium (HWE) analyses evidenced heterozygote deficits in
171 each group tested: whole population, slope/altitude groups (except group S1), altitude groups,
172 month groups, stations (except ST03, ST06, ST09 and ST19) and microgeographic
173 populations (except ST03, ST06, ST09, ST13A, ST17A, ST18A, ST18B and ST19) (Tables 3
174 and 4). These data showed clearly a Wahlund effect at the level of large subdivisions (slopes,
175 groups of altitude) but also at the station level suggesting smaller spatial population
176 subdivisions. This observation was confirmed by the detection of the highest number of
177 populations in HWE at the level of microgeographic subdivisions (Table 4).

178

Table 3. Genetic variability and Hardy-Weinberg equilibrium estimators for global population, slope groups, altitude groups, and month groups for 9 microsatellite loci. Nb: Number of samples; Na: mean number of alleles; Rs: mean allelic richness based on the smallest sample size; Ho: mean observed heterozygosity; He: mean unbiased expected heterozygosity; F_{IS} : Weir and Cockerham F_{IS} ; HWE : Hardy-Weinberg equilibrium.

		Nb	Na	Rs	Ho	He (\pm SD)	F_{IS}	HWE
Total		1692	7.56	7.43	0.38	0.40 (\pm 0.23)	0.037	
Slope / altitude	S1	55	3.89	2.73	0.38	0.40 (\pm 0.21)	0.040	*
	S2	470	5.89	3.03	0.40	0.41 (\pm 0.23)	0.016	
	S3	471	6.22	3.01	0.36	0.38 (\pm 0.24)	0.062	
	S4	310	5.89	3.10	0.39	0.41 (\pm 0.22)	0.049	
	S5	374	6.00	2.82	0.37	0.38 (\pm 0.25)	0.006	
	Outgroup	12	2.67	2.67	0.37	0.45 (\pm 0.23)	0.198	
Altitude	A2	406	5.89	4.53	0.37	0.38 (\pm 0.24)	0.014	
	A3	671	6.56	4.95	0.39	0.40 (\pm 0.23)	0.023	
	A4	132	4.89	4.63	0.42	0.43 (\pm 0.24)	0.036	
	A5	483	6.33	5.01	0.36	0.39 (\pm 0.24)	0.067	
Month	May	32	3.44	2.75	0.37	0.39 (\pm 0.23)	0.044	
	June	495	5.67	2.65	0.33	0.37 (\pm 0.25)	0.100	
	July	804	7.00	2.78	0.38	0.38 (\pm 0.23)	0.017	
	August	352	5.67	3.02	0.46	0.45 (\pm 0.23)	-0.022	
	September	9	2.56	2.56	0.47	0.41 (\pm 0.24)	-0.147	\$
Station	ST03	55	3.89	2.73	0.38	0.40 (\pm 0.21)	0.040	*
	ST04	83	4.56	3.02	0.40	0.42 (\pm 0.25)	0.028	
	ST05	142	4.89	3.01	0.39	0.40 (\pm 0.24)	0.017	
	ST06	114	4.78	2.96	0.40	0.41 (\pm 0.23)	0.015	*
	ST07	131	5.00	2.94	0.41	0.41 (\pm 0.24)	-0.003	
	ST09	39	4.00	2.93	0.39	0.39 (\pm 0.25)	-0.008	*
	ST11	221	5.22	2.98	0.37	0.38 (\pm 0.24)	0.031	
	ST12	97	4.56	2.95	0.33	0.38 (\pm 0.25)	0.131	
	ST13	114	4.78	3.05	0.36	0.40 (\pm 0.24)	0.091	
	ST14	132	4.89	3.14	0.42	0.43 (\pm 0.24)	0.036	
	ST15	60	4.78	3.18	0.38	0.40 (\pm 0.24)	0.060	
	ST16	118	4.67	2.84	0.37	0.38 (\pm 0.22)	0.039	
	ST17	23	4.11	3.34	0.43	0.43 (\pm 0.22)	-0.019	
	ST18	150	4.89	2.84	0.38	0.39 (\pm 0.24)	0.022	
	ST19	70	4.00	2.62	0.36	0.35 (\pm 0.27)	-0.045	*
	ST20	131	4.33	2.76	0.36	0.37 (\pm 0.26)	0.017	
ST21	12	2.67	2.67	0.37	0.45 (\pm 0.23)	0.198		

SD = Standard deviation

\$ = not applicable due to the low number of samples ($n < 10$)

* = Hardy-Weinberg equilibrium.

Table 4. Genetic variability and Hardy-Weinberg equilibrium estimators of microgeographic population for 9 microsatellite loci. Nb: Number of samples; Na: mean number of alleles; Rs: mean allelic richness based on the smallest sample size; Ho: mean observed heterozygosity; He: mean unbiased expected heterozygosity; F_{IS} : Weir and Cockerham F_{IS} ; HWE: Hardy-Weinberg equilibrium.

Microhabitat	Nb	Na	Rs	Ho	He (\pm SD)	F_{IS}	HWE
ST03	55	3.89	2.40	0.38	0.40 (\pm 0.21)	0.040	*
ST04	83	4.56	2.61	0.40	0.42 (\pm 0.25)	0.028	
ST05	142	4.89	2.58	0.39	0.40 (\pm 0.24)	0.017	
ST06	114	4.78	2.59	0.41	0.41 (\pm 0.23)	0.005	*
ST07	131	5.00	2.51	0.40	0.40 (\pm 0.24)	0.006	
ST09	39	4.00	2.50	0.39	0.39 (\pm 0.25)	-0.008	*
ST11A	129	4.89	2.49	0.36	0.38 (\pm 0.25)	0.054	
ST11B	92	4.22	2.51	0.38	0.37 (\pm 0.24)	-0.006	
ST12	97	4.56	2.48	0.33	0.38 (\pm 0.25)	0.131	
ST13A	27	3.11	2.42	0.36	0.39 (\pm 0.24)	0.079	*
ST13B	87	4.78	2.62	0.36	0.40 (\pm 0.24)	0.093	
ST14	132	4.89	2.70	0.42	0.43 (\pm 0.24)	0.036	
ST15	60	4.78	2.64	0.38	0.40 (\pm 0.24)	0.060	
ST16	118	4.67	2.44	0.37	0.38 (\pm 0.22)	0.039	
ST17A	17	3.77	2.77	0.45	0.44 (\pm 0.22)	-0.036	*
ST17B	6	2.67	2.67	0.39	0.42 (\pm 0.28)	0.071	\$
ST18A	60	3.89	2.51	0.42	0.40 (\pm 0.25)	-0.060	*
ST18B	50	3.56	2.30	0.35	0.36 (\pm 0.24)	0.042	*
ST18C	40	4.11	2.52	0.35	0.40 (\pm 0.23)	0.121	
ST19	70	4.00	2.29	0.36	0.35 (\pm 0.27)	-0.045	*
ST20	131	4.33	2.37	0.36	0.37 (\pm 0.26)	0.017	
ST21	12	2.67	2.47	0.37	0.45 (\pm 0.23)	0.198	

SD = Standard deviation

\$ = not applicable due to the low number of samples ($n < 10$)

* = Hardy-Weinberg equilibrium.

179 **Linkage Disequilibrium**

180 The LD results are detailed in Supplementary Table 2. We found significant LD
 181 between 7 loci pairs in the global population. We found also LD between 7, 8, 5, 5 and 4 loci
 182 pairs in the slope/altitude groups, altitude groups, month groups, stations and
 183 microgeographic populations, respectively. These data showed LD at each scale of study with
 184 a decreasing gradient of loci pairs in LD from the whole population to the microgeographic
 185 subdivisions. These results suggest the existence of smaller subdivisions.

186

187 **Population structure: F_{ST} and Mantel test**

188 Pairwise F_{ST} values were low but significant for the slope groups, except between 2
 189 group pairs (Table 5). The significant F_{ST} values varied between 0.0012 and 0.0422.

Table 5. Pairwise F_{ST} for the slope groups obtained with 9 microsatellite loci. Bold: significant values. Slopes and altitude groups: S1 (Northern slope, 100-300m); S2 (Northern slope, 300 et 500 m); S3 (Northern slope, >500 m); S4 (Southern slope, 300 et 500 m); S5 (Southern slope, 300-100 m) and S6 (Outgroup).

	S2	S3	S4	S5	S6
S1	0.0152	0.0104	0.0134	0.0117	0.0422
S2		0.0025	0.0012	0.0049	0.0175
S3			0.0028	0	0.0282
S4				0.0052	0.0118
S5					0.0302

190 For altitude groups, pairwise F_{ST} values were also small but significant between all
 191 groups except the pair A2-A4 and varied between 0.0010 and 0.0214 (Table 6).

Table 6. Pairwise F_{ST} for the altitude groups obtained with 9 microsatellite loci. Bold: significant values; Altitude groups: A1 (100-200m); A2 (200-300m); A3 (300-400m); A4 (400-500m) and A5 (>500)

	A3	A4	A5
A2	0.0037	0.0002	0.0214
A3		0.0010	0.0099
A4			0.0149

192 For month groups pairwise F_{ST} were significant for 6 pairs out of 10 and their values
 193 varied between 0.0014 and 0.0275 (Table 7).

Table 7. Pairwise F_{ST} for month groups obtained with 9 microsatellite loci. Bold: significant values.

	June	July	August	September
May	0.0070	0.0015	0.0080	0
June		0.0014	0.0275	0.0226
July			0.0190	0.0106
August				0.0065

194 Considering the station subdivisions, pairwise F_{ST} values were significant for 91
 195 (66.9%) out of 136 comparisons, with values varying between 0.0005 and 0.048. Population
 196 ST03, ST14 and ST21 were different from almost any other collection sites, whereas ST9,
 197 ST13 and ST17 were not differentiated from other stations (Table 8).

198 Considering microgeographic subdivisions, pairwise F_{ST} values were significant for
 199 123 (53.2%) out of 231 comparisons, with values varying between 0.0002 and 0.048 (Table
 200 9).

201 The correlation between geographic and genetic distances was not significant
 202 (intercept = 0.0394746, slope = -0.00254977, $P(\text{slope}) = 0.7281$).

Table 8. Genetic and geographic distances between pairs of stations with 9 microsatellite loci. Below the diagonal: geographic distances in meters. Above the diagonal: pairwise F_{ST} . Bold: significant values after Adjusted P -value for 5% nominal level. ST21: Outgroup

	ST03	ST04	ST05	ST06	ST07	ST09	ST11	ST12	ST13	ST14	ST15	ST16	ST17	ST18	ST19	ST20	ST21
ST03		0.0150	0.0219	0.0160	0.0160	0.0098	0.0084	0.0158	0.0111	0.0269	0.0127	0.0096	0.0214	0.0098	0.0131	0.0115	0.0422
ST04	988.69		0.0078	0.0075	0	0.0043	0.0074	0.0103	0.0011	0.0142	0.0059	0.0046	0.0068	0.0051	0.0127	0.0091	0.0211
ST05	1077.42	181.91		0.0063	0.0078	0.0050	0.0061	0.0067	0.0043	0.0078	0.0117	0.0084	0.0009	0.0056	0.0114	0.0137	0.0246
ST06	1143.22	290.10	108.99		0.0062	0.0012	0.0031	0.0073	0.0006	0.0088	0.0056	0.0020	0.0014	0	0.0063	0.0083	0.0168
ST07	1170.81	197.40	133.52	179.50		0.0045	0.0071	0.0105	0.0015	0.0108	0.0097	0.0022	0.0064	0.0045	0.0141	0.0123	0.0155
ST09	2061.29	1308.38	1411.99	1465.00	1281.80		0	0	0	0.0190	0	0	0	0	0	0	0.0306
ST11	2178.56	1503.98	1616.82	1679.95	1503.95	241.20		0.0017	0.0021	0.0156	0.0005	0.0004	0.0027	0	0	0.0015	0.0331
ST12	2227.11	1633.83	1761.94	1832.67	1651.78	461.21	214.86		0.0042	0.0169	0.0019	0.0050	0	0.0009	0	0	0.0208
ST13	2733.29	2076.32	2181.19	2237.78	2073.31	777.54	568.63	508.45		0.0134	0.0049	0	0.0014	0	0.0048	0.0043	0.0223
ST14	3721.08	3100.00	3194.64	3252.12	3090.14	1788.64	1588.78	1492.87	1019.30		0.0168	0.0155	0.0109	0.0117	0.0274	0.0271	0.0049
ST15	3941.32	3262.27	3367.88	3398.88	3242.32	1953.21	1772.62	1712.10	1210.90	339.11		0.0014	0	0.0023	0.0016	0.0013	0.0227
ST16	4045.02	3291.49	3379.92	3424.86	3251.11	2002.20	1848.75	1821.61	1312.27	615.74	307.68		0.0045	0	0.0020	0.0043	0.0247
ST17	4646.64	3898.83	3955.18	3993.61	3831.96	2604.79	2457.00	2435.09	1922.03	1110.75	778.88	608.65		0.0020	0.0077	0.0056	0.0112
ST18	5156.03	4358.52	4403.08	4416.58	4267.79	3103.58	2988.98	2996.83	2488.21	1771.21	1438.72	1213.74	663.00		0.0009	0.0009	0.0202
ST19	5632.39	4860.43	4922.82	4946.04	4787.26	3601.98	3472.63	3447.73	2940.68	2117.06	1793.06	1634.47	1025.11	544.45		0	0.0480
ST20	5783.72	4996.72	5069.75	5081.88	4927.42	3734.26	3604.88	3578.85	3064.35	2222.97	1909.53	1754.40	1147.62	701.38	149.34		0.0341
ST21	1899.49	2032.49	2227.76	2336.33	2180.48	1773.69	1642.50	1488.65	1865.83	2495.68	2820.73	3040.49	3610.73	4234.87	4612.65	4719.86	

Table 9. Pairwise F_{ST} according to microgeographic populations obtained with 9 microsatellite loci. Bold: significant values after adjusted P -value for 5% nominal level. ST21: Outgroup.

	ST04	ST05	ST06	ST07	ST09	ST11A	ST11B	ST12	ST13A	ST13B	ST14	ST15	ST16	ST17A	ST17B	ST18A	ST18B	ST18C	ST19	ST20	ST21
ST03	0.0150	0.0219	0.0182	0.0141	0.0098	0.0057	0.0141	0.0158	0.0213	0.0108	0.0269	0.0127	0.0096	0.0178	0.0158	0.0091	0.0089	0.0118	0.0131	0.0115	0.0422
ST04		0.0078	0.0081	0	0.0043	0.0062	0.0098	0.0103	0.0109	0.0003	0.0142	0.0059	0.0046	0.0010	0.0060	0	0.0125	0.0040	0.0127	0.0091	0.0211
ST05			0.0074	0.0069	0.0050	0.0068	0.0050	0.0067	0.0123	0.0044	0.0078	0.0117	0.0084	0	0	0.0051	0.0107	0.0001	0.0114	0.0137	0.0246
ST06				0.0063	0.0025	0.0042	0.0065	0.0096	0.0061	0.0021	0.0086	0.0070	0.0031	0	0.0058	0.0005	0.0041	0	0.0090	0.0109	0.0155
ST07					0.0032	0.0060	0.0046	0.0083	0.0039	0.0023	0.0110	0.0083	0.0013	0.0005	0.0067	0.0008	0.0062	0.0028	0.0113	0.0098	0.0168
ST09						0	0	0	0.0037	0	0.0190	0	0	0	0.0012	0	0	0	0	0	0.0306
ST11A							0.0016	0.0012	0.0161	0	0.0157	0.0002	0.0006	0.0012	0	0	0	0	0	0	0.0303
ST11B								0.0027	0.0134	0.0022	0.0151	0.0017	0.0005	0	0	0.0061	0	0.0011	0.0020	0.0057	0.0364
ST12									0.0167	0.0026	0.0169	0.0019	0.0050	0	0	0.0060	0	0	0	0	0.0208
ST13A										0.0092	0.0154	0.0239	0.0062	0.0056	0.0150	0.0067	0.0121	0.0048	0.0230	0.0235	0.0229
ST13B											0.0144	0.0004	0	0	0	0	0.0018	0	0.0025	0.0011	0.0228
ST14												0.0168	0.0155	0.0041	0.0116	0.0094	0.0172	0.0062	0.0274	0.0271	0.0049
ST15													0.0014	0	0	0.0046	0.0005	0.0006	0.0016	0.0013	0.0227
ST16														0	0.0030	0.0006	0	0.0001	0.0020	0.0043	0.0247
ST17A															0	0.0032	0	0	0.0067	0.0039	0.0010
ST17B																0.0083	0.0023	0	0.0022	0	0.0099
ST18A																	0.0049	0	0.0046	0.0041	0.0255
ST18B																		0	0	0	0.0220
ST18C																			0.0006	0	0.0051
ST19																				0	0.0480
ST20																					0.0341

203 **Advanced analyses at microgeographic scale**

204 *Analyses per month*

205 The results detailed above suggest a population structuring at microgeographic scale.
206 Nevertheless, all the subdivisions were not at HWE at this scale (see Table 4). It was thus
207 interesting to test if we can detect an effect of month of capture on the structuring. We only
208 took into account the months with the largest number of specimens, June and July. All the
209 analyses were thus performed on microgeographic subpopulations ($n \geq 10$) per month, June
210 and July respectively.

211 Genetic variability and HWE estimators for the microgeographic populations for June
212 and July analyzed separately are synthetized in Tables 10 and 11. Mean intrapopulation
213 diversity (H_s) was 0.363 and 0.379 for June and July, respectively. There were more
214 subpopulations in HWE in July than in June (15/20 *versus* 7/17, respectively) and also than
215 subpopulations analyzed without month subdivision (8/21).

216 The pairwise F_{ST} were also calculated per month only between microgeographic
217 subpopulations with a number of specimens superior or equal to 10. For June, pairwise F_{ST}
218 values were significant for 18 (13.2%) out of 136 comparisons, and values varying between
219 0.001 and 0.0349 (Table 12). The number of migrants after correction for size (Nm) was 14.8.
220 In July, F_{ST} were significant for 113 (59.5%) out of 190 comparisons, and values varying
221 between 0.0005 and 0.0704 (Table 13). Nm was 8.9. We observed an augmentation of the
222 percentage of pairwise F_{ST} significant values in July in comparison with June and the two
223 months together (53.2%).

Table 10. Genetic variability and Hardy-Weinberg equilibrium estimators of microgeographic populations for 9 microsatellite loci in June. Nb: Number of samples; Na: mean number of alleles; Rs: mean allelic richness based on the smallest sample size; Ho: mean observed heterozygosity; He: mean unbiased expected heterozygosity; F_{IS} : Weir and Cockerham F_{IS} ; HWE: Hardy-Weinberg equilibrium.

Microhabitat	Nb	Na	Rs	Ho	He (\pm SD)	F_{IS}	HWE
ST03	10	2.67	1.34	0.34	0.34 (\pm 0.28)	0.012	*
ST04	24	3.67	1.40	0.37	0.40 (\pm 0.27)	0.071	
ST05	33	3.11	1.33	0.27	0.33 (\pm 0.26)	0.181	
ST06	30	3.11	1.34	0.30	0.34 (\pm 0.26)	0.127	
ST07	13	2.67	1.35	0.36	0.35 (\pm 0.25)	-0.048	*
ST09	21	3.33	1.40	0.38	0.40 (\pm 0.27)	0.033	*
ST11A	44	3.44	1.35	0.31	0.35 (\pm 0.27)	0.105	
ST11B	14	2.89	1.39	0.42	0.39 (\pm 0.25)	-0.074	*
ST12	33	3.44	1.35	0.28	0.35 (\pm 0.28)	0.211	
ST13A	6	1.89	1.34	0.16	0.34 (\pm 0.24)	0.556	\$
ST13B	31	2.89	1.35	0.27	0.35 (\pm 0.25)	0.251	
ST14	40	3.33	1.37	0.32	0.37 (\pm 0.26)	0.132	
ST15	25	2.89	1.38	0.32	0.38 (\pm 0.24)	0.172	
ST16	57	3.67	1.38	0.34	0.38 (\pm 0.20)	0.087	
ST17A	2	1.78	1.37	0.44	0.37 (\pm 0.37)	-0.333	\$
ST17B	1	1.33	1.33	0.33	0.33 (\pm 0.50)	NA	\$
ST18A	10	2.89	1.35	0.38	0.35 (\pm 0.29)	-0.084	*
ST18B	16	3.11	1.38	0.37	0.38 (\pm 0.26)	0.003	*
ST18C	11	2.67	1.39	0.34	0.39 (\pm 0.24)	0.140	*
ST19	6	2.22	1.32	0.36	0.32 (\pm 0.28)	-0.110	\$
ST20	68	4.00	1.36	0.35	0.36 (\pm 0.26)	0.035	

SD = Standard deviation

\$ = not applicable due to the low number of samples ($n < 10$)

* = Hardy-Weinberg equilibrium.

Table 11. Genetic variability and Hardy-Weinberg equilibrium estimators of microgeographic populations for 9 microsatellite loci in July. Nb: Number of samples; Na: mean number of alleles; Rs: mean allelic richness based on the smallest sample size; Ho: mean observed heterozygosity; He: mean unbiased expected heterozygosity; F_{IS} : Weir and Cockerham F_{IS} ; HWE: Hardy-Weinberg equilibrium.

Microhabitat	Nb	Na	Rs	Ho	He (\pm SD)	F_{IS}	HWE
ST03	45	3.56	2,03	0.39	0.41 (\pm 0.21)	0.044	*
ST04	57	4.11	2,12	0.41	0.42 (\pm 0.24)	0.013	
ST05	49	3.78	2,00	0.40	0.38 (\pm 0.24)	-0.031	*
ST06	49	3.89	2,08	0.39	0.39 (\pm 0.23)	0.012	*
ST07	59	4.33	2,04	0.38	0.39 (\pm 0.25)	0.037	
ST09	18	3.22	2,01	0.40	0.38 (\pm 0.23)	-0.049	*
ST11A	70	4.00	2,05	0.38	0.39 (\pm 0.24)	0.022	
ST11B	64	3.78	1,92	0.34	0.34 (\pm 0.25)	0.009	*
ST12	44	3.56	1,99	0.32	0.37 (\pm 0.25)	0.123	
ST13A	19	3.00	2,07	0.45	0.41 (\pm 0.24)	-0.099	*
ST13B	35	3.78	2,03	0.39	0.38 (\pm 0.26)	-0.044	*
ST14	24	3.44	2,17	0.41	0.43 (\pm 0.26)	0.047	
ST15	18	3.44	2,25	0.46	0.45 (\pm 0.22)	-0.020	*
ST16	42	3.56	1,97	0.35	0.37 (\pm 0.24)	0.049	*
ST17A	11	3.44	2,14	0.41	0.49 (\pm 0.25)	-0.028	*
ST17B	3	1.67	1,67	0.30	0.24 (\pm 0.32)	-0.333	\$
ST18A	35	3.11	1,99	0.42	0.38 (\pm 0.26)	-0.097	*
ST18B	27	2.67	1,82	0.31	0.33 (\pm 0.26)	0.064	*
ST18C	16	3.11	2,01	0.35	0.37 (\pm 0.28)	0.068	*
ST19	64	4.00	1,93	0.36	0.35 (\pm 0.27)	-0.046	*
ST20	55	3.78	1,93	0.36	0.36 (\pm 0.26)	-0.014	*

SD = Standard deviation

\$ = not applicable due to the low number of samples ($n < 10$)

* = Hardy-Weinberg equilibrium

Table 12. Pairwise F_{ST} according to microgeographic populations obtained with 9 microsatellite loci in June. Bold: significant values after adjusted P -value for 5% nominal level.

	ST04	ST05	ST06	ST07	ST09	ST11A	ST11B	ST12	ST13B	ST14	ST15	ST16	ST18A	ST18B	ST18C	ST20
ST03	0.0082	0.0002	0	0	0.0030	0	0	0.0068	0	0	0.0127	0	0.0161	0	0.0045	0
ST04		0.0040	0.0116	0.0071	0	0.0016	0.0002	0.0015	0	0.0070	0	0.0206	0.0035	0.0000	0	0.0016
ST05			0.0133	0.0113	0	0.0059	0.0232	0.0203	0.0039	0.0001	0.0349	0.0306	0.0162	0.0035	0	0.0078
ST06				0	0	0	0	0	0	0.0017	0.0049	0	0.0091	0.0191	0.0037	0
ST07					0.0056	0	0	0.0078	0	0	0.0002	0	0.0044	0	0.0010	0.0021
ST09						0.0022	0	0.0006	0	0	0.0038	0.0057	0.0068	0.0032	0	0
ST11A							0	0	0	0.0038	0.0036	0.0017	0.0084	0.0104	0.0040	0
ST11B								0	0	0	0	0.0010	0	0	0.0006	0
ST12									0	0.0167	0	0.0128	0.0215	0.0260	0.0105	0
ST13B										0.0029	0.0011	0.0003	0	0.0092	0	0
ST14											0.0164	0.0112	0	0	0	0.0072
ST15												0.0038	0.0049	0.0163	0.0006	0.0072
ST16													0.0186	0.0240	0.0099	0.0105
ST18A														0	0	0.0174
ST18B															0	0.0141
ST18C																0.0032

Table 13. Pairwise F_{ST} according to microgeographic populations obtained with 9 microsatellite loci in July. **Bold:** significant values after adjusted P -value for 5% nominal level.

	ST04	ST05	ST06	ST07	ST09	ST11A	ST11B	ST12	ST13A	ST13B	ST14	ST15	ST16	ST17A	ST18A	ST18B	ST18C	ST19	ST20
ST03	0.0185	0.0510	0.0225	0.0106	0.0033	0.0053	0.0203	0.0211	0.0185	0.0154	0.0513	0.0337	0.0197	0.0070	0.0158	0.0203	0.0227	0.0195	0.0151
ST04		0.0529	0.0156	0.0007	0.0072	0.0097	0.0226	0.0142	0	0.0016	0.0475	0.0197	0.0042	0.0052	0.0094	0.0312	0.0217	0.0209	0.0168
ST05			0.0348	0.0349	0.0261	0.0326	0.0362	0.0351	0.0426	0.0289	0.0448	0.0322	0.0182	0.0157	0.0507	0.0545	0.0449	0.0383	0.0386
ST06				0.0039	0	0.0018	0.0074	0.0109	0.0106	0.0094	0.0399	0.0242	0.0069	0	0.0082	0.0178	0.0201	0.0044	0.0090
ST07					0	0	0.0039	0.0025	0.0017	0	0.0444	0.0200	0.0001	0	0.0010	0.0064	0.0066	0.0012	0
ST09						0	0	0	0.0137	0	0.0357	0.0154	0	0	0	0.0070	0	0	0
ST11A							0.0035	0.0004	0.0092	0.0001	0.0378	0.0216	0.0005	0	0.0009	0.0067	0.0043	0	0
ST11B								0.0068	0.0243	0.0071	0.0611	0.0421	0.0032	0	0.0170	0.0185	0.0110	0.0021	0.0051
ST12									0.0140	0.0047	0.0472	0.0265	0	0	0.0056	0.0130	0	0	0
ST13A										0.0044	0.0466	0.0275	0.0073	0.0121	0.0220	0.0270	0.0332	0.0245	0.0237
ST13B											0.0479	0.0229	0	0	0.0119	0.0215	0.0130	0.0072	0.0042
ST14												0	0.0419	0.0381	0.0491	0.0677	0.0704	0.0545	0.0537
ST15													0.0199	0.0223	0.0295	0.0330	0.0403	0.0305	0.0278
ST16														0	0.0114	0.0238	0.0108	0.0038	0.0029
ST17A															0.0012	0.0121	0	0	0
ST18A																0.0201	0.0063	0.0055	0.0018
ST18B																	0.0114	0.0028	0.0010
ST18C																		0.0017	0
ST19																			0

224 *Analyses per month and gender*

225 Finally, in order to investigate the population genetic structure according to the gender,
226 analyses were performed on microgeographic subpopulations per sex and per month (June
227 and July separately).

228 The genetic variability of microgeographic populations according to the month and to
229 the sex (Number of samples; mean number of alleles; mean allelic richness based on the
230 smallest sample size) are presented in Tables 14, 15, 16 and 17. The mean number of alleles
231 was more important for males, particularly in July (Table 18).

232 The pairwise F_{ST} were calculated only between microgeographic subpopulations when
233 the number of specimens was superior or equal to 10. For females in June, pairwise F_{ST}
234 values were significant for 0 (0%) out of 10 comparisons (Table 19). The number of migrants
235 (Nm) after correction for size was 8.0. In July, they were significant for 11 (24.4%) out of 45
236 comparisons, with ST04 differentiated from all other stations and values varying between
237 0.0090 and 0.0569 (Table 20). Nm was 5.8.

238 In contrast for male in June, F_{ST} values were significant for 21 (26.9%) out of 78
239 comparisons, with values varying between 0.001 and 0.037 (Table 21). The mean number of
240 migrants was 5.3. In July, F_{ST} values were significant for 79 (58.1%) out of 136 comparisons,
241 with values varying between 0.0022 and 0.0847 (Table 22). Nm was 3.7.

Table 14. Genetic variability of microgeographic populations obtained with 9 microsatellite loci for females in June. MP: Microgeographic populations; Nb: Number of samples; Na: mean number of alleles; Rs: mean allelic richness based on the smallest sample size.

MP	ST03	ST04	ST06	ST07	ST09	ST11A	ST11B	ST12	ST13B	ST15	ST16	ST17A	ST18A	ST18C	ST19	ST20
Nb	4	22	19	1	11	34	1	7	2	1	3	2	1	3	5	33
Na	1.89	3.56	2.89	1.56	2.44	3.00	1.33	2.33	1.89	1.33	2.00	1.78	1.33	2.00	1.78	3.22
Rs	1.33	1.40	1.35	1.56	1.33	1.35	1.33	1.37	1.46	1.33	1.37	1.37	1.33	1.33	1.27	1.35

Table 15. Genetic variability of microgeographic populations obtained with 9 microsatellite loci for females in July. MP: Microgeographic populations; Nb: Number of samples; Na: mean number of alleles; Rs: mean allelic richness based on the smallest sample size.

MP	ST03	ST04	ST05	ST06	ST07	ST09	ST11A	ST11B	ST12	ST14	ST15	ST16	ST17A	ST18B	ST18C	ST19	ST20
Nb	9	24	11	36	18	15	59	28	7	2	1	1	7	3	11	28	19
Na	2.56	3.67	2.56	3.67	3.11	3.00	3.89	3.22	2.22	1.78	1.44	1.44	2.78	1.78	3.11	3.33	3.33
Rs	1.39	1.45	1.29	1.39	1.37	1.38	1.40	1.31	1.32	1.37	1.44	1.44	1.38	1.32	1.40	1.34	1.38

Table 16. Genetic variability of microgeographic populations obtained with 9 microsatellite loci for males in June. MP: Microgeographic populations; Nb: Number of samples; Na: mean number of alleles; Rs: mean allelic richness based on the smallest sample size.

MP	ST03	ST04	ST05	ST06	ST07	ST09	ST11A	ST11B	ST12	ST13A	ST13B	ST14	ST15	ST16	ST17B	ST18A	ST18B	ST18C	ST19	ST20
Nb	6	2	33	11	12	10	13	26	6	29	40	24	54	1	9	16	8	1	35	35
Na	2.33	1.78	3.11	2.33	2.33	3.00	2.56	2.67	3.44	1.89	2.78	3.33	2.89	3.67	1.33	2.89	3.11	2.56	1.67	3.44
Rs	1.34	1.33	1.33	1.32	1.33	1.46	1.32	1.39	1.34	1.34	1.35	1.37	1.38	1.38	1.33	1.36	1.38	1.43	1.67	1.37

Table 17. Genetic variability of microgeographic populations obtained with 9 microsatellite loci for males in July. MP: Microgeographic populations; Nb: Number of samples; Na: mean number of alleles; Rs: mean allelic richness based on the smallest sample size.

MP	ST03	ST04	ST05	ST06	ST07	ST09	ST11A	ST11B	ST12	ST13A	ST13B	ST14	ST15	ST16	ST17A	ST17B	ST18A	ST18B	ST18C	ST19	ST20
Nb	36	33	38	13	41	3	11	36	37	19	35	22	17	41	4	3	35	24	5	36	36
Na	3.44	3.11	3.33	3.11	3.89	2.00	2.67	3.44	3.33	3.00	3.78	3.44	3.33	3.56	2.33	1.67	3.11	2.56	1.78	3.22	3.11
Rs	2.028	2.01	2.01	2.12	2.07	2.00	1.89	2.01	2.02	2.07	2.03	2.18	2.24	1.98	2.15	1.67	1.99	1.82	1.67	1.93	1.87

Table 18. Mean, minimum and maximum number of alleles (N_a) for males and females in June and July obtained with 9 microsatellite loci for males in July. Min: minimum; Max: maximum.

N_a	June		July	
	Males	Female	Males	Female
Mean	2.66	2.15	3.01	2.76
Min	1.33	1.33	1.67	1.44
Max	3.67	3.56	3.89	3.89

Table 19. Pairwise F_{ST} according to the microgeographic populations for females in June obtained with 9 microsatellite loci. Bold: significant values after adjusted P -value for 5% nominal level.

	ST06	ST09	ST11A	ST20
ST04	0.0001	0.0061	0	0.0009
ST06		0	0	0.0039
ST09			0.0010	0.0029
ST11A				0.0031

Table 20. Pairwise F_{ST} according to the microgeographic populations for females in July obtained with 9 microsatellite loci. Bold: significant values after adjusted P -value for 5% nominal level.

	ST05	ST06	ST07	ST09	ST11A	ST11B	ST18C	ST19	ST20
ST04	0.0602	0.0341	0.0235	0.0225	0.0258	0.0569	0.0442	0.0533	0.0287
ST05		0.0346	0.0119	0.0023	0.0090	0.0154	0.0256	0.0093	0
ST06			0	0	0.0009	0.0176	0.0393	0	0.0052
ST07				0	0	0.0025	0.0323	0	0
ST09					0	0	0.0061	0	0
ST11A						0.0088	0.0107	0	0
ST11B							0.0352	0.0060	0.0019
ST18C								0.0337	0
ST19									0

Table 21. Pairwise F_{ST} according to the microgeographic populations for males in June obtained with 9 microsatellite loci. Bold: significant values after adjusted P -value for 5% nominal level.

	ST06	ST07	ST09	ST11A	ST11B	ST12	ST13B	ST14	ST15	ST16	ST18B	ST20
ST05	0.0046	0.0154	0.0298	0.0035	0.0260	0.0305	0.0035	0.0001	0.0370	0.0329	0.0035	0.0181
ST06		0	0.0181	0	0	0	0	0	0.0130	0	0.0146	0
ST07			0.0259	0.0024	0	0	0	0	0	0	0	0
ST09				0.0236	0.0015	0.0022	0.0050	0.0147	0	0.0195	0.0220	0.0122
ST11A					0	0	0	0.0064	0	0.0200	0.0135	0
ST11B						0	0	0.0012	0	0.0045	0	0
ST12							0	0.0187	0	0.0108	0.0311	0
ST13B								0.0010	0	0.0009	0.0076	0
ST14									0.0210	0.0122	0	0.0095
ST15										0.0071	0.0213	0.0035
ST16											0.0243	0.0041
ST18B												0.0151

Table 22. Pairwise F_{ST} according to the microgeographic populations for males in July obtained with 9 microsatellite loci. Bold: significant values after adjusted P -value for 5% nominal level.

	ST04	ST05	ST06	ST07	ST11A	ST11B	ST12	ST13A	ST13B	ST14	ST15	ST16	ST18A	ST18B	ST19	ST20
ST03	0.0163	0.0722	0.0252	0.0178	0.0105	0.0166	0.0279	0.0284	0.0221	0.0736	0.0416	0.0271	0.0247	0.0320	0.0207	0.0229
ST04		0.0538	0.0025	0	0	0.0045	0.0043	0	0	0.0641	0.0175	0.0017	0.0014	0.0127	0.0001	0.0036
ST05			0.0356	0.0559	0.0619	0.0491	0.0546	0.0487	0.0473	0.0652	0.0439	0.0376	0.0687	0.0746	0.0559	0.0653
ST06				0.0062	0.0124	0	0.0061	0.0157	0.0059	0.0664	0.0211	0	0.0151	0.0263	0.0022	0.0191
ST07					0	0	0.0026	0	0	0.0628	0.0287	0	0.0035	0.0130	0	0.0019
ST11A						0	0.0083	0.0108	0	0.0519	0.0247	0.0042	0.0014	0	0	0
ST11B								0	0.0138	0.0558	0.0351	0	0.0042	0.0196	0	0
ST12									0.0117	0.0612	0.0283	0.0014	0.0055	0.0154	0	0.0041
ST13A									0.0044	0.0585	0.0327	0.0068	0.0220	0.0272	0.0217	0.0239
ST13B										0.0604	0.0257	0	0.0119	0.0243	0.0032	0.0064
ST14											0.0050	0.0538	0.0621	0.0847	0.0715	0.0695
ST15												0.0241	0.0350	0.0346	0.0320	0.0344
ST16													0.0117	0.0260	0	0.0061
ST18A														0.0233	0	0.0037
ST18B															0.0073	0
ST19																0

242 Discussion

243 **Genetic variability and population structure of *P. ariasi* at different spatiotemporal** 244 **scales**

245 The nine microsatellite markers used in this study, after detection of null alleles in two
246 loci (*aria2* and *aria15*), were polymorphic and revealed similar degrees of diversity at all
247 spatial scales of the transect. The genetic differentiation investigated according to a gradient
248 of spatial partitions along the transect, from large subdivisions (slopes and groups of altitude)
249 to small ones (stations and microhabitats), revealed a microgeographic population structure.
250 In agreement with this structure, the highest numbers of subpopulations at HWE were found
251 at the level of stations and microhabitats. Nevertheless, even at the small spatial scale several
252 subpopulations still showed heterozygote deficits (Wahlund effect). These data suggest the
253 existence of smaller subdivisions not detected in our sampling. The results obtained for the
254 Linkage Disequilibrium (LD) also support the microgeographic population structure, with
255 significant LD at large scale and no LD for a large part of subdivisions at small scale. Thus,
256 from all these analyses, our assumption is that *P. ariasi* is organized in microgeographic
257 populations that could be a hutch, a poultry farm, a wall or even a “barbacane”. Previous
258 studies on *P. papatasi* [36] (microsatellite markers), *Lutzomyia intermedia* [37] (Randomly
259 amplified polymorphic DNA) and *L. anduzei* [38] (*COI* and *Cytb* loci), also suggest a certain
260 degree of structuring at small scale. The population structure seems to be directly linked to the
261 biology of the insects, since some of them revealed very low structuring such as *Aedes*
262 *albopictus* [39], whereas some others present a microstructuring such as *Glossina palpalis* and
263 sandflies in our study [40].

264 This population genetic study evokes for the first time that the reproductive units for *P.*
265 *ariasi* corresponds to microgeographic subpopulations.

266

267 **The microgeographic scale**

268 At the microgeographic scale, the fixation index (F_{ST}) revealed small but significant
269 values of genetic differentiation for 53.2% of subpopulation comparisons. It is worth noting
270 that if we consider separately the two main months of sandfly activities, June and July,
271 whereas very few values were significant for June (13.2%), the percentage of significant
272 values was much higher in July (59.5%). This temporal structuring is confirmed by a higher
273 number of subpopulations at HWE in July than in June. Even if we can detect isolated
274 subpopulations, our data suggest gene flux between these microgeographic subpopulations
275 and particularly high in June. Migration between subpopulations is also supported by the
276 absence of genetic isolation by distance. These migration events can be linked to several
277 factors such as the resource accessibility (sugar meal for both genders and blood meal for
278 females), the availability of mating partners [5] and the dispersion abilities of *P. ariasi*. For
279 the latter aspect, this species is known to be able to flight more than 1km [41] and the
280 distances between the neighboring stations of capture are between 109 and 1282 meters.

281 When we considered females and males collected in June or in July separately, the
282 results showed different spatiotemporal structuring. The weaker spatial structuring patterns
283 observed in females than in males could be explained by differential behaviors according to
284 the gender but also by different dispersal capabilities. Because of blood meal necessity, the
285 female sandflies could fly on larger distances than males. This hypothesis is supported by the
286 estimated numbers of migrants between microgeographic subpopulations which are more
287 important for females (between 5.8 and 8.0) than for males (between 3.7 and 5.3).

288

289 **Ecological and epidemiological consequences of microgeographic population structure**

290 This structure in microgeographic populations has undoubtedly implications on the
291 ecology and evolution of sandflies. We expect that this structure favors the stability of sandfly

292 populations in space and time. The homogeneity of our data between the subpopulations in
293 terms of diversity suggests a demographic stability of the sandfly populations in this area.
294 Because of this structuring, local environmental changes would have no or low effect on the
295 whole population since the microgeographic subpopulations would be affected independently.
296 Furthermore, the migration of specimens could quickly generate new populations to replace
297 the old one. Migrants can also play a role in the expansion of sandfly distribution that we
298 currently observe in France [16]. Nevertheless, compared to *Aedes albopictus*, the expansion
299 process of sandflies seems to be very slow because of their weak dispersal capacities and the
300 low number of migrants found at small scales.

301 This structure has also certainly an effect on transmission of infectious diseases and in
302 this case of *Leishmania*. First, this organization in microgeographic subpopulations is in
303 agreement with previous studies dealing with *Leishmania* population analyses [42, 43]. These
304 works suggest the existence of different imbricated transmission cycles at very small scale
305 and the authors underline the need to pursue the analyses at the smallest possible spatio-
306 temporal and ecological scales.

307 Second, in term of transmission, the existence of infected hosts within a
308 microgeographic sandfly subpopulation can produce a fast increase of the prevalence of
309 infected sandflies and thus locally increase also the risk of transmission. This hypothesis is
310 supported by a study carried out in Senegal in which the prevalence of infected females was
311 significantly associated to the presence of infected dogs [44]. Furthermore, because of
312 migration events between the subpopulations, *Leishmania* infection can spread out gradually
313 to the neighboring subpopulations of sandflies.

314 Conclusion

315 This study demonstrates for the first time that *P. ariasi* presents a genetic structure in
316 microgeographic subpopulations. These data highlight the necessity to consider sandfly
317 population at microspatial and temporal scale to study the ecology of these insects and the
318 impact on *Leishmania* transmission.

319 This structure explains the stability of sandfly populations in space and time and the
320 functioning of *Leishmania* transmission cycles. Nevertheless, further analyses need to be done
321 to confirm this structure in other sandfly vector species.

322

323 Acknowledgments

324 This study was funded by EU grant FP7-261504 EDENext and is catalogued by the
325 EDENext Steering Committee as EDENextXXX (<http://www.edenext.eu>). The contents of
326 this publication are the sole responsibility of the authors and do not necessarily reflect the
327 views of the European Commission. We are particularly grateful to Mr Lacoste, IRD (Institut
328 de Recherche pour le Développement) and CNRS for financial support.

References

- 329 1. Dolmatova AV, Demina NA: Les phlébotomes (Phlebotominae) et les maladies qu'ils
330 transmettent, vol. 181966.
- 331 2. Bichaud L, Souris M, Mary C, Ninove L, Thirion L, Piarroux R, Piarroux R, De Lamballerie X,
332 Charrel R: Epidemiologic Relationship between Toscana Virus Infection and *Leishmania*
333 *infantum* Due to Common Exposure to *Phlebotomus perniciosus* Sandfly Vector. *PLoS*
334 *Neglected Tropical Diseases* 2011, 5(9):e1328.
- 335 3. Rioux JA, Killick-Kendrick R, Perieres J, Turner D, Lanotte G: Ecologie des Leishmanioses dans
336 le Sud de la France. 13. Les sites de "flanc de coteau", biotopes de transmission privilégiés
337 de la Leishmaniose viscérale en Cévennes. *Annales de parasitologie* 1980, 55(4):445-453.
- 338 4. Rioux JA, Carron S, Dereure J, Perieres J, Zeraia L, Franquet E, Babinot M, Gallego M,
339 Prudhomme J: Ecology of leishmaniasis in the South of France. 22. Reliability and
340 representativeness of 12 *Phlebotomus ariasi*, *P. perniciosus* and *Sergentomyia minuta*
341 (Diptera: Psychodidae) sampling stations in Vallespir (eastern French Pyrenees region).
342 *Parasite (Paris, France)* 2013, 20:34.
- 343 5. Rioux JA, Golvan Y, Croset H, Tour S, Hovin R, Abonnenc E, Petitdidier M, Vollhardt Y, Dedet
344 JP, Albaret JL et al: Epidémiologie des leishmanioses dans le Sud de la France, vol. 37:
345 Monographie de l'Institut National de la Santé et de la Recherche Médicale; 1969.
- 346 6. Lewis D: A taxonomic review of the genus *Phlebotomus* (Diptera: Psychodidae). *Bulletin of*
347 *the British Museum (Natural History)* 1982, 45(2):121-209.
- 348 7. Rossi E, Rinaldi L, Musella V, Veneziano V, Carbone S, Gradoni L, Cringoli G, Maroli M:
349 Mapping the main *Leishmania* phlebotomine vector in the endemic focus of the Mt.
350 Vesuvius in southern Italy. *Geospatial health* 2007, 1(2):191-198.
- 351 8. Ballart C, Barón S, Alcover MM, Portus M, Gallego M: Distribution of phlebotomine sand
352 flies (Diptera: Psychodidae) in Andorra: First finding of *P. perniciosus* and wide distribution
353 of *P. ariasi*. *Acta Tropica* 2012, 122(1):155-159.
- 354 9. Ballart C, Guerrero I, Castells X, Barón S, Castillejo S, Alcover M, Portús M, Gállego M:
355 Importance of individual analysis of environmental and climatic factors affecting the
356 density of *Leishmania* vectors living in the same geographical area: the example of
357 *Phlebotomus ariasi* and *P. perniciosus* in northeast Spain. *Geospatial health* 2014, 8(2):389-
358 403.
- 359 10. Boussaa S, Neffa M, Pesson B, Boumezzough A: Phlebotomine sandflies (Diptera:
360 Psychodidae) of southern Morocco: results of entomological surveys along the Marrakech-
361 Ouarzazat and Marrakech-Azilal roads. *Annals of tropical medicine and parasitology* 2010,
362 104(2):163-170.
- 363 11. Franco F, Morillas-Marquez F, Barón S, Morales-Yuste M, Gálvez R, Díaz-Sáez V, Pesson B,
364 Alves-Pires C, Depaquit J, Molina R et al: Genetic structure of *Phlebotomus (Larrousius)*
365 *ariasi* populations, the vector of *Leishmania infantum* in the western Mediterranean:
366 Epidemiological implications. *International Journal for Parasitology* 2010, 40(11):1335-1346.
- 367 12. Ready P: Leishmaniasis emergence in Europe. *Euro surveillance : bulletin Européen sur les*
368 *maladies transmissibles = European communicable disease bulletin* 2010, 15(10):19505.
- 369 13. Branco S, Alves-Pires C, Maia C, Cortes S, Cristovão J, Gonçalves L, Campino L, Afonso M:
370 Entomological and ecological studies in a new potential zoonotic leishmaniasis focus in
371 Torres Novas municipality, Central Region, Portugal. *Acta Tropica* 2013, 125(3):339-348.
- 372 14. Barón SD, Morillas-Márquez F, Morales-Yuste M, Díaz-Sáez V, Irigaray C, Martín-Sánchez J:
373 Risk maps for the presence and absence of *Phlebotomus perniciosus* in an endemic area of
374 leishmaniasis in southern Spain: implications for the control of the disease. *Parasitology*
375 2011, 138(10):1234-1244.

- 376 15. Boudabous R, Amor S, Khayech F, Marzouk M, Bdira S, Mezhoud H, Azaiez R, Sfar M, Babba:
377 **The phlebotomine fauna (Diptera: Psychodidae) of the eastern coast of Tunisia.** *Journal of*
378 *medical entomology* 2009, **46(1):1-8.**
- 379 16. Dedet J-P: **Les leishmanioses en France métropolitaine.** *BEH Hors-série* 2010:9-12.
- 380 17. Depaquit J, Grandadam M, Fouque F, Andry P-E, Peyrefitte C: **Arthropod-borne viruses**
381 **transmitted by Phlebotomine sandflies in Europe: a review.** *Euro surveillance : bulletin*
382 *Européen sur les maladies transmissibles = European communicable disease bulletin* 2010,
383 **15(10):19507.**
- 384 18. Kamhawi S, Molyneux D, Killick-Kendrick R, Milligan P, Phillips A, Wilkes T, Killick-Kendrick M:
385 **Two populations of *Phlebotomus ariasi* in the Cévennes focus of leishmaniasis in the south**
386 **of France revealed by analysis of cuticular hydrocarbons.** *Medical and Veterinary*
387 *Entomology* 1987, **1(1):97-102.**
- 388 19. Pesson B, Wallon M, Floer M, Kristensen A: **[Isoenzymatic studies of Mediterranean**
389 **populations of sandflies of the subgenus *Larroussius*].** *Parassitologia* 1991, **33:471-476.**
- 390 20. Martin-Sanchez J, Gramiccia M, Pesson B, Morillas-Marquez F: **Genetic polymorphism in**
391 **sympatric species of the genus *Phlebotomus*, with special reference to *Phlebotomus***
392 ***perniciosus* and *Phlebotomus longicuspis* (Diptera, Phlebotomidae).** *Parasite (Paris, France)*
393 **2000, 7(4):247-254.**
- 394 21. Selkoe K, Toonen R: **Microsatellites for ecologists: a practical guide to using and evaluating**
395 **microsatellite markers.** *Ecology Letters* 2006, **9(5):615-629.**
- 396 22. Mahamdallie S, Pesson B, Ready P: **Multiple genetic divergences and population expansions**
397 **of a Mediterranean sandfly, *Phlebotomus ariasi*, in Europe during the Pleistocene glacial**
398 **cycles.** *Heredity* 2010, **106(5):714-726.**
- 399 23. Aransay A, Malarky G, Ready P: **Isolation (with enrichment) and characterization of**
400 **trinucleotide microsatellites from *Phlebotomus perniciosus*, a vector of *Leishmania***
401 ***infantum*.** *Molecular Ecology Notes* 2001, **1:176-178.**
- 402 24. Prudhomme J, Toty C, Kasap O, Rahola N, Vergnes B, Maia C, Campino L, Antoniou M,
403 Jimenez M, Molina R *et al*: **New microsatellite markers for multi-scale genetic studies on**
404 ***Phlebotomus ariasi* Tonnoir, vector of *Leishmania infantum* in the Mediterranean area.**
405 *Acta Tropica* 2015, **142:79-85.**
- 406 25. Daget P: **Le bioclimat Méditerranéen: Analyse des formes climatiques par le système**
407 **d'Emberger.** *Vegetation* 1977, **34(2):87-103.**
- 408 26. Alten B, Ozbel Y, Ergunay K, Kasap O, Cull B, Antoniou M, Velo E, Prudhomme J, Molina R,
409 Bañuls A *et al*: **Sampling strategies for phlebotomine sand flies (Diptera: Psychodidae) in**
410 **Europe.** *Bulletin of entomological research* 2015:1-15.
- 411 27. Abonnenc E: **Les phlébotomes de la région éthiopienne (Diptera, Psychodidae).** 1972:1-290.
- 412 28. Killick-Kendrick R, Tang Y, Killick-Kendrick M, Sang D, Sirdar M, Ke L, Ashford R, Schorscher J,
413 Johnson R: **The identification of female sandflies of the subgenus *Larroussius* by the**
414 **morphology of the spermathecal ducts.** *Parassitologia* 1991, **33:335-347.**
- 415 29. Wang Q, Wang X: **Comparison of methods for DNA extraction from a single chironomid for**
416 **PCR analysis.** *Pakistan Journal of Zoology* 2012, **44(2):421-426.**
- 417 30. Zapata S, Mejía L, Le Pont F, León R, Pesson B, Ravel C, Bichaud L, Charrel R, Cruaud C, Trueba
418 G: **A study of a population of *Nyssomyia trapidoi* (Diptera: Psychodidae) caught on the**
419 **Pacific coast of Ecuador.** *Parasites & vectors* 2012, **5(1):1-8.**
- 420 31. Van Oosterhout C, Hutchinson WF, Wills DP, Shipley P: **MICRO-CHECKER: software for**
421 **identifying and correcting genotyping errors in microsatellite data.** *Molecular Ecology Notes*
422 **2004, 4(3):535-538.**
- 423 32. Goudet J: **FSTAT, a program to estimate and test gene diversities and fixation indices**
424 **(version 2.9.3).** 2001.
- 425 33. Belkhir K, Borsa P, Chikhi L, Raufaste N, Catch F: **GENETIX 4.04, software under Windows TM**
426 **for the genetics of the populations. Laboratory Genome, Populations, Interactions, CNRS**
427 **UMR 5000, University of Montpellier II, Montpellier, France.** 2009.

- 428 34. Rousset F: **genepop'007: a complete re-implementation of the genepop software for**
429 **Windows and Linux.** *Molecular Ecology Resources* 2008, **8**(1):103-106.
- 430 35. Dakin E, Avise J: **Microsatellite null alleles in parentage analysis.** *Heredity* 2004, **93**(5):504-
431 509.
- 432 36. Khalid NM, Aboud MA, Alrabba FM, Elnaiem D, Tripet F: **Evidence for genetic differentiation**
433 **at the microgeographic scale in Phlebotomus papatasi populations from Sudan.** *Parasites &*
434 *Vectors* 2012, **5**(1):249.
- 435 37. Rocha LDS, Falqueto A, Dos Santos CB, Grimaldi Jr G, Cupolillo E: **Genetic structure of**
436 **Lutzomyia (Nyssomyia) intermedia populations from two ecologic regions in Brazil where**
437 **transmission of Leishmania (Viannia) braziliensis reflects distinct eco-epidemiologic**
438 **features.** *The American journal of tropical medicine and hygiene* 2007, **76**(3):559-565.
- 439 38. Scarpassa VM, da Silva Figueiredo A, Alencar RB: **Genetic diversity and population structure**
440 **in the Leishmania guyanensis vector Lutzomyia anduzei (Diptera, Psychodidae) from the**
441 **Brazilian Amazon.** *Infection, Genetics and Evolution* 2015, **31**:312-320.
- 442 39. Zawani M, Abu H, Sazaly A, Zary S, Darlina M: **Population genetic structure of Aedes**
443 **albopictus in Penang, Malaysia.** *Genetics and molecular research: GMR* 2014, **13**(4):8184.
- 444 40. De Meeûs T: **Initiation à la génétique des populations naturelles: Applications aux parasites**
445 **et à leurs vecteurs:** IRD Editions; 2012.
- 446 41. Killick-Kendrick R, Rioux JA, Bailly M, Guy M, Wilkes T, Guy F, Davidson I, Knechtli R, Ward R,
447 Guilvard E *et al*: **Ecology of leishmaniasis in the south of France. 20. Dispersal of**
448 **Phlebotomus ariasi Tonnoir, 1921 as a factor in the spread of visceral leishmaniasis in the**
449 **Cévennes.** *Annales de parasitologie humaine et comparée* 1984, **59**(6):555-572.
- 450 42. Rougeron V, De Meeûs T, Hide M, Le Falher G, Bucheton B, Dereure J, El-Safi SH, Dessein A,
451 Bañuls A-L: **Multifaceted population structure and reproductive strategy in Leishmania**
452 **donovani complex in one Sudanese village.** *PLoS Negl Trop Dis* 2011, **5**(12):e1448.
- 453 43. Rougeron V, BAÑULS AL, Carme B, Simon S, Couppie P, Nacher M, Hide M, De Meeûs T:
454 **Reproductive strategies and population structure in Leishmania: substantial amount of sex**
455 **in Leishmania Viannia guyanensis.** *Molecular ecology* 2011, **20**(15):3116-3127.
- 456 44. Senghor MW, Niang AA, Depaquit J, Faye MN, Elguero E, Gaye O, Alten B, Perktas U, Cassan
457 C, Faye F *et al*: **Transmission of Leishmania infantum by Phlebotomine sandflies of the**
458 **genus Sergentomyia (Diptera: Psychodidae): ecological, parasitological and molecular**
459 **evidences in the canine leishmaniasis focus of Mont-Rolland, Senegal.** *PLoS Negl Trop Dis*
460 Accepted with modifications

461

Supplementary table 1. Presence (Y) or absence (N) of null alleles by group according to MICRO-CHECKER. Slopes and altitudes groups: S1 (Northern slope, 100-300m); S2 (Northern slope, 300-500 m); S3 (Northern slope, >500 m); S4 (Southern slope, 300-500 m); S5 (Southern slope, 300-100 m) and S6 (Outgroup). Altitude groups: A1 (100-200m); A2 (200-300m); A3 (300-400m); A4 (400-500m) and A5 (>500)

		aria1	aria2	aria3	aria4	aria5	aria10	aria11	aria12	aria13	aria14	aria15
Slope/ altitude groups	S 1	Y	Y	N	N	N	N	N	N	N	N	Y
	S 2	N	Y	N	N	Y	N	N	N	N	Y	Y
	S 3	Y	Y	Y	N	N	Y	N	N	Y	Y	Y
	S 4	Y	Y	N	N	N	Y	N	N	N	Y	Y
	S 5	N	Y	N	N	N	Y	N	N	N	Y	Y
	S6	N	N	N	N	N	N	N	N	N	Y	Y
Altitude Groups	A 2	Y	Y	N	N	N	N	N	N	N	Y	Y
	A 3	Y	Y	N	N	N	Y	N	N	N	Y	Y
	A 4	Y	Y	Y	N	N	Y	N	N	N	Y	Y
	A 5	Y	N	N	N	N	Y	N	N	N	Y	Y
Station	ST03	Y	Y	N	N	N	N	N	N	N	N	Y
	ST04	N	Y	N	N	N	Y	N	N	N	N	Y
	ST05	N	Y	N	Y	N	N	N	N	N	Y	Y
	ST06	N	Y	N	N	Y	N	N	N	N	N	Y
	ST07	N	N	N	N	N	N	N	N	N	N	Y
	ST09	Y	Y	N	N	N	N	N	N	N	N	N
	ST11	Y	Y	N	N	N	N	N	N	N	N	Y
	ST12	N	N	Y	Y	N	N	N	N	Y	N	Y
	ST13	N	Y	N	N	N	Y	N	N	N	Y	Y
	ST14	Y	N	N	N	N	Y	N	N	N	Y	Y
	ST15	Y	N	N	N	N	N	N	N	N	N	Y
	ST16	N	Y	N	N	N	N	N	N	N	Y	Y
	ST17	N	N	N	N	N	Y	N	N	N	N	Y
	ST18	N	Y	N	N	N	N	N	N	N	Y	Y
	ST19	N	Y	N	N	N	N	N	N	N	N	Y
ST20	N	N	N	N	N	Y	N	N	N	N	Y	
ST21	N	N	N	N	N	N	N	N	N	Y	Y	

Supplementary table 2. *P*-value of the linkage disequilibrium measured with 9 microsatellite loci using FSTAT 2.9.3. Bold: significant values after adjusted *P*-value for 5% nominal level.

	Global	Slope groups	Altitude groups	Month groups	Station groups	Microgeographic populations
Ajusted <i>P</i> -value	0.001389	0.000231	0.000347	0.000278	0.000082	0.000063
Msat1 X Msat3	0.00694	0.11852	0.03472	0.02389	0.05074	0.07304
Msat1 X Msat4	1.00000	0.68611	0.95972	0.87833	0.78807	0.82847
Msat1 X Msat5	0.67500	0.75000	0.71146	0.83861	0.85654	0.87967
Msat1 X Msat10	0.90833	0.89745	0.87465	0.93722	0.75931	0.74848
Msat1 X Msat11	0.00278	0.00463	0.00139	0.02389	0.04232	0.03030
Msat1 X Msat12	0.29722	0.02870	0.11146	0.26444	0.12312	0.13119
Msat1 X Msat13	0.34444	0.06690	0.18576	0.29278	0.02345	0.01938
Msat1 X Msat14	0.34722	0.40903	0.39549	0.01639	0.43619	0.38535
Msat3 X Msat4	0.07361	0.08727	0.05868	0.11806	0.60596	0.59129
Msat3 X Msat5	0.02917	0.10671	0.12153	0.06222	0.06871	0.09306
Msat3 X Msat10	0.45833	0.42407	0.48889	0.38583	0.22737	0.30701
Msat3 X Msat11	0.28472	0.47361	0.46493	0.21361	0.72721	0.70846
Msat3 X Msat12	0.02917	0.01481	0.01701	0.01472	0.17549	0.12412
Msat3 X Msat13	0.00139	0.00023	0.00035	0.00528	0.00033	0.00044
Msat3 X Msat14	0.01528	0.07454	0.01111	0.00111	0.40408	0.38712
Msat4 X Msat5	0.64583	0.47269	0.52917	0.70944	0.27696	0.25189
Msat4 X Msat10	0.82222	0.64375	0.76910	0.89861	0.81609	0.81105
Msat4 X Msat11	0.17917	0.21551	0.19167	0.71639	0.41830	0.56016
Msat4 X Msat12	0.15556	0.33935	0.30903	0.00306	0.65065	0.72008
Msat4 X Msat13	0.64583	0.87245	0.71597	0.86972	0.84306	0.76963
Msat4 X Msat14	0.88194	0.90556	0.86146	0.88556	0.94142	0.93504
Msat5 X Msat10	0.00139	0.00046	0.00035	0.00056	0.00049	0.00025
Msat5 X Msat11	0.27500	0.04676	0.10417	0.42222	0.03284	0.04646
Msat5 X Msat12	0.84167	0.51944	0.50382	0.15222	0.36487	0.43157
Msat5 X Msat13	0.20972	0.25116	0.28924	0.21667	0.37034	0.07727
Msat5 X Msat14	0.05694	0.04861	0.14479	0.22639	0.22574	0.27973
Msat10 X Msat11	0.00139	0.00023	0.00035	0.00028	0.00008	0.00006
Msat10 X Msat12	0.27917	0.23519	0.19514	0.11278	0.08627	0.08996
Msat10 X Msat13	0.00139	0.00023	0.00035	0.00028	0.00008	0.00006
Msat10 X Msat14	0.26528	0.21667	0.20417	0.12583	0.13505	0.32039
Msat11 X Msat12	0.00139	0.00023	0.00035	0.00028	0.00008	0.00006
Msat11 X Msat13	0.00139	0.00023	0.00035	0.00028	0.00008	0.00006
Msat11 X Msat14	0.04306	0.09907	0.05972	0.00944	0.02794	0.07992
Msat12 X Msat13	0.34028	0.09861	0.10764	0.16833	0.15760	0.40082
Msat12 X Msat14	0.00139	0.00023	0.00035	0.00028	0.00008	0.00044
Msat13 X Msat14	0.07778	0.10972	0.12813	0.02194	0.24265	0.38857
Msat1 X Msat3	0.00694	0.11852	0.03472	0.02389	0.05074	0.07304
Msat1 X Msat4	1.00000	0.68611	0.95972	0.87833	0.78807	0.82847
Msat1 X Msat5	0.67500	0.75000	0.71146	0.83861	0.85654	0.87967

Msat1 X Msat10	0.90833	0.89745	0.87465	0.93722	0.75931	0.74848
Msat1 X Msat11	0.00278	0.00463	0.00139	0.02389	0.04232	0.03030
Msat1 X Msat12	0.29722	0.02870	0.11146	0.26444	0.12312	0.13119
Msat1 X Msat13	0.34444	0.06690	0.18576	0.29278	0.02345	0.01938
Msat1 X Msat14	0.34722	0.40903	0.39549	0.01639	0.43619	0.38535
Msat3 X Msat4	0.07361	0.08727	0.05868	0.11806	0.60596	0.59129
Msat3 X Msat5	0.02917	0.10671	0.12153	0.06222	0.06871	0.09306
Msat3 X Msat10	0.45833	0.42407	0.48889	0.38583	0.22737	0.30701
Msat3 X Msat11	0.28472	0.47361	0.46493	0.21361	0.72721	0.70846
Msat3 X Msat12	0.02917	0.01481	0.01701	0.01472	0.17549	0.12412
Msat3 X Msat13	0.00139	0.00023	0.00035	0.00528	0.00033	0.00044
Msat3 X Msat14	0.01528	0.07454	0.01111	0.00111	0.40408	0.38712
Msat4 X Msat5	0.64583	0.47269	0.52917	0.70944	0.27696	0.25189
Msat4 X Msat10	0.82222	0.64375	0.76910	0.89861	0.81609	0.81105
Msat4 X Msat11	0.17917	0.21551	0.19167	0.71639	0.41830	0.56016
Msat4 X Msat12	0.15556	0.33935	0.30903	0.00306	0.65065	0.72008

Discussion générale

Des insectes sensibles aux facteurs environnementaux mais des populations stables au cours du temps.

L'étude de l'écologie des phlébotomes dans une région où la leishmaniose circule est une source d'information pour explorer l'organisation et le fonctionnement des populations d'espèces vectrices. Dans le contexte du changement climatique et en particulier de l'élévation de la température, notre hypothèse de travail était que la population de phlébotomes augmente au cours du temps et que l'aire de répartition de certaines espèces s'étende. Cependant, notre étude n'a pas montré de changements en 30 ans ni en termes d'espèces, ni de distribution ou de proportion relative de ces dernières le long du transect que nous avons étudié dans la région de Montpellier. Ces données suggèrent une stabilité spatio-temporelle des populations de phlébotomes malgré les changements environnementaux importants qui se sont opérés dans notre zone d'étude. Cette stabilité peut s'expliquer par une grande capacité d'adaptation de ces insectes.

Il est intéressant de noter que le transect étudié se caractérise par des fluctuations climatiques et environnementales. Les analyses spatio-temporelles des populations ont mis en évidence que la température, l'humidité relative, l'altitude, le versant et l'orientation des murs influençaient significativement la présence et l'abondance de *P. ariasi* et de *S. minuta* avec pour chacune d'elles des caractéristiques environnementales plus ou moins spécifiques. Ce modèle semble être la règle pour ces insectes. En effet, d'autres espèces sont également connues pour vivre en sympatrie avec des niches écologiques spécifiques comme par exemple au Sénégal : *P. duboscqi* (principalement collectée en milieu rural), *S. dubia* (au niveau intradomiciliaire) and *S. schwetzi* (ubiquiste mais principalement au niveau péridomiciliaire) [8]. Lorsque l'on considère *P. ariasi*, principal vecteur de *L. infantum* dans la région, il apparaît que cette espèce a été collectée tout au long du transect avec des variations d'abondance et de densité en fonction des facteurs climatiques ou environnementaux. Cette espèce est connue pour sa capacité d'adaptation et sa grande ubiquité [17] (Figure 10). Dans ce contexte, nous avons voulu explorer si ces caractéristiques étaient liées à un polymorphisme phénotypique par une approche de morphométrie géométrique sur les ailes.

Une plasticité phénotypique liée aux facteurs environnementaux.

Les analyses de morphométrie géométrique sur les ailes de spécimens de *P. ariasi*, capturés le long du transect, ont montré, dans un premier temps, un dimorphisme sexuel, phénomène fréquent chez beaucoup d'espèces d'insectes [95-98]. Ce dimorphisme peut être classiquement expliqué par le comportement différent des mâles et des femelles (repas sanguin, ponte, attractivité de la lumière, etc.).

Dans un second temps, nous avons observé une différence de forme et de taille des ailes des femelles et uniquement de forme pour celles des mâles entre les mois de juin et juillet. Les adultes capturés au cours de ces deux mois n'ont certainement pas émergé à la même période de l'année. Par conséquent, il est fort probable que leurs stades larvaires ont été soumis à des conditions climatiques différentes, générant un polymorphisme phénotypique chez les adultes. Il est connu que les paramètres climatiques influencent le développement des larves d'insectes et donc le phénotype de l'adulte [99]. De plus, comme un grand nombre d'espèces vivant en région tempérée, *P. ariasi* présente une diapause au 4^{ème} stade larvaire

[18] pour survivre pendant l'hiver et retarder la reproduction jusqu'à l'arrivée de conditions climatiques favorables. Il est donc probable que les conditions climatiques en fin et début de diapause ont également joué un rôle dans la différenciation phénotypique observée chez les adultes en juin et juillet [100].

Des variations de taille et de forme des ailes pour les femelles et de forme pour les mâles en fonction des groupes d'altitudes, ainsi que des différences de forme pour les femelles en juin par versant et par station ont été également observées. Ces résultats suggèrent que les pressions de sélection agissant sur les deux sexes sont différentes. Chez les mâles, la compétition entre ces derniers peut entraîner des variations phénotypiques sur l'aile, comme il a été montré chez les mouches *Drosophila simulans* [101]. En particulier, les modifications de l'aile pourraient avoir des conséquences sur la fréquence des battements. Les mâles phlébotomes produisent des vibrations alaires durant la copulation et non pendant la parade nuptiale comme observé chez les drosophiles [102]. Ces déformations pourraient donc induire des fréquences de battement alaire variées chez les phlébotomes et par conséquent avoir un effet sur l'accouplement [103]. Ces pressions de sélection peuvent également être d'origine environnementale. [72], Kasap et al. [104] ont démontré pour *P. papatasi* et Aytekin et al. [74] pour *Anopheles superpictus*, l'effet remarquable de la température et de l'humidité, dans des conditions de laboratoire, sur la déformation des ailes. Ces déformations alaires peuvent donc également être expliquées par la variabilité des conditions climatiques liée à l'altitude, au versant et aux stations (voir chapitre 3 – Analyses des données climatiques). En effet, l'analyse des données climatiques a montré des différences significatives de température et d'humidité relative en fonction des différentes subdivisions spatiales que nous avons réalisées. Cependant, une analyse fine des données de morphométrie géométrique et des données de température et d'humidité relative, permettra de valider cette hypothèse.

Le dimorphisme sexuel est l'exemple classique d'un polymorphisme génétique entre les mâles et les femelles. Par contre, les différenciations observées en fonction des paramètres environnementaux semblent être la conséquence d'une grande plasticité phénotypique chez ces insectes. En effet, des travaux démontrent que des individus d'une même espèce peuvent présenter des différences phénotypiques indépendamment du génotype [105]. De plus, des études en laboratoire sur des lignées de phlébotomes ont mis en évidence des déformations chez les adultes liées aux conditions climatiques testées lors du développement larvaire [72-74, 104, 106]. Bien qu'il soit désormais démontré que la plasticité phénotypique comporte souvent une base génétique [107], un des objectifs de ce travail était de déterminer s'il existe une indépendance entre le génotype des individus et la variabilité phénotypique pour les populations de *P. ariasi* de notre région d'étude.

Dans le but de compléter l'étude écologique de *P. ariasi* et d'étudier le lien entre diversité phénotypique et génétique, nous avons ensuite exploré la structure génétique de ces populations à l'aide de marqueurs microsatellites afin de comprendre leur organisation et leur dynamique.

Une structure génétique en sous-populations micro-géographiques et temporelles.

Nos résultats révèlent une variabilité génétique similaire à toutes les échelles spatiales permettant de réaliser des analyses de génétique des populations même au sein des petites subdivisions. Les données montrent que les populations de phlébotomes présentent une structure génétique en sous-populations micro-géographiques avec des événements de migration entre elles. Cette structuration est renforcée lorsque l'on considère les mois de collecte séparément en particulier pour le mois de juillet. Concernant la migration, nous supposons qu'elle se réalise majoritairement de proche en proche. En effet, les phlébotomes

étant de mauvais voiliers, leur distance de dispersion maximum s'approche du km [108]. On peut remarquer, cependant, que les mâles sont plus structurés que les femelles avec des nombres estimés de migrants plus faibles. Cette observation peut s'expliquer par le comportement différent et une capacité de dispersion plus importante chez les femelles. Ce résultat est en accord avec la différenciation phénotypique observée entre sexes, notamment pour la taille des ailes (voir ci-dessus). Excepté cette concordance, les résultats des analyses génétiques ne montrent pas d'adéquation avec la variabilité phénotypique détectée. Cela confirme que l'origine du polymorphisme sur les ailes est due à une plasticité phénotypique indépendante du génotype. Il n'en reste pas moins que des analyses supplémentaires sont indispensables pour tester l'existence d'une corrélation entre diversité génétique et phénotypique.

Toutes nos données démontrent clairement que *P. ariasi* est organisé en populations micro-géographiques et temporelles. L'existence de migrations entre ces sous-populations maintient une homogénéité génétique sur l'ensemble du transect. Cette structuration présente des avantages évolutifs certains en permettant le maintien des populations dans l'espace (colonisation et recolonisation rapide des environnements sur une petite échelle) et dans le temps (adaptation aux modifications environnementales).

Concernant l'épidémiologie des leishmanioses, cette structuration en sous-populations micro-géographiques et temporelles a inévitablement une incidence sur les cycles de transmission des *Leishmania*. En effet, par exemple cette structure peut expliquer l'émergence ou la réémergence de foyers de leishmaniose consécutivement à la migration de phlébotomes infectés [6] mais aussi les prévalences élevées d'individus infectés dans les micro-foyers [13]. Cependant, en raison de cette structuration micro-géographiques et des faibles capacités de dispersion des phlébotomes, il est probable que l'extension de la leishmaniose en France soit due aux déplacements des hôtes infectés et non des vecteurs. En effet, la colonisation de nouveaux foyers par les phlébotomes doit s'opérer principalement sur de courtes distances. Par conséquent, l'extension des aires de distribution des phlébotomes à l'échelle d'un pays doit être un processus très lent, contrairement à d'autres insectes tels qu'*Aedes albopictus*. De plus, contrairement aux moustiques qui présentent une dispersion passive importante liée aux activités humaines (transports maritimes, aériens et routiers), les phlébotomes sont peu sujets à ce genre de dispersion en raison de leur cycle de développement peu associé à ce genre d'activités.

Conclusion et perspectives

Pour progresser dans la connaissance de l'écologie des phlébotomes, vecteurs de *Leishmania*, ce travail avait pour objectif d'étudier la structure des populations de phlébotomes d'un point de vue génétique et phénotypique à différentes échelles géographiques et de déterminer l'impact des facteurs environnementaux et climatiques sur l'organisation spatio-temporelle de ces insectes. En effet, l'organisation des populations de phlébotomes dans les écosystèmes et les paramètres qui les structurent sont, à l'heure actuelle, très peu étudiés.

Ce travail de thèse a permis de valider certaines hypothèses et d'apporter des informations inédites sur l'écologie et l'évolution des populations de phlébotomes en France et en particulier de l'espèce *P. ariasi*, un des vecteurs principaux du pathogène responsable de la leishmaniose dans la région étudiée. Nous avons, tout d'abord, observé une grande **stabilité des populations** au cours du temps qui suggère une importante **capacité d'adaptation**. Nous avons également mis en évidence **les effets des facteurs environnementaux et climatiques** sur l'organisation des populations et le phénotype de *P. ariasi* le long d'un transect caractérisé par des fluctuations écosystémiques. Ces résultats démontrent leur importante **plasticité phénotypique** mais soulignent encore une fois leur **capacité d'adaptation à un écosystème changeant**. Le dernier aspect de ce travail concerne la structure génétique des populations de *P. ariasi*. Les analyses ont révélé pour la première fois chez les phlébotomes **une structure en sous-populations micro-géographiques**. Cependant, la **migration** semble également jouer un rôle important dans **le maintien des populations** sur le plan démographique et génétique à l'échelle du transect. Il est à noter que la dispersion apparaît plus marquée pour les femelles, ce qui suggère un rôle majeur de ces dernières dans les phénomènes de colonisation et de recolonisation.

Il est probable que toutes ces caractéristiques biologiques, écologiques et populationnelles ont **un impact sur les cycles de transmission des leishmanioses**. La stabilité, la plasticité, la capacité d'adaptation, la structure micro-géographique des populations, l'homogénéité génétique, les phénomènes de migration, toutes ces propriétés réunies semblent faites pour optimiser la transmission, favoriser l'endémicité mais également, dans une moindre mesure, la réémergence d'anciens foyers ou bien l'émergence de nouveaux foyers de leishmanioses.

En perspectives, pour valider nos hypothèses sur la structure des populations, nous réaliserons des analyses sur des tailles d'échantillons plus importantes par micro-habitats et par mois. L'analyse phénotypique et génétique sur plusieurs années permettra également de confirmer la stabilité, l'homogénéité génétique et la plasticité phénotypique.

Enfin, dans le but de valider notre modèle pour les espèces de phlébotomes en général, nous effectuerons des études similaires sur d'autres espèces vectrices ou non vectrices collectées en France mais également dans d'autres pays. Pour cela, nous pourrions utiliser les échantillons capturés au cours de ma thèse au Sénégal et en Turquie.

Au Sénégal, les échantillons proviennent de la commune de Mont Rolland, constituée de 18 villages et connue pour la circulation de la leishmaniose canine à *L. infantum*. Cette commune est caractérisée par différents écosystèmes et présente une distribution hétérogène des espèces de phlébotomes en fonction des villages et de l'environnement. La transmission

du parasite varie également en fonction des écosystèmes et les espèces majeures suspectées vectrices sont *Sergentomyia dubia* et *S. schwetzi* [13].

En Turquie, nous avons collecté des échantillons dans deux régions où circule *P. tobbi*, vecteur de l'espèce *L. infantum* [109]. Dans la première région (Adana), le parasite est endémique et circule chez l'homme et le chien alors que dans la deuxième région (Izmir), le parasite est absent. Cette étude nous permettra d'étudier dans quelle mesure le parasite a un impact sur la structure des populations de ces insectes.

Toutes ces études complémentaires permettront de définir de manière solide les bases de l'écologie et de la structure des populations des phlébotomes et de déterminer si le parasite *Leishmania* joue un rôle dans l'organisation et l'évolution des populations naturelles de ces insectes.

Références bibliographiques

1. Rioux JA, Killick-Kendrick R, Perieres J, Turner D, Lanotte G: Ecologie des Leishmanioses dans le Sud de la France. 13. Les sites de "flanc de coteau", biotopes de transmission privilégiés de la Leishmaniose viscérale en Cévennes. *Annales de parasitologie* 1980, 55(4):445-453.
2. Desjeux P: Worldwide increasing risk factors for leishmaniasis. *Medical microbiology and immunology* 2001, 190(1-2):77-79.
3. Franco F, Morillas-Marquez F, Barón S, Morales-Yuste M, Gálvez R, Díaz-Sáez V, Pesson B, Alves-Pires C, Depaquit J, Molina R *et al*: Genetic structure of *Phlebotomus (Larroussius) ariasi* populations, the vector of *Leishmania infantum* in the western Mediterranean: Epidemiological implications. *International Journal for Parasitology* 2010, 40(11):1335-1346.
4. Ready P: Leishmaniasis emergence in Europe. *Euro surveillance : bulletin Européen sur les maladies transmissibles = European communicable disease bulletin* 2010, 15(10):19505.
5. Rougeron V, Banuls A-L, Carme B, Simon S, Couppie P, Nacher M, Hide M, De Meeûs T: Reproductive strategies and population structure in *Leishmania*: substantial amount of sex in *Leishmania Viannia guyanensis*. *Molecular ecology* 2011, 20(15):3116-3127.
6. Rougeron V, De Meeûs T, Hide M, Le Falher G, Bucheton B, Dereure J, El-Safi SH, Dessein A, Bañuls A-L: Multifaceted population structure and reproductive strategy in *Leishmania donovani* complex in one Sudanese village. *PLoS Negl Trop Dis* 2011, 5(12):e1448.
7. Ready P: Biology of Phlebotomine Sand Flies as Vectors of Disease Agents. *Annual Review of Entomology* 2013, 58(1):227-250.
8. Senghor M, Faye M, Faye B, Diarra K, Elguero E, Gaye O, Bañuls A, Niang A: Ecology of phlebotomine sand flies in the rural community of Mont Rolland (Thiès region, Senegal): area of transmission of canine leishmaniasis. *PLoS ONE* 2011, 6(3):e14773.
9. Berdjane-Brouk Z, Koné A, Djimdé A, Charrel R, Ravel C, Delaunay P, Giudice P, Diarra A, Doumbo S, Goita S *et al*: First Detection of *Leishmania major* DNA in *Sergentomyia (Spelaemyia) darlingi* from Cutaneous Leishmaniasis Foci in Mali. *PLoS ONE* 2012, 7(1):e28266.
10. Campino L, Cortes S, Dionísio L, Neto L, Afonso MO, Maia C: The first detection of *Leishmania major* in naturally infected *Sergentomyia minuta* in Portugal. *Memórias do Instituto Oswaldo Cruz* 2013, 108(4):516-518.
11. Kanjanopas K, Siripattanapipong S, Ninsaeng U, Hitakarun A, Jitkaew S, Kaewtaphaya P, Tan-ariya P, Mungthin M, Charoenwong C, Leelayoova S: *Sergentomyia (Neophlebotomus) gemmea*, a potential vector of *Leishmania siamensis* in southern Thailand. *BMC Infectious Diseases* 2013, 13(1):333.
12. Nzelu C, Kato H, Pupilampu N, Desewu K, Odoom S, Wilson M, Sakurai T, Katakura K, Boakye D: First detection of *Leishmania tropica* DNA and *Trypanosoma* species in *Sergentomyia* sand flies (Diptera: Psychodidae) from an outbreak area of cutaneous leishmaniasis in Ghana. *PLoS Neglected Tropical Diseases* 2014, 8(2):e2630.
13. Senghor MW, Niang AA, Depaquit J, Faye MN, Elguero E, Gaye O, Alten B, Perktas U, Cassan C, Faye F *et al*: Transmission of *Leishmania infantum* by

- Phlebotomine sandflies of the genus *Sergentomyia* (Diptera: Psychodidae): ecological, parasitological and molecular evidences in the canine leishmaniasis focus of Mont-Rolland, Senegal. *PLoS Negl Trop Dis* Accepted with modifications
14. Mathis A, Depaquit J, Dvořák V, Tuten H, Bañuls A-L, Halada P, Zapata S, Lehrter V, Hlavačková K, Prudhomme J *et al*: Identification of phlebotomine sand flies using one MALDI-TOF MS reference database and two mass spectrometer systems. *Parasites & Vectors* 2015, 8(1).
 15. El-Hossary S: Morphological characteristics for sand fly taxonomy: Research and training center on vectors of diseases Ain Shams University; 2006.
 16. Lewis D: Phlebotomid sandflies. *Bulletin of the World Health Organization* 1971, 44(4):535-551.
 17. Abonnenc E: Les phlébotomes de la région éthiopienne (Diptera, Psychodidae). 1972:1-290.
 18. Volf P, Volfova V: Establishment and maintenance of sand fly colonies. *Journal of vector ecology : journal of the Society for Vector Ecology* 2011, 36 Suppl 1:S1-9.
 19. Alexander B: Sampling methods for phlebotomine sandflies. *Medical and Veterinary Entomology* 2000, 14(2):109-122.
 20. Killick-Kendrick R: The biology and control of phlebotomine sand flies. *Clinics in dermatology* 1999, 17(3):279-289.
 21. Lane R: Sandflies (phlebotominae). In: *Medical insects and arachnids*: Springer; 1993: 78-119.
 22. Alten B, Ozbel Y, Ergunay K, Kasap O, Cull B, Antoniou M, Velo E, Prudhomme J, Molina R, Bañuls A *et al*: Sampling strategies for phlebotomine sand flies (Diptera: Psychodidae) in Europe. *Bulletin of entomological research* 2015:1-15.
 23. Fischer D, Moeller P, Thomas S, Naucke T, Beierkuhnlein C: Combining Climatic Projections and Dispersal Ability: A Method for Estimating the Responses of Sandfly Vector Species to Climate Change. *PLoS Neglected Tropical Diseases* 2011, 5(11):e1407.
 24. Naucke T, Menn B, Massberg D, Lorentz S: Sandflies and leishmaniasis in Germany. *Parasitology Research* 2008, 103(S1):65-68.
 25. Alexander B: A review of bartonellosis in Ecuador and Colombia. *The American journal of tropical medicine and hygiene* 1995, 52(4):354-359.
 26. Chamberlin J, Laughlin L, Romero S, Solórzano N, Gordon S, Andre R, Pachas P, Friedman H, Ponce C, Watts D: Epidemiology of endemic *Bartonella bacilliformis*: a prospective cohort study in a Peruvian mountain valley community. *The Journal of infectious diseases* 2002, 186(7):983-990.
 27. Alkan C, Bichaud L, Lamballerie X, Alten B, Gould E, Charrel R: Sandfly-borne phleboviruses of Eurasia and Africa: epidemiology, genetic diversity, geographic range, control measures. *Antiviral Research* 2013, 100(1):54-74.
 28. Charrel R, Bichaud L, De Lamballerie X: Emergence of Toscana virus in the mediterranean area. *World Journal of Virology* 2012, 1(5):135.
 29. Bichaud L, Souris M, Mary C, Ninove L, Thirion L, Piarroux R, Piarroux R, De Lamballerie X, Charrel R: Epidemiologic Relationship between Toscana Virus Infection and *Leishmania infantum* Due to Common Exposure to *Phlebotomus perniciosus* Sandfly Vector. *PLoS Neglected Tropical Diseases* 2011, 5(9):e1328.
 30. Fontenille D, Lagneau C, Lecollinet S, Lefait-Robin R, Setbon M, Tirel B, Yébakima A: La lutte antivectorielle en France: IRD Editions; 2009.
 31. Kamhawi S: Phlebotomine sand flies and *Leishmania* parasites: friends or foes? *Trends in parasitology* 2006, 22(9):439-445.

32. Harhay M, Olliaro P, Costa DL, Costa CHN: Urban parasitology: visceral leishmaniasis in Brazil. *Trends in Parasitology* 2011, 27(9):403-409.
33. Bañuls AL, Bastien P, Pomares C, Arevalo J, Fisa R, Hide M: Clinical pleiomorphism in human leishmaniasis, with special mention of asymptomatic infection. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* 2011, 17(10):1451-1461.
34. Lachaud L, Dedet J, Marty P, Faraut F, Buffet P, Gangneux J, Ravel C, Bastien P: Surveillance of leishmaniasis in France, 1999 to 2012. *Euro surveillance : bulletin Européen sur les maladies transmissibles = European communicable disease bulletin* 2013, 18(29):20534.
35. Rioux JA, Golvan Y, Croset H, Tour S, Hovin R, Abonnenc E, Petitdidier M, Vollhardt Y, Dedet JP, Albaret JL *et al*: Epidémiologie des leishmanioses dans le Sud de la France, vol. 37: Monographie de l'Institut National de la Santé et de la Recherche Médicale; 1969.
36. Dedet J-P: Les leishmanioses en France métropolitaine. *BEH Hors-série* 2010:9-12.
37. Alten B, Maia C, Afonso O, Campino L, Jiménez M, González E, Molina R, Bañuls AL, Prudhomme J, Vergnes B *et al*: Seasonal dynamics of phlebotomine sand fly species proven vectors of Mediterranean leishmaniasis caused by *Leishmania infantum*. *PLOS Neglected Tropical Diseases* *Accepté avec révision*.
38. Langeron M, Nitzulescu V: *Phlebotomus larrouseï* n. sp. nouvelle espèce européenne de phlébotome. *Ann Parasitol Hum Comp* 1931, 10:72-76.
39. Langeron M, Nitzulescu V: Revision des Phlebotomes de France. *Annis Parasit hum comp* 1932, 10.
40. Rioux JA, Houin R, Baudouy J, Croset H, Tour S: Presence en Corse de *Phlebotomus (Paraphlebotomus) sergenti* Parrot, 1917. *Annales de parasitologie* 1970, 45(3):343-356.
41. Rioux JA, Jarry D, Maazoun R, Wallbanks K: Confirmation de l'existence en France continentale de *Phlebotomus sergenti* Parrot, 1917. *Annales de parasitologie* 1982, 57(6):647-648.
42. Rioux JA, Carron S, Dereure J, Perieres J, Zeraia L, Franquet E, Babinot M, Gallego M, Prudhomme J: Ecology of leishmaniasis in the South of France. 22. Reliability and representativeness of 12 *Phlebotomus ariasi*, *P. perniciosus* and *Sergentomyia minuta* (Diptera: Psychodidae) sampling stations in Vallespir (eastern French Pyrenees region). *Parasite (Paris, France)* 2013, 20:34.
43. Rioux JA, Golvan Y, Croset H, Houin R: Les Leishmanioses dans le "midi" méditerranéen. Résultats d'une enquête écologique. *Bulletin de la société de pathologie exotique* 1969, 62(2):332-333.
44. Chamailé L, Tran A, Meunier A, Bourdoiseau G, Ready P, Dedet J-P: Environmental risk mapping of canine leishmaniasis in France. *Parasites & Vectors* 2010, 3(1):31.
45. Rioux JA, Croset H, Aboukler JP, Papierok B: Ecologie des Leishmanioses dans le sud de la France. 4. - Infestation d'une population naturelle de *Phlebotomus ariasi* Tonnoir, 1921. *Annales de parasitologie* 1972, 47(2):325-330.
46. Naucke T, Menn B, Massberg D, Lorentz S: Winter activity of *Phlebotomus (Transphlebotomus) mascittii*, Grassi 1908 (Diptera: Psychodidae) on the island of Corsica. *Parasitology Research* 2008, 103(2):477-479.
47. Naucke T, Lorentz S, Rauchenwald F, Aspöck H: *Phlebotomus (Transphlebotomus) mascittii* Grassi, 1908, in Carinthia: first record of the

- occurrence of sandflies in Austria (Diptera: Psychodidae: Phlebotominae). *Parasitology Research* 2011, 109(4):1161-1164.
48. Bede-Fazekas Á, Trájer A: Potential urban distribution of *Phlebotomus mascittii* Grassi and *Phlebotomus neglectus* Tonn.(Diptera: Psychodidae) in 2021–50 in Budapest, Hungary. *Journal of vector borne diseases* 2015, 52(3):213-218.
 49. Berdjane-Brouk Z, Charrel R, Bitam I, Hamrioui B, Izri A: Record of *Phlebotomus (Transphlebotomus) mascittii* Grassi, 1908 and *Phlebotomus (Larroussius) chadlii* Rioux, Juminer & Gibily, 1966 female in Algeria. *Parasite* 2011, 18(4):337.
 50. Grimm F, Gessler M, Jenni L: Aspects of sandfly biology in southern Switzerland. *Medical and veterinary entomology* 1993, 7(2):170-176.
 51. Selkoe K, Toonen R: Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. *Ecology Letters* 2006, 9(5):615-629.
 52. Depaquit J: Molecular systematics applied to Phlebotomine sandflies: Review and perspectives. *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases* 2014, 28:744-756.
 53. Kamhawi S, Molyneux D, Killick-Kendrick R, Milligan P, Phillips A, Wilkes T, Killick-Kendrick M: Two populations of *Phlebotomus ariasi* in the Cévennes focus of leishmaniasis in the south of France revealed by analysis of cuticular hydrocarbons. *Medical and Veterinary Entomology* 1987, 1(1):97-102.
 54. Pesson B, Wallon M, Floer M, Kristensen A: [Isoenzymatic studies of Mediterranean populations of sandflies of the subgenus *Larroussius*]. *Parassitologia* 1991, 33:471-476.
 55. Martin-Sanchez J, Gramiccia M, Pesson B, Morillas-Marquez F: Genetic polymorphism in sympatric species of the genus *Phlebotomus*, with special reference to *Phlebotomus perniciosus* and *Phlebotomus longicuspis* (Diptera, Phlebotomidae). *Parasite (Paris, France)* 2000, 7(4):247-254.
 56. Mahamdallie S, Pesson B, Ready P: Multiple genetic divergences and population expansions of a Mediterranean sandfly, *Phlebotomus ariasi*, in Europe during the Pleistocene glacial cycles. *Heredity* 2010, 106(5):714-726.
 57. Zelditch ML, Swiderski DL, Sheets HD: Geometric morphometrics for biologists: a primer: Academic Press; 2012.
 58. Belen A, Alten B, Aytakin A: Altitudinal variation in morphometric and molecular characteristics of *Phlebotomus papatasi* populations. *Medical and Veterinary Entomology* 2004, 18(4):343-350.
 59. Aransay A, Malarky G, Ready P: Isolation (with enrichment) and characterization of trinucleotide microsatellites from *Phlebotomus perniciosus*, a vector of *Leishmania infantum*. *Molecular Ecology Notes* 2001, 1:176-178.
 60. Mahamdallie S: Effects of Environmental Change on the Genetic Diversity and Distribution of *Phlebotomus ariasi*, a Vector of Visceral Leishmaniasis in Southwest Europe. London School of Hygiene & Tropical Medicine; 2010.
 61. Bagnères AG: Les hydrocarbures cuticulaires des insectes sociaux: détermination et rôle dans la reconnaissance spécifique, coloniale et individuelle. Paris 6; 1989.
 62. Morakchi S, Aribi N, Farine J-P, Soltani N: Analyse des hydrocarbures cuticulaires chez *Blattella germanica*: Effets de divers groupes de pesticides. *Travaux de l'Institut Scientifique, Série Zoologie, Rabat* 2010(47):141-145.
 63. Killick-Kendrick R, Rioux JA, Bailly M, Guy M, Wilkes T, Guy F, Davidson I, Knechtli R, Ward R, Guilvard E *et al*: Ecology of leishmaniasis in the south of France. 20. Dispersal of *Phlebotomus ariasi* Tonnoir, 1921 as a factor in the

- spread of visceral leishmaniasis in the Cévennes. *Annales de parasitologie humaine et comparée* 1984, 59(6):555-572.
64. Daget P: Le bioclimat Méditerranéen: Analyse des formes climatiques par le système d'Emberger. *Vegetation* 1977, 34(2):87-103.
 65. Lewis D: A taxonomic review of the genus *Phlebotomus* (Diptera: Psychodidae). *Bulletin of the British Museum (Natural History)* 1982, 45(2):121-209.
 66. Killick-Kendrick R, Tang Y, Killick-Kendrick M, Sang D, Sirdar M, Ke L, Ashford R, Schorscher J, Johnson R: The identification of female sandflies of the subgenus *Larrousius* by the morphology of the spermathecal ducts. *Parassitologia* 1991, 33:335-347.
 67. Team RC: R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, 2012. ISBN 3-900051-07-0; 2014.
 68. Zuur AF, Ieno EN, Elphick CS: A protocol for data exploration to avoid common statistical problems. *Methods in Ecology and Evolution* 2010, 1:3-14.
 69. Hoef J, Boveng P: Quasi-Poisson vs. negative binomial regression: how should we model overdispersed count data? *Ecology* 2007, 88(11):2766-2772.
 70. Zuur AF, Saveliev AA, Ieno EN: A beginner's guide to generalised additive mixed models with R: Highland Statistics Limited; 2014.
 71. Rohlf FJ, Slice D: Extensions of the Procrustes method for the optimal superimposition of landmarks. *Systematic Biology* 1990, 39(1):40-59.
 72. Kasap O, Alten B: Comparative demography of the sand fly *Phlebotomus papatasi* (Diptera: Psychodidae) at constant temperatures. *Journal of vector ecology : journal of the Society for Vector Ecology* 2006, 31(2):378-385.
 73. Aytakin A, Alten B, Caglar SS, Ozbel Y, Kaynas S, Simsek F, Kasap O, Belen A: Phenotypic variation among local populations of phlebotomine sand flies (Diptera: Psychodidae) in southern Turkey. *Journal of vector ecology : journal of the Society for Vector Ecology* 2007, 32(2):226-234.
 74. Aytakin S, Aytakin A, Alten B: Effect of different larval rearing temperatures on the productivity (R₀) and morphology of the malaria vector *Anopheles superpictus* Grassi (Diptera: Culicidae) using geometric morphometrics. *Journal of vector ecology : journal of the Society for Vector Ecology* 2009, 34(1):32-42.
 75. Rohlf FJ: tpsUtil, file utility program. version 1.60. . Department of Ecology and Evolution, State University of New York at Stony Brook 2015.
 76. Rohlf FJ: tpsDIG2 version 2.18. . Stony Brook, NY: Department of Ecology and Evolution, State University of New York 2015.
 77. Arnqvist G, Martensson T: Measurement error in geometric morphometrics: empirical strategies to assess and reduce its impact on measures of shape. *Acta Zoologica Academiae Scientiarum Hungaricae* 1998, 44(1-2):73-96.
 78. MoMe-CLIC. Morphometrics in Medical Entomology – Collection of Landmark for Identification and Characterization. [<http://mome-clic.com/>]
 79. Bookstein FL: Morphometric tools for landmark data: Cambridge univ. press Cambridge etc; 1991.
 80. Rohlf F: Shape statistics: Procrustes superimpositions and tangent spaces. *Journal of Classification* 1999, 16:197-223.
 81. Rohlf F: Relative warp analysis and an example of its application to mosquito. *Contributions to morphometrics* 1993, 8:131.
 82. Alibert P, Moureau B, Dommergues J-L, David B: Differentiation at a microgeographical scale within two species of ground beetle, *Carabus auronitens*

- and *C. nemoralis* (Coleoptera, Carabidae): a geometrical approach. *Zoologica Scripta* 2001, 30(4):299-311.
83. Wang Q, Wang X: Comparison of methods for DNA extraction from a single chironomid for PCR analysis. *Pakistan Journal of Zoology* 2012, 44(2):421-426.
 84. Zapata S, Mejía L, Le Pont F, León R, Pesson B, Ravel C, Bichaud L, Charrel R, Cruaud C, Trueba G: A study of a population of *Nyssomyia trapidoi* (Diptera: Psychodidae) caught on the Pacific coast of Ecuador. *Parasites & vectors* 2012, 5(1):1-8.
 85. Edwards KJ: Miniprep procedures for the isolation of plant DNA. In: *Molecular tools for screening biodiversity*: Springer; 1998: 22-24.
 86. Malausa T, Gilles A, Megléczy E, Blanquart H, Duthoy S, Costedoat C, Dubut V, Pech N, Castagnone-Sereno P, Délye C *et al*: High-throughput microsatellite isolation through 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries. *Molecular Ecology Resources* 2011, 11(4):638-644.
 87. Goudet J: FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). 2001.
 88. Belkhir K, Borsa P, Chikhi L, Raufaste N, Catch F: GENETIX 4.04, software under Windows TM for the genetics of the populations. Laboratory Genome, Populations, Interactions, CNRS UMR 5000, University of Montpellier II, Montpellier, France. 2009.
 89. Rousset F: genepop'007: a complete re-implementation of the genepop software for Windows and Linux. *Molecular Ecology Resources* 2008, 8(1):103-106.
 90. Ceccarelli M, Galluzzi L, Migliazzi A, Magnani M: Detection and characterization of *Leishmania* (*Leishmania*) and *Leishmania* (*Viannia*) by SYBR green-based real-time PCR and high resolution melt analysis targeting kinetoplast minicircle DNA. *PLoS ONE* 2014, 9(2).
 91. Almeida P, Sciamarelli A, Batista P, Ferreira A, Nascimento J, Raizer J, Filho J, Gurgel-Goncalves R: Predicting the geographic distribution of *Lutzomyia longipalpis* (Diptera: Psychodidae) and visceral leishmaniasis in the state of Mato Grosso do Sul, Brazil. *Memórias do Instituto Oswaldo Cruz* 2013, 108(8):992-996.
 92. Pigott D, Bhatt S, Golding N, Duda K, Battle K, Brady O, Messina J, Balard Y, Bastien P, Pratlong F *et al*: Global distribution maps of the leishmaniasis. *eLife* 2014, 3.
 93. Tantely M, Rakotoniaina J-C, Tata E, Andrianaivolambo L, Fontenille D, Elissa N: Modification of *Anopheles gambiae* distribution at high altitudes in Madagascar. *Journal of vector ecology : journal of the Society for Vector Ecology* 2012, 37(2):402-406.
 94. Prudhomme J, Toty C, Kasap O, Rahola N, Vergnes B, Maia C, Campino L, Antoniou M, Jimenez M, Molina R *et al*: New microsatellite markers for multi-scale genetic studies on *Phlebotomus ariasi* Tonnoir, vector of *Leishmania infantum* in the Mediterranean area. *Acta Tropica* 2015, 142:79-85.
 95. Wormington JD, Juliano SA: Sexually dimorphic body size and development time plasticity in *Aedes mosquitoes* (Diptera: Culicidae). *Evolutionary Ecology Research* 2014, 16:223-234.
 96. Bitner-Mathá B, Klaczko L: Plasticity of *Drosophila melanogaster* wing morphology: effects of sex, temperature and density. *Genetica* 1999, 105(2):203-210.
 97. Virginio F, Vidal P, Suesdek L: Wing sexual dimorphism of pathogen-vector culicids. *Parasites & Vectors* 2015, 8(1).

98. Gilchrist GW, Huey RB: Plastic and genetic variation in wing loading as a function of temperature within and among parallel clines in *Drosophila subobscura*. *Integrative and Comparative Biology* 2004, 44:461-470.
99. Lawyer P, Young D: Diapause and quiescence in *Lutzomyia diabolica* (Diptera: Psychodidae). *Parassitologia* 1991, 33:353-360.
100. Ready P, Croset H: Diapause and laboratory breeding of *Phlebotomus perniciosus* Newstead and *Phlebotomus ariasi* Tonnoir (Diptera: Psychodidae) from southern France. *Bulletin of entomological research* 1980, 70:511-523.
101. Debat V, Savriama Y, Gidaszewski N, David JR: Variation individuelle, asymétrie et modularité le long d'un gradient de température. In: *7eme Symposium Morphométrie et Evolution des Formes*. Lyon (France)2012.
102. Kyriacou C: Sex and rhythms in sandflies and mosquitoes: an appreciation of the work of Alexandre Afranio Peixoto (1963-2013). *Infection, genetics and evolution* 2014, 28:662-665.
103. Araki AS, Ferreira G, Mazzoni CJ, Souza NA, Machado RC, Bruno RV, Peixoto AA: Multilocus analysis of divergence and introgression in sympatric and allopatric sibling species of the *Lutzomyia longipalpis* complex in Brazil. *PLoS Negl Trop Dis* 2013, 7(10):e2495.
104. Kasap O, Alten B: Laboratory estimation of degree-day developmental requirements of *Phlebotomus papatasi* (Diptera: Psychodidae). *Journal of vector ecology : journal of the Society for Vector Ecology* 2005, 30(2):328-333.
105. Boussaa S, Perrotey S, Boumezzough A, Harrak R, Hilali S, Pesson B: Isoenzymatic characterization of *Phlebotomus papatasi* (Diptera: Psychodidae) of the Marrakech area, Morocco. *Journal of Medical Entomology* 2008, 45(3):370-374.
106. Bates M: The development and longevity of Haemagogus mosquitoes under laboratory conditions. *Annals of the Entomological Society of America* 1947, 40(1):1-12.
107. Agrawal AA: Phenotypic plasticity in the interactions and evolution of species. *Science* 2001, 294(5541):321-326.
108. Rioux JA, Killick-Kendrick R, Leaney A, Turner D, Bailly M, Young C: Ecologie des Leishmanioses dans le sud de la France. 12. Dispersion horizontale de *Phlebotomus ariasi* Tonnoir, 1921. Experiences préliminaires. *Annales de parasitologie* 1979, 54(6):673-682.
109. Svobodova M, Alten B, Zídková L, Dvorak V, Hlavačková J, Myšková J, Seblova V, Kasap O, Belen A, Votypka J *et al*: Cutaneous leishmaniasis caused by *Leishmania infantum* transmitted by *Phlebotomus tobbi*. *International Journal for Parasitology* 2009, 39(2):251-256.

Annexe 1

Annexe 1 : Significativité des tests post-hoc (tests de Mann-Whitney avec correction de Bonferroni) réalisés sur les comparaisons de température / d'humidité relative entre stations par mois (*: significatif, NS: Non Significatif).

		Température / Humidité relative																			
Mois	Station	ST01	ST02	ST03	ST04	ST05	ST06	ST07	ST08	ST09	ST10	ST11	ST12	ST13	ST14	ST15	ST16	ST17	ST18	ST19	
Mai	ST2	NA / NA																			
	ST3	NA / NA	NS / NS																		
	ST4	NA / NA	NS / NS	NS / NS																	
	ST5	NA / NA	NS / *	* / NS	* / NS																
	ST6	NA / NA	NS / NS	NS / NS	NS / NS	NS / NS	NS / NS														
	ST7	NA / NA	NS / NS	* / NS	* / NS	* / NS	NS / NS	NS / NS													
	ST8	NA / NA	NS / NS	NS / NS	NS / NS	NS / NS	* / NS	NS / NS	* / NS												
	ST9	NA / NA	* / NS	NS / NS	NS / NS	NS / NS	* / NS	* / NS	* / NS	NS / NS											
	ST10	NA / NA	NS / NS	NS / NS	NS / NS	NS / NS	NS / NS	NS / NS	NS / NS	NS / NS	NS / NS										
	ST11	NA / NA	* / NS	NS / NS	NS / NS	NS / NS	* / NS	* / NS	* / NS	NS / NS	NS / NS	NS / NS									
	ST12	NA / NA	NS / *	* / NS	* / NS	* / NS	NS / NS	NS / NS	NS / NS	* / NS	* / NS	NS / NS	* / NS								

Annexe 2 - Article 8

Article 8 : « Mathis A, Depaquit J, Dvořák V, Tuten H, Bañuls A-L, Halada P, Zapata S, Lehrter V, Hlavačková K, **Prudhomme J.**, Volf P., Sereno D., Kaufmann C. Identification of phlebotomine sand flies using one MALDI-TOF MS reference database and two mass spectrometer systems. *Parasites & Vectors* 2015, 8(1) » [14]

RESEARCH

Open Access

Identification of phlebotomine sand flies using one MALDI-TOF MS reference database and two mass spectrometer systems

Alexander Mathis^{1*}, Jérôme Depaquit², Vit Dvořák³, Holly Tuten^{1,9}, Anne-Laure Bañuls⁴, Petr Halada⁵, Sonia Zapata⁶, Véronique Lehrter², Kristýna Hlavačková³, Jorian Prudhomme⁴, Petr Volf³, Denis Sereno⁴, Christian Kaufmann¹, Valentin Pflüger⁷ and Francis Schaffner^{1,8}

Abstract

Background: Rapid, accurate and high-throughput identification of vector arthropods is of paramount importance in surveillance programmes that are becoming more common due to the changing geographic occurrence and extent of many arthropod-borne diseases. Protein profiling by MALDI-TOF mass spectrometry fulfils these requirements for identification, and reference databases have recently been established for several vector taxa, mostly with specimens from laboratory colonies.

Methods: We established and validated a reference database containing 20 phlebotomine sand fly (Diptera: Psychodidae, Phlebotominae) species by using specimens from colonies or field-collections that had been stored for various periods of time.

Results: Identical biomarker mass patterns ('superspectra') were obtained with colony- or field-derived specimens of the same species. In the validation study, high quality spectra (i.e. more than 30 evaluable masses) were obtained with all fresh insects from colonies, and with 55/59 insects deep-frozen (liquid nitrogen/-80 °C) for up to 25 years. In contrast, only 36/52 specimens stored in ethanol could be identified. This resulted in an overall sensitivity of 87 % (140/161); specificity was 100 %. Duration of storage impaired data counts in the high mass range, and thus cluster analyses of closely related specimens might reflect their storage conditions rather than phenotypic distinctness. A major drawback of MALDI-TOF MS is the restricted availability of in-house databases and the fact that mass spectrometers from 2 companies (Bruker, Shimadzu) are widely being used. We have analysed fingerprints of phlebotomine sand flies obtained by automatic routine procedure on a Bruker instrument by using our database and the software established on a Shimadzu system. The sensitivity with 312 specimens from 8 sand fly species from laboratory colonies when evaluating only high quality spectra was 98.3 %; the specificity was 100 %. The corresponding diagnostic values with 55 field-collected specimens from 4 species were 94.7 % and 97.4 %, respectively.

Conclusions: A centralized high-quality database (created by expert taxonomists and experienced users of mass spectrometers) that is easily amenable to customer-oriented identification services is a highly desirable resource. As shown in the present work, spectra obtained from different specimens with different instruments can be analysed using a centralized database, which should be available in the near future via an online platform in a cost-efficient manner.

Keywords: Arthropod identification, Bruker, Centralized reference database, Cross reference, Phlebotominae, Protein Profiling, Shimadzu, Spectra processing, Validation

* Correspondence: alexander.mathis@uzh.ch

¹Swiss National Centre for Vector Entomology, Institute of Parasitology, University of Zürich, Winterthurerstrasse 266a, 8057 Zürich, Switzerland

Full list of author information is available at the end of the article

Background

The geographic occurrence and extent of arthropod-borne diseases is changing, due to globalisation and environmental alterations. Most spectacular is the emergence of invasive species, e.g. *Aedes* mosquitoes [1, 2], with associated new disease threats [3]. Other arthropod vectors such as ticks (Ixodida: Ixodidae) [4, 5], biting midges (Diptera: Ceratopogonidae) [6] and phlebotomine sand flies (Diptera: Psychodidae) [7–9] are gradually expanding their distribution ranges in Europe. Because these changes in vector distribution are associated with changes in the risk of exposure to the pathogens they transmit, monitoring the presence and abundances of arthropod vectors is of growing importance in many regions. Guidelines for surveillance and options for control have so far only been developed for mosquitoes, both native and invasive species [2, 10, 11]. Regularly updated distribution maps for Europe are provided by ECDC (www.ecdc.europa.eu) for a number of vectors (eight sand fly species, five *Aedes* mosquito species, four tick species).

Undoubtedly, reliable, rapid and cost-effective identification of vector arthropods is a key element of surveillance programmes. Morphological identification requires at least some degree of proficiency and can be time-consuming, e.g. requiring slide preparations and in-depth analysis of characteristics such as the morphology of pharynx and genitalia in the case of sand flies [12]. However, morphological identification can be difficult or impossible in many instances (e.g. due to specimens of sibling or cryptic species; damaged or incomplete specimens; life stages with few morphological features like eggs or larvae; poor preservation methods or damage during shipment). In the case of sand flies, which are the focus of this paper, morphological identification is sometimes hampered by the lack of comprehensive keys, minute species-distinctive characters in some subgenera and the existence of phenotypic plasticity among populations [13, 14]. PCR-based approaches are established in different formats for a number of phlebotomine species (compiled by [15, 16]), but their validity may be impaired due to genetic variability at the target locus [17, 18]. Further, these approaches are generally considered expensive and time-consuming.

As an alternative, protein profiling by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS), which is routinely used in clinical diagnostics of bacteria and fungi [19, 20] with high interlaboratory reproducibility [21], has recently been applied for the identification of a number of arthropods, including adult stages of vector taxa [15, 22–33]. In addition, protein profiles have been determined for larvae of holometabolous *Culicoides* biting midges and Culicidae [34, 35], and for eggs of nine aedine mosquito species [36]. MALDI-TOF MS was capable of identifying sister taxa and cryptic species [26, 28], and the method proved reliable for large

scale species identification of *Culicoides* biting midges (correct identification of 98.9 % of 1,200 field-collected specimens) as well as in the surveillance of invasive mosquito species (identification of eggs from ovitraps; [28]).

This paper elaborates on the suitability of MALDI-TOF MS to identify adult phlebotomine sand flies, the biological vectors of a number of important protozoan and viral pathogens of medical or veterinary importance [7, 37, 38], extending the method from identifying specimens from laboratory colonies [15] to identifying specimens collected in the field and stored for a prolonged period of time. In addition, we evaluated whether raw mass spectra obtained with the instrument of one company (Ultraflex III, Bruker, Germany) allow species identification by using the software and the reference database established on an instrument of another company (Axima Confidence, Shimadzu, Japan), whereby the two most widespread instrument series were involved. The ability to cross-reference specimens with non-parent databases would strongly enhance the value and availability of in-house reference databases established at different institutions.

Methods

Sand fly collection and morphological identification

Mass spectra were determined from sand fly specimens of 20 species obtained from field collections (9 species), colonies (7) or both (4) (see Table 1 for origin, year of collection). Sand flies were captured using CDC miniature light traps (John W. Hock Co. FL, USA) or sticky papers. Specimens were killed using carbon dioxide or liquid nitrogen and stored as described in Table 1. For morphological identification, head and genitalia of each specimen were processed and mounted on slides following traditional procedures [39] and the species determined using standard keys [40–42]. The taxonomic abbreviations used are as described [43].

Generation of MALDI-TOF MS biomarker mass sets, validation study

Thoraxes with wings and legs were manually homogenized, mixed with matrix and spotted on steel target plates as described [26]. Protein mass fingerprints were obtained using a MALDI-TOF Mass Spectrometry Axima™ Confidence machine (Shimadzu-Biotech Corp., Kyoto, Japan) and analysed with SARAMIS™ Premium software (spectral archive and microbial identification system, AnagnosTec, Potsdam-Golm, Germany) as described elsewhere [36]. Biomarker mass patterns, called 'superspectra', were calculated using the SARAMIS™ SuperSpectra™ tool with 5 specimens per species (Table 1) with 4 technical replicates each (quadruplicates). For SuperSpectra™ validation, 161 specimens (Table 1) were analysed (also in quadruplicates), and the generated mass fingerprints were imported into SARAMIS™ software for automated identification with

Table 1 Features of the phlebotomine sand flies investigated in the experimental part of the study

Species (morphological ID)	Origin (field; colony)	Collection year; storage until analyses in 2014	No. used for calculating reference database	No. used in validation study
<i>Lutzomyia (Lutzomyia) longipalpis</i> ¹	Brazil (colony ³)	2014; 70 % EtOH for few days	5	5
<i>Nyssomyia trapidoi</i> ¹	Ecuador (field)	2013; 96 % EtOH	5	5
<i>Phlebotomus (Adlerius) arabicus</i> ¹	Israel (colony ³)	2014; 70 % EtOH for few days	5	5
<i>Phlebotomus (Euphlebotomus) argentipes</i> ¹	India (colony ³)	2014; 70 % EtOH for few days	5	5
<i>Phlebotomus (Larroussius) ariasi</i> ¹	Southern France (field)	2011; 70 % EtOH, -20 °C	5	7
<i>Phlebotomus (Euphlebotomus) barguesae</i> ¹	Thailand (field)	2012; 96 % EtOH	5	6
<i>Phlebotomus (Phlebotomus) duboscqi</i>	Senegal (colony)	2002; N ₂ then -80 °C ⁴ .	0	12
<i>Phlebotomus (Phlebotomus) duboscqi</i> ¹	Senegal (colony ³)	2014; 70 % EtOH for few days	5	5
<i>Phlebotomus (Transphlebotomus) mascittii</i> ¹	France (colony)	2004; N ₂ then -80 °C ⁴ .	5	8
<i>Phlebotomus (Larroussius) neglectus</i> ¹	Italy (field)	1991; N ₂ then -80 °C ⁴ .	5	2
<i>Phlebotomus (Larroussius) orientalis</i> ¹	Ethiopia (colony ³)	2014; 70 % EtOH for few days	5	5
<i>Phlebotomus (Phlebotomus) papatasi</i>	Saudia Arabia (colony)	1994; N ₂ then -80 °C ⁴ .	0	7
<i>Phlebotomus (Phlebotomus) papatasi</i> ¹	Turkey (colony ³)	2014; 70 % EtOH for few days	5	5
<i>Phlebotomus (Larroussius) perfiliewi transcaucasicus</i> ¹	Iran (field)	2010; 70 % EtOH	5	4
<i>Phlebotomus (Larroussius) perniciosus</i>	Southern France (field)	2011; 70 % EtOH, -20 °C	0	6
<i>Phlebotomus (Larroussius) perniciosus</i> ²	Italy (field)	1991; N ₂ then -80 °C ⁴ .	0	11
<i>Phlebotomus (Larroussius) perniciosus</i> ²	Malta (field)	1989; N ₂ then -80 °C ⁴ .	0	12
<i>Phlebotomus (Larroussius) perniciosus</i> ¹	Spain (colony ³)	2014; 70 % EtOH for few days	5	5
<i>Phlebotomus (Paraphlebotomus) sergenti</i>	Iran (field)	2010; 70 % EtOH		2
<i>Phlebotomus (Paraphlebotomus) sergenti</i> ¹	Turkey, Israel (colony ³)	2014; 70 % EtOH for few days	5	5
<i>Phlebotomus (Larroussius) tobbi</i> ²	Iran (field)	2010; 70 % EtOH	0	10
<i>Phlebotomus (Larroussius) tobbi</i> ¹	Turkey (colony ³)	2014; 70 % EtOH for few days	5	5
<i>Phlebotomus (Larroussius) tobbi</i>	Greece (field)	1990; N ₂ then -80 °C ⁴	0	2
<i>Phlebotomus</i> sp.	Iran (field)	2010; 70 % EtOH	0	1
<i>Psathyromyia (Foratiniella) aragai</i> ¹	Ecuador (field)	2013; 96 % EtOH	5	0
<i>Psychodopygus panamensis</i> ¹	Ecuador (field)	2013; 96 % EtOH	5	2
<i>Sergentomyia (Sergentomyia) dentata</i> ¹	Israel (field)	2000; N ₂ then -80 °C ⁴ .	5	7
<i>Sergentomyia (Sergentomyia) minuta</i> ¹	Southern France (field)	2011; 70 % EtOH, -20 °C	5	7
<i>Sergentomyia (Sergentomyia) schwetzi</i> ¹	Ethiopia (colony ³)	2014; 70 % EtOH for few days	5	5
		Total	100	161

¹Used for calculating reference database²Used for calculating alternative reference database (replacing spectra from colony insects, see also Fig. 2)³Colonies maintained at Department of Parasitology, Charles University, Prague, Czech Republic [15]⁴-80 °C since 2012

SuperSpectra™. The threshold for identification was set at 75 % biomarker matches based on the reference data set, according to the SARAMIS™ user guideline. Spectra containing less than 30 data counts were considered low quality. A dendrogram was produced as described [27].

Analyses of raw data mass spectra obtained on Bruker mass spectrometer with Shimadzu software

Mass fingerprints generated using automatic routine procedures on an Ultraflex III MALDI TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) [15] were analysed in a blinded manner with the SARAMIS™ Premium software. Bruker mass lists were exported as mzXML files, adapted to the SARAMIS™ Premium file format by use of an in-house python script and imported for automated identification. Mass lists obtained with insect thoraxes were available for 312 specimens of *Phlebotomus arabicus* (n = 13), *Ph. argentipes* (n = 20), *Ph. duboscqi* (n = 10), *Ph. orientalis* (n = 10), *Ph. papatasi* (n = 84), *Ph. perniciosus* (n = 94), *Ph. sergenti* (n = 22), *Ph. tobbi* (n = 59) (from colonies maintained in Prague and analysed after storage in 70 % EtOH overnight [15]) and from 55 field-collected (Karpathos island, Greece, stored in 70 % EtOH for several weeks) *Ph. neglectus* (n = 44); *Ph. similis* (n = 8); *Ph. alexandri* (n = 1), *Ph. galilaeus* (n = 2) (total 367 specimens).

Genetic analyses

DNA was isolated from the remains of the abdomens with a kit (Qiamp DNA mini kit, Qiagen, Hildesheim, Germany) according to the manufacturer's instruction and after mechanical homogenization as previously described [44]. Genetic characterisation by PCR/sequencing was mainly done at the mitochondrial cytochrome b gene (cyt b). The primers CB3_PDRmod (5'-CTC CYC ATA TYC AAC CWG AAT G-3') and CB_R06mod (TAT CTA ATG KTT TCA AAA CAA TTG C-3') were modified from described ones [45,46]. Species for which no corresponding cyt b sequence was available in GenBank were characterized at the mitochondrial cytochrome c oxidase subunit I gene (COI) using primers LCO1490/HCO2198 [47] or at the small subunit nuclear ribosomal RNA gene using forward primer F2 [48] and the new reverse primer R2_new (5'-GTC CTA TTC CAT TAT TCC ATG C-3'). Direct sequencing of the amplicons was performed by a private company (Synergene, Schlieren, Switzerland).

Results

Reference database

Biomarker mass patterns ('superspectra') were calculated for 20 sand fly species, by using 5 insects per species. Specimens from colonies (10 species) and insects collected in the field (10 species) were used (Table 1). Reference mass peaks were in the range of 4000 and, depending on the species, 9800 to 11400 Da. Identification of the field-

collected species could be confirmed by PCR/sequencing with 1 specimen per species for 8 of these 10 species. The two remaining species were *Ph. barguesae* and *Psathyromyia aragaoi*. In the case of *Ph. barguesae*, the partial (around 570 bp) sequence of the COI gene obtained from the field-collected specimens best matched with the two GenBank sequences ascribed to this species (acc. nos. FJ348734-5), but differed by 7 %. Another 5 specimens morphologically attributed to this species were then genetically characterized, yielding identical sequences. No single sequence was available in GenBank for *Ps. aragaoi*, and partial cyt b and COI gene sequences have been deposited [GenBank: KP763471, GenBank: KP763472].

Superspectra were calculated for the species *Ph. perniciosus* and *Ph. tobbi* with 5 specimens each from either colonies or the field (Table 1), yielding identical masses.

Validation

The reference database was validated with 161 specimens from 19 species (Table 1). Eighty-four specimens were field-collected and stored in various media, 50 specimens were fresh from ongoing colonies of 10 species (that were also used to create the reference database), and 27 specimens were stored deep-frozen from earlier colonies. Altogether, 140 insects were correctly identified, including 77 specimens from colonies. Poor quality spectra (less than 30 data counts) were obtained with 20 field-collected specimens, of which 17 yielded no result upon automated identification with SuperSpectra™. The analyses of the other 3 of these 20 low data count specimens produced discrepant identifications (in 2 of the 4 technical replicates, no identification in the other two) as compared to morphological and genetic identification. Further, 2 insects probably were mislabelled as *Ph. perniciosus*, as both mass spectrometry and DNA sequencing identified them as *Ph. ariasi*, and specimens of this species had been obtained from the same source. Finally, one specimen yielded a novel spectrum but was identified by morphology as *Ph. perfliewi*, which is included in the database, and as belonging to the *Ph. perfliewi* complex by DNA analyses.

Thus, overall sensitivity of MALDI-TOF MS was 87 % (140/161); specificity (defined as 100 minus percentage of wrong identifications as compared to morphological identification, all specimens considered that yielded an MS identification) was 97 % (139/144) or 100 % (when consequently omitting low data count specimens and considering the probable mislabelling in 2 cases).

In addition to the specimens with discrepant identification, 22 arbitrarily chosen specimens were genetically analysed, confirming morphological and MALDI-based identification. The specimen with inconclusive morphological identification (Table 1) was among the specimens with low data count but turned out to belong to the *Ph. perfliewi* complex based on DNA sequence analysis.

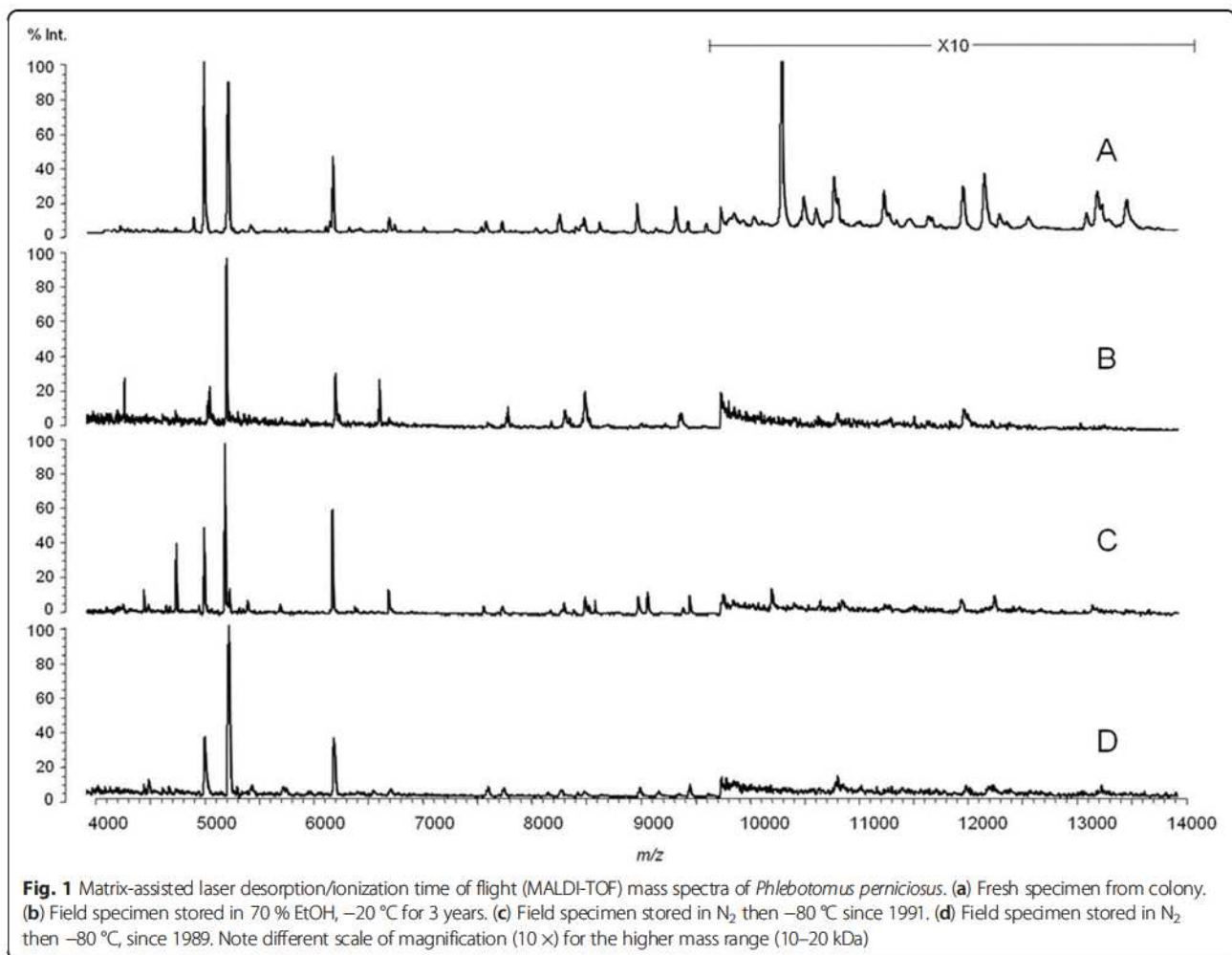
High quality spectra (i.e., equal to or more than 30 data counts) were obtained with all ($n = 50$) fresh insects from colonies, and with 55/59 insects from colonies or the field and stored deep-frozen (liquid nitrogen/ -80°C) for up to 25 years. In contrast, only 36/52 specimens stored in EtOH (70 or 95 %; room temperature or -20°C) for only a few years could be identified (Table 1). Duration of storage impaired data counts in the high mass range as exemplified in Fig. 1.

Different batches of sand fly specimens (colony, wild catches from different geographical origins and stored under different conditions and/or for different periods of time) were available for a few species. For example, a dendrogram of *Ph. perniciosus* groups is given in Fig. 2. The spectra from colony insects (originally from Spain) form a distinct cluster, whereas spectra of specimens from two geographical origins (Italy, Malta) and stored under comparable conditions do not. A single spectrum available from a more recently collected *Ph. perniciosus* from France, which was stored differently, is placed on a distinct

branch, separated from both the colony- but also the other field-derived spectra.

Analyses of mass lists obtained on a Bruker instrument with a reference database (SARAMIS™) and reference spectra acquired on a Shimadzu instrument

Raw data from a total of 312 specimens from 8 sand fly species from laboratory colonies were analysed, resulting in the correct identification of 297 individuals. No identification was obtained for 15 specimens, of which 10 had low data count spectra (less than 30). Five spectra with a sufficient number of data counts (30 or more) could not be identified. No misidentification occurred (overall sensitivity 95.2; sensitivity considering only high quality spectra 98.3 %; specificity 100 %). Thirty-six of the 55 field-collected specimens from 4 species were identified in concordance with the morphological identification. The single specimen of *Ph. alexandri* had a high quality spectrum (data count of 57), but yielded no identification as this species was not included in the database. Seventeen



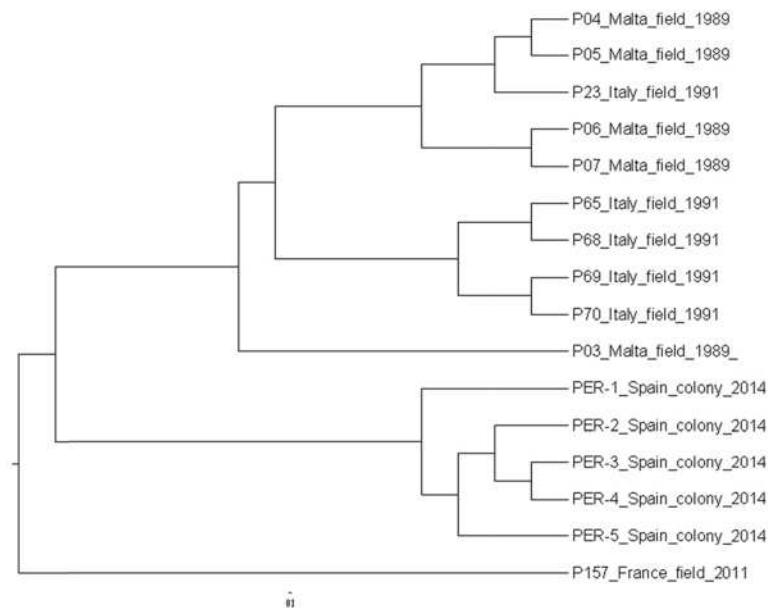


Fig. 2 Dendrogram of matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectra (paired-group dice algorithm) of *Phlebotomus perniciosus* specimens from different groups (colony, geographical origin of field specimens; for conditions and time of storage, see Table 1)

spectra contained insufficient data counts and were not identified. One specimen morphologically identified as *Ph. galilaeus* yielded a spectrum identified as *Ph. perniciosus*. Thus, diagnostic parameters with the field-collected specimens differed slightly from laboratory collections (sensitivity considering only high quality spectra 94.7 %; specificity 97.4 %).

Discussion

MALDI-TOF MS reference database and validation

A MALDI-TOF MS database was established with 20 phlebotomine species. In selecting our study species, we took into account i) their vectorial role - in order to include many species which are proven vectors of *Leishmania* spp., and ii) their taxonomic position - in order to compare closely related species and species belonging to different genera (Table 1). An evaluation of the database with 161 specimens revealed a robust sensitivity and a very high specificity of this approach. Twenty specimens were not ($n = 17$) or incorrectly ($n = 3$) identified due to low spectra quality (data count below 30). Thus, a threshold of 30 data counts, based on our experience with mass spectrometry analyses of Ceratopogonidae biting midges [26, 27], seems a useful criterion to exclude spectra from future analyses.

The major factor impairing mass spectrum quality seems to be the way the insects were stored rather than the duration of storage, though this aspect has not systematically been addressed in the present study. Thus, e.g. 22/23 specimens of *Ph. perniciosus* stored for 25 years in liquid nitrogen/ -80 °C could be identified, but only 3/6 insects of the

same species stored since 2011 in 70 % EtOH at -20 °C could be identified. This confirms earlier findings that freezing is superior to EtOH as a storage medium, with EtOH at a concentration of 70 % being more suitable than higher concentrations, as experimentally determined with sand flies stored up to 75 days [15]. Thus, this 'storage constraint' limits the value of mass spectrometry for the identification of insects, as ethanol is the widely preferred storage medium. However, a high reliability of mass spectrometry (98.9 % good quality spectra) was found with 1,200 field-collected *Culicoides* specimens stored in 70 % EtOH at 4 °C for one year [49]. Most other studies on mass spectrometry as a tool for the identification of arthropods relied on fresh colony-derived specimens. Successful identification by MALDI-TOF MS with field-collected specimens was reported with ticks (stored in liquid nitrogen, [25]), tsetse flies (using air-dried wings, [50]; and mosquitoes (analysis of legs, storage conditions not described, [31]).

As also shown in our study, storage reduces the higher weight masses (Fig. 1). Identification to species level is unaffected by this constraint as reference masses are in the lower mass range. Identification of specimens from different geographic origins (given they were properly stored) was equally reliable by using superspectra derived with either insects fresh from colonies or wild-catches from storage (Table 1). However, higher resolution (e.g., 'fine typing' with regard to geographical origin of specimens or lower taxonomic levels) might be critical as the topography of a dendrogram created in cluster analyses could reflect the storage conditions of the analysed specimens rather than

phenotypic distinctness (see also Fig. 2). The identification of cryptic insect species (*Anopheles* spp.) and even the classification of specimens from different laboratory colonies were possible with a standardized approach (fresh specimens, same feed etc.) [28], but seem improbable with field-collected specimens. Thus, care should be taken not to over-interpret cluster analyses of mass spectra obtained from field-collected and stored specimens.

A novel spectrum was obtained for a specimen that morphologically was identified as a species (*Ph. perfiliewi*) that is included in the database. This species is known to exist as a complex of species [12] which might explain variability in mass spectra. All morphologically identified *Ph. barguesae* specimens had considerable genetic differences at the barcoding locus from corresponding GenBank entries. Genetic variability of this species, which is characterized by a unique morphology of the female spermathecae [51], has been observed among populations from different caves (Depaquit, unpublished). Further studies employing mass spectrometry to different populations of freshly collected specimens and the comparison with results of DNA-based molecular approaches might contribute to elucidate taxonomic relationships. This technique shall be especially considered when dealing with species complexes like *Ph. perfiliewi* or *Ph. major* which are incriminated in transmission of medically important *Leishmania* species and represent a taxonomic challenge.

Analyses of mass lists obtained on a Bruker instrument with a reference database (SARAMIS™) and reference spectra acquired on a Shimadzu instrument

Comparative studies have shown that the performance of the two systems (Shimadzu, Bruker) for the identification of microorganisms in clinical laboratories is comparable [52, 53]. Exchangeability of data obtained on the two different instruments, however, has to the best of our knowledge not been reported so far. Here, we show that fingerprints of phlebotomine sand flies obtained by automatic routine procedure on a Bruker instrument allow for reliable (high values of diagnostic parameters) determination of species by using the database and the software (SARAMIS™) established on a Shimadzu instrument. The sensitivity of this analysis was high despite slight differences in sample preparation [15] and mass ranges considered (Bruker: 2–25 kDa; Shimadzu: 3–20 kDa). Non-identifiable spectra mostly contained few data counts ($n = 10$) or low intensity peaks ($n = 3$), and these spectra could not be identified with the Bruker Biotyper software either. Two spectra of good quality did not have sufficient matches for automated identification with superspectra, though their identification (*Ph. argentipes*, *Ph. perniciosus*) was possible with manual full comparison.

Conclusions

The power of mass spectrometric identification of organisms depends on the quality of the available database (i.e., reference quality, taxonomic coverage) and its accessibility. The first aspect requires the participation of expert taxonomists, particularly when dealing with closely related species, and experienced users of mass spectrometers who keep a high quality standard for generating the spectra. It further advocates for a centralized, comprehensive database, rather than scattered in-house ones. Accessibility (i.e., the analysis of specimens by third parties) might be an issue at scientific institutions where capacity for analysis, availability and willingness of an operator could be limiting factors. Therefore, a centralized database and analyses at a private company, which provides a customer-oriented service, is a valuable alternative for greater sustainability. As shown in the present work, spectra obtained with different instruments can be analysed using such a centralized database, and this should be possible in the near future via an online platform in a cost-efficient manner.

Competing interests

V.P. is employed by Mabritec SA, a commercial service laboratory. The other authors declare that they have no competing interests.

Authors' contributions

AM, VP and FS conceived the study; JD, VD, ALB, SZ, VL, KH, JP, PV, DS performed field collections and morphological identification of specimens; VD, KH and PV were involved in insect rearing; AM and HT performed genetic identification; PH and VP carried out the mass spectrometry analyses; AM, HT, CK, VP and FS performed the data analyses; AM and HT drafted the manuscript. All authors contributed to writing the manuscript and have approved the final version.

Acknowledgments

We thank Jeannine Hauri for excellent technical support, and greatly acknowledge the Swiss Food Safety and Veterinary Office as sponsor of the Swiss National Centre for Vector Entomology. We also thank Bernard Pesson and Mohammad Akhondi for providing us many specimens. The work of AM, VD, HT, CK and FS was done under the frame of EurNegVec COST Action TD1303. The work of VD, PH, PV and KH was supported by the Czech Science Foundation (project GACR 15-04329S). The work of PH was also supported by the Institutional Research Project of the Institute of Microbiology (RVO61388971). The work of ALB, JP and DS has been supported by the EDENext project (reference nr. EDENext329). We are also grateful to the PhDB group of Montpellier for the field collection.

Author details

¹Swiss National Centre for Vector Entomology, Institute of Parasitology, University of Zürich, Winterthurerstrasse 266a, 8057 Zürich, Switzerland. ²Université de Reims Champagne-Ardenne, ANSES, EA4688 - USC "transmission vectorielle et épidémiologie de maladies parasitaires (VECPAR)", SFR Cap Santé, 51, rue Cognacq-Jay, 51096 Reims, France. ³Department of Parasitology, Faculty of Sciences, Charles University in Prague, Vinicna 7, 128 44 Prague 2, Czech Republic. ⁴MIVEGEC, UMR CNRS-IRD-Université de Montpellier Centre IRD, 911 Avenue Agropolis, BP 64501, Cedex 34394 Montpellier, France. ⁵Laboratory of Molecular Structure Characterization, Institute of Microbiology, The Czech Academy of Sciences, Videnska 1083, 142 20 Prague 4, Czech Republic. ⁶Instituto de Microbiología, Universidad San Francisco de Quito, Diego de Robles S/N, Quito, Ecuador. ⁷Mabritec SA, Lörracherstrasse 50, 4125 Riehen, Switzerland. ⁸Avia-GIS, Risschotlei 33, 2980 Zoersel, Belgium. ⁹Current address: NSF Center for Integrated Pest Management, 1730 Varsity Drive, Suite 110, Venture IV Building, Raleigh, North Carolina 27606, USA.

Received: 9 March 2015 Accepted: 29 April 2015

Published online: 10 May 2015

References

- Schaffner F, Kaufmann C, Hegglin D, Mathis A. The invasive mosquito *Aedes japonicus* in central Europe. *Med Vet Entomol.* 2009;23:448–51.
- Medlock JM, Hansford KM, Schaffner F, Versteir V, Hendrickx G, Zeller H, et al. A review of the invasive mosquitoes in Europe: ecology, public health risks, and control options. *Vector Borne Zoonotic Dis.* 2012;12:435–47.
- Schaffner F, Mathis A. Dengue and dengue vectors in the WHO European region: past, present, and scenarios for the future. *Lancet Infect Dis.* 2014;14:1271–80.
- Jaenson TGT, Jaenson DGE, Eisen L, Petersson E, Lindgren E. Changes in the geographical distribution and abundance of the tick *Ixodes ricinus* during the past 30 years in Sweden. *Parasit Vectors.* 2012;5:8.
- Dergousoff SJ, Galloway TD, Lindsay LR, Curry PS, Chilton NB. Range expansion of *Dermacentor variabilis* and *Dermacentor andersoni* (Acari: Ixodidae) near their northern distributional limits. *J Med Entomol.* 2013;50:510–20.
- Nolan DV, Dallas JF, Pierny SB, Mordue Luntz AJ. Incursion and range expansion in the bluetongue vector *Culicoides imicola* in the Mediterranean basin: a phylogeographic analysis. *Med Vet Entomol.* 2008;22:340–51.
- Maroli M, Feliciangeli MD, Bichaud L, Charrel RN, Gradoni L. Phlebotomine sandflies and the spreading of leishmaniasis and other diseases of public health concern. *Med Vet Entomol.* 2013;27:123–47.
- Poepl W, Obwaller AG, Weiler M, Burgmann H, Mooseder G, Lorentz S, et al. Emergence of sandflies (Phlebotominae) in Austria, a Central European country. *Parasitol Res.* 2013;112(12):4231–7.
- Medlock JM, Hansford KM, van Bortel W, Zeller H, Alten B. A summary of the evidence for the change in European distribution of phlebotomine sand flies (Diptera: Psychodidae) of public health importance. *J Vector Ecol.* 2014;39:72–7.
- Schaffner F, Bellini R, Petric D, Scholte E-J, Zeller H, Marrama Rakotoarivony L. Development of guidelines for the surveillance of invasive mosquitoes in Europe. *Parasit Vectors.* 2013;6:209.
- European Centre for Disease Prevention and Control: Guidelines for the surveillance of native mosquitoes in Europe. Stockholm: ECDC Technical Report; 2014. doi:10.2900/37227
- Depaquit J, Bounamous A, Akhouni M, Augot D, Sauvage F, Dvorak V, et al. A taxonomic study of *Phlebotomus (Larrousius) perilliewi* s. l. *Infect Genet Evol.* 2013;20:500–8.
- Prudhomme J, Unay F, Rahola N, Ouaini F, Guernaoui S, Boumezzough A, et al. Wing size and shape variation of *Phlebotomus papatasi* (Diptera: Psychodidae) populations from the south and north slopes of the Atlas Mountains in Morocco. *J Vector Ecol.* 2012;37:137–47.
- Ready PD. Biology of phlebotomine sand flies as vectors of disease agents. *Ann Rev Entomol.* 2013;58:227–50.
- Dvorak V, Halada P, Hlavackova K, Dokianakis E, Antoniou M, Volf P. Identification of phlebotomine sand flies (Diptera: Psychodidae) by matrix-assisted laser desorption/ionization time of flight mass spectrometry. *Parasit Vectors.* 2014;7:21.
- Depaquit J. Molecular systematics applied to Phlebotomine sandflies: Review and perspectives. *Infect Genet Evol.* 2014;28:744–56.
- Depaquit J, Ferte H, Leger N, Lefranc F, Alves-Pires C, Hanafi H, et al. ITS 2 sequences heterogeneity in *Phlebotomus sergenti* and *Phlebotomus similis* (Diptera, Psychodidae): possible consequences in their ability to transmit *Leishmania tropica*. *Int J Parasitol.* 2002;32:1123–31.
- Dvorak V, Votycka J, Aytekin A, Alten B, Volf P. Intraspecific variability of natural populations of *Phlebotomus sergenti*, the main vector of *Leishmania tropica*. *J Vector Ecol.* 2011;36:549–57.
- Croxatto A, Prod'homme G, Greub G. Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology. *FEMS Microbiol Rev.* 2012;36:380–407.
- Posteraro B, De Carolis E, Vella A, Sanguinetti M. MALDI-TOF mass spectrometry in the clinical mycology laboratory: identification of fungi and beyond. *Expert Rev Proteomic.* 2013;10:151–64.
- Mellmann A, Bimet F, Bizet C, Borovskaya AD, Drake RR, Eigner U, et al. High interlaboratory reproducibility of matrix-assisted laser desorption ionization-time of flight mass spectrometry-based species identification of nonfermenting bacteria. *J Clin Microbiol.* 2009;47:3732–4.
- Campbell PM. Species differentiation of insects and other multicellular organisms using matrix-assisted laser desorption/ionization time of flight mass spectrometry protein profiling. *Syst Entomol.* 2005;30:186–90.
- Feltens R, Gorner R, Kalkhof S, Groger-Arndt H, von Bergen M. Discrimination of different species from the genus *Drosophila* by intact protein profiling using matrix-assisted laser desorption ionization mass spectrometry. *BMC Evol Biol.* 2010;10:95.
- Hoppenheit A, Murugaiyan J, Bauer B, Steuber S, Clausen PH, Roesler U. Identification of Tsetse (*Glossina* spp.) using matrix-assisted laser desorption/ionization time of flight mass spectrometry. *PLoS Negl Trop Dis.* 2013;7, e2305.
- Karger A, Kampen H, Bettin B, Dautel H, Ziller M, Hoffmann B, et al. Species determination and characterization of developmental stages of ticks by whole-animal matrix-assisted laser desorption/ionization mass spectrometry. *Ticks Tick Borne Dis.* 2012;3:78–89.
- Kaufmann C, Schaffner F, Ziegler D, Pfluger V, Mathis A. Identification of field-caught *Culicoides* biting midges using matrix-assisted laser desorption/ionization time of flight mass spectrometry. *Parasitology.* 2012;139:248–58.
- Kaufmann C, Ziegler D, Schaffner F, Carpenter S, Pfluger V, Mathis A. Evaluation of matrix-assisted laser desorption/ionization time of flight mass spectrometry for characterization of *Culicoides nubeculosus* biting midges. *Med Vet Entomol.* 2011;25:32–8.
- Muller P, Pfluger V, Wittwer M, Ziegler D, Chandre F, Simard F, et al. Identification of cryptic *Anopheles* mosquito species by molecular protein profiling. *PLoS One.* 2013;8, e57486.
- Perera MR, Vargas RDF, Jones MGK. Identification of aphid species using protein profiling and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Entomol Exp Appl.* 2005;117:243–7.
- Yssouf A, Flaudrops C, Drali R, Kernif T, Socolovschi C, Berenger JM, et al. Matrix-assisted laser desorption ionization-time of flight mass spectrometry for rapid identification of tick vectors. *J Clin Microbiol.* 2013;51:522–8.
- Yssouf A, Socolovschi C, Flaudrops C, Ndiath MO, Sougoufara S, Dehecq JS, et al. Matrix-assisted laser desorption ionization-time of flight mass spectrometry: an emerging tool for the rapid identification of mosquito vectors. *PLoS One.* 2013;8:e72380.
- Yssouf A, Socolovschi C, Leulmi H, Kernif T, Bitam I, Audoly G, et al. Identification of flea species using MALDI-TOF/MS. *Comp Immunol Microbiol Infect Dis.* 2014;37:153–7.
- Yssouf A, Parola P, Lindstrom A, Lilja T, Lambert G, Bondesson U, et al. Identification of European mosquito species by MALDI-TOF MS. *Parasitol Res.* 2014;113:2375–8.
- Steinmann IC, Pfluger V, Schaffner F, Mathis A, Kaufmann C. Evaluation of matrix-assisted laser desorption/ionization time of flight mass spectrometry for the identification of ceratopogonid and culicid larvae. *Parasitology.* 2013;140:318–27.
- Dieme C, Yssouf A, Vega-Rúa A, Berenger J-M, Failloux A-B, Raoult D, et al. Accurate identification of Culicidae at aquatic developmental stages by MALDI-TOF MS profiling. *Parasit Vectors.* 2014;7:544.
- Schaffner F, Kaufmann C, Pfluger V, Mathis A. Rapid protein profiling facilitates surveillance of invasive mosquito species. *Parasit Vectors.* 2014;7:142.
- Alkan C, Bichaud L, de Lamballerie X, Alten B, Gould EA, Charrel RN. Sandfly-borne phleboviruses of Eurasia and Africa: epidemiology, genetic diversity, geographic range, control measures. *Antiviral Res.* 2013;100:54–74.
- Alvar J, Velez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. WHO Leishmaniasis Control Team: Leishmaniasis worldwide and global estimates of its incidence. *PLoS One.* 2012;7, e35671.
- Zapata S, Mejia L, Le Pont F, Leon R, Pesson B, Ravel C, et al. A study of a population of *Nyssomyia trapidoi* (Diptera: Psychodidae) caught on the Pacific coast of Ecuador. *Parasit Vectors.* 2012;5:144.
- Dedet JP, Addadi K, Belazzoug S. Les phlébotomes (Diptera, Psychodidae) d'Algérie. *Cah ORSTOM, Sér Ent Méd Parasitol.* 1984;22:99–127.
- Lewis D. A taxonomic review of the genus *Phlebotomus*. *Bull Brit Mus Nat Hist (Entomol).* 1982;45:121–209.
- Young DG, Duncan MA: Guide to the identification and geographic distribution of *Lutzomyia* sand flies in Mexico, the West Indies, Central and South America (Diptera: Psychodidae). *Memoirs of the American Entomological Institute*, vol. 54. Associated Publishers, Gainesville, Florida, USA; 1994, 881 pp. www.dtic.mil/dtic/tr/fulltext/u2/a285737.pdf
- Marcondes CB. A proposal of generic and subgeneric abbreviations for Phlebotomine sandflies (Diptera: Psychodidae: Phlebotominae) of the World. *Entomol News.* 2007;118:351–6.
- Wenk C, Kaufmann C, Schaffner F, Mathis A. Molecular characterisation of Swiss Ceratopogonidae (Diptera) and evaluation of real-time PCR assays for the identification of *Culicoides* biting midges. *Vet Parasitol.* 2012;184:258–66.

45. Essegir S, Ready PD, Killick-Kendrick R, Ben-Ismael R. Mitochondrial haplotypes and phylogeography of *Phlebotomus* vectors of *Leishmania major*. *Insect Mol Biol*. 1997;6:211–25.
46. Parvizi P, Ready PD. Molecular investigation of the population differentiation of *Phlebotomus papatasi*, important vector of *Leishmania major*, in different habitats and regions of Iran. *Iranian Biomed J*. 2006;10:69–77.
47. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol Mar Biol Biotech*. 1994;3:294–9.
48. Aransay AM, Scoulica E, Tselentis Y, Ready PD. Phylogenetic relationships of phlebotomine sandflies inferred from small subunit nuclear ribosomal DNA. *Insect Mol Biol*. 2000;9:157–68.
49. Kaufmann C, Steinmann IC, Hegglin D, Schaffner F, Mathis A. Spatio-temporal occurrence of *Culicoides* biting midges in the climatic regions of Switzerland, along with large scale species identification by MALDI-TOF mass spectrometry. *Parasit Vectors*. 2012;5:246.
50. Hoppenheit A, Murugaiyan J, Bauer B, Clausen P-H, Roesler U. Analysis of *Glossina palpalis gambiense* and *Glossina tachinoides* from two distant locations in Burkina Faso using MALDI TOF MS. *Parasitol Res*. 2014;113:723–6.
51. Depaquit J, Muller F, Léger N. *Phlebotomus (Euphlebotomus) barguesae* n. sp. from Thailand (Diptera-Psychodidae). *Parasit Vectors*. 2009;2:5.
52. Alby K, Gilligan PH, Miller MB. Comparison of matrix-assisted laser desorption ionization-time of flight (maldi-tof) mass spectrometry platforms for the identification of gram-negative rods from patients with cystic fibrosis. *J Clin Microbiol*. 2013;51:3852–4.
53. Lohmann C, Sabou M, Moussaoui W, Prevost G, Delarbre J-H, Candolfi E, et al. Comparison between the Biflex III-Biotyper and the Axima-SARAMIS systems for yeast identification by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J Clin Microbiol*. 2013;51:1231–6.

Submit your next manuscript to BioMed Central and take full advantage of:

- Convenient online submission
- Thorough peer review
- No space constraints or color figure charges
- Immediate publication on acceptance
- Inclusion in PubMed, CAS, Scopus and Google Scholar
- Research which is freely available for redistribution

Submit your manuscript at
www.biomedcentral.com/submit



Annexe 3 - Article 9

Article 9 : « Rioux J.A., Carron S., Dereure J., Périères J., Zeraia L. Franquet E., Babinot M., Gallego M., **Prudhomme J.** 2013. *Ecology of leishmaniasis in the South of France. 22. Reliability and representativeness of 12 Phlebotomus ariasi, P. perniciosus and Sergentomyia minuta (Diptera: Psychodidae) sampling stations in Vallespir (eastern French Pyrenees region)*. Parasite. 20(34) » [42].

Ecology of leishmaniasis in the South of France. 22. Reliability and representativeness of 12 *Phlebotomus ariasi*, *P. perniciosus* and *Sergentomyia minuta* (Diptera: Psychodidae) sampling stations in Vallespir (eastern French Pyrenees region)

Jean-Antoine Rioux^{1,*}, Stéphane Carron^{2,†}, Jacques Dereure¹, José Périères¹, Lamri Zeraia³, Evelyne Franquet⁴, Michel Babinot², Montserrat Gállego⁵, and Jorian Prudhomme⁶

¹ Faculté de Médecine, Université Montpellier 1, 1, rue École de médecine, 34000 Montpellier, France

² Entente Interdépartementale pour la Démoustication du Littoral Méditerranéen (EID-Méditerranée), 165 rue Paul Rimbaud, 34000 Montpellier, France

³ Office National des Forêts (ONF), 505 rue de la Croix Verte, Parc Euromédecine, 34094 Montpellier, France

⁴ Université Aix-Marseille, IMBE, Pôle de l'Étoile, Saint Jérôme, 13397 Marseille Cedex 20, France

⁵ Parasitology Laboratory, Faculty of Pharmacy, Av. Joan XXIII, 08028 Barcelona, Spain

⁶ UMR MIVEGEC (IRD 224 – CNRS 5290), Universités Montpellier 1 et 2, 911 avenue Agropolis, 34394 Montpellier, France

Received 1 June 2013, Accepted 18 September 2013, Published online 11 October 2013

Abstract – This study was conducted around Céret (Pyrénées-Orientales, mean elevation 200 m) to test the statistical reliability of 12 stations devoted to sampling the *Leishmania infantum* vectors *Phlebotomus ariasi* and *P. perniciosus* in the South of France. Each station included a retaining wall and the surrounding phytoecological environment (total area: 2,000 m²). The wall had rectangular drainage cavities (weep holes) in which flight interception traps (sticky paper) were inserted and stretched every 10 days from May to October. For both vector species, the statistical analysis of 10-day and annual frequencies led to the following conclusions: (1) *P. ariasi* densities were significantly higher than *P. perniciosus* densities, (2) densities per species were significantly different at the 12 stations : none of them could be considered as representative of local vector densities, which depend on the wall structure (exposure, shade, vertebrate hosts), (3) the 10-day variation trends were not significantly different between stations, indicating that these variations are not determined by the station structure but rather by a common external factor (likely meteorological) and (4) the phytoecological features at the stations were not correlated with the sandfly densities. Most of the observations obtained with *P. ariasi* and *P. perniciosus* are also relevant for the non-vectorial species *S. minuta*. In conclusion, future research on the dynamics of leishmaniasis outbreaks relative to climate change and agricultural-silvicultural modifications should be very cautiously carried out, while focusing especially on the vector sampling quality and the use of phytoecological maps as vector density indicators.

Key words: Leishmaniasis, Pyrénées-Orientales, Ecoepidemiology, Vector sampling, Phytoecological indicator, Climate change, Zero point.

Résumé – Écologie de la leishmaniose dans le Sud de la France. 22. Fiabilité et représentativité de douze stations d'échantillonnage de *Phlebotomus ariasi*, *P. perniciosus* et *Sergentomyia minuta* (Diptera: Psychodidae) en Vallespir (Pyrénées-Orientales). La présente étude, réalisée aux environs de Céret (Pyrénées-Orientales, altitude moyenne 200 m) avait pour objectif d'éprouver la fiabilité de 12 stations de *Phlebotomus ariasi* et de *P. perniciosus*, vecteurs de *Leishmania infantum* dans le Sud de la France. Chaque station comportait un mur de soutènement et son environnement phyto-écologique (surface totale : 2000 m²). Le mur était pourvu de cavités rectangulaires dans lesquelles ont été insérés des pièges d'interception (papiers adhésifs) tendus tous les 10 jours, de mai à octobre. Pour les deux vecteurs étudiés, les analyses statistiques ont conduit aux déductions suivantes : 1) les densités de *P. ariasi* sont significativement supérieures à celle de *P. perniciosus*, 2) les densités de chaque espèce sont significativement différentes pour les 12 stations : aucune d'elles ne peut donc être considérée comme représentative des densités vectorielles locales; ces valeurs dépendent de la structure du mur (exposition, ombre

*Corresponding author: j.a.rioux@wanadoo.fr

† Deceased.

portée, hôtes vertébrés), 3) le sens des fluctuations décennales n'est pas significativement différent d'une station à l'autre : ces fluctuations ne sont donc pas conditionnées par la structure des stations, mais par un facteur commun extérieur, vraisemblablement de nature météorologique, 4) la phyto-écologie des stations n'est pas corrélée avec leur richesse en Phlébotomes. La plupart des observations obtenues avec *P. ariasi* et *P. perniciosus* sont également pertinentes pour l'espèce non-vectorielle *S. minuta*. En conclusion, les recherches sur la dynamique des foyers leishmaniens, en rapport avec les changements climatiques ou les modifications agro-sylvicoles, devraient être conduites avec beaucoup de prudence : une attention particulière devrait être accordée à l'échantillonnage des vecteurs et à l'utilisation des phytocénoses comme indicateurs des stations.

« Il faut laisser l'expérience à sa liberté : c'est la tenir captive que de n'en montrer que le côté qui prouve et que de voiler le côté qui contredit ».¹

Denis Diderot

Introduction

For several decades, the epidemiology of leishmaniasis has benefited from ecology-based scientific concepts and methods. The aim of this approach was to investigate epidemiological cycles in terms of "parasitic systems". The ecoepidemiological method developed on this occasion [11, 23] enabled identification of key factors that govern the structure and function of leishmaniasis outbreaks. The disciplines involved in this approach include: 1° mesology (biotopes, biogeography, bioclimatology, predators and pathogens), 2° taxonomy, genetics and evolution, 3° trophic and sexual behaviours of sandflies (hematophagy, trophogonic cycle, "eurystenogamy", fertility, fecundity and diapause). 4° morphophysiological modifications in parasites during the intravectorial cycle (multiplication, fusion, parietal attachment, metacyclogenesis), 5° *Leishmania* virulence in vertebrate reservoirs (receptivity, inoculation chancre, visceralization and immunity), 6° the last step of the approach, rational control, involves several techniques (physical, chemical and biological) targeting the cycle overall, i.e. the parasite, vectors and reservoirs.

This ecoepidemiological approach was applied in the Mediterranean region, which gave rise to the vector pre-eminence concept, i.e. the sandfly vector is the main factor responsible for the structure and dynamics of leishmaniasis outbreaks [23, 28].

Global warming was recently taken into account with respect to the emergence and expansion of these outbreaks, thus giving new impetus to this type of research [5, 14, 16, 26]. However, to clearly confirm the involvement of climatic factors, project leaders have stressed the need to very precisely determine the conventional zero point. In Languedoc-Roussillon, where mean annual temperatures increased by around 2 °C between 1946 and 2004 (Figure 1), these recommendations prompted us to reassess the statistical quality of the trapping initiatives conducted in 1981 in the eastern Pyrenees region where both *Phlebotomus ariasi* and *P. perniciosus*, i.e. sandfly vectors of *Leishmania infantum*, are found. The aim of the present study was to detect potential sampling bias, which could call into question the conclusions of certain previous studies [22].

Material and methods

General points

The survey was carried out at Vallespir, in the vicinity of Céret (P.O., France), in an area of mixed oak, including *Quercus ilex*, *Q. pubescens* and *Q. suber* (Figure 2), which is typical subhumid Mediterranean climate [4, 8, 9, 13, 20].

In the study area (mean elevation 200 m), 12 sampling stations located 2–3 km apart were selected using a purposive sampling method [12]. All stations except No. 1, 8 and 10 were located in wooded rural or periurban areas. Each had a retaining wall with drainage cavities (weep holes). Sandfly samples were obtained using flight interception traps (20 × 20 cm sticky paper that were stretched vertically in the weep holes) (Figure 3). From 3 June to 12 November 1981, the 12 stations were sampled on a 10-day basis from March to November (total number of traps: 4,263; total area: 341.04 m²; mean number of traps per station: 355.25; mean area per station: 28.42 m²; mean number of traps per 10-day period: 284). Sampled sandflies were placed in a 90° alcohol solution and identified. For each species, imago densities were calculated on the basis of the number of sandflies (P♂ + ♀) counted per m² of sticky paper [2, 23, 27, 32]. The statistical analysis was focused on *P. ariasi* and *P. perniciosus*, the only confirmed vectors sampled at the site, using Kolmogorof-Smirnov, Wilcoxon and Friedman, Cochran and McNemar tests (SYSTAT[®]9 software) [33, 35].

List of sampling stations and coordinates

Station No. 1 – Le Vila-locality. GPS coordinates (Coord.): latitude North (N) 42° 29,912', Longitude east (E) 02° 42,651'. Elevation (Elev.): 150 m. Wall exposure (exp.): east southeast. Forest vegetation (For. veg.): *Quercus ilex* (*Qi*), *Q. suber* (*Qs*). Number of sampled traps (Tot. traps): 489.

¹ "Experience must be allowed its freedom: only showing the side that proves while hiding the side that contradicts is to maintain it captive". Denis Diderot – Pensées sur l'interprétation de la nature, 1774, § 47 [6].

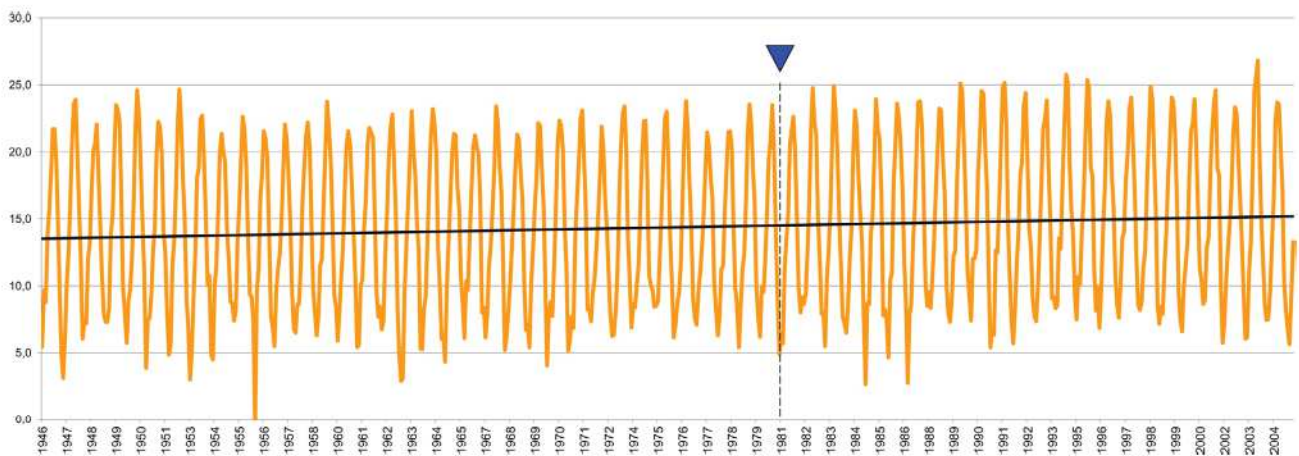


Figure 1. Variations in mean monthly temperatures at the Montpellier-Fréjorgues (France) meteorological station between 1946 and 2004 (blue triangle: 1981, year of the study). The data have a normal distribution and the regression is significant (trend curve: $Y = 0.0024, X + 13.511$). The temperature increase over the considered period was 1.895 ± 0.068 °C.

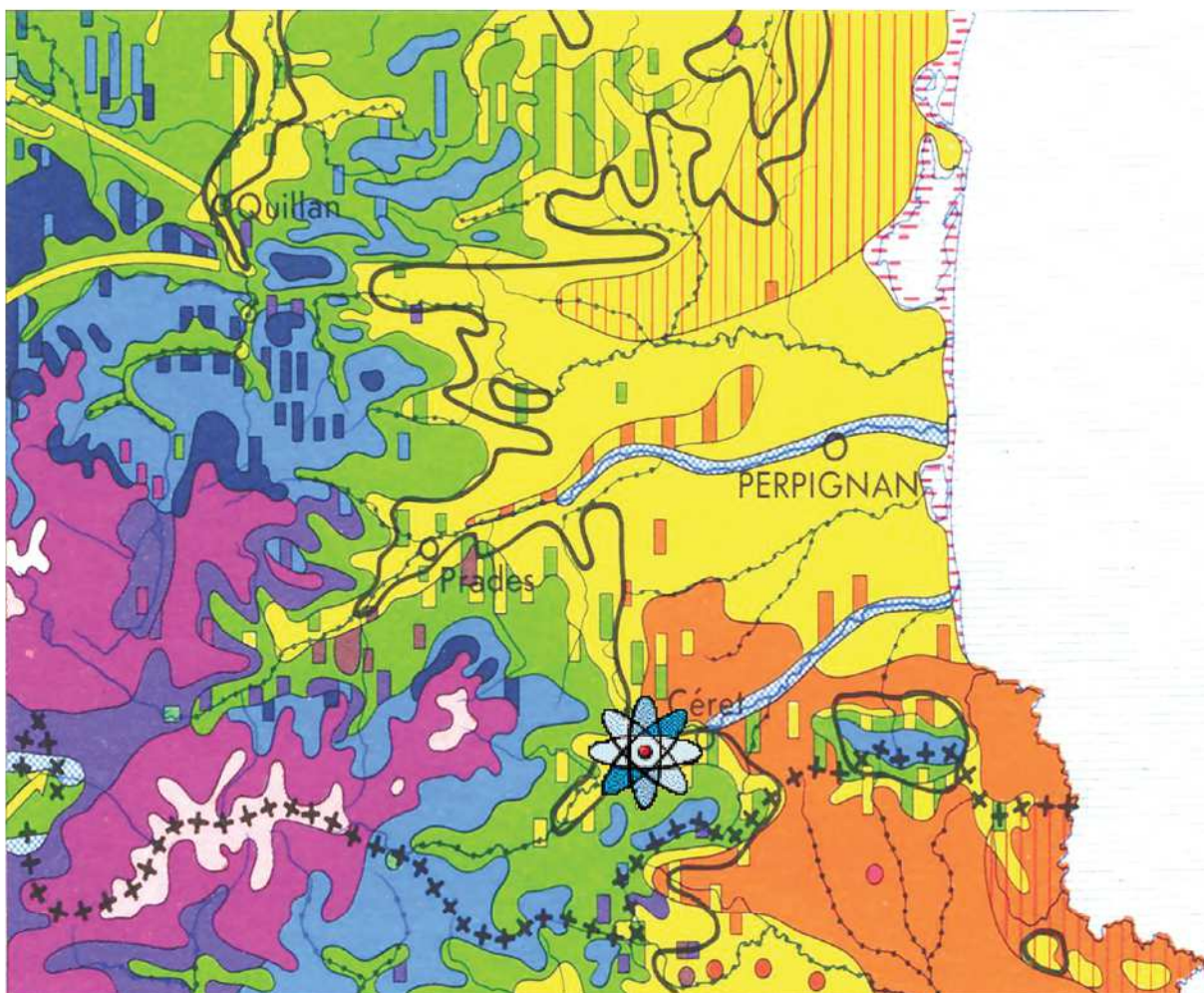



Figure 2. Map of botanical successions around Perpignan (*in*: H. Gaussen : Carte de la végétation de la France au 1 / 200 000^e, CNRS). In orange: cork oak. In yellow: holm oak. In green: white oak. In light blue: beech. In dark blue: fir. In purple: Scots pine. In red: mountain pine. In pink: alpine storey. Sampling area, , located in the Mediterranean holm oak and white oak succession. There were patches of cork oak.



Figure 3. Station No. 3. Trapping wall with weep holes (or so-called “barbacanes” in French). Inset: a weep hole containing a sticky trap. Forefront: *Brachypodium phoenicoides* meadow along the edge of a road. Background: *Robinia pseudacacia* and *Quercus ilex*.

Station No. 2 – Road (R) D15. Coord.: N 42° 29,901', E 02° 42,528'. Elev.: 140 m. Exp.: E. For. veg.: *Qi*, *Qs*; Tot. traps: 73.

Station No. 3 – R D618 (Le Vila at Palalda). Coord.: N 42° 29,441', E 02° 41,020'. Elev.: 280 m. Exp.: S. For. veg.: *Qi*, *Qs*. Tot. traps: 386. (Figure 3).

Station No. 4 – R D618 (Palalda 2 km). Coord.: N 42° 29,290', E 02° 40,622'. Elev.: 260 m. Exp.: S. For. veg.: *Qi*. Tot. traps: 254.

Station No. 5 – Palalda-locality. Coord.: N 42° 29,087', E 02° 40,437'. Elev.: 220 m. Exp.: SSE. For. veg.: *Qi*. Tot. traps: 332.

Station No. 6 – Palalda-locality. Coord.: N 42° 29,067', E 02° 40,315'. Elev.: 200 m. Exp.: SE. Tot. traps: 678.

Station No. 7 – R D618 (Palalda-Amélie). Coord.: N 42° 28,920', E 02° 40,203'. Elev.: 180 m. Exp.: SE. For. veg.: *Qi*, *Q. pubescens* (*Qp*). Tot. traps: 543.

Station No. 8 – Arles-localité. Coord.: N 42° 27,746', E 02° 38,294'. Elev.: 200 m. Exp.: SE. Tot. traps: 712.

Station No. 9 – R F13 (Céret-locality). Coord.: N 42° 29,322', E 02° 44,705'. Elev.: 120 m. Exp.: NE. Tot. traps: 203.

Station No. 10 – Céret-locality. Coord.: N 42° 29,430', E 02° 44,800'. Elev.: 120 m. Exp.: E. Tot. traps: 305.

Station No. 11 – R D63 (Le Vila-Taillet). Coord.: N 42° 30,194', E 02° 42,487'. Elev.: 235 m. Exp.: S. For. veg.: *Qs*, *Qp*, *Qi*. Tot. traps: 144.

Station No. 12 – R D63 (Le Vila-Taillet). Coord.: N 42° 30,182', E 02° 42',506'. Elev.: 240 m. Exp.: S. For. veg.: *Qs*, *Qi*, *Qp*. Tot. traps: 144.

Corine biotope habitats

A 2,000 m² area was delineated around each sampling wall to integrate the different natural or manmade habitats that could have an impact on sandfly abundance. These habitats (including walls and access roads) were classified according to Corine Biotope codes [1, 36]. Most of them corresponded to synsystematic units (phytosociological classification of J. Braun-Blanquet). For each station, the cover of each habitat was expressed as a percentage occupation within the delineated areas. The weep-hole retaining walls and access roads accounted for 18% of the overall area.

Habitat/station/sandfly relationships were described by normalized principal component analysis (PCA) [7, 34] on a matrix designed to correlate 12 sampling station records with 19 Corinne Biotope habitats (Table 1, Figures 9 and 10). In

Table 1. Frequency (% cover per 2,000 m²) of Corine Biotope habitats in the 12 sampling stations (including trapping walls and access roads). These stations were highly modified by human activities, but holm oaks were still well represented in most of them (20–40%).

Corine Biotope codes	Corine Biotope habitats	St.	1	2	3	4	5	6	7	8	9	10	11	12	Average (%)
		Elev. Exp.	150 m S.E.	140 m E.	280 m S.	260 m S.	220 m S.E.	200 m S.E.	180 m S.E.	200 m S.E.	120 m N.E.	120 m E.	235 m S.	240 m S.	
86.43	Open areas		15%	15%	20%	30%	15%	15%	15%	20%	25%	20%	15%	15%	18%
86	Towns, villages		30	30	•	15	50	25	•	•	60	60	•	•	18
45.3	<i>Quercion ilicis</i>		20	20	35	•	10	40	30	•	•	•	20	40	26
85.3	Gardens		•	20	•	•	•	•	•	15	15	20	•	•	6
41.85	<i>Celtis australis</i>		•	15	•	15	•	20	•	•	•	•	•	•	4
84.1	Tree rows		•	•	3	•	10	•	15	•	•	•	•	•	4
87.2	Ruderal communities		•	•	15	•	•	•	•	15	•	•	•	10	3
83.15	Fruit orchards		20	•	•	25	•	•	•	•	•	•	•	•	4
83.324	<i>Robinia pseudacacia</i>		10	•	15	•	•	•	•	30	•	•	•	•	3
32.21C	<i>Osyris</i> brush		•	•	•	•	5	•	5	•	•	•	•	•	0.8
45.216	<i>Quercus suber</i>		•	•	7	•	•	•	•	•	•	•	5	35	4
83.211	Traditional vineyards		•	•	•	15	•	•	•	•	•	•	•	•	1
83.3113	<i>European cypress</i>		•	•	•	•	10	•	•	•	•	•	•	•	0.8
62.2	Vegetated siliceous cliffs		•	•	•	•	•	•	35	•	•	•	•	•	2
44.5	<i>Osmundo-Alnion</i>		•	•	•	•	•	•	•	15	•	•	•	•	1
31.831	Bramble thickets		•	•	•	•	•	•	•	5	•	•	•	•	0.4
83.3112	Native pine plantations		•	•	•	•	•	•	•	•	•	•	30	•	2.5
41.714	<i>Quercus pubescens</i> forest		•	•	5	•	•	•	•	•	•	•	30	•	2.5
32.A	<i>Spartium junceum</i>		5	•	•	•	•	•	•	•	•	•	•	•	0.4

Table 2. Annual frequencies of the three sandfly species (*Phlebotomus ariasi*, *P. perniciosus*, *Sergentomyia minuta*) caught at the 12 selected sampling stations (“rational choice” method). There were considerable differences in sampled sandfly abundances between these stations (1.36–268.57 P/m²).

Stations	1	2	3	4	5	6	7	8	9	10	11	12	Total
Number of traps	489	73	386	254	332	678	543	712	203	305	144	144	4263
Surface area (m ²)	39.12	5.84	30.88	20.32	26.56	54.24	43.44	56.96	16.24	24.4	11.52	11.52	341.04
<i>P. ariasi</i>	47	27	382	1049	44	60	53	16	46	11	103	459	2297
P/m ²	1.2	4.62	12.37	51.62	1.65	1.1	1.22	0.28	2.83	0.45	8.94	39.84	6.73
<i>P. perniciosus</i>	38	8	32	188	10	25	8	5	19	12	26	12	383
P/m ²	0.97	1.36	1.03	9.25	0.37	0.46	0.18	0.08	1.16	0.49	2.25	1.04	1.12
<i>S. minuta</i>	408	125	280	911	556	7	16	57	89	84	2965	102	5600
P/m ²	10.42	21.4	9.06	44.83	20.93	0.12	0.36	1.00	5.48	3.44	257.37	8.85	16.42
P. total	493	160	694	2148	610	92	77	78	154	107	3094	573	8280
P/m ²	12.6	27.39	22.47	105.70	22.96	1.96	1.77	1.36	9.48	4.38	268.57	49.73	24.27

order to determine potential links between vegetation complexes and vector productivity, total sandfly densities were projected on the first PCA axis which pooled all of the main floristic characteristics in the records (ADE[®]4 software).

Results and Discussion

1. At the end of the survey, 8,280 sandflies (P) were caught in a total of 4,263 traps (Table 2). When calculated in terms of m² of sticky paper (both sides), the annual cumulative frequency of *P. ariasi*, *P. perniciosus* and *Sergentomyia minuta* was 24.27 P/m². *S. minuta* was more abundant (5,600 specimens: 16.42 P/m²), followed by

P. ariasi (2,297 specimens: 6.73 P/m²), and *P. perniciosus* (383 specimens: 1.12 P/m²). Stations with the highest abundance of *P. ariasi* (>8 P/m², No. 3, 4, 11, 12) were south-facing. For *S. minuta*, stations 4 and 11 also had the highest abundances (44.83 P/m² and 257.37 P/m², respectively).

2. The cumulative monthly total for the 12 stations was used to plot annual activity curves for both vectors. At Vallespir, *P. ariasi* thus had a bimodal distribution pattern, with the second peak in early autumn, whereas this species had a unimodal distribution in the Cévennes region (Figure 4). This pattern was likely due to bioclimatic differences, as indicated by the presence of cork oak, since the Pyrénées-Orientales stations had much milder

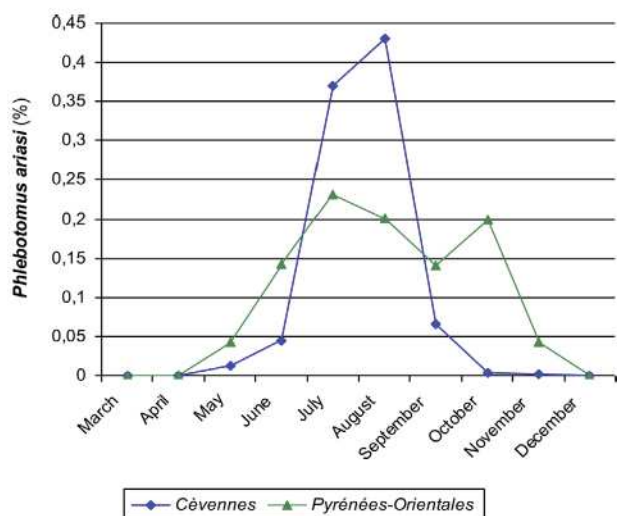


Figure 4. Monthly frequency distribution of *Phlebotomus ariasi* ♂ + ♀ (sampling with sticky-paper traps): in the Cévennes region in 1960 and in Pyrénées-Orientales region in 1981. The subhumid bioclimatic conditions in the Pyrénées-Orientales could explain the bimodal distribution pattern and the autumn frequency spread.

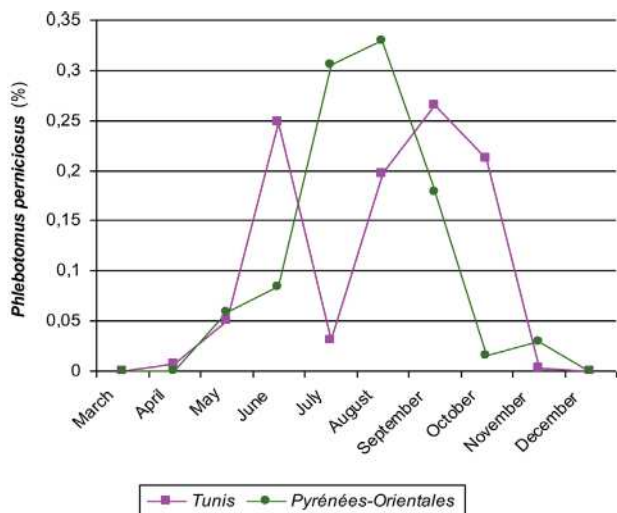


Figure 5. Monthly frequency dynamics of *Phlebotomus perniciosus* in Pyrénées-Orientales region (1981) and in Tunisia (1960). In the vicinity of Tunisia, under a semiarid Mediterranean bioclimate, these frequencies had a biphasic distribution pattern (summer diapause?). At Vallespir, in subhumid bioclimatic conditions, a unimodal distribution was noted.

climatic conditions than the southern Cévennes. These differences could be explained by the fact that *P. ariasi* thrives in humid-subhumid climatic conditions [23]. A similar phenomenon was observed with *P. perniciosus*, which is the most thermophilic sandfly species. At Vallespir, as in the Cévennes, this species had a unimodal distribution, while it has a bimodal pattern in the vicinity of Tunisia, where semiarid climatic conditions prevail (Figure 5) [3]. However, there could have been a temporal shift in the maximum activity peaks between

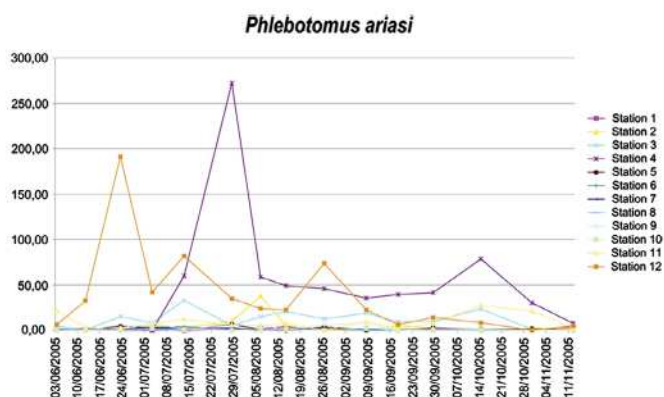


Figure 6. 10-day frequency distribution for *Phlebotomus ariasi* at the 12 sampling stations. The stations with the highest abundance (No. 4 and No. 12) showed a marked shift in maximum peak abundance: in July-June for No. 14 and July for No. 4. There was a similar shift in the lower second peak. This shift was likely related to the characteristics of the two trapping walls.

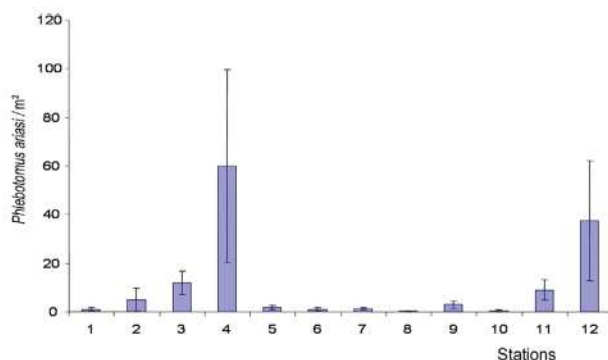


Figure 7. *Phlebotomus ariasi*: mean annual density per station (P ♂ + ♀ / m²). These frequencies did not have a normal distribution (Kolmogorov-Smirnov test, $p < 0.01$). The Friedman test, $p < 0.001$ (nonparametric, matched by date), showed that the frequencies were significantly different between the 12 stations, i.e. each station had its own specific sandfly density.

stations. At station No. 12, the peak for *P. ariasi* was thus around 20 June, whereas it occurred around 20 July at station No. 4 (Figure 6). Excluding the fact that the corresponding walls had identical exposure, this shift could have been due to other factors, such as the extent of solar radiation or the presence of vertebrate hosts.

3. *P. ariasi* was clearly the most abundant of the two vectors present at the site: for 4,263 sticky paper traps (surface area 341.04 m²), a total of 2,297 specimens (6.73 P/m²) were sampled, as compared to only 383 for *P. perniciosus* (1.12 P/m²). Moreover, there were substantial between-station differences in densities of both species. At stations No. 4 and No. 12, for *P. ariasi*, a total of 51.62 P/m² and 39.84 P/m², respectively, were sampled, whereas at the 10 other sites, the frequencies never exceeded 13 P/m². For *P. perniciosus*, the two stations with the greatest densities (No. 4, No. 11) had a total of 9.25 P/m² and 2.25 P/m²,

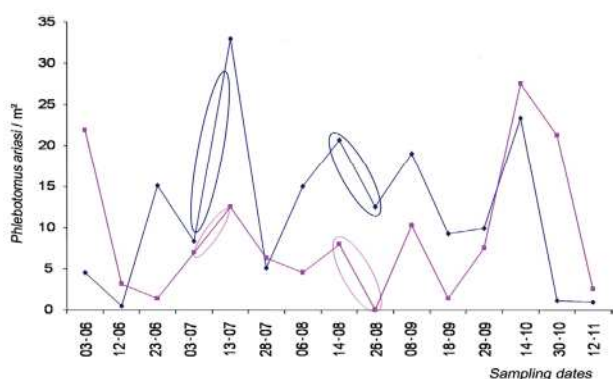


Figure 8. 10-day variations in *Phlebotomus ariasi* densities—sampling results for stations No. 3 (red) and No. 11 (blue). The 10-day variation patterns did not significantly differ between stations (Cochran’s Q test, $p = 0.949$, nonparametric, matched by date).

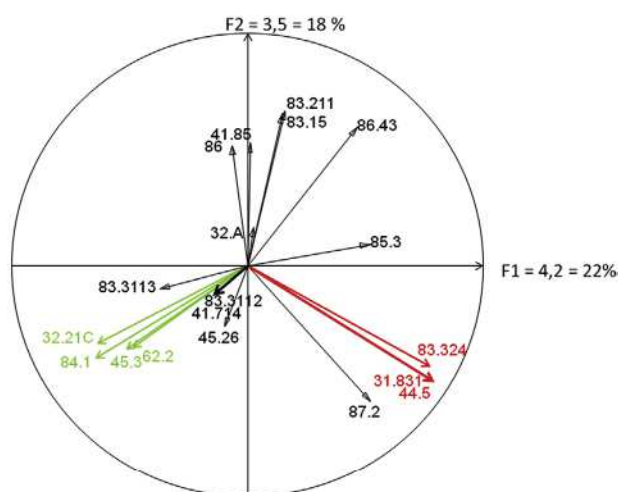


Figure 9. Circle of correlation from a normalized PCA based on the data in Table 1 (19 Corine Biotope codes \times 12 sampling station records). These correlations led to the identification of the Corine Biotope habitats most frequently associated in each of the 12 records. The F1 axis thus compares records containing wild *Robinia* stands (83.324), western Mediterranean riparian forests (44.5) and brambles (31.831) with those containing *Osyris alba* brush (32.216), tree rows (84.1), and holm oak thickets (45.3) and vegetation cover on siliceous rocks (62.2). The F2 axis compares records that associate orchards (83.15) and vineyards (83) with those that do not. The method used to differentiate the stations according to their phytoecological profile ... and nothing else!

respectively, whereas the frequencies at the remaining stations ranged from 0.08 P/m² (No. 8) and 1.36 P/m² (No. 2) (Table 2).

4. The statistical analyses confirmed these findings. The comparison of vector densities revealed significant differences between the 12 stations, i.e. each had a specific sandfly abundance pattern with respect to both *P. ariasi* (Figure 7) and *P. perniciosus*.
5. Moreover, although the sandfly densities were specific to each station, this was not the case for their variations. Between samplings (10-day sampling periods), the

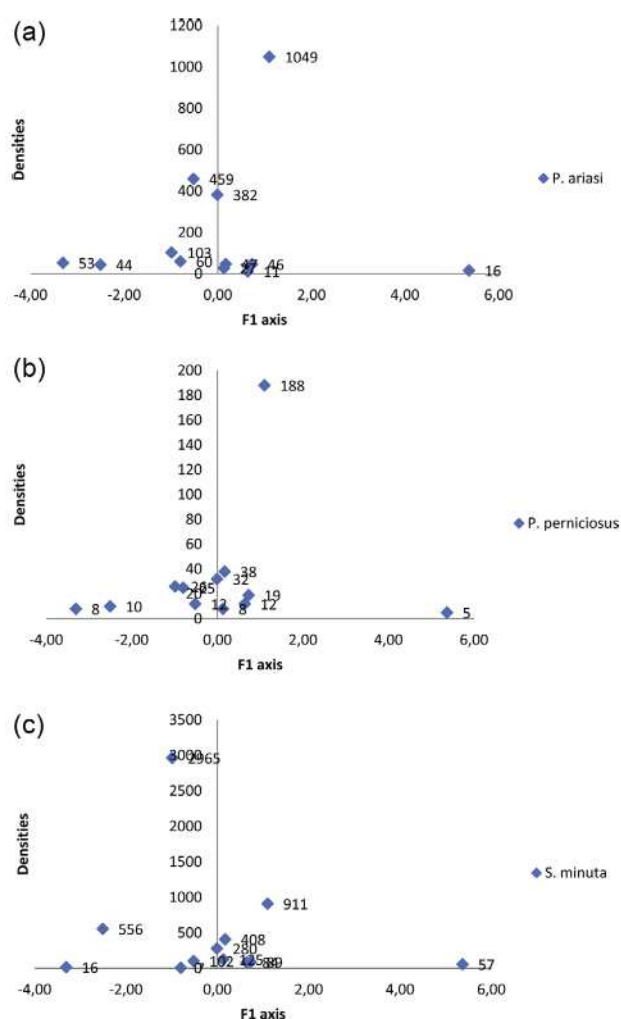


Figure 10. Projection of *Phlebotomus ariasi* (a), *P. perniciosus* (b) and *Sergentomyia minuta* (c) densities at sampling stations on the normalized PCA F1 axis. Among the readings grouped at the origin on the F1 axis, there are two habitat groups that are opposed in Figure 9 (circle of correlations). There is therefore no gradual relationship between the vector abundance and the environmental (habitat) trends. Corine Biotope habitats thus do not seem to be suitable for drawing up a vector sampling plan.

variation trends were not significantly different: the curves were parallel and in the same direction for the 12 stations (Figure 8).

6. Projection of the vector abundances on the first PCA axis did not reveal any relationship between the sampled sandfly abundance of the trapping walls and their phytoecological environment, expressed in terms of the cover of the different Corine Biotope habitats (Figures 9, 10).

Conclusion and prospects

The present results led to the following conclusions:

- At the study site, the per-station *P. ariasi* abundances were significantly higher than those of *P. perniciosus*. However, this species was not as scarce as it was in the southern

Cévennes region. This difference could be explained by the bioclimatic conditions, i.e. humid Mediterranean climate in the Cévennes and subhumid at Vallespir. These climatic differences could also explain the *P. ariasi* variation patterns, i.e. monophasic in the Cévennes and diphasic at Vallespir.

- The significant between-station differences in sampled sandfly abundance were due to the specific environmental conditions at each station (geopedology, orientation and shadows cast on the trapping wall, animal occupation of the weep holes, nearby livestock) [18, 28]. Note that the weep holes served as roosting sites, not as breeding sites
- During many surveys, our attempts to isolate larvae or nymphs in weep-hole soil were always fruitless. However, the constant high abundance sampled in some especially attractive walls could be explained by the high movement capacity of imagos [30].
- The parallelism in the 10-day variations could have been due to a single factor, i.e. likely weather related (storm, heat wave, etc.), taking place accidentally and sporadically at all stations.
- The multivariate analysis did not reveal any association between sandfly frequencies and the phytoecological environment of the sampling stations (Figures 9 and 10). As we noted, these associations occurred with certain physical or biotic properties specific to each wall. More generally, the cartographic typology of the area according to the Corine Biotope system or its derivatives (Corine Land Cover, EUNIS [15, 17]) cannot be recommended as a vector abundance indicator, especially in the definition of zero points. By their phytosociological nature, the corresponding maps should be used for studies on landscape modifications resulting from climate change, fire, flooding, cropping or non-native plant invasions. However, these maps have a real value in applied research, e.g. in forest management (see French Forestry Office). Finally, they can still be used as density indicators to characterize certain zoological groups on the condition that they are closely tailored to specific botanical taxa or syntaxa. This category includes strict pests, specific pollinators, specialized herbivores and animals with a narrow ecological niche. In other cases, caution is necessary [21], especially since Corine Biotope maps are not the only way to express vegetation-indicator relationships. This was the case in studies carried out in Morocco to identify the preferred bioclimatic conditions of sandflies. In this country, the only phytoclimatic map, drawn up on the basis of vegetation layers (from humid to arid), made it possible to attribute a bioclimatic value to each inventoried sandfly species, and especially to specify the current or future geographical distribution (global warming) of *Leishmania* spp. vectors [23]. Otherwise, in metropolitan France, a very successful phytoecological map was drawn up of biotopes for pre-adults of *Aedes* spp. (Diptera-Culicidae). The detection of eggs in the litter of halophyte plants revealed a close relationship between some plant species (*Salicornia* spp., *Scirpus maritimus*) and certain Aedinae species (*Aedes caspius*, *A. detritus*). This relationship was dependent on the egg-laying behaviour of gravid females, which were found to often only lay

eggs on one or two species of plants growing along narrow strips in lagoon environments. The focus has thus readily shifted from “egg laying sites” to the phytoecological mapping of “breeding sites”, which has turned out to be a remarkable operational tool that has been very successful in the control of pest mosquitoes in Mediterranean coastal regions [10, 24, 25].

- Finally, the current results should be considered as a starting point for further research. At Vallespir, after more than 30 years, the same protocol should be applied at the same sampling stations. This type of operation was recently undertaken in the lower Cévennes region [19, 31], which generated promising results. At Vallespir, if the sandfly density modifications and geographical distributions noted here are confirmed, it would be of interest to supplement this work by more in-depth taxonomic and genetic analyses, both in terms of specific taxa (*P. sergenti* is already present in Pyrénées-Orientales region [29]), and within the same species (modification in population-based polymorphism, selection of thermophilic variants, drift [30], etc.). Future epidemiological research focused on the impact of climate change or of agronomic-silvicultural modifications should be very cautiously carried out, especially with respect to vector sampling and the use of phytoclimatic maps as vector density indicators.

Acknowledgements. We warmly thank Anne-Laure Bañuls, Bulent Alten, Nathalie Barras, Jean Cousserans, Henri Descamps, Jean-Charles Gantier, Christian Jean, Nicole Léger, Michele Maroli, Georges Metge, Pierre Quézel, Joseph Travé and Marco Zito.

References

1. Bissardon M, Guibal L. 1997. Corine biotopes, Version originale. Types d'habitats français. École Nationale du Génie Rural, Nancy.
2. Croset H, Rioux JA, Léger N, Houin R, Cadi-Soussi M, Benmansour N, Maistre M. 1977. Les méthodes d'échantillonnage des populations de Phlébotomes en Région méditerranéenne, Écologie des leishmanioses. Centre National de la Recherche Scientifique, Paris, 239, 139–151.
3. Croset H, Rioux JA, Juminer B, Tour S. 1970. Fluctuations annuelles des populations de *Phlebotomus perniciosus* Newstead, 1911, *Phlebotomus perfiliewi* Parrot, 1930 et *Sergentomyia minuta parroti* (Adler et Theodor, 1927) [Diptera : Psychodidae] en Tunisie du Nord, Archives de l'Institut Pasteur de Tunis, 47, 43–56.
4. Daget P. 1977. Le bioclimat méditerranéen: analyse des formes climatiques par le système d'Emberger. Vegetatio, 34, 87–103.
5. Dereure J, Vanwambeke S, Malé P, Martinez S, Pralong F, Balard Y, Dedet JP. 2009. The potential effects of global warming on changes in canine leishmaniasis in a focus outside the classical area of diseases in southern France. Vector-borne and zoonotic diseases, 9, 687–694.
6. Diderot D. 1753. Pensées sur l'interprétation de la nature, Paris, 99 p.
7. Dray S, Dufour AB. 2007. The ade4 package: implementing the duality diagram for ecologists. Journal of Statistical Software, 22, 1–20.

8. Emberger L. 1930. Sur l'étage de végétation. Comptes rendus de l'Académie des Sciences, 191, 389–390.
9. Emberger L. 1955. Une classification biogéographique des climats. Recherches et Travaux du Laboratoire de Botanique, Géologie, et Zoologie de la Faculté des Sciences de Montpellier, 7, 3–43.
10. Gabinaud A. 1975. Écologie de deux *Aedes* halophiles du littoral méditerranéen français, *Aedes (Ochlerotatus) caspius* (Pallas, 1771), *Aedes (Ochlerotatus) detritus* (Haliday, 1833) (Nematocera Culicidae). Utilisation de la végétation comme indicateur biotique pour l'établissement d'une carte écologique. Application en dynamique des populations, Thèse Sciences, Université des Sciences et techniques (USTL), Montpellier, (enregistrée au Centre National de la Recherche Scientifique A.O.11990), 451 p.
11. Giraudoux P, Raoul F, Pleydell D, Craig PS. 2008. Multidisciplinary studies, systems approaches and parasite eco-epidemiology: something old, something new. Parasite, 15, 469–476.
12. Gounot M. 1969. Méthodes d'étude quantitative de la végétation. Masson, Paris, 314 p.
13. Gaussen H. 1970. Carte de la végétation de la France. 2ème édition, Centre National de la Recherche Scientifique (CNRS), Toulouse, 75.
14. Hartemink N, Vanwambeke S, Heesterbeek H, Rogers D, Morley D, Pesson B, Davies C, Mahamdallie S, Ready P. 2011. Integrated mapping of establishment risk for emerging vector-borne infections: a case study of canine leishmaniasis in southwest France. PLoS One, 6, e20817.
15. Institut Français de l'Environnement. 2011. L'utilisation de Corine land cover 2000, Institut Français de l'Environnement (IFEN), Orléans, 2007–2011.
16. La Rocque de S, Rioux JA, Slingenbergh J. 2008. Climate change: effects on animal disease systems and implications for surveillance and control. Revue des sciences et des techniques, Office International des Epizooties, 27, 339–354.
17. Louvel J, Gaudillat V, Poncet L. 2013. EUNIS, Système d'information européen sur la nature, Classification des habitats, Traduction française. Muséum National d'Histoire Naturelle: Paris, 289 p.
18. Maroli M, Rossi L, Baldelli R, Capelli G, Ferroglio E, Genchi C, Gramiccia M, Mortarino M, Pietrobelli M, Gradoni L. 2008. The northward spread of leishmaniasis in Italy: evidence from retrospective and ongoing studies on the canine reservoir and phlebotomine vector. Tropical Medicine and International Health, 13, 256–264.
19. Prudhomme J, Rahola N, Senghor M, Elgara E, Alten B, Serano D, Banuls AL. 2012. Spatiotemporal dynamic and bio-ecology of sandflies in the region of Montpellier. E-Sove, the 18th International Conference, Montpellier, p. 17.
20. Quézel P, Médail F. 2003. Écologie et biogéographie des forêts du Bassin méditerranéen. Elsevier, 571 p.
21. Quézel P, Verdier P. 1953. Les méthodes de la phytosociologie sont-elles applicables à l'étude des groupements animaux ? Quelques associations rupicoles de Carabiques dans le Midi de la France et leurs rapports avec les groupements végétaux correspondants. Vegetatio, 4, 165–181.
22. Ready P, Ready PA. 1981. Prévalence of *Phlebotomus* spp. in southern France: sampling bias due to different man-biting habits and autogeny. Annals of Tropical medicine and Parasitology, 75, 475–476.
23. Rioux JA. 2001. Trente ans de coopération franco-marocaine sur les leishmanioses : dépistage et analyse des foyers. Facteurs de risque. Changements climatiques et dynamiques nosogéographiques. Association des Anciens Élèves de l'Institut Pasteur, 168, 90–100.
24. Rioux JA, Cousserans J. 2008. La « démostication » du littoral méditerranéen : base écologique, stratégie opérationnelle, devenir. Bulletin de l'Académie des Sciences et Lettres de Montpellier, NS, 39, 343–356.
25. Rioux JA, Croset H, Corre JJ, Simmoneau P, Gras G. 1968. Phyto-ecological basis of mosquito control: Cartography of larval biotopes. Mosquito News, 28, 572–582.
26. Rioux JA, La Rocque de S. 2003. Climats, leishmanioses et trypanosomoses. Changements climatiques, maladies infectieuses et allergiques, Sciences Médicales, Paris, 41–62.
27. Rioux JA, Golvan YJ, Croset H, Houin R, Juminer B, Bain O, Tour S. 1967. Écologie des leishmanioses dans le Sud de la France. 1. Les Phlébotomes. Échantillonnage, éthologie. Annales de Parasitologie humaine et comparée, 42, 561–603.
28. Rioux JA, Golvan YJ, Croset H, Tour S, Houin R, Abonnenc E, Petitdidier M, Vollhardt Y, Dedet JP, Albaret JL, Lanotte G, Quilici M. 1969. Épidémiologie des leishmanioses dans le Sud de la France, Préface N. Ansari, Monographie de l'Institut National de la Santé et de la Recherche Médicale (INSERM), Paris, 217 p.
29. Rioux JA, Jarry D, Maazoun R, Wallbanks K. 1982. Confirmation de l'existence en France continentale de *Phlebotomus sergenti* Parrot, 1917. Annales de Parasitologie humaine et comparée, 57, 647–648.
30. Rioux JA, Killick-Kendrick R, Leaney AJ, Turner DP, Bailly M, Young CJ. 1979. Écologie des leishmanioses dans le Sud de la France. 12. Dispersion horizontale de *Phlebotomus ariasi* Tonnoir, 1921. Expériences préliminaires. Annales de Parasitologie humaine et comparée, 54, 673–682.
31. Rioux JA, Killick-Kendrick R, Périères J, Turner DP, Lanotte G. 1980. Écologie des leishmanioses dans le Sud de la France. 13. Les sites de « flanc de coteau », biotopes de transmission privilégiés de la leishmaniose viscérale en Cévennes. Annales de Parasitologie humaine et comparée, 55, 445–453.
32. Rioux JA, Périères J, Killick-Kendrick R, Lanotte G, Bailly M. 1982. Écologie des leishmanioses dans le Sud de la France. 17. Échantillonnage des Phlébotomes par le procédé des pièges adhésifs. Comparaison avec la technique de capture sur appât humain. Annales de Parasitologie humaine et comparée, 57, 631–648.
33. Siegel S, Castellan NJ. 2000. Nonparametric statistics for the behavioral sciences, 2nd ed. McGraw-Hill, USA, 399 p.
34. Thioulouse J, Chessel D, Dolédec S, Olivier JM. 1997. ADE-4: a multivariate analysis and graphical display software. Statistics and Computing, 7, 75–83.
35. Zar JH. 1999. Biostatistical Analysis, 4th ed. Prentice-Hall: USA, 663 p.
36. Zeraia L. 2007. Inventaire floristique, écologique et cartographique des groupements végétaux des sites « Ausières » et « Quatorze » en vue de la mise en place de deux puits de carbone par reboisement adapté. Rapport de l'Office National des Forêts (ONF), 49 p.

Cite this article as: Rioux J.-A., Carron S, Dereure J, Périères J, Zeraia L, Franquet E, Babinot M, Gállego M & Prudhomme J: Ecology of leishmaniasis in the South of France. 22. Reliability and representativeness of 12 *Phlebotomus ariasi*, *P. perniciosus* and *Sergentomyia minuta* (Diptera: Psychodidae) sampling stations in Vallespir (eastern French Pyrenees region). Parasite, 2013, 20, 34.



An international open-access, peer-reviewed, online journal publishing high quality papers on all aspects of human and animal parasitology

Reviews, articles and short notes may be submitted. Fields include, but are not limited to: general, medical and veterinary parasitology; morphology, including ultrastructure; parasite systematics, including entomology, acarology, helminthology and protistology, and molecular analyses; molecular biology and biochemistry; immunology of parasitic diseases; host-parasite relationships; ecology and life history of parasites; epidemiology; therapeutics; new diagnostic tools.

All papers in Parasite are published in English. Manuscripts should have a broad interest and must not have been published or submitted elsewhere. No limit is imposed on the length of manuscripts.

Parasite (open-access) continues **Parasite** (print and online editions, 1994-2012) and **Annales de Parasitologie Humaine et Comparée** (1923-1993) and is the official journal of the Société Française de Parasitologie.

Editor-in-Chief:
Jean-Lou Justine, Paris

Submit your manuscript at
<http://parasite.edmgr.com/>