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Ankara, January 15 , 2025

Subject: Report regarding PhD thesis of Kamal Eddine Benallal entitled *"**The life cycle of leishmaniasis in Algeria**"*
Supervisor: doc. RNDr. Vitek Dvorak, Ph.D
Co-supervisor: doc. RNDr. Jovana Sadlova, Ph.D

Dear Prof. Jan Votypka
Chairman of the examination board

After evaluation of the thesis of Kamal Eddine Benallal entitled *'"The life cycle of leishmaniasis in Algeria"*, please find in attachment the assessment report prepared by me. I, with great pleasure, report that the thesis makes a significant contribution to knowledge, makes a significant contribution to the understanding of the subject with which deals, demonstrates the candidate's capacity to carry out independent research, contains material worthy of publications, and that the format and literary presentation of the thesis are satisfactory.

I recommend that the candidate be awarded the degree of PhD.

I enclose a list of my overall assessment, comments, corrections, and questions according to the rules for PhD thesis at the Charles University.

Prof.Dr. Salih Bülent Alten
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REPORT:

A) Overview and summary of the thesis

The document is a Ph.D. thesis on *"The life cycle of leishmaniasis in Algeria,"* authored by Kamal Eddine Benallal from the Department of Parasitology at Charles University. It primarily investigates leishmaniasis, a neglected tropical disease, focusing on the parasite *Leishmania*, its transmission, and associated vector, the sand fly. Here is an initial overview:

1. **Abstract**:
    - Summarizes four projects related to sand flies' fauna in Algeria, the parasite-vector-host interaction, potential reservoir hosts, and drug resistance in *Leishmania major*.
    - Highlights the identification of new sand fly species, the use of DNA barcoding and MALDI-TOF for species and blood meal source identification, and insights into drug-resistant strains in sand flies.
2. **Introduction and Background**:
    - Provides an in-depth look at *Leishmania* parasites, various forms of leishmaniasis (cutaneous, mucocutaneous, and visceral), and its prevalence globally and specifically in Algeria.
    - Discusses the sand fly lifecycle, the biology of *Leishmania* transmission, and drug resistance challenges.
3. **Research Goals**:
    - Aimed at expanding the database of sand fly species, understanding the epidemiology of *Leishmania major*, and assessing asymptomatic carriers' roles in disease transmission.
4. **Findings and Discussions**:
    - Presents experimental findings, including potential new reservoir hosts for *Leishmania major* and evidence for sand flies supporting the transmission of drug-resistant strains.

B) Detailed Review and Critique of Ph.D. Thesis on *The Life Cycle of Leishmaniasis in Algeria*

1. Methodology

Strengths:

- **DNA Barcoding and MALDI-TOF**: The thesis employs advanced DNA barcoding alongside MALDI-TOF mass spectrometry to accurately identify sand fly species and analyze their blood meal sources. This combination of methods provides a dual-layer approach, enhancing the precision of species identification and the accuracy in determining feeding behavior. This is particularly important in regions with diverse vector and host populations.

- **Use of Experimental Infections and Xenodiagnosis**: The author uses laboratory-controlled infection and transmission studies to explore the host-parasite interactions and confirm reservoir competency of certain rodent species. This rigorous experimental approach provides robust data on host-parasite dynamics and is a strength of this work.

Potential Limitations and Suggestions:

- **Species Misidentification and Detection Sensitivity**: While DNA barcoding is highly accurate, discussing potential limitations, such as difficulty in distinguishing closely related species or detection threshold, would add transparency. For example, misidentification can occur if reference libraries are incomplete, or if variations within the *Leishmania* species are not accounted for.

- **Sample Representativeness and Statistical Power**: Further explanation regarding the sample sizes for sand flies and host species across diverse environments (e.g., rural vs. urban) could add to the reliability of the findings. Small sample sizes may limit the statistical power and could bias results, particularly in identifying opportunistic feeding patterns.

2. Key Findings and Analysis

New Species Discovery:

- **Significance**: The discovery of *Sergentomyia (Sergentomyia) imihra* n. s.p., a new sand fly species, expands the taxonomy of vector species in Algeria and potentially broadens the scope of vector studies in North Africa. The work contributes to the understanding of vector biodiversity, which is essential for developing region-specific control strategies.

- **Ecological and Epidemiological Insights**: The thesis suggests that several sand fly species feed on a wide range of hosts, including livestock and humans. This behavior indicates high zoonotic transmission potential, especially in settings with close human-animal interactions. Quantifying these feeding preferences by geographic region would enhance the thesis's impact by identifying areas of higher zoonotic risk.

Host-Reservoir Interaction:

- **Role of *Gerbillus amoenus* as a Reservoir**: The experimental infection studies demonstrate that *Gerbillus amoenus* could act as a reservoir for *Leishmania major*, filling gaps where other known reservoirs are absent. This finding holds significant epidemiological value and suggests that disease modeling in Algeria may need to be revised to include this species.

- **Prevalence in Wild Populations**: While the thesis confirms reservoir competence experimentally, additional research into the natural infection rates of *Gerbillus amoenus* in the wild would bolster the claim. Including prevalence data would give more insight into how often this rodent acts as a reservoir in natural settings versus under controlled conditions.

Drug Resistance Findings:

- **Transmission of Drug-Resistant *Leishmania major* Strains**: The research provides crucial evidence that sand flies can host and transmit Sb(III)-resistant strains of *Leishmania major*, shedding light on the persistence and transmission potential of resistant strains in the wild. This finding is vital for understanding resistance dynamics and supports the need for drug-resistance monitoring.

- **Environmental and Genetic Influences on Resistance**: The thesis could further examine environmental or genetic factors affecting resistance development. For example, geographic differences in resistance patterns may inform targeted interventions and could be explored in future work.

3. Implications and Recommendations

Public Health Recommendations:

- **Targeted Vector Control**: Given the opportunistic feeding behaviors of sand flies on various hosts, public health strategies could focus on vector control measures tailored to high-risk settings. For example, in areas with close human-livestock proximity, control efforts may include animal-specific measures alongside traditional human-focused interventions.

- **Inclusion of *Gerbillus amoenus* in Epidemiological Models**: Including this rodent species as a potential reservoir in disease modeling for Algeria could improve predictions of leishmaniasis outbreaks and inform reservoir-targeted control strategies, such as habitat management or targeted baiting campaigns.

Future Research Directions:

- **Expansion on Host-Reservoir Dynamics**: Exploring the role of asymptomatic carriers and environmental influences on vector distribution could provide a more holistic view of leishmaniasis transmission. This might involve broader field studies across diverse ecological zones to better understand the distribution and prevalence of reservoir hosts.

- **Monitoring of Drug-Resistant Strains**: Establishing regular monitoring of drug-resistant *Leishmania* strains in both vectors and mammalian hosts could aid in early detection and containment of resistant parasites. Public health authorities could use this data to develop resistance management programs, potentially delaying or preventing resistance spread.

Summary

Overall, this thesis offers valuable insights into leishmaniasis in Algeria, with significant contributions to vector and reservoir understanding, as well as drug resistance dynamics. The work's use of advanced identification techniques, controlled experiments, and analysis of host-vector-parasite relationships makes it a notable contribution to parasitology and epidemiology. By addressing the outlined areas for further research and considering a few methodological expansions, future studies could build on this work to enhance leishmaniasis control efforts in Algeria and similar endemic regions.

C) Papers: (unordered)

Paper 1:

The document is a research article titled *"Phlebotomine sand fly survey, blood meal source identification, and description of Sergentomyia imihra n. sp. in the central Sahara of Algeria"* published in *Parasites & Vectors*. It details an entomological study on sand flies in Algeria, focusing on species identification, blood meal sources, and the discovery of a new sand fly species, *Sergentomyia imihra n. sp.*

Here is a comprehensive critique and feedback based on the study's methodology, findings, and implications:

Review and Feedback

1. Methodology

- **Sample Collection**: The study collected 640 sand fly specimens from Ghardaïa and Illizi, using light traps over multiple nights, which is robust for capturing species diversity. The detailed geographic and environmental context provided for each location strengthens the study's foundation.

- **Identification Techniques**: Combining morphological assessment with DNA barcoding and MALDI-TOF mass spectrometry (MS) enhances the reliability of species identification. This integrative approach addresses morphological ambiguities, particularly useful in cryptic species like those in the *Sergentomyia* genus.

- **Blood Meal Analysis**: The use of peptide mass mapping (PMM)-based MALDI-TOF MS for blood meal identification is innovative, yielding high-quality spectra that identified a range of hosts. The authors effectively demonstrate the versatility of PMM-MALDI-TOF in detecting blood sources, even with advanced digestion stages, though further validation against traditional methods (e.g., ELISA) might strengthen findings.

Strengths:

- **Comprehensive Species Identification**: The study combines morphological assessment, DNA barcoding, and MALDI-TOF mass spectrometry (MS) protein profiling to identify sand fly species. This integrative approach is a strength, especially given that morphological identification alone can be insufficient for cryptic species. By leveraging DNA barcoding and MALDI-TOF, the researchers ensure accurate species identification, which is crucial for understanding sand fly diversity and taxonomy.

- **Blood Meal Analysis with MALDI-TOF PMM**: The use of peptide mass mapping (PMM) MALDI-TOF MS for blood meal identification is innovative, allowing detection of low-quantity or partially digested blood, which may be difficult with other techniques. This method adds significant value to the study, given its efficiency and accuracy in determining blood sources across different host species.

Limitations:

- **Sampling Locations and Representation**: The study collected sand flies from two provinces (Ghardaïa and Illizi), which, while providing valuable insights, may not fully represent the diversity across Algeria's varied habitats. Additional sampling in more diverse ecological zones could yield a more comprehensive understanding of sand fly fauna and host feeding behavior.

- **Temporal Limitations**: Sampling was conducted only during two months (June and October 2021), which may not account for seasonal variations in sand fly populations and feeding behavior. Sand fly activity can be highly seasonal, and year-round sampling could help to capture these fluctuations, potentially revealing species or feeding behaviors missed in the current study.

2. Key Findings

- **Species Composition and Discovery**: Fourteen sand fly species were identified, with a significant finding being the description of *Sergentomyia imihra n. sp.*, a new species distinct from related species based on both morphology and molecular data. This discovery expands the known biodiversity of sand flies in Algeria, adding to the baseline knowledge needed for future taxonomic and epidemiological studies.

- **Host Feeding Patterns**: The identification of diverse blood meal sources—goats, humans, camels, and other mammals—indicates opportunistic feeding in certain sand fly species (*Phlebotomus papatasi* and *Ph. alexandri*), a behavior that can increase zoonotic transmission risk in human-sand fly interactions. However, the study might benefit from more detailed analysis on the proportion of anthropophilic vs. zoophilic feeding patterns across different environments.

3. Analysis and Discussion

- **New Species Description**: The meticulous morphological description of *Sergentomyia imihra* (e.g., unique cibarium and pharyngeal armature features) aligns well with molecular evidence, supporting its designation as a new species. The use of ABGD and TCS network analysis adds robustness to the genetic data, confirming distinct haplotypes that support this taxonomic addition.

- **Implications for Disease Transmission**: While no *Leishmania* parasites were detected in the screened sand flies, the presence of potential vectors in areas with human leishmaniasis cases underscores the need for ongoing surveillance. The study rightly recommends further investigation into the vector competence of *Sergentomyia* species, which remains inconclusive.

4. Implications and Recommendations

- **Public Health Relevance**: The study emphasizes the zoonotic potential of sand flies in Algeria, particularly in regions with emerging leishmaniasis cases. Future research could explore ecological variables (e.g., human activity, livestock density) that influence vector feeding patterns, which would aid in targeted vector control efforts.

- **Molecular and Proteomic Techniques in Taxonomy**: The success of MALDI-TOF MS in identifying species and blood meals suggests that it could become a standard in sand fly research, complementing DNA barcoding. Expanding MALDI-TOF reference libraries to include other vectors could enhance identification accuracy and efficiency across different vector species.

Conclusion

Overall, this study makes significant contributions to vector biology and epidemiology in Algeria by characterizing sand fly fauna and revealing opportunistic feeding behaviors that impact zoonotic transmission risks. Future research should continue to focus on the ecological dynamics of sand fly populations in Algeria and the implications for leishmaniasis transmission, particularly under changing environmental conditions.

Paper 2:

The article is a systematic review focusing on phlebotomine sand flies in the Maghreb region, particularly their distribution, morphology, and roles as vectors for various pathogens, including the parasites that cause leishmaniasis.

1. Purpose and Scope

- **Objective**: The review aims to consolidate fragmented information about the sand fly species in the Maghreb (Mauritania, Morocco, Algeria, Tunisia, and Libya), creating an updated list with maps of their distribution and morphological characteristics.
- **Justification**: Leishmaniasis is a significant health problem in the Maghreb, transmitted by sand flies. These flies also carry pathogens like *Bartonella bacilliformis* and *Phlebovirus*. Regional studies on the role of sand flies in disease transmission are limited, so this review fills a critical gap.

2. Methodology

- The authors follow a systematic review protocol (PRISMA-P) and survey literature from 1900 to 2020. Data sources include both scholarly databases and institutional archives.
- **Mapping and Distribution**: Geographic data from Google Earth and ArcGIS are used to create distribution maps, illustrating the bioclimatic conditions favorable for different sand fly species.
- **Morphological Analysis**: Detailed illustrations are compiled from various sources to highlight species-specific traits, focusing on morphological differences important for taxonomic identification.

3. Findings and Discussion

- **Species Diversity**: A total of 32 sand fly species are identified, with species counts differing across countries in the Maghreb (e.g., 24 in Algeria, 23 in Morocco). Unique species are noted in each country, emphasizing regional biodiversity.
- **Vectorial Roles**: Some sand flies are confirmed vectors of *Leishmania* (causing cutaneous and visceral leishmaniasis), while others are suspected based on circumstantial evidence. The study classifies sand flies by their potential or confirmed roles in disease transmission cycles for pathogens like *Phlebovirus* and *Leishmania*.
- **Morphological Differentiation**: The review addresses morphological characteristics, especially for closely related species (e.g., *Phlebotomus papatasi* vs. *Phlebotomus bergeroti*). This is critical for accurate identification, as it impacts understanding of species-specific roles in disease spread.

4. Strengths and Limitations

- **Strengths**: The extensive period covered (120 years) and comprehensive use of morphological and distributional data provide a rich resource for researchers. The review also incorporates modern molecular identification techniques like MALDI-TOF MS, supplementing traditional morphological methods.
- **Limitations**: Data gaps remain, especially for Mauritania, where accurate distribution information is lacking. The review also notes possible underreporting of leishmaniasis cases, affecting the perceived distribution and vector roles of certain species.

  Reliance on Morphological Characteristics: The review largely depends on morphological characteristics to classify and differentiate sand fly species, a traditional but often limited approach. Sand flies show significant morphological overlap, especially in closely related species, leading to possible misidentification. The review acknowledges this and highlights the utility of molecular techniques, but it doesn't fully incorporate these modern methods or discuss their potential application in resolving ambiguous cases. Integration of molecular data and deeper discussion of its reliability could enhance the review's accuracy and taxonomic depth.

  Mapping and Distribution Limitations: The maps provided use ArcGIS and Google Earth data, which are effective tools for visualizing distribution. However, these maps may not be entirely reliable due to (a) patchy data availability, particularly for Mauritania, and (b) underreporting or absence of field data in certain regions. The review could have benefited from more robust spatial analysis, possibly using environmental data layers like climate and land use to predict potential sand fly habitats and areas of disease transmission.

5. Implications for Public Health and Future Research

- The findings underscore the need for targeted vector control and surveillance programs in the Maghreb. Detailed knowledge of sand fly species distributions and vectorial capacity can aid in mitigating the spread of leishmaniasis and other sand fly-borne diseases.
- Future research is recommended to focus on the ecological and environmental drivers influencing sand fly distribution and vectorial capacity, especially under climate change pressures which may alter habitats.

Conclusion

This systematic review provides a foundational understanding of sand flies in the Maghreb, crucial for public health efforts against leishmaniasis. It highlights the value of integrating morphological and molecular data to advance taxonomy and epidemiology in the region.

Paper 3:

This article, titled "*Infectiousness of Asymptomatic Meriones shawi, Reservoir Host of Leishmania major,*" focuses on understanding the role of asymptomatic *Meriones shawi* (a North African rodent) as a potential reservoir host in the transmission of *Leishmania major*, the causative agent of cutaneous leishmaniasis (CL). The study aims to analyze the infectiousness of these asymptomatic animals to sand flies, which are vectors for the disease.

Key elements of the study and scientific analysis are as follows:
Study Background and Relevance

Leishmania major, causing CL, is known to have rodent reservoirs, and sand flies transmit the parasite to humans. Previous assumptions were that transmission was primarily from symptomatic hosts showing skin lesions. However, this study investigates if asymptomatic animals, without visible skin lesions, also contribute to transmission.

This finding is significant because if asymptomatic animals can transmit *L. major*, they represent hidden carriers that complicate disease control efforts. It parallels findings in visceral leishmaniasis, where asymptomatic hosts have been shown to contribute significantly to transmission dynamics.

Methodology
The researchers infected 32 *Meriones shawi* with *L. major* through sand flies carrying the parasite, mimicking natural transmission. They conducted xenodiagnosis (exposing sand flies to potentially infected hosts) to determine the infectiousness of both symptomatic and asymptomatic rodents.

- **Experimental Setup**: Different infection doses were used, and animals were observed weekly for symptoms and underwent xenodiagnosis at intervals.
- **Xenodiagnostic Testing**: This involved using live sand flies to test if they could acquire the parasite from the host.
- **qPCR Analysis**: Tissue samples post-mortem confirmed the presence of *L. major* across various tissues, including skin and lymph nodes.

Key Findings and Implications

1. **High Rate of Asymptomatic Infectiousness**: Around 67% of the animals were infectious to sand flies, and 45% were repeatedly infectious, regardless of visible symptoms. This suggests that asymptomatic rodents are still capable of transmitting the parasite, challenging the notion that only animals with lesions contribute to transmission.
2. **Presence in Multiple Body Tissues**: The parasite was found in various body tissues beyond the primary inoculated site, showing its ability to disseminate within the host.
3. **No Significant Infectiousness Difference in Symptomatic vs. Asymptomatic Hosts**: Statistical analysis indicated no substantial difference in transmission rates from asymptomatic versus symptomatic animals. This undermines prior assumptions that skin lesions are necessary for effective transmission.

Scientific and Epidemiological Impact
The study highlights the potential epidemiological importance of asymptomatic hosts in sustaining and spreading *L. major*. In field settings, relying on symptom-based identification of carriers would likely miss a substantial portion of infectious animals. This could lead to underestimating the reservoir capacity of rodent populations, affecting control strategies aimed at reducing transmission.

Paper 4:

The article, *"Identification of blood source preferences and Leishmania infection in sand flies (Diptera: Psychodidae) in north-eastern Algeria,"* by Messahel et al., 2022, investigates the blood-feeding behavior and potential role of local sand fly species as vectors for *Leishmania infantum*, the causative agent of canine leishmaniasis (CanL) and a zoonotic pathogen affecting humans. This study is critical for understanding sand fly ecology in Algeria, where leishmaniasis remains a significant public health challenge, particularly in rural and agricultural areas.

Scientific Analysis

1. **Study Goals and Importance**
    - This study examines sand fly species composition, feeding behavior, and *Leishmania* infection rates in north-eastern Algeria. It is essential as it provides data on the blood-feeding preferences of sand flies, which helps clarify whether cattle or other domestic animals may serve as blood sources for sand flies and potential indirect contributors to *Leishmania* transmission.
2. **Methodology**
    - **Sampling and Identification**: Sand flies were collected using CDC light traps over the active season. They were identified morphologically, with certain specimens confirmed via DNA barcoding, ensuring accuracy in species classification.
    - **Blood Meal Analysis**: The study used peptide mass mapping (PMM)-based MALDI-TOF mass spectrometry to identify the blood sources in engorged female sand flies. This innovative approach provided high accuracy even with heavily digested samples, establishing it as a reliable tool in blood meal analysis compared to traditional PCR.
    - **Leishmania Detection**: To assess infection, *Leishmania* DNA was screened using ITS1 and kDNA primers. Pooled DNA samples from sand flies were tested, but no *Leishmania* DNA was detected, indicating low or absent infection rates in this area.
3. **Key Findings**
    - **Predominant Species and Feeding Patterns**: *Phlebotomus perniciosus* dominated the captured specimens, consistent with its role as a known vector in Algeria. Interestingly, almost all identified blood meals came from cattle, with only two samples (1.3%) from dog and sheep blood. This suggests a marked preference for cattle in rural environments.
    - **Absence of *Leishmania* in Sand Flies**: The lack of detected *Leishmania* infection among sampled sand flies could suggest a low infection rate or a dilution effect from non-competent hosts (e.g., cattle), reducing the likelihood of vector-human transmission.
4. **Implications and Further Research**
    - **Zoonotic Reservoirs and Control Measures**: The observed feeding preference for cattle raises questions about whether cattle might inadvertently influence the epidemiology of CanL in Algeria. Since cattle are not proven reservoirs of *L. infantum*, their role may be limited to providing blood meals rather than directly spreading the parasite.
    - **Enhanced Vector Surveillance**: Future studies could benefit from expanding sampling to more diverse environments and animal species to capture broader feeding patterns and vector dynamics.

- **Epidemiological Impact**: The study provides foundational data on the ecology of sand flies in Algeria, which could inform targeted vector control strategies, especially in rural areas where cattle rearing is prevalent.

In conclusion, this study offers valuable insights into the feeding behavior of sand flies in Algeria and introduces PMM-based MALDI-TOF MS as a robust method for blood meal analysis in hematophagous insects. The findings underscore the need for continued research on potential animal reservoirs and vector ecology to develop effective leishmaniasis control strategies in Algeria

Paper 5:

The article titled *"Host competence of Algerian Gerbillus amoenus for Leishmania major"* explores the potential role of the gerbil species *Gerbillus amoenus* as a reservoir host for *Leishmania major* (*L. major*), which causes cutaneous leishmaniasis (CL). The study assesses the gerbil's susceptibility to *L. major* infection, its ability to sustain and transmit the parasite to sand flies, and the parasite's spread in various tissues of the infected rodents. This work adds to the understanding of disease ecology, especially in areas where established reservoirs are absent.

Scientific Analysis

1. **Background and Relevance**

    - CL is a significant health issue in North Africa, particularly in Algeria, with more than 5,000 cases reported annually. Traditionally, rodents like *Psammomys obesus* and *Meriones shawi* have been considered primary reservoirs of *L. major*, but in regions where these species are absent, other rodents, including *G. amoenus*, may contribute to parasite maintenance.

    - This study focuses on the desert province of Illizi in Algeria, where CL cases have been documented despite the absence of known reservoir species, suggesting a possible role for *G. amoenus* as an alternative host.

2. **Methodology**

    - **Rodent Trapping and Identification**: The researchers trapped *G. amoenus* rodents in areas near human settlements. Morphological and genetic analyses, including CytB gene sequencing, confirmed the species' identity.

    - **Experimental Infection**: Seven *G. amoenus* gerbils were intradermally inoculated with *L. major* parasites to observe their susceptibility and lesion development over six months.

    - **Xenodiagnosis**: Sand flies were exposed to infected gerbils to test whether *G. amoenus* could infect sand flies with *L. major*. Surviving sand flies were dissected for microscopic examination and PCR testing.

    - **qPCR Analysis**: Quantitative PCR was used to detect and quantify *L. major* in various tissues, providing insights into the parasite's dissemination throughout the gerbil's body.

3. **Key Findings**

    - **Susceptibility to *L. major***: The study demonstrated that *G. amoenus* is susceptible to *L. major* infection, similar to BALB/c mice (a model organism for leishmaniasis studies). Infected gerbils developed lesions at the site of inoculation, with lesion progression resembling those in mice.

- **Infectiousness to Sand Flies**: *G. amoenus* retained *L. major* for up to six months post-infection and was capable of transmitting the parasite to sand flies. A 3.89% infection rate was observed in exposed sand flies, underscoring the gerbil's potential role in maintaining the parasite in natural settings.

- **Tissue Dissemination**: qPCR confirmed the presence of *L. major* in multiple tissues, with some gerbils showing high parasite loads in the spleen, tail, and paws. This widespread tissue distribution indicates that *G. amoenus* can support the parasite's persistence over an extended period, enhancing its role as a reservoir host.

4. **Implications**

    - **Reservoir Host Criteria**: This study meets the essential criteria for reservoir hosts, as *G. amoenus* can harbor *L. major* long-term, develop infections without mortality, and transmit the parasite to sand flies. This fulfills Chaves et al.'s criteria for identifying reservoir species.

    - **Epidemiological Impact**: *G. amoenus* potentially plays a significant role in CL transmission in regions lacking primary reservoir species. By maintaining the parasite and facilitating its transfer to sand flies, *G. amoenus* could help sustain transmission cycles, especially in remote desert environments with sparse populations of other rodent hosts.

    - **Control and Surveillance**: The identification of *G. amoenus* as a competent host for *L. major* suggests the need for focused CL control measures in areas where this species resides. Monitoring *G. amoenus* populations and implementing vector control around human dwellings could be crucial in CL management efforts.

5. **Future Directions**

    - Expanding research to assess the natural infection rates of *G. amoenus* in the wild and in other regions could provide a more comprehensive view of its role in CL transmission.

    - Further studies could investigate co-infection dynamics with other pathogens or environmental factors influencing reservoir competence.

Conclusion
This study offers robust evidence that *G. amoenus* can serve as a competent reservoir for *L. major* in Algeria. The species' ability to maintain and transmit the parasite highlights its potential role in the epidemiology of CL in desert regions, underscoring the need for enhanced surveillance and targeted control strategies in affected areas.

Paper 6:

1. Research Aims and Hypotheses
The study investigates the impact of antimony (Sb(III))-resistant *Leishmania major* strains on their development and infectivity within their natural vector, *Phlebotomus papatasi*. The primary aim is to understand how resistance affects parasite fitness and transmissibility in sand flies, an aspect that remains poorly studied despite its potential significance in understanding disease spread in endemic regions. The study hypothesizes that the resistance trait may alter the parasite's development in vectors, impacting infection intensity, location within the sand fly gut, morphological forms, and fitness parameters like growth rate and infectivity.

2. Methodology and Experimental Design

The methodology is thorough, involving both in vitro and in vivo experimental setups. Key methodological strengths include:

- Parasite Lines and Resistance Induction: The study uses a stepwise induction method to develop two Sb(III)-resistant strains, ensuring that resistance levels differ (SbIIIR-0.5 mM and SbIIIR-5.5 mM), which allows for comparison at varying resistance intensities. This methodological approach is valuable for observing nuanced differences in resistance effects.
- Vector-Specific Investigations: By infecting *Ph. papatasi* sand flies with these resistant and wild-type strains, the study directly simulates natural vector-host interactions, enhancing the relevance of findings to field settings.
- Quantitative Analysis of Infection: Detailed assessments of parasite loads, infection intensity, and parasite distribution within the vector gut at different time points provide high-resolution data on parasite dynamics.
- Gene Expression and IC50 Measurement: By measuring IC50 values and gene expression changes post-infection, the study robustly examines if sand fly passage affects resistance markers and viability, shedding light on the stability of the resistance phenotype.

However, several limitations in methodology should be noted:

- Single Vector Species: While *Ph. papatasi* is a primary vector for *L. major*, exploring multiple vector species could have provided a broader perspective on resistance dynamics across different host-vector systems.
- Lack of Longitudinal Study Design: This study primarily focuses on infection dynamics within one vector cycle. A longitudinal analysis across multiple sand fly generations could better illustrate the persistence of resistant strains and adaptation over time.
- Limited Environmental Simulation: Although the study attempts to replicate natural infection conditions, other environmental factors (temperature variation, sand fly immune responses) could influence the results.

3. Results and Analysis

Key Findings

- Infection and Colonization: Both resistant and wild-type strains successfully infected *Ph. papatasi*, but resistant strains exhibited reduced colonization of critical sites like the stomodeal valve, which is essential for transmission.
- Reduced Parasite Loads: Antimony resistance appears inversely correlated with parasite load in the vector. The higher the resistance, the lower the parasite density in late-stage infections, suggesting a potential trade-off between resistance and fitness within the vector.
- Stable Resistance Post-Passage: Both resistant strains retained their antimony resistance after sand fly passage, with an increased IC50, indicating the resilience of resistance traits in vector conditions. This finding raises concerns about potential field transmission of resistant parasites.
- Gene Expression Patterns: Genes associated with antimony resistance (*LmMRPA*, *LmTDR1*, *LmPRX*) were upregulated in resistant strains, with some markers (e.g., *LmMRPA*) retaining increased expression post-sand fly passage. This suggests these genes play essential roles in enabling resistance and may be potential targets for intervention.

Statistical Rigor

The study utilizes robust statistical analysis, including ANOVA and Chi-square tests, to compare infection rates, parasite loads, and morphological forms. However, some findings might benefit from additional validation via larger sample sizes or alternative statistical models to control for potential confounders (e.g., variability in vector responses to infection).

4. Interpretation and Broader Implications

This study has important implications for understanding the spread of drug-resistant *Leishmania* in endemic areas:

- Transmission Potential: The finding that resistant *L. major* strains can still infect and reach transmissible stages in sand flies, albeit at lower intensities, highlights a concerning risk of drug-resistant parasites circulating in the field. Although the fitness costs in the vector might limit resistance spread, the stable resistance phenotype post-sand fly passage implies that these strains could still contribute to treatment failure in humans and animals.

- Vector-Induced Selection Pressure: The study's observation that sand fly passage may select for even higher resistance levels (as seen in the IC50 increase post-vector passage) suggests that vectors could inadvertently enhance resistance in natural settings, which could complicate control efforts. This indicates the need for integrated control strategies that consider both host and vector dynamics.
- Drug Resistance Management: Identifying gene markers (*LmMRPA*, *LmTDR1*) that remain stable through the vector life cycle could aid in developing molecular tools for monitoring and controlling resistant strains in endemic regions, particularly if resistance-associated genes contribute to enhanced survival under stress.

5. Limitations and Suggested Future Directions

Despite the valuable insights, this study has several limitations that could be addressed in future research:

- Mechanistic Studies on Gene Function: While gene amplification and expression data are provided, further mechanistic studies on how these genes contribute to resistance would strengthen our understanding of parasite-vector interactions.
- Multi-Vector Analysis: Expanding this research to other sand fly species could offer insights into whether different vectors exert distinct pressures on resistant *Leishmania*, affecting resistance transmission dynamics.
- Field Studies for Ecological Validity: Field-based studies involving natural parasite and vector populations could validate these findings in real-world conditions, offering more comprehensive risk assessments for the spread of resistant *Leishmania*.
- Investigation of Resistance Trade-Offs: While resistance appears to lower infection intensities in sand flies, the broader ecological costs of resistance on parasite fitness in both hosts and vectors remain unexplored. Studies that assess trade-offs over multiple generations could provide deeper insights into resistance evolution.

6. Conclusions

This study provides valuable insights into how antimony-resistant *L. major* parasites interact with sand fly vectors, demonstrating that resistance traits are stably maintained post-vector passage and suggesting potential selection pressure for even higher resistance levels. The findings underscore the complex dynamics of resistance transmission in vector-borne diseases and the need for integrated control strategies that consider both drug-resistance management and vector ecology. Future studies exploring gene functionality, vector diversity, and longitudinal effects will be crucial to fully understanding and mitigating the spread of resistant *Leishmania* strains in endemic areas.

D) Specific comments on the thesis

1) The thesis study has been conducted in accordance with the professional requirements of the field, using modern and appropriate methods and techniques. It has been observed that a high level of effort was devoted by the thesis student and the supervising academics during the course of the thesis work. For me, establishing a connection between the developed ideas, framework, methods, and findings in a scientific study is extremely important. In this thesis study, this connection has been successfully established.
2) The thesis's writing language, English, demonstrates highly successful grammar usage. Apart from a few typographical errors, no significant issues have been identified linguistically.
3) The design and format of the thesis are very good and have been prepared in accordance with the rules. The figures and tables used in the thesis and articles aid in a better understanding of the thesis content and are sufficient.

QUESTIONS:

(*Please answer 7 of the following questions that you consider more important than the others*)

Thesis in general:

1. What potential limitations should be discussed to add transparency to DNA barcoding, such as challenges in distinguishing closely related species or issues with detection thresholds?
2. How might incomplete reference libraries or unaccounted variations within the *Leishmania* species contribute to misidentification?
3. How could further explanation of sample sizes for sand flies and host species across diverse environments (e.g., rural vs. urban) enhance the reliability of findings?
4. In what ways might small sample sizes limit statistical power and introduce bias, especially in identifying opportunistic feeding patterns?

Paper 1:

5. How might the collection of sand flies from only two provinces (Ghardaïa and Illizi) limit the representation of Algeria's diverse habitats in the study?
6. Could additional sampling in more varied ecological zones provide a more comprehensive understanding of sand fly fauna and host feeding behavior?
7. How might the two-month sampling period (June and October 2021) limit the study's ability to account for seasonal variations in sand fly populations and feeding behavior?
8. Could year-round sampling better capture these seasonal fluctuations and reveal species or feeding behaviors that may have been missed?

Paper 2:

9. What data gaps remain, particularly for Mauritania, where accurate distribution information is lacking?
10. How might possible underreporting of leishmaniasis cases affect the perceived distribution and vector roles of certain species?
11. How reliable are the maps provided using ArcGIS and Google Earth data for visualizing distribution, given challenges like (a) patchy data availability, particularly in Mauritania, and (b) underreporting or lack of field data in certain regions?
12. Could the review have benefited from more robust spatial analysis, incorporating environmental data layers such as climate and land use to better predict potential sand fly habitats and areas of disease transmission?

Paper 6:

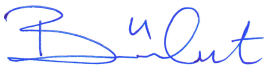
13. How could further mechanistic studies on gene amplification and expression enhance our understanding of how these genes contribute to resistance in parasite-vector interactions?
14. How might expanding this research to include other sand fly species provide insights into whether different vectors exert distinct pressures on resistant *Leishmania*, potentially influencing resistance transmission dynamics?
15. What are the broader ecological costs of resistance on parasite fitness in both hosts and vectors, given that resistance seems to lower infection intensities in sand flies?
16. Could studies assessing trade-offs over multiple generations offer deeper insights into the evolution of resistance?

Statement:

The reviewer believes that the PhD candidate, Kamal Eddine Benallal, has demonstrated his qualification for scientific work at the PhD level. The thesis fulfills qualitative requirements for a PhD dissertation and fulfills also the formal rule as far as the number of published papers in peer-reviewed and impacted journals is concerned.

In conclusion, I want to congratulate Kamal and her supervisor Doc. Vitek Dvorak and co-supervisor Doc. Jovana Sadlova for the excellent study. I find the study of Kamal Eddine Benallal and his thesis fully deserving of a PhD. I recommend that the candidate be awarded the degree of Doctor of Philosophy.

Sincerely yours



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January 15th, 2025