UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

Molecular studies in the genus *Bombus:* population analyses and defence mechanisms in conditions of thermal stress and infection by the parasite *Crithidia bombi*

Estudios moleculares en el género *Bombus*: análisis de poblaciones y de los mecanismos de defensa en condiciones de estrés térmico e infección por el parásito *Crithidia bombi*

Nuria Blasco Lavilla

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Escuela Internacional de Doctorado

Unversidad de Murcia

Directora:

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Este trabajo se ha realizado en el Área de Biología Animal del Departamento de Zoología y Antropología Física de la Universidad de Murcia, bajo la financiación obtenida en la convocatoria de Grupos de Excelencia Regional de la Fundación Séneca de la Región de Murcia (19908/GERM/15) y la obtenida en la convocatoria del Programa Estatal de I+D+i orientado a los Retos de la Sociedad del Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (E-RTA2014-00003-C03) por el grupo de "Filogenia y Evolución Animal".

La autora ha disfrutado de un contrato predoctoral FPU (FPU/05189) del Ministerio de Ciencia, Innovación y Universidades de España para la realización de la tesis, de una ayuda Erasmus (+) Prácticas para la realización de una estancia de tres meses en el Dpto. de Genética y Biología Genómica de la Universidad de Leicester (Reino Unido), y de una ayuda para estancias breves FPU del Ministerio de Ciencia, Innovación y Universidades para la realización de una estancia en el Instituto de Biología Integrativa de la Universidad de Liverpool (Reino Unido).

Agradecimientos / Acknowledgements

Empezando por el principio, agradezco a mis profesores del instituto y de la universidad, Yolanda, Marimar, Pepín y Marisa el entusiasmo que me transmitieron en sus clases y especialmente a Marisa, las innumerables veces en las que fue guía y apoyo durante mi etapa universitaria. También a mi amigo José, cuyas motivadas clases de Entomología aficionada no sólo disminuyeron mi fobia a los insectos, sino que me animaron a estudiarlos.

Ya en el desarrollo de la tesis, agradezco a Pilar el haberme posibilitado diseñar en buena medida mi plan de investigación a la vez que me ha guiado y ofrecido soluciones a los mil problemas que hemos encontrado por el camino. También al resto del grupo de Biología Animal: Andrés, Alejandro, Alberto, Javier, José, Vicente, Irene, Carlos, Obdulia, Finuqui, Julia, Claudia, Laura, Diego, Daniel, Micaela y Yolanda, por su apoyo técnico y moral, su amabilidad y su gran sentido del humor día a día. Mención aparte a Ana, por su increíble ayuda técnica en un grupo tan numeroso y con todo tipo de tareas. También a Pepe Galián y Pepe Serrano, por darnos consejo y ánimo cuando asoma el síndrome impostor.

Outside my university, I'm grateful to Eamonn and Seth for allowing me to be in their labs, not only learning techniques that have been essential for my thesis, but also meeting wonderful people. Thanks to Boris, Lys and Jon, for making my stay in Leicester unforgettable. Thanks Anthony, Robin, Thiago for being marvellous company in Liverpool. Thanks to Hande for being such a cool colleague, supportive host, and fantastic cook. And thanks Seth, Lauren and Rama, for making it possible that, after two years of trying, I got to study *Bombus* infection by *Crithidia,* with the difficulties and hurry of doing the experimental part in such a short time, and the inconvenience of supervising me from overseas once my stay had finished.

Gracias a todos los abejorros que han hecho que esta tesis sea posible.

Gracias enormes a las personas que siempre son un apoyo y por supuesto también lo han sido con la tesis, escuchando mis problemas aunque en muchas ocasiones no los entendiesen: mis amigas, Patricia y Umayma, y mi familia. Gracias a mis padres, a mis abuelos, a mi hermano, y a Helena, que además de escuchar mis frustraciones con experimentos sobre bichos, tienen que aceptar que esté lejos de ellos para hacerlos. Y sobre todo gracias a mi padre, por confiar en mí, y enseñarme a perseverar y a tener una paciencia infinita siempre.

Por último, gracias a Jon,

"I am glad you are here with me. Here at the end of all things."

"It's like in the great stories Mr. Frodo. The ones that really mattered. Full of darkness and danger they were, and sometimes you didn't want to know the end. (…) A new day will come. And when the sun shines it will shine out the clearer."

J. R. R. Tolkien

Contribution statement

Chapter 1

This chapter is published in the journal Graellsia. I share coauthorship with Concepción Ornosa and Pilar De la Rúa, who provided the samples and contributed to manuscript writing. Andreas Bertsch contributed with manuscript suggestions, Ana Isabel Asensio with technical support and José Herrera with English edition. I carried out the sample and data analysis and wrote the manuscript.

Chapter 2

This chapter is published in the Journal of Insect Conservation. I share coauthorship with Concepción Ornosa, Pilar De la Rúa and Denis Michez, who provided the samples and contributed to manuscript writing. Pilar De la Rúa also helped with experimental design and assisted with Irene Muñoz and Diego Cejas in microsatellite raw data analysis. Carlos Ruíz contributed with manuscript suggestions, Ana Isabel Asensio with technical support and Jonathan Smith with English edition. I contributed with experimental design, analysed the samples and data, and wrote the manuscript.

Chapter 3

This chapter is under review in Apidologie. I share coauthorship with Andrés García-Reina and Pilar De la Rúa, who contributed to experimental design and manuscript writing. Andrés García-Reina also assisted in RNA extractions, raw qPCR data and statistical analysis. Ana Isabel Asensio provided technical support and Jonathan Smith helped with English edition. I contributed with experimental design, performed the experiment, analysed the samples and data, and wrote the manuscript.

Chapter 4

The manuscript corresponding to this chapter is in preparation for publication. Seth Barribeau contributed to experimental design, provided the *Crithidia bombi* cultures, assisted in experiment performance, helped with statistical analysis and contributed to manuscript writing. Lauren Mee assisted with RNA extractions and provided technical support with Rama Bhatia. Seth Barribeau and Jonathan Smith helped with English edition. I contributed with experimental design, carried out the experiment, analysed the samples and data, and wrote the manuscript.

Dr. Gherardo Bhogo and Dr. Silvio Erler kindly reviewed this thesis and provided suggestions for improvement.

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Resumen
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Introducción general

Los abejorros (Hymenoptea: Apoidea) son un grupo de insectos pertenecientes al género *Bombus* (Latreille, 1802). Son particularmente grandes y la mayoría de ellos están cubiertos de pelo. Estas características hacen que sean capaces de generar calor (endotermos) y que estén bien adaptados al frío (Heinrich 1993). Se estima que su origen ocurrió hace 40-25 millones de años en Asia (Hines 2008). Actualmente comprenden unas 250 especies que se encuentran en las zonas templadas, alpinas y árticas de Asia, América y Europa, con algunas excepciones en los trópicos de baja latitud del Sureste Asiático, Sudamérica y América Central (Williams 1998).

Ciclo vital y organización social

Los abejorros son insectos eusociales (Wilson 1971). En la mayoría de las especies, la reina fecundada emerge de la hibernación en el invierno tardío o la primavera y busca un lugar adecuado para fundar el nido. A continuación, la reina trae polen y néctar al nido y forma una masa esférica sobre la cual deposita los primeros huevos (hembras que darán lugar a obreras). La reina incuba la cría, forrajea, y alimenta a las larvas jóvenes. Cuando el primer grupo de obreras emerge, ellas toman la tarea de forrajear, ayudar a la reina a atender la cría y regular la temperatura del nido. La colonia continúa creciendo hasta que comienza la producción de individuos reproductivos: machos y nuevas reinas. Una vez emergen, las reinas forrajean y acumulan reservas de grasa, mientras que los machos abandonan el nido en unos pocos días y se alimentan fuera hasta que se aparean y mueren. Después de ser fecundadas, las reinas continúan alimentándose y buscan un sitio adecuado en el que hibernar durante el invierno. Por otro lado, la reina y las obreras del antiguo nido envejecen y mueren (Goulson 2010).

Para entender el altruismo responsable del funcionamiento de un nido de abejorros, es necesario decir que los abejorros son organismos haplodiploides. Esto significa que los machos solo tienen una copia de cada cromosoma (son haploides) y se forman de huevos sin fecundar, mientras que las hembras tienen dos copias de cada cromosoma (son diploides) y se forman de huevos fecundados. Por esta razón, como todos los espermatozoides producidos por el macho son idénticos, las hijas comparten una mayor proporción de sus genes en comparación con los organismos diploides (0.75 *vs* 0.5). Cómo resultado, las hembras están más emparentadas con sus hermanas que con sus hijas y de ahí que propaguen mejor sus genes ayudando a sus hermanas que produciendo su propia descendencia (Heimpel & De Boer 2008).

Importancia polinizadora

Los abejorros tienen distintas características que les confieren un gran valor económico así como una gran importancia ecológica: son capaces de forrajear en condiciones de frío (Corbet et al. 1993), tienen una gran variación en la longitud de su probóscide (o lengua) lo que les permite polinizar un amplio rango de flores (Fussell & Corbet 1991), tienen la habilidad de realizar la polinización "con vibración", lo que consiste en mover sus músculos de las alas para liberar el polen de las anteras en ciertas plantas que no lo liberarían de otra forma (Buchmann 1985). Los nidos de abejorros comerciales se usan en agricultura para polinizar cultivos de gran valor económico cómo la fresa, el kiwi, las manzanas y los tomates. Por otro lado, los abejorros silvestres son responsables de la mayor parte de la polinización de muchas flores silvestres en zonas templadas, árticas y alpinas del hemisferio norte, algunas de las cuáles solo se encuentran en poblaciones pequeñas y fragmentadas (Goulson 2010).

Declive

Mientras que algunas especies de abejorros continúan siendo abundantes, otras han reducido su rango de distribución o su abundancia en Europa, Asia, Sudamérica y Norteamérica a lo largo de las últimas décadas (Cameron & Sadd 2019).

Las principales causas que se han señalado como responsables del declive de abejorros son:

1. **La pérdida de hábitat:** ha contribuido a lo largo de mucho tiempo al declive de abejorros por la conversión de hábitats naturales y seminaturales ricos en flores en tierras de cultivo durante el s. XX (Goulson et al. 2015).
2. **Los plaguicidas:** tienen efectos tóxicos directos sobre las abejas que pueden ser letales o subletales. De los 161 detectados en colmenas de la abeja de la miel, los neonicotinoides y los organofosforados son los que se cree que tendrían un efecto más negativo por su toxicidad, su frecuencia de uso y las concentraciones encontradas (Goulson et al. 2015).
3. **El cambio climático:** también podría estar afectando a ciertas especies. Como los abejorros están en general adaptados al frío, las predicciones de tendencia al calentamiento y eventos climáticos extremos como las olas de calor, podrían tener un efecto directo en las poblaciones de abejorros, pero también indirecto a través de cambios en la diversidad, el tiempo de floración y la distribución de las plantas (Cameron & Sadd 2019).
4. **La introducción de abejas que no son nativas y el uso de especies comerciales:** especies del género *Bombus* y otros géneros como *Osmia* y *Megachile* se han introducido en nuevos países como polinizadores comerciales. Incluso donde son nativas, especies manejadas de abejas como *Apis mellifera* alcanzan unas densidades más altas de lo que lo harían

sin intervención humana. Esto provoca el desplazamiento de las especies de abejorros nativas por competencia por los recursos, la introgresión donde hay especies emparentadas, y la propagación de patógenos con mayor frecuencia o a especies en las que no eran nativos ("pathogen spillover"; Goulson 2003, 2010).

5. **Los patógenos** que se han propagado recientemente o han incrementado su transmisión a través de los procesos "pathogen spillover" y "spillback" entre especies de abejas introducidas y comerciales (Cameron & Sadd 2019). Los patógenos mejor caracterizados son:

    - **Virus:** las infecciones virales pueden ser asintomáticas hasta que el virus "se activa", mostrando síntomas genéricos o convirtiéndose en letal (Ball 1996). Este grupo incluye el virus de las alas deformadas (DWV; Genersch et al. 2006), el virus israelí de la parálisis aguda (IAPV) y el virus Kashmir de las abejas (KBV; Meeus et al. 2014).

    - **Hongos microsporidios:** se sabe que *Nosema bombi* disminuye el bienestar del nido, reduciendo la supervivencia de las obreras y comprometiendo la producción de individuos reproductivos (Otti & Schmid-Hempel 2008). Además, *Nosema ceranae* se ha detectado en abejorros recientemente, como resultado de la propagación (spillover) desde la abeja melífera. Aunque sus efectos son menos conocidos, parece ser altamente patógeno colonizando el epitelio del intestino medio y reduciendo la supervivencia de las abejas (Graystock et al. 2013).

    - **Protozoos:** este grupo comprende a *Apicystis bombi*, un neogregarino que reside en el cuerpo graso del hospedador causando su reducción y una moderada mortalidad (Graystock et al. 2016), y los tripanosomátidos *Crithidia bombi* y *Crithidia expoeki*,

dos protozoos flagelados que residen en el intestino medio de los abejorros (Schmid-Hempel & Tognazzo 2010). *C. bombi* afecta al bienestar de las poblaciones reduciendo el bienestar de las reinas fundadoras en primavera, disminuyendo el tamaño de las colonias y la producción de individuos reproductores, e incrementando la mortalidad en obreras estresadas (Brown et al. 2000, 2003). La asociación entre *C. bombi* y *B. terrestris* se ha convertido en un modelo de las interacciones parásito-hospedador.

La preocupación sobre la importancia de los abejorros y su declive ha aumentado el interés científico sobre este género con un incremento de 12 veces en los artículos publicados por año durante los últimos 20 años (Goulson et al. 2011). Además, con el objetivo de poner el conocimiento científico en práctica, se han creado varios programas y organizaciones para evaluar el estado de amenaza de las especies y establecer y comunicar pautas de manejo. Algunos ejemplos son los programas Bumble Bee Watch en Norteamérica, y Beewatch (Goulson et al. 2015) o la asociación The Bumblebee Conservation Trust (https://www.bumblebeeconservation.org/) en el Reino Unido.

Diversidad y taxonomía de abejorros

Como un primer paso para conservar a los abejorros, es necesario conocer su diversidad. Esto es particularmente difícil ya que su taxonomía es especialmente compleja debido a la homogeneidad morfológica del género (Michener 1990). La genitalia del macho ha sido el carácter más útil para la clasificación en subgéneros, y su fiabilidad se confirmó con la primera filogenia basada en marcadores moleculares (Cameron et al. 2007). Esta filogenia fue elaborada con las secuencias de cuatro genes nucleares y uno mitocondrial de 218 especies de abejorros y apoyó la clasificación morfológica previa, sugiriendo la separación en dos clados principales: los abejorros "de cara corta" y los abejorros "de cara

larga". Estos clados se corresponden ampliamente con diferencias en la morfología de la cabeza y también en su ecología (Cameron et al. 2007).

El complejo de especies *Bombus-lucorum* en la península Ibérica

La taxonomía del subgénero *Bombus* s. str. es especialmente complicada. En Europa Occidental cuatro especies conforman el género: *B. terrestris, B. lucorum, B. magnus* y *B. cryptarum* (Williams et al. 2012). Aunque es difícil de diferenciar, *B. terrestris* puede ser identificada morfológicamente (Wolf et al. 2010). Sin embargo, *B. lucorum, B. magnus* y *B. cryptarum* han sido aceptadas como especies separadas en base a su ADN y a los perfiles de las secreciones de las glándulas labiales de los machos. No hay carácteres morfológicos que permitan diferenciar estas tres especies crípticas con certeza, y por ello se las ha agrupado en el llamado complejo *B. lucorum* (Bossert 2015).

La península Ibérica tiene aproximadamente un 60% de las especies de abejorros europeas. Sin embargo, hay poca información acerca de su distribución (Penado et al. 2016). Además, no hay información actualizada respecto a las especies del complejo *B. lucorum.* Los registros históricos no son fiables ya que las herramientas moleculares que permiten la correcta identificación de las especies han sido desarrolladas recientemente (Bossert 2015). La información actual indica que *B. cryptarum* estaría ausente de la península Ibérica, el límite sur de la distribución de *B. magnus* se encontraría en los Pirineos y las poblaciónes de *B. lucorum* llegarían hasta la mitad norte peninsular (Ornosa & Ortiz-Sánchez 2004; Bossert 2015). Con el pronóstico de que el cambio climático pueda afectar a estas poblaciones, que se encuentran cerca de sus límites climáticos (Rasmont et al. 2015; Penado et al. 2016) es necesario actualizar su distribución para poder evaluar el estado de conservación de las especies del complejo *B. lucorum.* Por tanto, la primera parte de esta tesis se ha enfocado en la identificación de estas especies crípticas en un muestreo realizado a lo largo de la península Ibérica

utilizando herramientas moleculares. Adicionalmente, los individuos de *B. lucorum* se caracterizaron con marcadores moleculares y un análisis de morfometría geométrica para entender major la diversidad dentro de esta especie. A continuación, se explican estas herramientas utilizadas para la identificación y la caracterización de las especies.

Marcadores moleculares usados en estudios de abejorros

A lo largo de los últimos 40 años, se han usado muchos marcadores moleculares para aclarar la taxonomía y la diversidad genética de los abejorros (Al-Samarai & Al-Kazaz 2015; Woodard et al. 2015):

- **Aloenzimas** o variantes de enzimas cuyas diferencias alélicas se pueden visualizar mediante una electroforesis de proteínas (Owen et al. 1992).
- **Secuencias del ADN mitocondrial** con altas tasas de mutación que son útiles para inferir la historia evolutiva (Williams et al. 2012).
- **Polimorfismos de longitud de fragmentos de restricción (RFLPs por sus siglas en inglés)** definidos como alelos alternativos en el ADN que son reconocidos y digeridos por enzimas de restricción (Murray et al. 2008).
- **Amplificación aleatoria de ADN polimórfico (RAPD por sus siglas en inglés)** que consiste en la amplificación de secuencias aleatorias usando cebadores cortos que se unen a diferentes partes del ADN molde dependiendo de la existencia de ciertos polimorfismos (Owen & Whidden 2013).
- **Microsatélites** o *loci* de repeticiones cortas en tándem que están presentes en el genoma nuclear (Duennes et al. 2017).
- **Polimorfismos de nucleótido único (SNPs por sus siglas en inglés)** como resultado de mutaciones en un solo nucleótido que se detectan por la

hibridación de ciertos fragmentos de ADN con arrays que tienen una alta densidad de sondas de ADN (Theodorou et al. 2018).

De todos estos marcadores, los más ampliamente usados en abejorros son los microsatélites y los marcadores mitocondriales, especialmente un fragmento del gen *citocromo c oxidasa I* (*cox1;* Woodard et al. 2015). Por tanto, hemos usado estos marcadores moleculares en esta tesis.

El gen *citocromo c oxidasa I* (*cox1*) como código de barras de ADN en los abejorros

Los códigos de barras de ADN emergieron como secuencias cortas de ADN estandarizadas que proporcionan un marcador de identificación único para cada especie. Tienen diferentes aplicaciones, entre ellas, el reconocimiento de especies crípticas, los estudios evolutivos, el análisis de la dieta, la cuantificación de la diversidad genética, y la identificación fiable de especies con valor económico. En general, el código de barras de ADN más usado es una secuencia de 600 pb del gen *citocromo c oxidasa subunidad I* (*cox1*). Sin embargo, no funciona tan bien en algunos grupos de organismos que tienen bajas tasas de sustitución nucleotídica en el ADN mitocondrial, complicaciones en los polimorfismos ancestrales, hibridación e introgresión (Kress et al. 2015), como las plantas (Fazekas et al. 2009) y los hongos (Seifert 2009).

En abejorros, varios estudios han secuenciado la región del código de barras del gen *cox1* con diferentes objetivos habiéndose depositado cientos de secuencias en la base de datos GenBank, proporcionando una herramienta muy buena para la identificación de especies o los estudios filogeográficos. Algunos ejemplos incluyen la filogenia mundial del subgénero *Bombus* s. str. (Williams et al. 2012), el estudio poblacional de las especies del complejo *B. ephippiatus* a lo largo de México y América Central (Duennes et al. 2012), o la filogeografía de *B. lapidarius* a lo largo de Europa (Lecocq et al. 2013). Además, hay varias publicaciones

utilizando las secuencias de *cox1* como identificadores e indicadores de diversidad en el complejo de especies *B. lucorum* a lo largo de Europa (Murray et al. 2008; Bertsch 2009; Carolan et al. 2012; Bossert et al. 2016). En consecuencia, hemos usado la secuenciación de esta región del gen *cox1* para identificar individuos del complejo de especies crípticas *B. lucorum* en la península Ibérica y caracterizar la diversidad genética de la especie *B. lucorum* en comparación con otras poblaciones europeas.

Microsatélites para estudios poblacionales en abejorros

Los microsatélites son repeticiones cortas en tándem de 1-6 nucleótidos con una alta tasa de mutación, que se encuentran con una alta frecuencia en el genoma de los procariotas y eucariotas. El hecho de que sean hipervariables, codominantes, y muchas veces estén conservados en diferentes especies, los ha convertido en unos marcadores convenientes para los estudios poblacionales. Permiten analizar procesos genéticos como el aislamiento de poblaciones o la conectividad, su expansión o contracción, la recolonización, la extinción, eventos pasados de deriva génica o cuellos de botella, migraciones, la frecuencia de apareamiento, endogamia o la selección de determinadas características, ayudando a la identificación de unidades evolutivamente significativas (ESUs por sus siglas en inglés; Schmid-Hempel & Schmid-Hempel 2000; Oliveira et al. 2006; Selkoe & Toonen 2006).

Por otro lado, los microsatélites también tienen inconvenientes: alelos que tienen el mismo tamaño pero derivan de distintos linajes (homoplasia) y son indetectables, problemas de amplificación para alelos específicos que dan como resultado alelos nulos (indetectables), errores en la determinación de alelos como resultado de amplificaciones deficientes o la interpretación incorrecta de los patrones de los electrofenogramas, *loci* que pueden tender a transmitirse juntos ("desequilibrio de ligamiento o gamético"), microsatélites que pueden no ser

neutrales por tener funciones o estar adyacentes a un gen bajo selección, y algunos factores causantes de patrones de herencia no mendeliana como los elementos transponibles o la deriva meiótica (Selkoe & Toonen 2006).

Afortunadamente, los microsatélites se han usado ampliamente en estudios poblacionales de muchas especies de abejorros de distintos subgéneros (Schmid-Hempel & Schmid-Hempel 2000; Erler & Lattorff 2010; Connop et al. 2011) y muchas publicaciones se han enfocado en el desarrollo y la validación de microsatélites en el género *Bombus* (Estoup et al. 1993; Reber Funk et al. 2006; Stolle et al. 2009, 2011). Además, en el caso de *B. lucorum* se ha validado un conjunto de cebadores y un protocolo de dos reacciones múltiples para amplificar distintos *loci* (Cejas et al. 2019). Por ello, hemos usado este conjunto de microsatélites para estudiar la estructura de las poblaciones de *B. lucorum* en un gradiente de distribución desde el norte de Europa hasta la península Ibérica.

La morfometría geométrica para analizar la diferenciación de poblaciones de abejorros

La morfometría geométrica es el análisis estadístico de la variación de la forma de las estructuras morfológicas y su correlación con otras variables. Para retener la geometría de la estructura de interés, se usa un conjunto de coordenadas o "landmarks" en dos o tres dimensiones que define la localización anatómica de características que representan atributos biológicos discretos. A continuación, se realiza un análisis Procrustres que superpone las configuraciones de los landmarks de distintos individuos en un sistema de coordenadas común y elimina el efecto de la localización, la rotación y el tamaño. Finalmente, se aplican distintos métodos de estadística multivariante con los datos de coordenadas para probar diferentes hipótesis biológicas (Adams et al. 2013).

La morfometría geométrica es útil para analizar la simetría individual, visualizar como varía la forma con el tamaño (alometría), cuantificar las trayectorias en el

cambio de forma como resultado de cambios evolutivos, desplazamientos ecológicos o trayectorias de movimiento, analizar los *loci* responsables de la variación en la forma e identificar qué características fenotípicas tienen una alta correlación para entender mejor la integración y la modularidad de los caracteres (Adams et al. 2013). La mayoría de los estudios usan como coordenadas las uniones de las venas longitudinales y cruzadas de las alas, que se asume que reflejan información filogenética y de desarrollo. En abejorros, la morfometría geométrica de la venación de las alas se ha usado para analizar la asimetría en condiciones de estrés (Gerard et al. 2018), distinguir machos diploides de haploides (Gerard et al. 2015), estudios de estructura de poblaciones (Duennes et al. 2012) y la resolución de problemas taxonómicos (Aytekin et al. 2007; Wappler et al. 2012), incluyendo las especies del complejo *B. lucorum* (De Meulemeester et al. 2009). Por tanto, se ha seleccionado este método como herramienta para el análisis de la estructura poblacional junto a los microsatélites.

El sistema inmune y la microbiota como defensas del abejorro

Como los patógenos se cree que podrían ser uno de los factores que está causando el declive de los abejorros (Goulson et al. 2015), muchos estudios se han enfocado en las interacciones de los abejorros con los patógenos, especialmente entre las especies comerciales *B. terrestris* y *B. impatiens* y los parásitos más extendidos, *Nosema* spp. y *Crithidia bombi* (Imhoof & Schmid-Hempel 1999; Schlüns et al. 2010; Conroy et al. 2016). Especificamente, la interacción entre *B. terrestris* y *C. bombi* ha sido estudiada con profundidad, mostrando que la expresión de genes del sistema inmune durante la infección puede ser específica y que la microbiota intestinal proporciona protección contra este parásito (Sadd & Barribeau 2013; Näpflin & Schmid-Hempel 2016). Por tanto, en esta tesis se consideran como

defensas del abejorro tanto los efectores del sistema inmune y como la microbiota intestinal.

Inmunidad en los insectos

Aunque la inmunidad en los insectos podría parecer simple, ha evolucionado a lo largo de millones de años para enfrentar un espectro muy diverso de patógenos. El sistema inmune de los insectos comprende múltiples tejidos y células que reconocen los patógenos como externos y nocivos cuando consiguen entrar al intestino medio, al hemocele o a los órganos internos. Los principales tejidos inmunitarios son el cuerpo graso, el intestino medio y las glándulas salivares, que, junto a las células inmunitarias de los insectos, los hemocitos, interaccionan para mediar la respuesta inmune. Esta respuesta incluye diversos procesos moleculares y celulares que reconocen y combaten los patógenos (Hillyer 2016).

En el comienzo de la infección, los patrones moleculares asociados a los patógenos (PMAPs), que son motivos conservados presentes en microbios, pero ausentes en insectos, y que son reconocidos por los receptores de reconocimiento de patógenos (RRPs) del hospedador. Los RRPs desencadenan procesos efectores y/o activan las rutas de señalización intracelulares. Las rutas de señalización mejor caracterizadas son las rutas Toll, Imd y JAK/STAT. Tanto la ruta de señalización Toll como la ruta Imd llevan a la transcripción de péptidos antimicrobianos (AMPs por sus siglas en inglés) y otros genes efectores inmunitarios. Las moléculas efectoras activadas por la vía Toll son efectivas principalmente contra bacterias Gram (+), virus y hongos, y los efectores asociados a la ruta Imd, en combatir bacterias Gram (-) y virus. La ruta JAK/STAT desencadena la transcripción de genes antimicrobianos como el que codifica la óxido nítrico sintasa, involucrada en las respuestas antibacteriana y antivírica. Otras rutas de señalización menos estudiadas son la JNK y la del factor de

crecimiento insulínico tipo 1, involucradas en la respuesta antimicrobiana, y las rutas de los ARN de interferencia involucradas en la respuesta antivírica (Hillyer 2016).

Los mecanismos efectores del sistema inmunitario de los insectos se clasifican como humorales y celulares, y consisten en los siguientes (Hillyer 2016):

- **La fagocitosis**: que consiste en la internalización y la digestión de pequeños organismos por los hemocitos.
- **La melanización**: que es la formación de una capa de melanina mediada por la enzima fenoloxidasa, para secuestrar un patógeno invasor y causar su muerte presumiblemente por daño oxidativo o inanición.
- **La encapsulación**: que está mediana por diferentes subgrupos de hemocitos que se unen formando una capa en torno al patógeno cuando este es demasiado grande para ser fagocitado, p. ej. en el caso de los huevos de las avispas parasitoides. Esta cápsula puede además ser melanizada.
- **La nodulación**: que consiste en la adherencia de distintos tipos de hemocitos alrededor de grandes agregados de bacterias. Los hemocitos liberan sus contenidos confinando a las bacterias en un material floculante y melanizando la estructura.
- **La lisis**: que involucra la muerte de un patógeno mediante la disrupción de su membrana celular. Puede ser llevada a cabo por AMPs, lisozimas y especies reactivas de oxígeno y nitrógeno.
- **Los ARN de interferencia (ARNi):** que conforman la respuesta antivírica en la que los ARNs de interferencia del hospedador se cargan en un complejo proteico de silenciamiento que destruye el ARN vírico que se une por complementariedad al complejo.

- **La autofagia**: que es la degradación de los materiales intracelulares ocurrida cuando los receptores de la membrana plasmática reconocen factores virales de patógenos intracelulares y se forma un autofagosoma que degrada el contenido engullido.
- **La apoptosis**: que consiste en la muerte celular programada de células infectadas para proteger al organismo.

El sistema inmune y los análisis de expresión génica en abejorros

Los genomas publicados de *B. terrestris* y *B. impatiens* mostraron que poseen un repertorio de genes inmunitarios que incluye los componentes centrales de todas las cascadas de señalización principales descritas en insectos, pero un número de genes inmunitarios reducido en comparación con las especies modelo de dípteros (Sadd et al. 2015). Esta reducción se ha encontrado también en otras especies de himenópteros (Gadau et al. 2012; Barribeau et al. 2015).

Por otro lado, los insectos sociales han desarrollado defensas colectivas conocidas en su conjunto como "inmunidad social". Los abejorros exhiben inmunidad social a través de distintas formas: comportamiento higiénico en el nido como la retirada de cadáveres (Munday & Brown 2018), evitar flores contaminadas con patógenos (Fouks & Lattorff 2011), la búsqueda de temperaturas bajas cuando están parasitados presumiblemente para alargar sus vidas (Müller & Schmid-Hempel 1993), e incluso el incremento de la actividad antibacteriana en los huevos de madres inmunizadas – "memoria inmune" transgeneracional (Sadd & Schmid-Hempel 2006, 2007).

Los análisis de expresión han jugado un papel importante en la exploración de la complejidad y la plasticidad de la respuesta inmune en abejorros. Concretamente, los análisis de PCR en tiempo real (RT-PCR por sus siglas en inglés) se han usado con muchos genes del sistema inmune de distintas clases por su sensibilidad y simplicidad para realizar análisis de expresión génica

mediante la cuantificación del ARN mensajero de genes diana. Estos análisis han mostrado la especificidad de la respuesta inmune a patógenos diferentes e incluso a distintas cepas (Barribeau & Schmid-Hempel 2013), la dinámica temporal de la respuesta inmune (Erler et al. 2011; Riddell et al. 2011), o la activación de la respuesta inmune tras el apareamiento en abejorros (Barribeau & Schmid-Hempel 2017). En esta tesis, hemos utilizado el conocimiento previo de expresión de genes del sistema inmune en *B. terrestris* para caracterizar la respuesta inmune mediante PCR cuantitativa en diferentes situaciones.

El efecto del estrés térmico en la actividad inmunitaria en insectos

La temperatura ambiental a través de su influencia en la temperatura corporal del hospedador, puede afectar al curso de una infección alterando el rendimiento del microorganismo y las defensas del hospedador (Thomas & Blanford 2003).

Respecto a la actividad inmune del hospedador, el estrés térmico puede ser beneficioso si los mecanismos de respuesta a los patógenos y al estrés por temperatura están compartidos para responder a ambas situaciones de estrés simultáneamente, o como parte de una respuesta general ante el estrés. Alternativamente, el estrés térmico podría suprimir la actividad inmunitaria como parte de un ahorro de energía para invertirla en protección térmica. En este sentido se han obtenido evidencias para ambos casos, ya que muchos insectos han mostrado un enlace entre ambos tipos de estrés con la activación de la respuesta inmune tras un tratamiento de estrés térmico (Wojda 2017), y también se ha observado un efecto de compensación entre ambas respuestas cuando los recursos son limitados (Karl et al. 2011; Triggs & Knell 2012).

Con el cambio climático en curso, se espera que los cambios de temperatura sean más frecuentes y de mayor amplitud (Easterling et al. 2000). Por consiguiente, es importante conocer cómo podrían afectar estos cambios de temperatura a la capacidad de los abejorros para defenderse ante las infecciones. Los estudios

previos que han analizado el efecto del estrés térmico en la actividad immune de las abejas han mostrado que, tanto las temperaturas moderadamente bajas como altas pueden incrementar la expresión de genes inmunitarios de diferentes clases en *Megachile rotundata* (Xu & James 2012), mientras que en *Apis mellifera* la respuesta a estrés térmico participa en la defensa antiviral (McMenamin et al. 2020) pero además es antagonista a la respuesta immune humoral (McKinstry et al. 2017). En los abejorros, aunque se ha visto que el incremento de temperatura puede disminuir la infección por *Crithidia bombi* (Palmer-Young et al. 2019a), no hay estudios que examinen la actividad immune del hospedador bajo distintas temperaturas. Por ello, en esta tesis se ha analizado el efecto del estrés térmico en la expresión de distintos genes del sistema immune para obtener una primera visión de cómo la temperatura cambiante podría afectar la habilidad de los abejorros para combatir patógenos.

La microbiota intestinal en los insectos

La microbiota intestinal comprende a todos los microorganismos que viven en el intestino, aunque en la mayoría de los estudios solo se ha caracterizado la comunidad bacteriana. Estos microorganismos pueden estar altamente adaptados a la vida dentro del insecto o ser adquiridos del medio ambiente, y pueden ser tanto beneficiosos como perjudiciales. Por ejemplo, las bacterias y los hongos están involucrados en la nutrición de las termitas mediante la degradación de la lignocelulosa (Brune & Dietrich 2015), y el simbionte *Serratia symbiotica* protege al pulgón de la pera *(Acyrthosiphon pisum)* del estrés por calor (Burke et al. 2010), mientras que la bacteria *Staphylococcus sciuri* produce compuestos de señalización que atraen a los depredadores de *A. pisum* (Leroy et al. 2011).

Varios estudios han relacionado la microbiota intestinal con una función protectora frente a patógenos aunque los mecanismos siguen sin estar claros. La

microbiota intestinal podría proteger al hospedador de dos formas principales: compitiendo por la colonización del nicho y la nutrición con el patógeno, e incrementando su competencia inmunitaria. Algunos casos bien conocidos incluyen el simbionte bacteriano *Enterococcus mundtii* que previene la invasión de patógenos en el intestino de la rosquilla negra *(Spodoptera littoralis)* mediante la secreción de un AMP (Shao et al. 2017), y el endosimbionte *Wigglesworthia* sp., que estimula la hematopoiesis en la mosca tsetse (*Glossina* spp.; Benoit et al. 2017).

La microbiota del abejorro

La microbiota de los abejorros ha sido muy estudiada en la última década debido a su simplicidad y a la alta adaptación al hospedador. Estudios en diferentes especies de *Bombus* han revelado que existen dos enterotipos principales: uno dominado por las especies potencialmente patógenas de la familia *Enterobacteriaceae* (*Hafnia, Serratia, Enterobacter*), y otro dominado por los géneros *Gilliamella* y *Snodgrasella*, que son endosimbiontes especializados restringidos a los géneros *Apis* y *Bombus* y que pueden ayudar a las abejas en la digestión del polen (Engel et al. 2012; Zheng et al. 2016). Ambos enterotipos también contienen distintas especies de *Lactobacillus* (Li et al. 2015; Praet et al. 2018). Se ha visto que la microbiota intestinal es capaz de conferir resistencia a los abejorros frente a *C. bombi* (Koch & Schmid-Hempel 2011; Mockler et al. 2018), pero las diferentes correlaciones encontradas entre las especies de microbiota y *C. bombi* no han aclarado qué simbiontes específicos podrían ejercer esta protección (Koch & Schmid-Hempel 2011; Cariveau et al. 2014; Mockler et al. 2018; Palmer-Young et al. 2019a). En lugar de la competición por recursos entre microorganismos en el intestino, es possible que la microbiota produzca sustancias que inhiban el crecimiento del parásito como se ha observado en algunas cepas de *Lactobacillus* de abejas (Forsgren et al. 2010; Palmer-Young et al. 2019b), o que estimule la actividad immune del hospedador como ocurre en *A. mellifera* (Kwong et al.

2017). Así, esta tesis ha explorado este tema analizando si la microbiota intestinal estimula la respuesta immune de *B. terrestris* durante la infección por *C. bombi.*

Objetivos e hipótesis

Esta tesis se ha centrado en estudios moleculares aplicados a la conservación de los abejorros (género *Bombus*) con dos objetivos principales claramente diferenciados:

- El **primer objetivo** ha sido clarificar el estado del complejo *B. lucorum* en la península Ibérica. Específicamente se han seguido los siguientes objetivos e hipótesis:
    - Muestrear e identificar las especies crípticas que forman el complejo *B. lucorum* mediante la secuenciación del fragmento génico *cox1* conocido como código de barras de ADN, con la hipótesis de que estas especies están presentes en áreas donde no están registradas dada la dificultad de distinguirlas morfológicamente.
    - Caracterizar la diversidad genética de *B. lucorum* en la península Ibérica en comparación con otras poblaciones de Europa, con la hipótesis de que la península actuó como un refugio durante el último máximo glaciar, observándose en sus poblaciones una mayor diversidad genética.
- El **segundo objetivo** ha sido analizar cómo interaccionan las defensas del abejorro con dos situaciones de estrés a las que se enfrentan los abejorros en la naturaleza: el estrés térmico y la infección por el parásito *Crithidia bombi.* Específicamente se han seguido los siguientes objetivos e hipótesis:

- Analizar el efecto del estrés térmico leve en la expresión de genes del sistema inmune de *B. terrestris* trabajando con la hipótesis de que el estrés térmico afecta a su actividad inmunitaria causando la sobreexpresión de genes del sistema inmune.
- Estudiar el efecto de la microbiota en la expresión de genes del sistema inmune en *B. terrestris* durante la infección con *Crithidia bombi*, con la hipótesis de que la microbiota protege frente a la infección estimulando la respuesta inmune del hospedador.

El primer objetivo se ha tratado en los dos primeros capítulos de la tesis, que corresponden a la primera parte, y el segundo objetivo se ha tratado en los dos últimos capítulos, englobados en la segunda parte de la tesis. Los resúmenes de cada capítulo, así como las conclusiones se detallan a continuación.

Resúmenes de los capítulos

Capítulo 1. El código de barras de DNA confirma la distribución de *Bombus magnus* (Vogt, 1911) (Hymenoptera: Apidae) en la península Ibérica

Bombus magnus (Vogt, 1911) (Hymenoptera: Apidae) es una de las tres especies crípticas pertenecientes al complejo *B. lucorum* junto con *B. lucorum* (Linnaeus, 1761) y *B. cryptarum* (Fabricius, 1775). En la península Ibérica solo se encuentran *B. lurocum* y *B. magnus*, pero la presencia de esta última no ha sido confirmada al sur de los Pirineos. Dada su similitud morfológica, usamos la región del código de barras de ADN para identificar 113 individuos de este complejo de especies en un muestreo ibérico. Los resultados confirman la presencia de *B. magnus* en los Pirineos y amplían su distribución actual hacia la meseta Norte ibérica. Dados estos resultados, sugerimos que ha de revisarse su distribución y el estado de conservación de esta especie en la península Ibérica.

Capítulo 2. Contrastando los patrones de diversidad genética y morfológica en el abejorro *Bombus lucorum* (Hymenoptera: Apidae: *Bombus)* a lo largo de un gradiente europeo

La península Ibérica ha actuado como refugio glaciar para muchas especies en Europa durante el Pleistoceno. Varios estudios filogeográficos realizados con el género *Bombus* han indicado la diferenciación genética de algunas especies en las penínsulas del sur de Europa. *Bombus lucorum* (Linnaeus, 1761) es una de las tres especies crípticas pertenecientes al complejo *B. lucorum.* En los últimos años, este complejo ha sido ampliamente estudiado; sin embargo, hay una falta de información respecto a la diversidad genética de esta especie y sus posibles eventos de recolonización posglaciares. Para cubrir esta falta de información, en este estudio se han caracterizado varias poblaciones situadas entre el centro de la península Ibérica y Bélgica, usando un marcador mitocondrial y varios nucleares (el código de barras *cox1* y 11 *loci* de microsatélites), así como la morfometría geométrica de las alas. Los resultados de *cox1* indican una diferenciación genética en la población de la Sierra de Guadarrama del centro de la península Ibérica, mientras que los análisis con los 11 *loci* de microsatélites y la morfometría geométrica no muestran ninguna estructura poblacional. Estos resultados señalan un evento de diferenciación pasada de *B. lucorum* en la península Ibérica, aunque también sugieren un flujo genético con poblaciones de Europa continental.

Capítulo 3. Impacto de la variación de temperatura en la expresión de genes del sistema inmune en el abejorro *Bombus terrestris*

Los cambios en la temperatura corporal pueden afectar a la habilidad de un insecto para luchar contra los patógenos alterando su actividad inmunitaria. Este trabajo ha evaluado el impacto del estrés térmico leve por frío y por calor en la expresión de distintos genes inmunitarios y de choque térmico en la especie

Bombus terrestris. Adicionalmente, un tratamiento de temperatura se repitió en condiciones de inanición para analizar un posible compromiso de la expresión de genes inmunitarios en favor de una respuesta a estrés térmico cuando los recursos energéticos están limitados. Los resultados señalan un papel de los genes *Hsc70* y *Aha1* en la tolerancia a temperaturas moderadamente altas. Los tratamientos con calor y con frío no tuvieron un impacto negativo en la expresión de genes del sistema inmune, y el receptor *BGRP1* se sobreexpresó con el tratamiento de frío, posiblemente indicando un aumento de la actividad inmunitaria celular. En condiciones de inanición, los tratamientos de calor y de frío causaron una mayor sobreexpresión en todos los genes analizados, sugiriendo un efecto de sinergia entre el estrés nutricional y el estrés térmico en la activación de genes de choque térmico e inmunitarios.

Capítulo 4. Efecto de la microbiota intestinal en la expresión de los genes de péptidos antimicrobianos y *Mucin-5AC* en *Bombus terrestris*

La microbiota intestinal de los géneros *Bombus* y *Apis* les confiere protección frente a sus patógenos naturales. En las especies de abejorros comerciales *B. terrestris* y *B. impatiens*, la microbiota aumenta su resistencia al parásito tripanosomátido común y virulento *Crithidia bombi*. Sin embargo, los mecanismos por los que los microorganismos intestinales protegen al hospedador siguen siendo desconocidos. Aquí, probamos la hipótesis de que la microbiota protege al hospedador mediante la estimulación de su respuesta inmunitaria o la protección de su epitelio intestinal. Para ello, redujimos la microbiota de las obreras y las expusimos a una dosis infectiva de *C. bombi.* Examinamos por qPCR la expresión de los genes de tres péptidos antimicrobianos (AMPs) y el gen *Mucin-5AC,* cuya función putativa es la protección del epitelio intestinal, tras la infección por *C. bombi*. Aunque se observó un efecto protector contra *C. bombi* en los abejorros con microbiota suplementada, no hubo diferencias en la expresión

del gen *Mucin-5AC* entre los tratamientos de microbiota, sugiriendo que la expresión de *Mucin-5AC* en el hospedador no está involucrada en los mecanismos de protección. Por otro lado, la presencia de microbiota no incrementó la expresión basal de los genes de AMPs en los abejorros, pero la interaccion con *C. bombi*, incrementó la expresión génica de los AMPs cuando el hospedador estaba infectado. Aunque este efecto por sí solo no explica el efecto protector observado, nuestros resultados sugieren que algunas cepas específicas de microorganismos intestinales podrían estimular la actividad inmunitaria del hospedador a través de la interacción con el parásito en el intestino, promoviendo quizá un ambiente más hostil para el establecimiento de *C. bombi.*

Conclusiones

Las conclusiones que se extraen de esta tesis son las siguientes:

- La especie críptica *Bombus magnus* está presente en la meseta norte de la península Ibérica, aunque es muy rara de encontrar.
- La península Ibérica actuó como un refugio glaciar para las poblaciones de *B. lucorum* donde se diferenciaron de las poblaciones de los Pirineos y del norte de Europa como puede verse en la variación del gen mitocondrial *cox1*.
- En la actualidad existe flujo génico entre la población de *B. lucorum* del centro peninsular y aquellas al norte de los Pirineos, tal como indican los análisis de microsatélites y de morfometría geométrica.
- La temperatura moderadamente alta (38 °C) provoca la sobreexpresión de los genes de choque térmico *Hsc70* y *Aha1* en abejorros de la especie comercial *Bombus terrestris.*
- La expresión de genes inmunitarios se mantiene a temperaturas moderadamente altas y bajas (38 °C y 9 °C) en *B. terrestris*, aunque el

mantenimiento 9 °C causa además la sobreexpresión del receptor *BGRP1*, relacionado con la respuesta inmunitaria celular.

- La inanición aumenta la expresión de genes de choque térmico e inmunitarios con los tratamientos de estrés térmico a 38 °C y a 9 °C, indicando un posible efecto sinérgico entre las condiciones de estrés nutricional y térmico.
- El efecto protector de la microbiota de *B. terrestris* frente a la infección intestinal del parásito *Crithidia bombi* no depende de la expression del gen *Mucin-5AC*, que codifica una proteína de la mucosa intestinal del hospedador.
- La microbiota intestinal endógena no protege a *B. terrestris* contra *C. bombi* a través del incremento de la expression génica basal de los AMPs *Abaecin, Defensin* e *Hymenoptaecin*. Sin embargo, algunas cepas específicas de microorganismos intestinales pueden interaccionar con *C. bombi* e incrementar la expression de los AMPs durante la infección. Este mecanismo puede proteger al hospedador frente a la infección del parásito, pero no explica por sí solo la resistencia que confiere la microbiota frente al parásito.

Introduction

General introduction

Bumblebees are a group of insects (Hymenoptera: Apoidea) belonging to the genus *Bombus* (Latreille, 1802). They are particularly large and most of them are covered in fur. These features make them capable of endothermy and well adapted to cool conditions (Heinrich 1993). Their origin is estimated to have happened 40-25 mya in Asia (Hines 2008). Currently, they comprise over 250 species found in temperate, alpine and arctic zones of Asia, America and Europe, with some exceptions in the lowland tropics of south-east Asia and Central and South America (Williams 1998).

Life cycle and social organization

Bumblebees are eusocial insects (Wilson 1971). In most species, the fertilised queen emerges from hibernation in late winter or spring and looks for a suitable nest site. Then, she brings pollen and nectar to the nest and forms a lump within the top of which she lays the first (female) eggs. The queen incubates the brood, forage, and feed the young larvae. When the first batch of workers hatch, they take the foraging duty, help the queen tending to the brood and regulate the temperature of the nest. The colony keeps growing until it switches to producing reproductive individuals: males and new queens. Once they emerge, queens forage to build their fat reserves and males leave the nest in a few days and feed outside until they mate and die. After being fertilised, queens keep feeding and look for a suitable site to hibernate during the winter, while the former queen and the workers become old and perish **(Fig. 1;** Goulson 2010).

To understand the altruism responsible of the bumblebee nest working, it is necessary to note that bumblebees are haplodiploid organisms. This means that males have only one copy of each chromosome (they are haploid) and are formed from unfertilised eggs, whereas females have two copies of each chromosome (they are diploid) and are formed from fertilised eggs. Therefore, all sperm cells

produced by the male are identical and daughters share a higher proportion of their genes than they would in diploid organisms (0.75 *vs* 0.5). As a result, females are more closely related to their sisters than to their daughters and propagate their genes better by helping to support their sisters than by producing their own offspring (Heimpel & De Boer 2008).

Fig. 1. Stages of the bumblebee lifecycle. 1) The queen hibernates 2) The queen looks for food and a suitable nest site 3) The queen lays the first eggs and incubates the brood 4) Emerged workers forage and look after the nest 5) New queens and males mate (Bumblebee Conservation Trust Picture).

Pollination importance

Bumblebees have different features that give them a great economical as well as ecological importance: they can forage in very cold conditions (Corbet et al. 1993),

their tongue length varies a lot between species allowing them to pollinate a wide range of plants (Fussell & Corbet 1991), and have the ability of buzz pollination, which consists of contracting their flight muscles to release pollen from anthera in some plants that otherwise would not release it (Buchmann 1985). Commercial bumblebee nests are used in agriculture to pollinate high value crops such as strawberry, kiwi, apples, and tomatoes. Also, wild bumblebees are mainly or exclusively responsible for the pollination of many wild flowers in temperate, arctic and alpine zones of the northern hemisphere, some of which only exist in small and fragmented populations (Goulson 2010).

Decline

Even if some bumblebee species remain very abundant, there are reports of species reducing their distribution range or abundance in Europe, Asia, South America, and North America over many decades **(Fig. 2;** Cameron & Sadd 2019).

Fig. 2. Rusty patched bumblebee *(Bombus affinis)*. First bumblebee declared endangered in U.S. Since the late 1990s, it has experienced a dramatic decline from being common across 28 states to have scattered populations in only 13 states. (Jill Utrup, U.S. Fish and Wildlife Service).

Below are shown the main pointed causes of bumblebee declines:

- **Habitat lost** has been a long-term contributor to bumblebee declines with the conversion of natural and semi-natural flower rich habitat to farmland during the 20th century (Goulson et al. 2015).
- **Pesticides** have direct toxic effects on the bees being lethal or sublethal. From the 161 detected in honey bee colonies, neonicotinoids and organophosphates have been predicted to have the biggest risk because of their toxicity, frequency and concentrations found (Goulson et al. 2015).
- **Climate change** may be affecting certain species too. As bumblebees are generally cold adapted, predicted warming trends and extreme weather events like heatwaves could have direct effect on bumblebee populations but also indirect effects changing the diversity, flowering time and distribution of plants (Cameron & Sadd 2019).
- **Introduction of non-native bees and commercial beekeeping.** Species of *Bombus* and other genus like *Osmia* and *Megachile* have been introduced in new countries as commercial pollinators. Even where they are native, commercial bee species like *Apis mellifera* reach higher densities than they would without human intervention. This results in competitive displacement of native bumblebee species, introgression where related species or subspecies occur, and the spreading of pathogens in higher abundances or to species where they were not native ("pathogen spillover") (Goulson 2003, 2010).
- **Pathogens** which have recently been spread or have increased their transmission through the processes of pathogen spillover and spillback from introduced and managed commercial bee species (Cameron & Sadd 2019). The best characterised pathogens are:

- **Viruses:** viral infections may be asymptomatic until the virus "activates," showing generic symptoms or becoming lethal (Ball 1996). This group includes the deformed wing virus (DWV) (Genersch et al. 2006), the Israeli acute paralysis virus (IAPV) and the Kashmir bee virus (KBV) (Meeus et al. 2014).
- **Microsporidian fungi:** *Nosema bombi* is known to decrease the fitness of the nest, reducing worker survival and compromising the production of reproductive individuals (Otti & Schmid-Hempel 2008). Additionally, *Nosema ceranae* has been detected in bumblebees more recently as the result of pathogen spillover from honey bees. Its effects are less known, but it seems to be highly pathogenic colonizing midgut epithelium and reducing bee survival (Graystock et al. 2013).
- **Protozoa:** this group comprises *Apicystis bombi,* a neogregarine that resides in the host fat body causing its reduction and a moderate mortality (Graystock et al. 2016), and the trypanosomatids *Crithidia bombi* **(Fig. 3)** and *Crithidia expoeki,* flagellated protozoan that reside in the bee midgut (Schmid-Hempel & Tognazzo 2010). *C. bombi* affects populations fitness by reducing the fitness of founding queens in spring, decreasing colony size and production of reproductives and increasing mortality in stressed workers (Brown et al. 2000, 2003). The association between *C. bombi* and *B. terrestris* has become a model to study host-parasite interactions.

Awareness of bumblebee importance and decline has risen the scientific interest in this genus with a 12-fold increase in articles published per year over the last 20 years (Goulson et al. 2011). Also, with the aim of putting scientific knowledge into practice, different programs and organizations have been created to assess the threat status of *Bombus* species and to establish and communicate

management guidelines. Some examples are the monitoring programs Bumble Bee Watch in North America, and Beewatch (Goulson et al. 2015) or The Bumblebee Conservation Trust (https://www.bumblebeeconservation.org) in the United Kingdom.

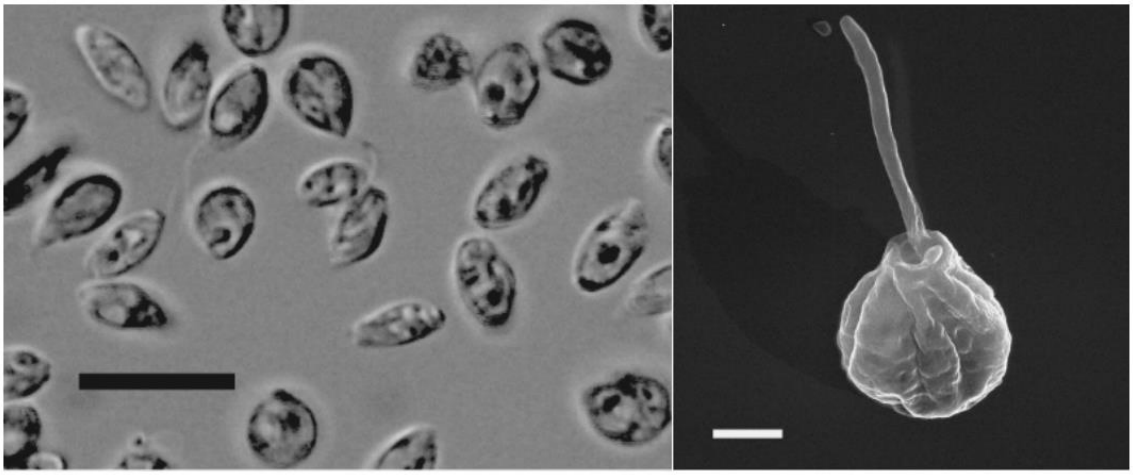

Fig. 3. *Crithidia bombi.* Differential interference contrast microscopy image to the left, scale bar = 10 um; and scanning electron microscopy image to the right, scale bar = 1 um. (Schmid-Hempel & Tognazzo, 2010).

Bumblebee diversity and taxonomy

As a first step to conserve bumblebees, it is mandatory to know their diversity. This seems particularly difficult as taxonomy of the genus is especially complex because of its morphological homogeneity (Michener 1990). Male genitalia was the most useful character to create a subgenera classification and its reliability was confirmed with the first highly resolved phylogeny using molecular tools (Cameron et al. 2007). This phylogeny was elaborated with the sequences of four nuclear and one mitochondrial gene for 218 bumblebee species. It supported previous morphological classifications and suggested the separation of two main clades, the short-faced and the long-faced bumblebees. These clades correspond broadly with differences in head morphology and ecology (Cameron et al. 2007).

Bombus lucorum species-complex in the Iberian Peninsula

Taxonomy is especially complicated in the subgenus *Bombus* s. str. In Western Europe four species conform the genus: *B. terrestris, B. lucorum, B. magnus* and *B. cryptarum* (Williams et al. 2012). Although difficult to differentiate, *B. terrestris* can be morphologically identified (Wolf et al. 2010). However, *B. lucorum*, *B. magnus* and *B. cryptarum* have been accepted as separated species based on their DNA and male labial gland secretions profiles. There are not morphological characters that allow a reliable identification of these three cryptic species and consequently, they have been grouped in the so-called *B. lucorum* complex (Bossert 2015).

The Iberian Peninsula has ca. 60% of the European bumblebee species. However, there is little information about their distribution (Penado et al. 2016). Furthermore, there is no updated information regarding the *B. lucorum* complex species. As molecular tools to correctly identify them have been only recently developed, historical records are not reliable (Bossert 2015). Current information indicates that *B. cryptarum* would be absent from the *Iberian Peninsula*, *B. magnus* south limit distribution may be the Pyrenees and *B. lucorum* populations may reach the northern half of the Iberian Peninsula (Ornosa & Ortiz-Sánchez 2004; Bossert 2015). With the prospect of climate change impacting these populations, which are near their climate limits (Rasmont et al. 2015; Penado et al. 2016) it is mandatory to update their distribution to assess the conservation of the *B. lucorum*-complex species. Therefore, the first part of this thesis has focused on identifying these cryptic species sampled throughout the Iberian Peninsula with molecular tools. Additionally, *B. lucorum* individuals, which were abundantly sampled, were characterised with molecular markers and geometric morphometrics analysis to better understand the diversity within this species. These tools used for species identification and characterisation are explained below.

Molecular markers used in bumblebee studies

Along the last 40 years, many molecular markers have been used to clarify bumblebee taxonomy and genetic diversity (Al-Samarai & Al-Kazaz 2015; Woodard et al. 2015):

- **Allozymes** or enzyme variants whose allelic differences can be visualised through protein electrophoresis (Owen et al. 1992).
- **mtDNA sequences** with high mutation rates useful in inferring evolutionary history (Williams et al. 2012).
- **Restriction Fragment Length Polymorphisms (RFLPs)** defined as alternative alleles in the DNA that are recognised and digested with restriction enzymes (Murray et al. 2008).
- **Random Amplfication of Polymorphic DNA (RAPD)** which consist in the amplification of random sequences using short primers that bind in different parts of the template depending on the existance of certain polymorphisms (Owen & Whidden 2013).
- **Microsatellite** or short tandem repeats *loci* present in the nuclear genome (Duennes et al. 2017).
- **Single-nucleotide polymorphisms (SNPs)** as result of single nucleotide mutations detected by the hybridization of certain DNA fragments with high-density DNA probe arrays (Theodorou et al. 2018).

From these markers, the most widely used in bumblebees are microsatellite *loci* and mitochondrial markers, especially the *cytochrome oxidase I* gene fragment (*cox1*; Woodard et al. 2015). Therefore, we have used these molecular markers in this thesis.

Cytochrome oxidase I (*cox1*) gene as DNA barcode for bumblebees

DNA barcodes emerged as standarised short DNA sequences providing a unique identification marker for each species. They have several applications: recognition of cryptic species, evolutionary studies, diet analysis, quantification of genetic diversity, and the reliable identification of species with an economic value. In general, a sequence of 600 bp from the gene *cytochrome c oxidase subunit I* (*cox1*) is the most used DNA barcode. However, it does not work so well in some groups of organisms with lower rates of mtDNA nucleotide substitution, complications of ancestral polymorphisms, hybridization or introgresion (Kress et al. 2015), such as plants (Fazekas et al. 2009) and fungi (Seifert 2009).

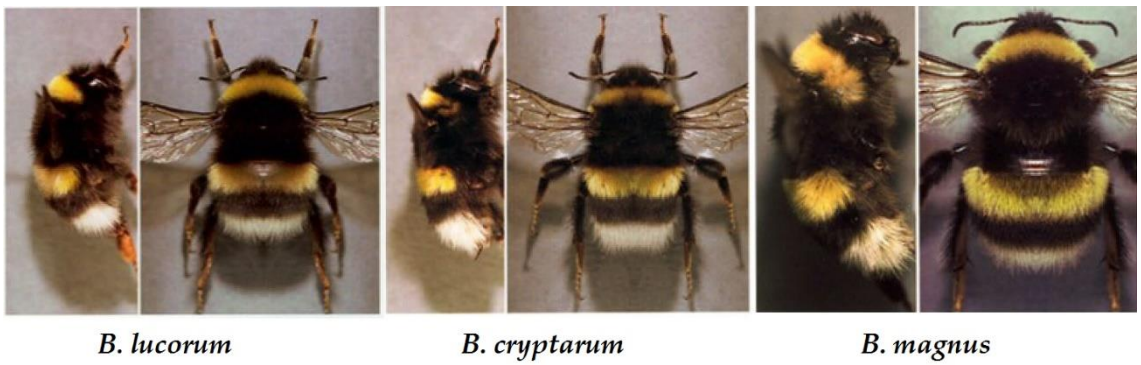

Fig. 4. Queens of the three cryptic member species from the *B. lucorum* complex. These species can be distinguished reliably with molecular markers but not with morphological characters (Bertsch et al. 2004).

In bumblebees, several studies have sequenced the *cox1* barcode region with different aims and hundreds of sequences have been deposited in the database GenBank, providing a great tool for species identification or phylogeography studies. Some examples include the worldwide phylogeny of *Bombus* s. str. subgenus (Williams et al. 2012), the population study of the *B. ephippiatus* species complex across Mexico and Central America (Duennes et al. 2012) or the phylogeography of *B. lapidarius* across Europe (Lecocq et al. 2013). Additionally, there are several papers reporting the *cox1* sequences as identifiers and diversity indicators of the *B. lucorum* species complex across Europe **(Fig. 4;** Murray et al.

2008; Bertsch 2009; Carolan et al. 2012; Bossert et al. 2016). Consequently, we used the *cox1* barcode sequencing to identify individuals of the *B. lucorum* complex of cryptic species in the Iberian Peninsula and to characterise the genetic diversity of *B. lucorum* in comparison to other European populations.

Microsatellite *loci* for bumblebee population studies

Microsatellites are short tandem repeats of 1-6 nucleotides with a high mutation rate found at high frequency in the genome of prokaryotes and eukaryotes. The fact that they are hypervariable, codominant and often transferable among related species, has made them convenient markers in population studies. They allow to analyse genetic processes such as population isolation or connectivity, expansion or contraction, recolonization, extinction, past events of genetic drift or bottlenecks, migrations, mating frequencies, inbreeding or selection of specific traits, helping to identify evolutionarily significant units (ESU, Schmid-Hempel & Schmid-Hempel 2000; Oliveira et al. 2006; Selkoe & Toonen 2006).

On the other hand, microsatellites can also have many drawbacks: alleles from the same size but of distinct lineages (homoplasy) can be undetectable, amplification problems for specific alleles can result in null (undetected) alleles, errors at scoring alleles as a result of poor amplifications or misinterpretations of electropherogram patterns, two *loci* can tend to be transmitted together ("linkage or gametic disequilibrium"), microsatellite *loci* could have functions or be adjacent to a gene under selection losing its neutrality, and some factors such as transposable elements or meiotic drive may cause non-Mendelian patterns of inheritance (Selkoe & Toonen 2006).

Fortunately, microsatellite *loci* have been broadly used in bumblebee population studies of many species from different subgenus (Schmid-Hempel & Schmid-Hempel 2000; Erler & Lattorff 2010; Connop et al. 2011) and many articles have focused on microsatellite development and validation in *Bombus* (Estoup et al.

1993; Reber Funk et al. 2006; Stolle et al. 2009, 2011). Also, in the case of *B. lucorum*, a set of bumblebee primers has been already validated and a protocol developed to amplify the different *loci* in two PCR multiplex reactions (Cejas et al. 2019). Thus, we used this set of microsatellite *loci* to study the structure of the *B. lucorum* populations in a distribution gradient from Northern Europe to the Iberian Peninsula.

Geometric morphometrics to analyse population differentiation in bumblebees

Geometric morphometrics is the statistical analysis of the shape variation of morphological structures and its covariation with other variables. To retain the geometry of the structure of interest, a set of coordinates or "landmarks" in two or three dimensions is used to define anatomical locations of traits representing discrete biological attributes. Next, a Procrustes analysis is performed to superimpose the landmark configurations of different individuals in a common coordinate system and remove the effect of location, rotation and size. Finally, multivariate statistical methods are used with the coordinate data to test different biological hypothesis (Adams et al. 2013).

Geometric morphometrics is useful to analyse individual symmetry, visualizing how shape changes with size (allometry), quantify trajectories of shape change as a result of evolutionary changes, ecological shifts or motion trajectories, analyse the *loci* underpinning shape variation and identify which phenotypic traits have a high shape correlation to better understand morphological integration and modularity of characters (Adams et al. 2013). The majority of studies use as coordinates the junctions of the longitudinal and the crossveins of the wings, which are assumed to reflect phylogenetic and developmental information. In bumblebees, the geometric morphometrics of wing venation has been used to analyse asymmetry under stressful conditions (Gerard et al. 2018),

distinguish diploid from haploid males (Gerard et al. 2015), study population structure (Duennes et al. 2012) and solve taxonomic problems (Aytekin et al. 2007; Wappler et al. 2012), including the *B. lucorum* complex species (De Meulemeester et al. 2009). Hence, this method was selected as a tool for population structure analysis together with microsatellite *loci*.

Immune system and microbiota as bumblebee defences

As pathogens are believed to be one of the factors causing bumblebee decline (Goulson et al. 2015), several studies have focused in bumblebee interactions with pathogens, especially between the commercial species *B. terrestris* and *B. impatients* and the most spread parasites, *Nosema* spp. and *Crithidia bombi* (Imhoof & Schmid-Hempel 1999; Schlüns et al. 2010; Conroy et al. 2016). Specifically, the interaction between *B. terrestris* and *C. bombi* has been deeply studied, showing that bumblebee immune genes expression upon infection can be specific and that gut microbiota provides protection to this parasite (Sadd & Barribeau 2013; Näpflin & Schmid-Hempel 2016). Therefore, in this thesis immune effectors and gut microbiota are considered as bumblebee defences.

Insect immunity

Although insect immunity may seem simple, it has evolved through millions of years to deal with a diverse spectrum of pathogens. The insect immune system comprises multiple tissues and cells that recognise pathogens as foreign and dangerous when they gain entry to the midgut, the hemocoel or the internal organs. Main immune tissues are the fat body, the midgut and the salivary glands, which together with insect immune cells, hemocytes, interact to mediate the immune response. This response includes diverse molecular and cellular processes to recognise and fight pathogens (Hillyer 2016).

In the beginning of the infection, pathogen-associated molecular patterns (PAMPs) which are conserved motifs present in microbes but absent in insects, are recognised by host pathogen recognition receptors (PRRs). PRRs elicit effector processes or/and activate intracellular signalling pathways. The best characterised signalling pathways are the Toll, the Imd, and the JAK/STAT pathways. Both Toll and Imd pathway signalling lead to the transcription of antimicrobial peptides (AMPs) and other immune effector genes. Toll activated effector molecules are principally effective in combating Gram (+) bacteria, viruses and fungi, and Imd associated effector molecules, in combating Gram (-) bacteria and viruses. The JAK/STAT pathway triggers the transcription of antimicrobial genes such as the gene codifying the nitric oxide synthase, involved in antibacterial and antiviral responses. Other signalling pathways less studied are the JNK and the insulin growth factor-1 signalling pathways involved in antimicrobial response, and the RNA interference pathways involved in the antiviral response (Hillyer 2016).

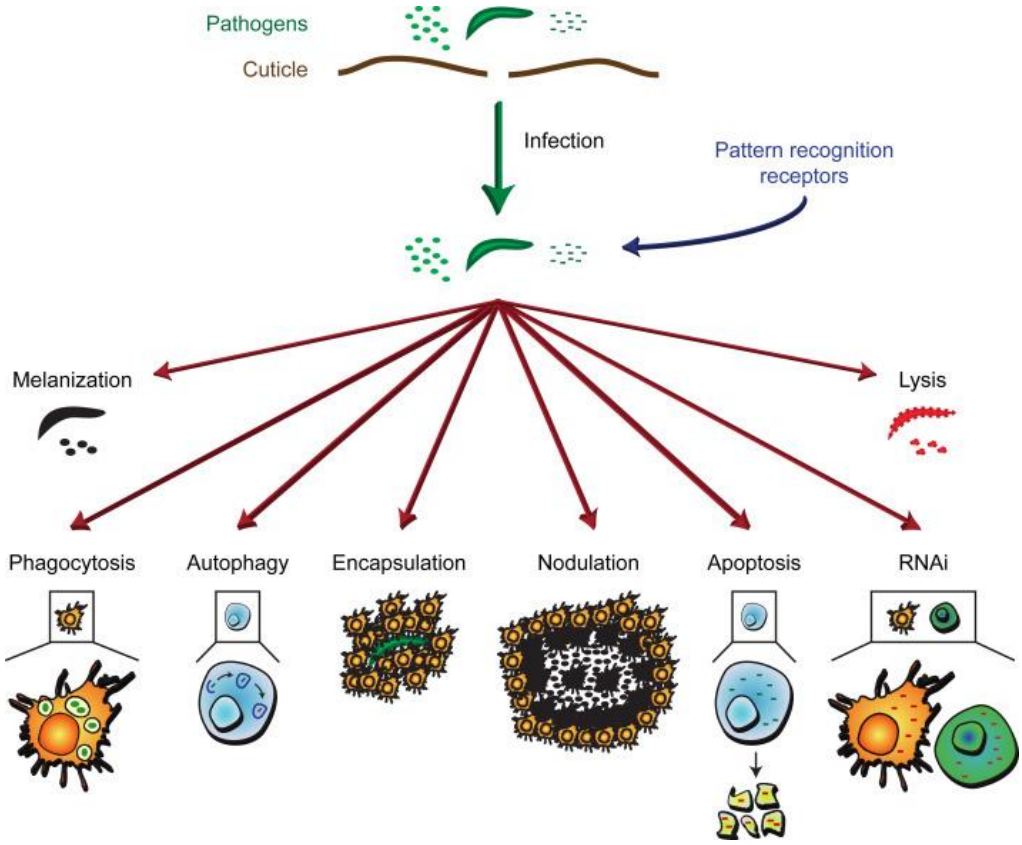

Fig. 5. Activation of the immune response and its mechanisms in insects. (Hillyer, 2016).

Insect immune effector mechanisms are generally classified as humoral or cellular, and consist of the following (**Fig. 5;** Hillyer 2016):

- **Phagocytosis** consists in the internalization and digestion of small organisms by hemocytes.
- **Melanisation** is the formation of a melanin layer mediated by the enzyme phenoloxidase that sequesters an invading pathogen causing its death presumably through oxidative damage or starvation.
- **Encapsulation** is mediated by different subgroups of hemocytes that attach forming a layer surrounding a pathogen that is too large to be phagocytosed, e. g. the eggs of parasitoid wasps. This capsule can also be melanised.
- **Nodulation** consists in the adherence of different kinds of hemocytes surrounding large aggregates of bacteria. The hemocytes release their contents confining the bacteria in a flocculent material and melanizing the structure.
- **Lysis** involves the death of a pathogen through disruption of its cellular membrane. It can be carried out by AMPs, lysozyme proteins, and reactive oxygen and nitrogen species.
- **RNAi** is an antiviral response where host interfering RNAs are loaded into a silencing protein complex that destroys the viral RNA binded by complementarity to this complex.
- **Autophagy** is the degradation of intracellular materials that occurs when plasma membrane receptors recognise viral factors of intracellular pathogens. An autophagosome is then formed to degrade the engulfed content.

- **Apoptosis** is the programmed cell death of infected cells to protect the organism.

Immune system and immune gene expression analyses in bumblebees

The published genomes of *B. terrestris* and *B. impatients* showed that they possess an immune gene repertoire that includes the core components of all major immune pathways described in insects, but a reduced number of immune genes in comparison with Dipteran model species (**Fig. 6;** Sadd et al. 2015). This reduction has also been found in other hymenopteran species (Gadau et al. 2012; Barribeau et al. 2015).

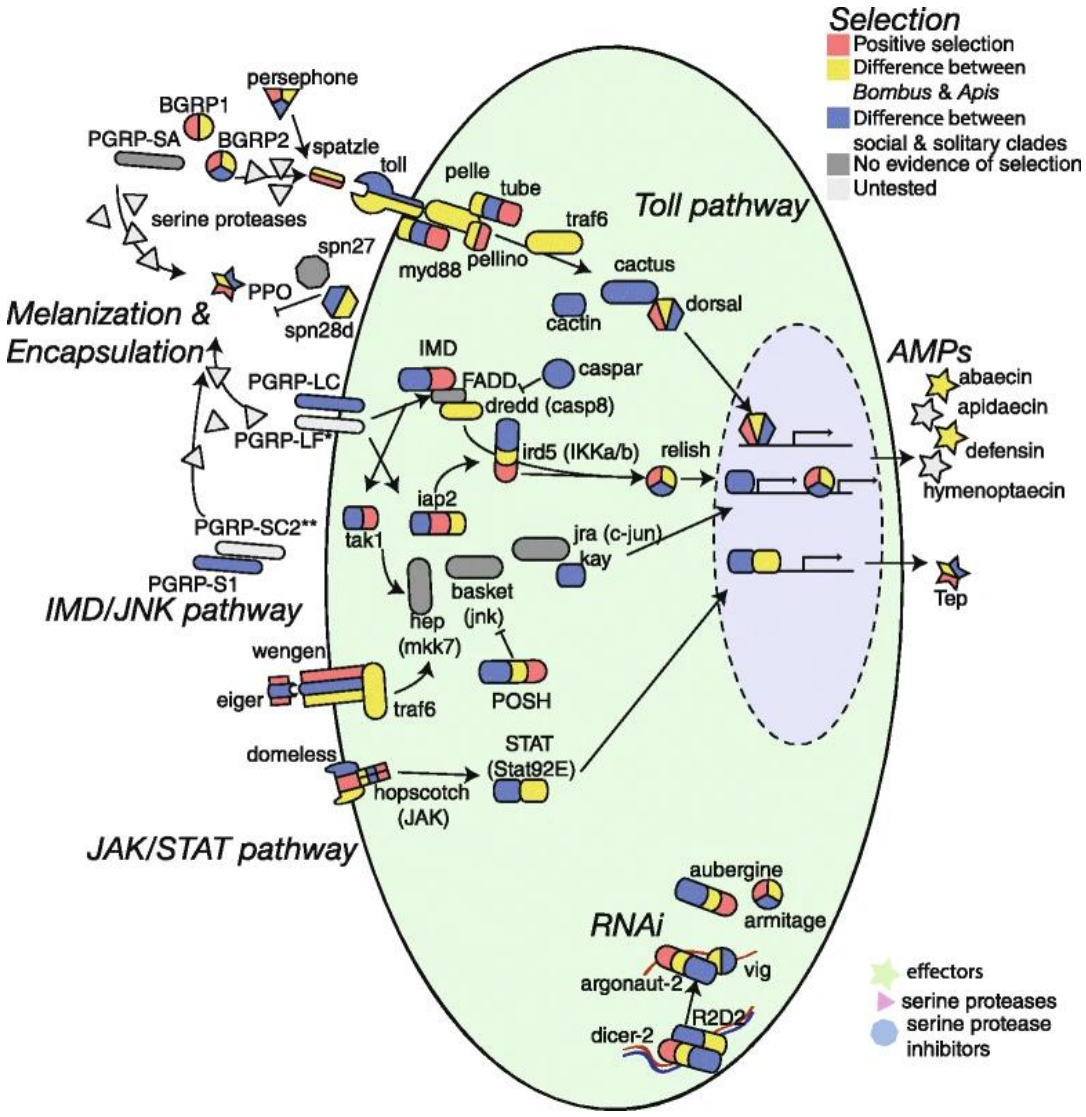

Fig. 6. Diagram of immune response in bees. Colors indicate detection of positive selection (Barribeau et al. 2015).

On the other hand, social insects have developed collective defences known as "social immunity". Bumblebees exhibit social immunity through several ways: nest hygiene behaviours like corpse removal (Munday & Brown 2018), avoidance of flowers contaminated with pathogens (Fouks & Lattorff 2011), seeking cold temperatures when they are parasitised presumably to prolong their lives (Müller & Schmid-Hempel 1993), even an increase in antibacterial activity in eggs from immune-challenged mothers – transgenerational immune priming (Sadd & Schmid-Hempel 2006, 2007).

Expression analyses have played an important role exploring the complexity and plasticity of the immune response in bumblebees. Specifically, quantitative real-time PCR (RT-PCR) has been widely used with many immune genes from different classes because of its sensitivity and simplicity to analyse gene expression through target mRNA quantification. These analyses have displayed the specificity in the immune response to different pathogens and even different strains (Barribeau & Schmid-Hempel 2013), the temporal dynamics of the immune response (Erler et al. 2011; Riddell et al. 2011), or the activation of the immune response after mating in bumblebees (Barribeau & Schmid-Hempel 2017). In this thesis, we have used the previous knowledge of immune gene expression in *B. terrestris* to characterise the immune response through quantitative RT-PCR in different settings.

The effect of thermal stress on immune activity in insects

Environmental temperature via its influence on host body temperature, can affect the course of infection by influencing the performance of the invading microorganism or the host's defences (Thomas & Blanford 2003).

Regarding host immune activity, thermal stress can be beneficial if the mechanisms to answer to pathogen and temperature stresses are shared either as an adaptive response to answer to both stress conditions simultaneously or as

part of a general stress response. Alternatively, thermal stress can suppress the immune activity as an energy saving to invest in thermal protection. Evidence has been obtained for both interactions, as many insects have showed a link between both stress responses with an activation of the immune response after a thermal stress treatment (Wojda 2017), but a trade-off has also been observed between both responses when resources are limited (Karl et al. 2011; Triggs & Knell 2012).

With ongoing climate change, shifts in temperature are predicted to be more frequent and of greater amplitude (Easterling et al. 2000). Hence, it is important to know how these temperature shifts might affect bumblebee's ability to fight infections. Previous studies analysing the effect of thermal stress on bee immune activity have shown that moderate low and high temperatures increased expression of different classes of immune genes in *Megachile rotundata* (Xu & James 2012). In *Apis mellifera,* the heat shock response is involved in antiviral defence (McMenamin et al. 2020) but is also antagonistic to the humoral immune response (McKinstry et al. 2017). In bumblebees, although increase of temperature has been shown to decrease infection of *Crithidia bombi* (Palmer-Young et al. 2019a), there are not studies that examine host immune activity under different temperatures. Therefore, in this thesis the effect of thermal stress has been analysed on the expression of different immune genes to obtain a first vision of how changing temperatures could affect bumblebee ability to fight pathogens.

Insect gut microbiota

Gut microbiota comprises all the microorganisms that live in the gut, although most studies have only characterised the bacterial community. These microorganisms can be highly adapted to living inside the insect or being acquired from the environment and have beneficial or harmful roles. For

example, bacteria and fungus are involved in termite nutrition through lignocellulose degradation (Brune & Dietrich 2015) and the symbiont *Serratia symbiotica* protects the pea aphid (*Acyrthosiphon pisum*) from heat stress (Burke et al. 2010), whereas the bacterium *Staphylococcus sciuri* produce signalling compounds that attract predators of *A. pisum* (Leroy et al. 2011).

Several studies have also related the gut microbiota with a protective function against pathogens, but the mechanisms are still not clear. The gut microbiota could protect the host mainly in two different ways: competing for niche colonisation or nutrition with the pathogen and increasing its immune competence. Some well-known cases include the bacterial symbiont *Enterococcus mundtii* which prevents invasion by pathogens in the gut of the cotton leafworm *(Spodoptera littoralis)* through secretion of one AMP (Shao et al. 2017) and the endosymbiont *Wigglesworthia*, that stimulates hematopoiesis in the tsetse fly (*Glossina* spp.; Benoit et al. 2017).

Bumblebee microbiota

The microbiota of the bumblebees has been widely studied in the last decade because of its simplicity and high adaptation to the host. Studies across different *Bombus* species have revealed that two main enterotypes exist: one dominated by potentially pathogenic species of the family *Enterobacteriaceae* (*Hafnia, Serratia, Enterobacter*), and the second dominated by the genus *Gilliamella* and *Snodgrasella,* which are specialised endosymbionts restricted to the *Apis* and *Bombus* genera and may help bees in pollen digestion (Engel et al. 2012; Zheng et al. 2016). Both enterotypes also contain different species of *Lactobacillus* (Li et al. 2015; Praet et al. 2018). Gut microbiota has been shown to confer resistance to bumblebees against *C. bombi* (Koch & Schmid-Hempel 2011; Mockler et al. 2018), but the different correlations found between microbiota species and *C. bombi* have not clarified which specific symbionts could exert this protection (Koch & Schmid-

Hempel 2011; Cariveau et al. 2014; Mockler et al. 2018; Palmer-Young et al. 2019a). Instead of a direct competition for resources between microorganisms inside the gut, it is possible that the microbiota produces substances that inhibit parasite growth as has been shown for bee *Lactobacillus* strains (Forsgren et al. 2010; Palmer-Young et al. 2019b), or that it stimulates the immune activity in the host as occurs in *Apis mellifera* (Kwong et al. 2017). Hence, this thesis has explored this topic by analysing whether gut microbiota stimulates the host's immune response during infection with *C. bombi*.

Aims and hypothesis

This thesis focused on the use of molecular tools for bumblebee conservation (genus *Bombus*) with two main aims clearly differentiated:

- The **first aim** was to clarify the state of the *B. lucorum* complex in the Iberian Peninsula. Specifically, I had the following aims and hypothesis:
    - Sampling and identification of the cryptic species conforming the *B. lucorum* complex through the sequencing of the *cox1* gene fragment (DNA barcode). The hypothesis was that these species are present in areas where they are not recorded because of the difficulty in distinguishing them morphologically.
    - Characterisation of the genetic diversity in Iberian populations of *B. lucorum* and its comparison with other European populations. The hypothesis was that the Iberian Peninsula has acted as a refugium during the Last Glacial Maximum, therefore its populations have a higher genetic diversity.
- The **second aim** was to analyse how bumblebee defences respond to two stress situations that they encounter in nature: thermal stress and infection

with the parasite *Crithidia bombi.* Especifically, I had the following aims and hypothesis:

- Analysing the effect of mild thermal stress on the expression of immune genes in *B. terrestris.* The hypothesis was that thermal stress alters immune activity causing the upregulation of immune genes.
- Studying the effect of the microbiota in the expresión of immune genes in *B. terrestris* upon the infection with *Crithidia bombi.* The hypothesis was that microbiota protects against pathogen infection through the stimulation of the immune response in the host.

The first aim was addressed in the first two chapters of this thesis, corresponding to the first part; while the second aim was addressed in the two last chapters of this tesis corresponding to the second part.

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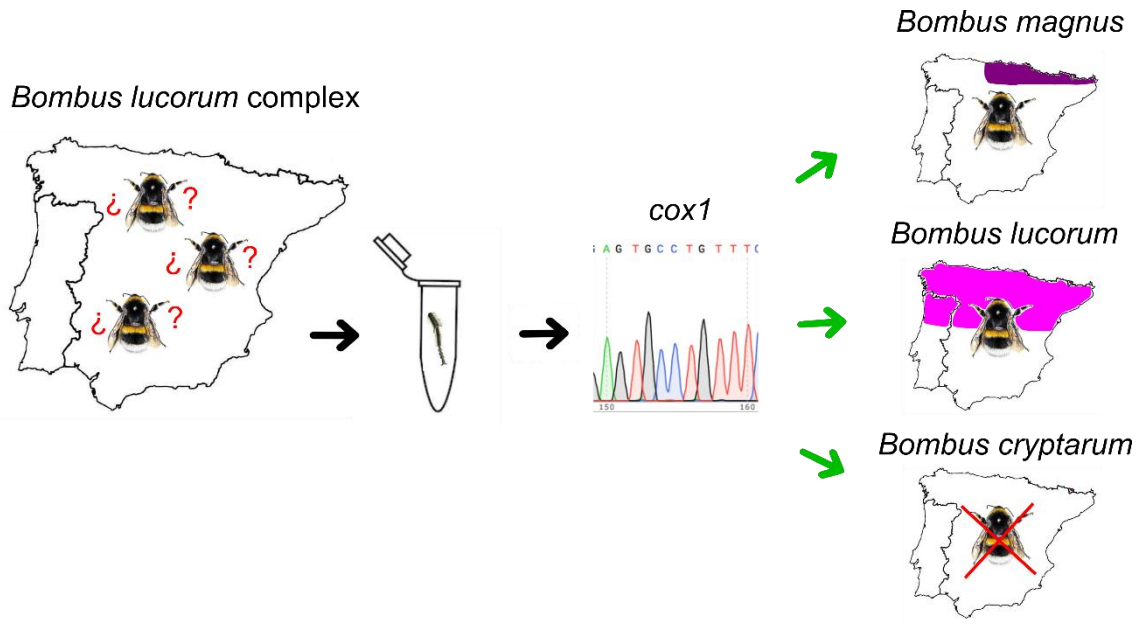
Part I

Chapter 1

DNA barcode confirms the distribution of *Bombus magnus* (Vogt, 1911) (Hymenoptera: Apidae) in the Iberian Peninsula



Abstract

Bombus magnus (Vogt, 1911) (Hymenoptera: Apidae) is one of the three cryptic species belonging to the *B. lucorum* complex besides *B. lucorum* (Linnaeus, 1761) and *B. cryptarum* (Fabricius, 1775). In the Iberian Peninsula, only *B. lucorum* and *B. magnus* are present but the presence of this last species south of the Pyrenees has not yet been confirmed. Given their morphological similarity, we used the DNA barcode region for the identification of 113 individuals of this species complex in an Iberian sampling. Results confirm the presence of *B. magnus* in the Pyrenees and extend its current distribution to the Northern Iberian Plateau. Given these results, we suggest that the distribution and conservation status of this species in the Iberian Peninsula should be revised.

Keywords: bumblebees; *Bombus magnus*; *B. lucorum* complex; Apidae; Hymenoptera; DNA barcode; *cox1*

Introduction

Bumblebee species (*Bombus* Latreille, 1802) were initially identified by their colour patterns. However, different species can show convergent colour patterns and furthermore, one given species can display a high intraspecific variation in different geographical locations (Williams 2007). This results in the presence of cryptic species very similar or identical in morphology which cannot be readily distinguished (Rasmont 1984). To overcome this, a molecular approach based on the sequence variation of a region of the mitochondrial *cytochrome c oxidase subunit I* (*cox1*) gene known as DNA barcode, has proved useful for revealing species identical to those recognised by traditional methods (Schmidt et al. 2015). DNA barcodes are included together with other data in an integrative taxonomy approach to delimit, discover and identify meaningful, natural species and taxa at all levels (Will et al. 2005), and have been widely used in bumblebee identification (Williams et al. 2012).

Within *Bombus*, the subgenus *Bombus s. str.* has presented more problems than other subgenera when it comes to species identification (Williams et al. 2012). In Western Europe, only two species were widely accepted until the mid-twentieth century: *B. terrestris* (Linnaeus, 1758) and *B. lucorum* (Linnaeus, 1761). However, a detailed review of the species *B. lucorum* based on morphological methods led to the confirmation of the species *B. magnus* (Vogt, 1911) by Krüger (1954) and *B. cryptarum* (Fabricius, 1775) by Rasmont (1984). Their status as species was supported by cross-breeding experiments (De Jonghe & Rasmont 1983) and mating observations that indicated reproductive isolation between them (Bučánková et al. 2011). Later, molecular data and male labial gland secretions contributed enough evidence to accept all three as separate species (Bossert 2015; McKendrick et al. 2017). This grouping is now known as the *B. lucorum* complex.

Nevertheless, there are not enough morphological characteristics to discriminate the species reliably. The first collar may be the only characteristic that allows discrimination of the three species (Williams 2000; Bossert 2015), but it is only useful for queens. Ecological data cannot distinguish the species as they live sympatrically, and there are no comparative studies about their habitat preferences over a wide geographic area (Bossert 2015). Consequently, Rasmont et al. (2015) has considered *B. magnus* as a taxonomically problematic species.

In the Iberian Peninsula, only two species from this complex are known to be present: *B. lucorum* and *B. magnus. Bombus magnus* in particular has been located in the northern half of the Iberian Peninsula: between altitudes of 100 and 2000 m in the Cantabrian Range, the Pyrenees, Sierra de Guadarrama and provinces of Guadalajara and Teruel (Rasmont 1984; Castro 1996; Ornosa et al. 2017). However, these reports have not been confirmed with molecular assays and have been the topic of further discussion (Williams et al. 2012; Ornosa et al. 2017). Only one report from a specimen collected in 1965 in Soria (also in the northern half of the Iberian Peninsula) has been confirmed with DNA barcoding (Williams et al. 2012).

The lack of certainty in the *B. magnus* records from the Iberian Peninsula south of the Pyrenees (Bossert 2015; Ornosa et al. 2017) reveals the need for further sampling to clarify the distribution of this species in the Iberian Peninsula. In addition, specimens from the Iberian Peninsula are of particular importance since there are indications that queens of *B. lucorum* exhibit a yellow thoracic collar coloration similar to *B. magnus* queens in central Spain (Bertsch 2009). *Bombus lucorum* queens in Spain can also be exceptionally large and this could lead to wrong identifications as *B. magnus* queens are supposedly larger, although specimens with medium and small size have been also observed (Bertsch com. pers. 2017). In this study, we used the DNA barcode to molecularly identify individuals of this species complex. Although this species is not considered to be

threatened in the IUCN Red List of European Bees (Rasmont et al. 2015), distribution models project a reduction of suitable areas by 2050, therefore an update of the distribution of *B. magnus* in the Iberian Peninsula is needed to propose conservation measures.

Materials and methods

Bumblebees were sampled from different areas of the northern half of the Iberian Peninsula between the summers of 2013 and 2017 **(Table 1)**. Individuals (N = 113) of different sexes and castes (Seven queens; 47 workers; 59 males) were preserved in absolute ethanol until analysed.

The posterior left leg was removed to extract the DNA with the Chelex method (Walsh et al., 1991). A fragment of the mitochondrial gene *cox1* of 614 bp was amplified with MyTaq™ Red Mix (Bioline) using two different methods. For the samplings between 2013 and 2016, we used the primers LCO/ HCO (Folmer et al. 1994) and the following program: initial denaturation at 94 °C for 3 min; 40 cycles of 94 °C for 30 s, 48/50 °C for 30 s, 72 °C for 1 min, and a final extension step at 72 °C for 10 min. For the sampling of 2017, we used the mini-barcode primers Barbee and MtD9 (Françoso & Arias 2013) and the following program: initial denaturation at 94 °C for 5 min; 35 cycles of 94 °C for 1 min, 46 °C for 1 min 20 s, 64 °C for 2 min, and a final extension step at 64 °C for 10 min.

PCR products were sequenced in Secugen (Madrid). Sequences were edited with Geneious 7.1 and species identification was performed by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 1. Samples of the *B. lucorum*-complex collected between the summers of 2013 and 2017 in the Iberian Peninsula. The samples of *B. magnus* are indicated in boldface (a.s.l. = above sea level; M = male, W = worker, Q = queen).

Species	Latitude/ Longitude	Collection date	Locality/Province	Elevation (m a.s.l.)	Sex or caste
B. lucorum	42.63683 0.00972	10/07/2013	Parque Nacional de Ordesa, Huesca	1720	1 W
B. lucorum	42.63483 -0.03838	11/07/2013	Parque Nacional de Ordesa, Huesca	1950	2 Q, 1 W
B. lucorum	42.61313 -0.20201	12/07/2013	Linas de Broto, Huesca	1420	1 M
B. lucorum	40.75388 -4.06388	20/08/2013	Cercedilla, Madrid	1323	3 M
B. lucorum	42.63896 -0.03253	08/08/2014	Torla, Huesca	1390	2 W
B. lucorum	42.63582 -0.02783	08/08/2014	Torla, Huesca	1407	1 M
B. lucorum	42.63527 -0.01416	08/08/2014	Torla, Huesca	1665	2 M, 1 W
B. lucorum	42.63519 -0.01220	08/08/2014	Torla, Huesca	1649	1 W
B. lucorum	42.63500 -0.01083	08/08/2014	Torla, Huesca	1648	1 M
B. lucorum	42.63767 -0.00165	08/08/2014	Torla, Huesca	1673	1 Q
B. lucorum	42.69796 -0.12189	09/08/2014	San Nicolás de Bujaruelo, Huesca	1477	1 W
B. lucorum	42.69847 -0.12945	09/08/2014	San Nicolás de Bujaruelo, Huesca	1591	2 M
B. magnus	**42.69847 -0.12945**	**09/08/2014**	**San Nicolás de Bujaruelo, Huesca**	**1591**	**1 M**
B. lucorum	42.70055 -0.11777	09/08/2014	San Nicolás de Bujaruelo, Huesca	1381	1 M
B. lucorum	42.63033 -0.31615	10/08/2014	Biescas, Huesca	914	1 M
B. lucorum	42.74025 -0.78453	11/08/2014	Ansó, Huesca	1053	2 M, 4 W

Species	Latitude/ Longitude	Collection date	Locality/Province	Elevation (m a.s.l.)	Sex or caste
B. lucorum	42.86482 -0.81805	11/08/2014	Ansó, Huesca	1216	1 M
B. lucorum	42.58361 1.05916	07/07/2014	Parque Nacional Aigüestortes, Lérida	1548	1 W
B. lucorum	42.58750 1.00111	08/07/2014	Parque Nacional Aigüestortes, Lérida	2035	1 W
B. lucorum	42.58194 1.01138	08/07/2014	Parque Nacional Aigüestortes, Lérida	2035	1 W
B. lucorum	42.58916 0.98927	08/07/2014	Parque Nacional Aigüestortes, Lérida	2152	4 W
B. lucorum	42.58111 1.12583	10/07/2014	Jou, Lérida	1369	1 W
B. lucorum	42.69388 0.93611	10/07/2014	Ruda, Lérida	1440	1 W
B. lucorum	42.66666 0.91694	10/07/2014	Colomers, Lérida	1589	1 W
B. lucorum	40.79194 -4.05972	29/07/2014	Puerto de la Fuenfría, Madrid	1797	4 M, 1 W
B. lucorum	40.78611 -4.05305	29/07/2014	Puerto de la Fuenfría, Madrid	1750	3 M
B. lucorum	40.75388 -4.06388	02/08/2014	Cercedilla, Madrid	1323	1 M
B. lucorum	40.82305 -3.96166	08/08/2014	Parque Natural de Peñalara, Madrid	1830	1 M
B. lucorum	40.77666 -4.05527	21/08/2014	Puerto de la Fuenfría, Madrid	1696	2 M
B. lucorum	40.79194 -4.05972	26/08/2014	Puerto de la Fuenfría, Madrid	1797	3 M
B. lucorum	40.78611 -4.05305	26/08/2014	Puerto de la Fuenfría, Madrid	1750	1 M
B. lucorum	42.58900 0.98700	02/09/2015	Clots de Rialba, Lérida	2174	2 W
B. lucorum	42.45116 1.07388	04/09/2015	Llessuí, Lérida	1405	1 Q

Species	Latitude/ Longitude	Collection date	Locality/Province	Elevation (m a.s.l.)	Sex or caste
B. lucorum	42.42666 2.26444	08/07/2015	Valle de Camprodón, Gerona	2175	1 W
B. lucorum	40.75388 -4.06388	01/05/20155	Cercedilla, Madrid	1260	1 Q
B. lucorum	42.38472 1.94361	09/07/2015	Estación La Molina, Gerona	1730	1 Q, 4 W
B. lucorum	42.32027 1.96777	09/07/2015	Estación La Molina, Gerona	1800	5 W
B. lucorum	40.79194 -4.05972	20/07/2015	Puerto de la Fuenfría, Madrid	1797	6 M, 3 W
B. lucorum	40.78611 -4.05305	04/08/2015	Fuente de Antón Velasco, Madrid	1750	16 M, 1 W
B. lucorum	40.79194 -4.05972	20/07/2015	Puerto de la Fuenfría, Madrid	1797	2 W
B. lucorum	40.79194 -4.05972	26/08/2016	Puerto de la Fuenfría, Madrid	1797	1 W
B. lucorum	40.75388 -4.06388	18/06/2017	Cercedilla, Madrid	1230	1 M
B. magnus	**42.44222 -4.26388**	**20/06/2017**	**Naveros de Pisuerga, Palencia**	**800**	**1 W**
B. lucorum	43.04138 -4.45861	21/06/2017	Piedrasluengas, Palencia	1355	1 W
B. lucorum	40.75388 -4.06388	25/06/2017	Cercedilla, Madrid	1230	1 W
B. lucorum	40.79194 -4.05972	26/07/2017	Puerto de la Fuenfría, Madrid	1797	1Q, 4M, 3W
B. lucorum	40.79194 -4.05972	01/09/2017	Puerto de la Fuenfría, Madrid	1797	1 M

Results and discussion

Both amplification methods amplified the same region of the gene *cox1*. DNA barcodes of two individuals showed an identity of 100% with the sequences GU705915 (*B. magnus* from Germany) and JN872621 (*B. magnus* from Denmark) from Genbank, therefore of 113 individuals sampled, 111 were found to be *B. lucorum* and two individuals firstly identified as *B. lucorum* by morphological data were found to be *B. magnus*. One of the individuals was a male caught in San Nicolás de Bujaruelo (Huesca) in the Pyrenees, at 1591 m above sea level, 9 VIII 2014 (P. De la Rúa leg.). The second individual was a worker sampled in Naveros de Pisuerga (Palencia), at 800 m above sea level, 20 VI 2017 (C. Ornosa leg.), foraging on *Rubus ulmifolius* Schott **(Fig. 1).**

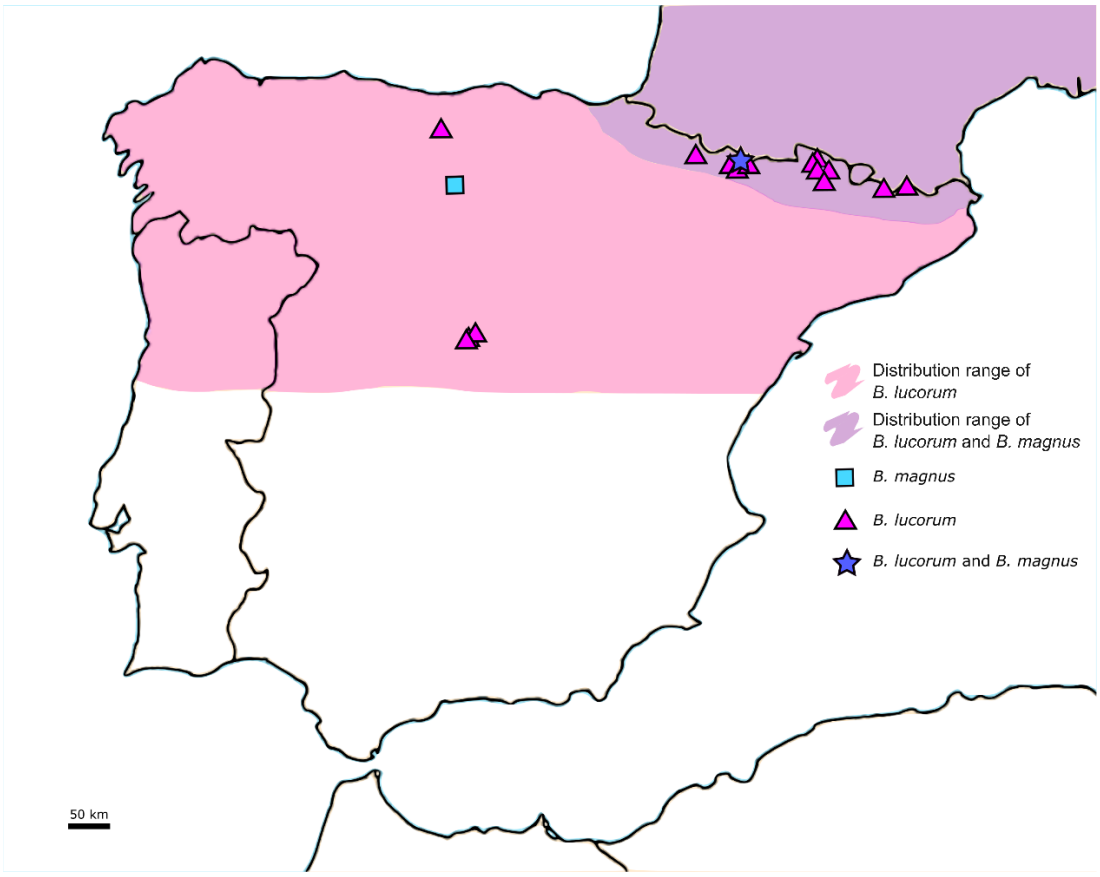


Fig. 1. Known distribution range of *B. magnus* (Vogt 1911) and *B. lucorum* (Linnaeus 1761) according to Bossert (2015) and Ornosa & Ortiz-Sánchez (2004), respectively, and sampling sites of the present study. A blue square indicates a site where only *B. magnus* was found, a pink triangle indicates sites where only *B. lucorum* was found and a purple star indicates a site where both species were found.

These new reports of *B. magnus* confirm the current distribution of the species in the Iberian Northern Plateau. The fact that only two individuals were sampled in four years strongly suggests that the species is rare and much less abundant than *B. lucorum.* Although both species are more widely distributed in Northern Europe, previous works have already reported that *B. lucorum* is more frequent than *B. magnus* in mainland Europe (Bossert 2015).

These reports agree with previous studies, which have pointed to a patchy distribution for *B. magnus*, with an association to heathlands with low diversity of plant species in Europe (Bossert 2015) and other Mediterranean landscapes in the Iberian Peninsula, where it was previously reported based on morphological data (Ornosa & Ortiz-Sánchez 2004). These results highlight the need for sampling more individuals in the Iberian Peninsula, and the utility of the DNA barcode region for identifying them. Obtaining more samples could help pinpoint both the period of the year when *B. magnus* nests are active and their habitat, facilitating the discrimination of the species from *B. lucorum*. It could also help to assess its conservation status in Spain which is unknown at present.

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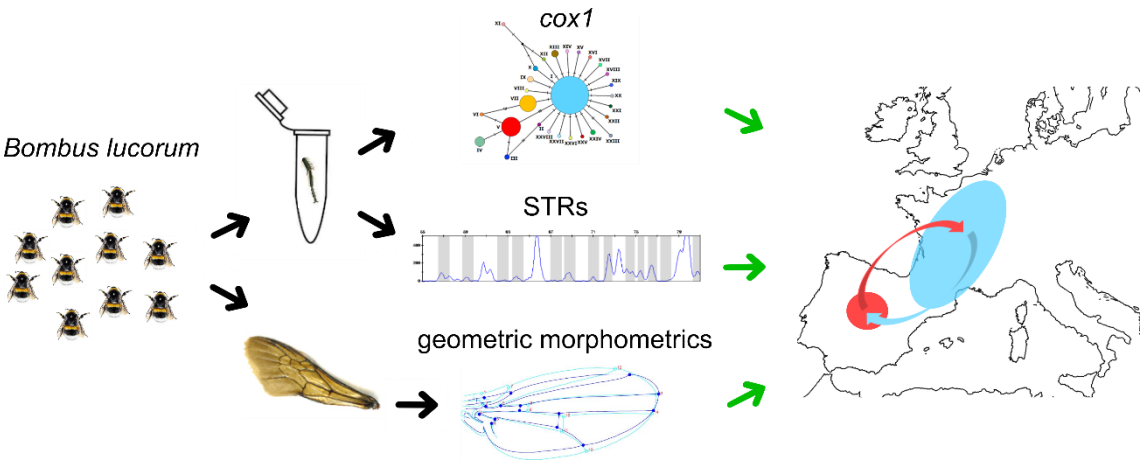
Chapter 2

Contrasting patterns of genetic and morphological diversity in the bumblebee *Bombus lucorum* (Hymenoptera: Apidae: *Bombus*) along a European gradient



Abstract

The Iberian Peninsula is known to have acted as a glacial refugium for many species during the Pleistocene in Europe. Several phylogeographical studies have been carried out within the genus *Bombus* which indicate a genetic differentiation of some of its species in the southern European peninsulas. *Bombus lucorum* (Linnaeus, 1761) is one of the three cryptic species belonging to the *B. lucorum* complex. In recent years, this complex has been widely studied; however, there is a lack of information about the genetic diversity of this species and its possible postglacial recolonisation events. To overcome this knowledge gap, in this study several populations from the centre of the Iberian Peninsula to Belgium have been characterised using mitochondrial and nuclear markers (*cox1* barcoding and 11 microsatellite *loci*) and the geometric morphometrics of the wings. Results from *cox1* indicate a genetic differentiation of the population of Sierra de Guadarrama at the centre of the Iberian Peninsula, while microsatellite *loci* and geometric morphometrics analyses do not show any population structure. These results point to a past event of genetic differentiation of *B. lucorum* in the Iberian Peninsula although they also suggest a current gene flow with populations from mainland Europe.

Keywords: *Bombus lucorum*; gene flow; genetic differentiation; population structure; glacial refugium; Iberian Peninsula

Introduction

The Iberian Peninsula is considered one of the most important glacial refugia of the Pleistocene in Europe. In addition, mountainous areas as well as the influence of both the Mediterranean Sea and the Atlantic Ocean create different climatic zones that are thought to have functioned as "microrefugia" in different parts of the Peninsula during the last maximum glacial (LMG). In this way, a wide variety of organisms is known to either have differentiated genetically in the Peninsula or have colonised the north and east of Europe from this region (Gómez & Lunt 2007).

Recently, several phylogeographic studies of the genus *Bombus* Latreille, 1802 have been carried out due to the increasing interest about the conservation of this group given their status as important pollinators of wild flora and crops (Widmer et al. 1998; Widmer & SchmidHempel 1999; Duennes et al. 2012; Lecocq et al. 2013a, b, 2015; Françoso et al. 2016). *Bombus lucorum* (Linnaeus, 1761) (subgenus *Bombus*) is a Palearctic species which extends from Southern Europe north to the Barents Sea coast, reaching Iceland to the west and the Pacific coast to the east (Rasmont et al. 2015). It is abundant all over Western, Central and Northern Europe but is restricted to the mountainous regions in Southern Europe (Bertsch et al. 2004). Recent studies (Bertsch et al. 2005; Bertsch 2009; McKendrick et al. 2017) have provided evidence that *B. lucorum* is a complex of three cryptic species: *B. lucorum*, *B. magnus* (Vogt 1911) and *B. cryptarum* (Fabricius 1775). Analyses of male labial gland secretions and sequence variation of the mitochondrial *cytochrome oxidase subunit I (cox1)* gene consistently distinguish the three species in Europe (Bertsch et al. 2004, 2005; Bertsch 2009; Carolan et al. 2012), but there is a lack of morphological characters to easily discriminate among them (Carolan et al. 2012). The form of the first collar in the queens may be the unique character to discriminate them in the field (Rasmont 1984; Bertsch 1997;

Bertsch et al. 2004). In *B. lucorum* the lateral border of the collar is higher than in *B. magnus* and *B. cryptarum* and is almost exclusively restricted to the pronotal lobes (Bertsch et al. 2004). Regarding their ecology and based on the most recent studies that used genetics-supported identification, *B. lucorum* is generally the most abundant of the three species in the complex at the majority of European sites (Murray et al. 2008; Vesterlund et al. 2014; Scriven et al. 2015; Bossert et al. 2016), with the exception of Waters et al. (2011) who found *B. cryptarum* as the most frequent species in northwestern Scotland. *B. lucorum* feeds on a wide range of flowers and seems to be most closely associated with urbanised areas than other members of the species complex (Murray et al. 2008; Waters et al. 2011; Scriven et al. 2015). However, the fact that the three species of the complex can be found living simpatrically in various habitats and the insufficient comparative studies across large geographic areas make it difficult to establish the factors determining the particular niche of *B. lucorum* (Bossert 2015).

Many *B. lucorum* populations have been characterised using *cox1* gene sequence variation throughout Europe with the aim of differentiating the three species of the *lucorum* complex (Bertsch et al. 2005; Murray et al. 2008; Bertsch 2009; Carolan et al. 2012; Williams et al. 2012). However, only one individual from the Iberian Peninsula was included in one of those studies (Bertsch 2009). The Iberian Peninsula is the southwest limit of the species distribution and it becomes less abundant here, being present only in the mountains of the northern half (Ornosa & Ortiz-Sánchez 2004). As stated in Penado et al. (2016), the lack of knowledge of bumblebee distribution in the Iberian Peninsula combined with the susceptibility to a rapid decline of these marginal populations, urge studies in this area. Indeed, *B. lucorum* is considered a species of Least Concern in the IUCN Red List of European Bees, but it has already become less abundant in Belgium and western France and a projected climatic scenario for 2050 has shown the reduction and even loss of the Iberian populations (Rasmont et al. 2015). Taking this into

account, and the possibility that the peninsula could have acted as a glacial refugium for bumblebee species (Widmer & Schmid-Hempel 1999; Lecocq et al. 2013a, 2015), it may be particularly informative to sample Iberian populations and perform molecular analyses to infer the genetic diversity of the species and to clarify possible postglacial recolonisation events.

In this work, several populations of *B. lucorum* ranging from the Iberian Peninsula to Belgium have been characterised by *cox1* sequencing adding published sequences to the data set. A median-Joining haplotype network was constructed to infer intraspecific relationships (Bandelt et al. 1999). Furthermore, individuals were genotyped with nine nuclear microsatellite *loci* to infer the intraspecific population structure, and finally the geometric morphometry of the wings has been analysed to resolve fine-scale variation (Aytekin et al. 2007; Kozmus et al. 2011; Schutze et al. 2012). As has been observed in other insect species (Cooper and Hewitt 1993; Schmitt & Seitz 2004; Habel et al. 2005; Lecocq et al. 2013a; 2015), a differentiation is expected of the Iberian individuals with respect to those from Central Europe.

Materials and methods

Sampling

Samples were collected along a latitudinal gradient from the central region of the Iberian Peninsula to Belgium. Ninety-nine individuals were collected from the Spanish National Parks Sierra de Guadarrama (central Spain), Aigüestortes and Ordesa (Pyrenees), and the Cantabrian mountain range (Spain) between the summers of 2013 and 2017. They were then preserved in absolute ethanol **(Fig. 1).** Forty-five individuals from the Pyrenees and Monts d'Ardèche (southern France), Namur and the High Fens (Belgium) sampled between 1986 and 2001

(Fig. 1) were provided from the collection of Dr. Denis Michez (University of Mons, Belgium) **(Table S1).**

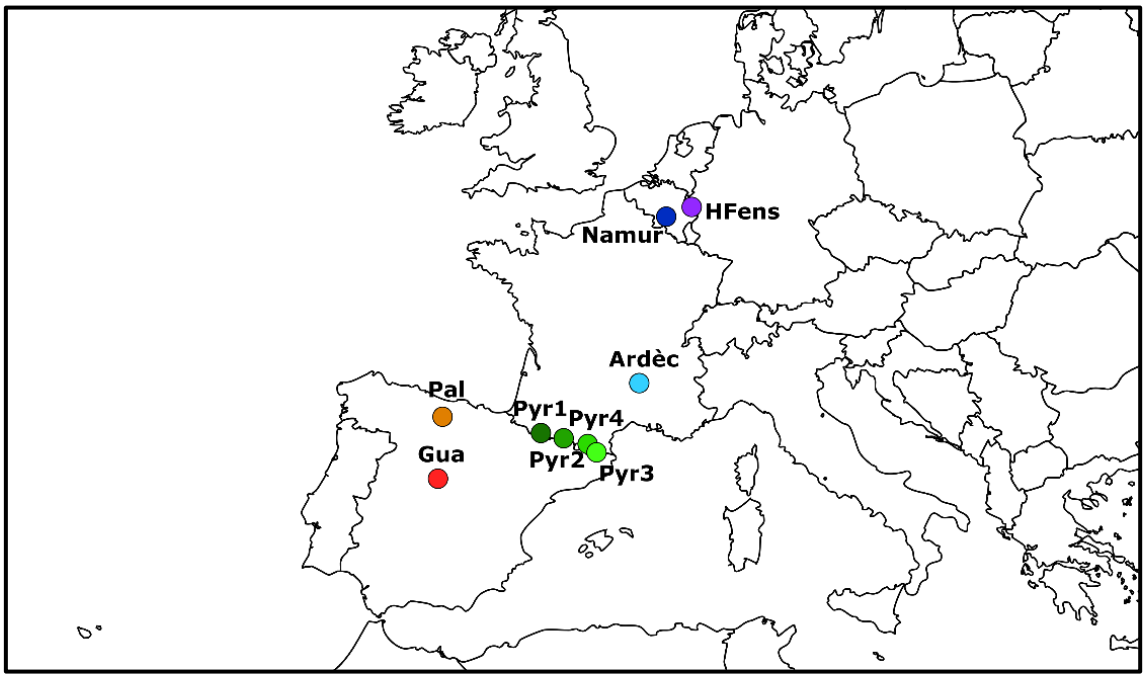


Fig. 1. Map showing the sampling locations of *Bombus lucorum: Hfens* High Fens, *Ardèc* Monts d'Ardèche, *Pyr1* Pyrenees1, *Pyr2* Pyrenees2, *Pyr3* Pyrenees3, *Pyr4* Pyrenees4, *Pal* Palencia, *Gua* Sierra de Guadarrama.

DNA extraction

DNA was extracted from a hind leg removed from every individual using the Chelex method (Walsh et al. 1991). In the case of the bumblebees from the Belgian collection, total DNA was extracted using the Canadian Centre for DNA Barcoding Glass Fiber Plate DNA Extraction Protocol (Ivanova et al. 2006).

cox1 amplification and sequencing

PCR amplification of a fragment of the *cox1* gene was carried out using the primers pair LCO1490/HCO2198 (Folmer et al. 1994) with an initial denaturation at 94 °C for 3 min; followed by 40 cycles of 94 °C for 30 s, 48/50 °C for 30 s, 72 °C for 1 min, and a final extension step at 72 °C for 10 min. As some individuals had degraded DNA, the *cox1* amplification was carried out for those individuals

using the mini barcode approach (Françoso & Arias 2013). PCR conditions included in this case an initial denaturation at 94 °C for 5 min; followed by 35 cycles of 94 °C for 1 min, 46 °C for 1 min 20 s, 64 °C for 2 min, and a final extension step at 64 °C for 10 min. Amplicons of the *cox1* fragments were Sanger sequenced (Secugen SL, Madrid, Spain).

Species identification and haplotype analysis

Bombus lucorum individuals were initially identified through morphological characters and lately confirmed by *cox1* sequencing and BLAST comparisons (https://blast.ncbi.nlm.nih.gov/Blast.cgi). *B. lucorum* sequences available for the same mitochondrial fragment were downloaded from GenBank (accession numbers in **Table S2**). Sequences were aligned using Geneious 7.1 and the final matrix comprised 216 sequences. Sequences were translated to check for aminoacid changes and to discard possible sequencing mistakes and the presence of NUMTs (nuclear mitochondrial DNA segments).

To study haplotype relationships, a haplotype network was constructed with PopArt 1.7 (Leigh & Bryant 2015) using the median-Joining distance method. Nucleotide divergence and differentiation coefficients (Φ_{ST}) were calculated using the package StrataG (Archer et al. 2017) in R software only in those sampling locations consisting in more than ten individuals (including both females and males). Bonferroni correction was applied to *p*-values.

Microsatellite amplification and genotyping

Eleven microsatellite *loci* (B10, B11, B96, B100, B118, B119, 121, B124, B126, B131, B132; Estoup et al. 1995, 1996) were amplified with two multiplex reactions (Cejas et al. 2019). PCR consisted in an initial denaturation at 95 °C for 5 min, followed by 30 cycles of 92 °C for 30 s, 54 °C for 30 s, 72 °C for 30 s, and a final extension step at 72 °C for 30 min. Allele discrimination was carried out on an AB3700

capillary genotyping system (Servei Central de Suport a la Investigació Experimental, University of Valencia, Spain). Alleles were scored using GeneMapper 4.8 (Applied Biosystems Inc.).

Microsatellite analysis

Only females from sampling locations with more than nine individuals were used in the microsatellite analysis. Putative full siblings were identified with the software Colony 2.0 (Jones & Wang 2010). Individuals with probabilities of sibship above 0.95 were discarded from further molecular and geometric morphometrics analyses.

The presence of null alleles, scoring error due to stuttering, and allele dropout were analysed with the software Micro-Checker 2.2 (Van Oosterhout et al. 2004). Genetic structure was estimated using two methods. Firstly, a Bayesian clustering algorithm was performed using the program STRUCTURE 2.3.4 (Pritchard et al. 2000). The admixture correlated frequency model was used and K (number of clusters) was tested from 1 to 10. Five independent runs were performed with a burn-in period of 250,000 and a run length of 750,000 iterations. The optimal number of clusters (K) was selected using Structure Harvester (Earl 2012) according to the ad hoc statistic deltaK. In a first step temporal stability of populations was tested performing an analysis only with Pyrenean samples collected in 2001 and 2013–2017. Then, a second analysis was done with all the samples independently of their geographical location. Additionally, a discriminant analysis of principal components (DAPC) (Jombart et al. 2010) was also performed with the package adegenet in R. Number of clusters was identified following the Bayesian Information Criterion (BIC) and an optimal number of principal components that explained more than 90% of the variance was selected.

Mean allele richness (A_R), number of private alleles (Npa), observed (H_O) and unbiased expected heterozigosity (uH_E), F_{IS} values, departure from Hardy–Weinberg equilibrium, linkage disequilibrium, migration rate, and differentiation coefficients (pairwise F_{ST}) were calculated using the software GenAlEx v6.5 (Smouse & Peakall 2012) and the packages GenePop (Rousset 2008), adegenet (Jombart 2008), StrataG (Archer et al. 2017) and diveRsity (Keenan et al. 2013) in R. Due to the different number of individuals of each group A_R values were corrected after rarefaction with diveRsity (Keenan et al. 2013). Bonferroni correction was applied to *p*-values when testing for linkage disequilibrium, Hardy–Weinberg equilibrium and F_{ST} pairwise comparison.

Wing morphometrics

Wing shape was considered to characterise the phenotype of the individuals. Wings are two dimensional structures where crossing veins and the maximum curve can be considered as homologous (e.g. Dehon et al. 2017). The left forewings were removed and mounted with water in glass slides with glass slipcovers. Only females were considered to avoid differences based on sexual dimorphism (Gerard et al. 2015). Pyrenean samples were grouped in this analysis given their geographical proximity. All slide-mounted wings were photographed with a Spot Insight Firewire camera and the application SPOT Advanced (SPOT Imaging). Images were converted to tps format with tpsUtil 1.74 (Rohlf 2017a). Eighteen landmarks were selected **(Fig. S1)** and manually plotted on the forewings images using tpsDig2 2.32 (Rohlf 2016). Correlation between the Procrustes distances in the shape space with the Euclidean distances in the tangent space was checked using the software tpsSmall 1.34 (Rohlf 2017b).

Geometric morphometrics analysis

The program MorphoJ 1.06d (Klingenberg 2011) was used for geometric morphometrics analyses. A Procrustres fit was performed to eliminate all the variations due to non-shape diferences and outliers were checked. Individuals were classifed by population clusters. A principal component analysis (PCA) was performed to visualise shape variation among groups. A canonical variance analysis (CVA) was performed and the Mahalanobis and Procrustres distances were computed for each group and tested by a permutation test applying Bonferroni correction. The separation of the groups was also tested in a discriminant function analysis (DFA) with a leave-one-out cross-validation test. ANOVA tests were performed to see if the origin of the individuals had any effect on the size and the shape of the wings.

Results

Although southern locations suitable for Iberian bumblebees were sampled, including the mountain ranges Sierra Nevada and Sierra Espuña, and the natural park Calares del Mundo y de la Sima (southeastern Spain), no *B. lucorum* individuals were found supporting its limited distribution to the northern half of the Iberian Peninsula (Ornosa & Ortiz-Sánchez 2004).

cox1 analyses

The resulting matrix of the partial *cox1* gene included 216 sequences of 564 base pairs (bp) with a total of 28 segregating variable sites, 11 parsimony-informative sites and a nucleotide diversity (π) of 0.0028. Sequences obtained in this study were submitted to GenBank (accession numbers MK279535–MK279653). All variable nucleotides corresponded to synonym mutations, thus discarding the presence of NUMTs. Eleven haplotypes were found in the locations analysed

with seven being private and only two of them (IV and V) being predominant in the individuals of the Iberian Peninsula. The private haplotype of Palencia was obtained from one sample of this location **(Fig. 2)**. Individuals from Sierra de Guadarrama showed a differentiation from the rest of the populations with two main private haplotypes, V and IV, observed in 92.7% of the samples of this location. These haplotypes, V and IV, were 3 and 4 mutation steps apart from the most common haplotype in Europe and Asia, respectively. Individuals from the locations of High Fens and the Pyrenees shared haplotypes I and VII with the rest of Europe with 83.3% and 91.9% of the samples, respectively. One sample of Monts d'Ardèche had the haplotype I and the two individuals sampled of Namur had a diferent haplotype, XIII. Remarkably, one sample with haplotype V was found in the Pyrenees and one sample with haplotype I was found in Sierra de Guadarrama. The haplotype IV found in Palencia occupied an intermediate position in the network between the haplotype VII found mainly in the Pyrenees and the haplotype V found mainly in Sierra de Guadarrama **(Fig. 2)**.

Only a single individual from Monts d'Ardèche yielded DNA of enough quality for sequencing, therefore this population was excluded from further analyses. Intra-population nucleotide divergence for the populations of this study was low, with a maximum value of 0.0014. Inter-population nucleotide divergence values were within the same range as intra-population values, except for the population of Sierra de Guadarrama, which had values of divergence ranging from 0.0056 to 0.0065 in relation to the rest of populations **(Table S3)**. The pairwise Φ_{ST} indicated a signifcant differentiation only in the population of Sierra de Guadarrama (Φ_{ST}=0.768–0.806) after applying the Bonferroni correction **(Table 1)**.

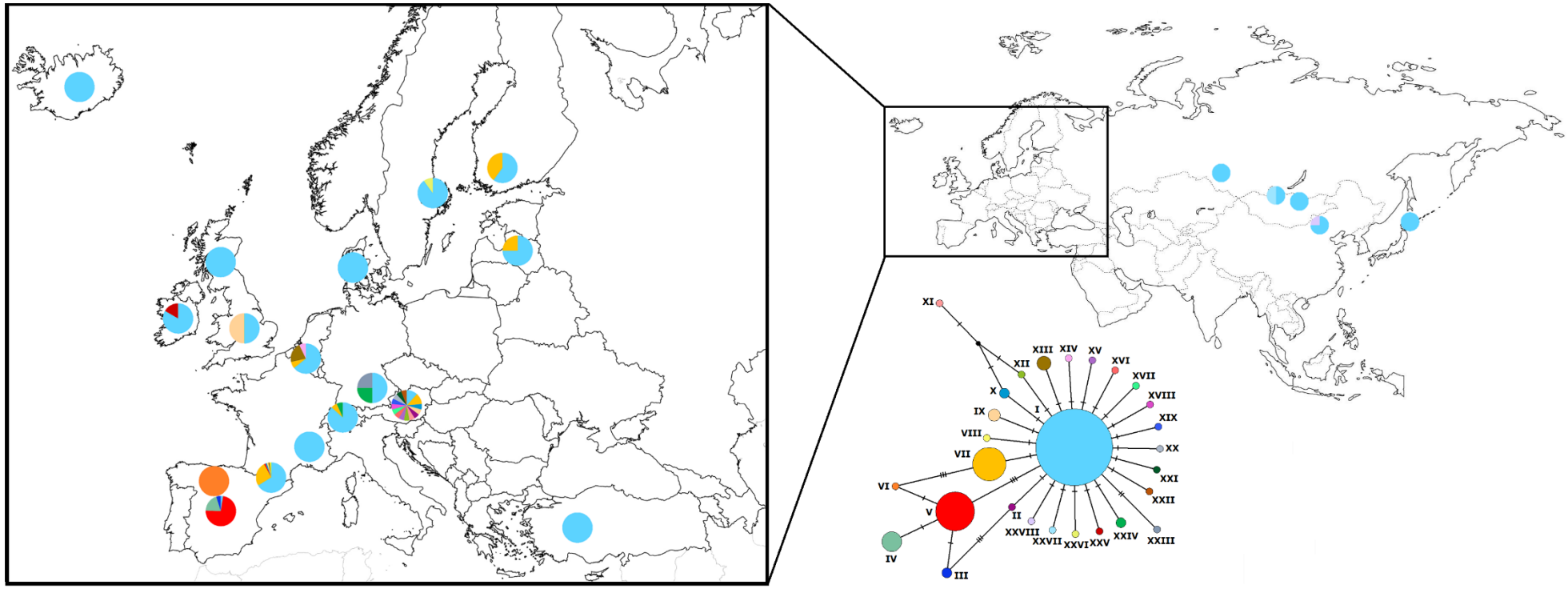


Fig. 2. *Bombus lucorum* haplotype frequencies and haplotype network based on *cox1* gene. Analysis included all *B. lucorum* sequences available in GenBank for the same *cox1* gene fragment.

Table 1. Differentiation test values (Φ_{ST}) between *B. lucorum* populations based on mitochondrial variation. *Pyr1* Pyrenees1, *Pyr2* Pyrenees2, *Pyr3* Pyrenees3, *Pyr4* Pyrenees4, *Gua* Sierra de Guadarrama, *Hfens* High Fens.

	Gua	**Pyr1**	**Pyr2**	**Pyr3**	**Pyr4**	**HFens**
Gua	NA	0.0009*	0.0009*	0.0009*	0.0009*	0.0009*
Pyr1	0.7682*	NA	0.6263	0.0819	0.6483	0.1488
Pyr2	0.7896*	-0.0284	NA	0.0349	0.2807	0.0129
Pyr3	0.8058*	0.0960	0.2747	NA	0.2467	0.9500
Pyr4	0.7907*	-0.0247	0.0010	0.0720	NA	0.4625
HFens	0.7890*	0.0374	0.1215	-0.0166	-0.0109	NA

Φ_{ST} coefcients are shown in the lower part of the matrix with their corresponding *p*-values for 1000 permutations in the upper part of the matrix. The asterisks indicate the signifcance at the 5% level after applying Bonferroni correction (*p*-value < 0.0033).

Microsatellite analysis

Three putative full sibling individuals were identified with a probability > 0.95 and discarded from the analyses. Locus B131 was monomorphic and *loci* B121 and B132 gave unclear peak patterns in the electropherograms, consequently they were discarded from further analyses. MicroChecker did not detect the presence of null alleles, nor found evidence for scoring error or allele dropout.

The lack of structure in the temporal analysis of the old (2001) and new (2013–2017) samples from the Pyrenees allowed us to gather all the samples independently of the sampling year in the following analyses (data not shown). Although STRUCTURE and Structure Harvester indicated an optimal number of groups with K=2, none of the populations showed a higher proportion of their individuals belonging to one of the two genetic groups, i.e. all individuals had a similar probability of belonging to both groups **(Fig. S2).**

The DAPC showed that the BIC reached its minimum value and an elbow in the curve at K = 3. The first 35 principal components explaining 92.4% of the variation and two discriminant functions were retained. Microsatellite *loci* B119 and B96, contributed more to define the discriminant function 1 and 2, respectively. DAPC classification of individuals in clusters was consistent with a membership probability > 91.9% in all cases. However, the clustering showed no correspondence between genetic clusters and spatial distribution of the samples **(Fig. 3a)**, with individuals from distant locations included in the three genetic clusters **(Fig. 3b)**.

Given the lack of structure, individuals were finally grouped according to their geographical proximity to calculate population genetics parameters. Allelic richness ranged from 3.8 in Monts d'Ardèche to 4.0 in High Fens. Populations of Sierra de Guadarrama and Pyrenees showed the same value of expected heterozigosity (H_E = 0.624) meanwhile the highest inbreeding coefficient value (F_{IS} = 0.466) was observed in Sierra de Guadarrama **(Table 2).**

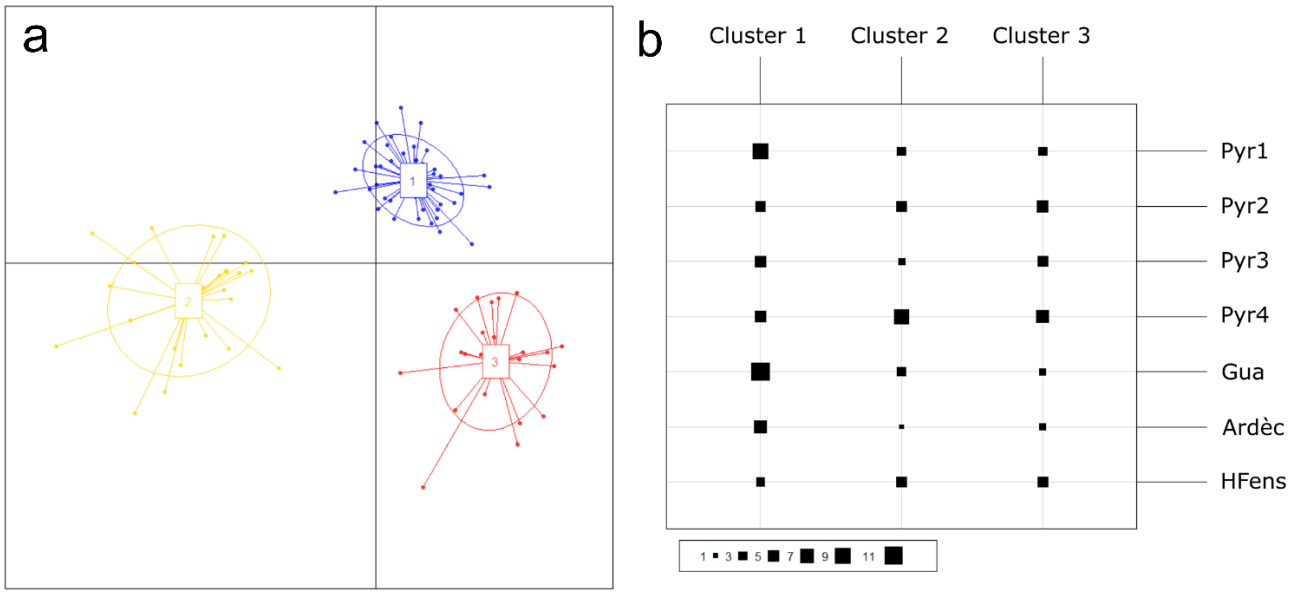


Fig. 3. Results of a nested discriminant analysis of principal components (DAPC). Three genetic clusters were identified following the Bayesian Information Criterion (BIC). **a)** Scatterplot of the frst two principal components based on microsatellite genotypes. **b)** Distribution of *B. lucorum* populations per cluster.

Signifcant linkage disequilibrium was not observed for any *loci* after applying the Bonferroni correction. Only the population of Sierra de Guadarrama showed a significant departure from Hardy–Weinberg equilibrium due to a significant heterozygosity deficiency in the *loci* B96, B100 and B124 **(Table 2).** Pairwise differentiation coefficients F_{ST} ranged from 0.0015 to 0.1277 and were not signifcant in any case after applying the Bonferroni correction (*p*-value < 0.0024) **(Table 3).** An overall migration rate of Nm = 3.40 was obtained using private alleles.

Table 2. Population genetic parameters for every *B. lucorum* population based on microsatellite variation.

	N	A_R	Npa	H_O	uH_E	F_{IS}	H-W
Sierra de Guadarrama	16	3.9	4	0.358	0.624	0.466	B96, B100, B124
Pyrenees	57	3.9	18	0.605	0.624	0.043	-
Monts d'Ardèche	9	3.8	1	0.500	0.535	- 0.111	-
High Fens	11	4.0	2	0.577	0.612	0.014	-

Number of individuals (N), allelic richness (A_R) after rarefaction, number of private alleles (Npa), observed (H_O) and unbiased expected heterozigosity (uH_E), inbreeding coefficient (F_{IS}) and *loci* showing a significant departure from Hardy-Weinberg equilibrium (H-W).

Table 3. Differentiation test values (pairwise F_{ST}) between *B. lucorum* populations based on microsatellite data.

	Sierra de Guadarrama	Pyrenees	Monts d'Ardèche	High Fens
Sierra de Guadarrama	NA	0.1878	0.4482	0.0710
Pyrenees	0.0136	NA	0.0370	0.0200
Monts d'Ardèche	0.0015	0.0483	NA	0.0221
High Fens	0.0576	0.0433	0.1277	NA

F_{ST} coefficients are shown in the lower part of the matrix with their corresponding *p*-values for 1000 permutations in the upper part of the matrix.

Geometric morphometrics

The same populations as above were considered in this analysis. When the PCA was performed, the first two principal components explained an accumulated variation of 34.7% (PC1=21.4%, PC2=13.3%). The scatterplot of variation with the two principal components did not show any clear diferentiation between the individuals of different populations **(Fig. 4a).**

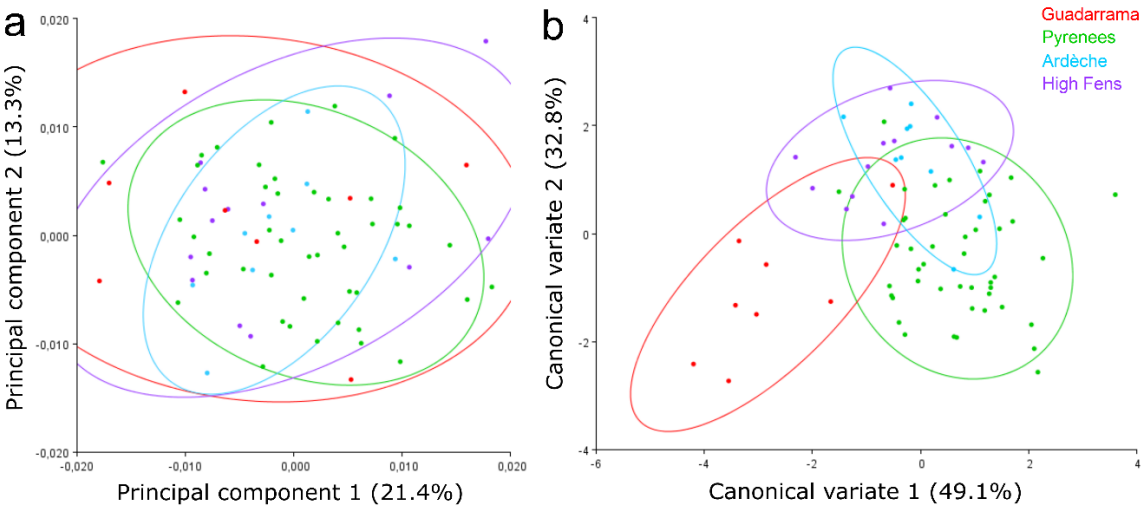


Fig. 4. Results from geometric morphometrics analysis of the wings of *B. lucorum* individuals. **a)** Scatterplot showing the first two principal components from the principal components analysis (PCA). **b)** Scatterplot showing the first two canonical variates from the canonical variance analysis (CVA).

The diferences between the Mahalanobis distances were signifcant in all the pairwise comparisons except between the populations from Monts d'Ardèche and High Fens **(Table S4).** The diferences between the Procrustres distances were not signifcant in any case after applying the Bonferroni correction **(Table S5).** The total accumulated variation explained by the first two canonical variates was 81.9% (CV1=49.1%, CV2=32.8%). The scatterplot of variation with the first two canonical variates showed an overlap in the shape of the wings for all the groups although the population of Sierra de Guadarrama seemed more differentiated with a higher within group variance **(Fig. 4b).** The discrimination between the

groups was assessed with a DFA by calculating the percentage of individuals correctly classified to one population of each pairwise comparison with a leave-one-out cross-validation test. The percentage of correct classifications ranged from 54.5 to 76.2% in all the pairwise comparisons **(Table S6).**

The results from the ANOVA analyses indicated that there was not a significant effect of the population origin of the individuals on the size of the wings (*p*-value = 0.8822) **(Table S7)** but there was a significant effect on the shape of the wings (*p*-value = 0.0022) that explained a 5.9% of the total sum of squares **(Table S8).**

Discussion

Mitochondrial and nuclear markers were analysed in *B. lucorum* individuals collected along a European gradient from the centre of the Iberian Peninsula to Belgium, with the aim of increasing our understanding of the genetic diversity and phylogeography of this bumblebee species. The results from the *cox1* gene sequence analysis resolved a divergence of *B. lucorum* from the central Iberian Peninsula from those of the Pyrenees and Central Europe. However, microsatellite and geometric morphometrics analyses failed to provide any additional evidence of differentiation.

The haplotype network based in the *cox1* gene showed a clear differentiation between the *B. lucorum* population from Sierra de Guadarrama and those from the rest of Europe, with evidence for an intermediate haplotype in the Cantabrian mountain range. Differentiation tests also supported this distinction with a highly significant Φ_{ST} value obtained between Sierra de Guadarrama and the other populations analysed. In contrast, the analysis of population structure using microsatellite data, supported no clear genetic differentiation. STRUCTURE analysis identified a similar composition of the two genetic clusters for all the individuals and DAPC analysis did not display a correlation between

the geographical location of the populations and the genetic clustering. The contrast between the degree of differentiation obtained with the *cox1* gene and the microsatellite *loci* could be explained by differences in coalescence times (Boursot & Bonhomme 1986). While the number of microsatellite *loci* and their high variability make them useful to study local gene flow and inbreeding (Queller et al. 1993), the mitochondrial marker *cox1* has a shorter coalescence time and is sex-biased because of the maternal inheritance of the mitochondrial molecule. Differentiation mirrored in *cox1* gene diversity could reflect a low dispersion of the queens. However, queens are known to migrate long distances (Mikkola 1984; Lepais et al. 2010) so the differentiation observed probably results from the short coalescence time and may reflect a differentiation event that took place in the past. Previous studies within the genus *Bombus* have already observed a contrast between the divergence of mitochondrial genes and the homogeneity with nuclear markers that could be explained due to a current gene flow between populations (Lecocq et al. 2013a, b; Moreira et al. 2015; Maebe et al. 2019).

In the case of the morphometric analysis, a slight differentiation could be seen in the CVA, but this analysis is known to maximise the differences between groups established a priori (Campbell & Atchley 1981). The DFA with a cross-validation test and the PCA are more reliable and in both cases did not indicate any population differentiation. PCA failed to resolve the different populations and DFA was unable to classify most individuals according to their population in the leave-one-out cross-validation. Furthermore, insufficient individuals from Sierra de Guadarrama were included in these analyses as most of them were males and only females were used to avoid inter-caste and inter-sex bias (Gerard et al. 2015). Although some studies have discriminated taxa at an intraspecific level with this method (Schachter-Broide et al. 2004; Toflski 2008; Francoy et al. 2011), there could be a convergent or stabilising selection on wing shape (Dockx 2007).

Nevertheless, given the lack of isolation between the populations as indicated by the microsatellite data, a differentiation in wing morphology would not be expected.

Several studies in other *Bombus* species have found a differentiation in the southern European peninsulas that support the hypothesis that they could have acted as glacial refugium during the Pleistocene (Widmer & Schmid-Hempel 1999; Lecocq et al. 2013a, 2015). In the case of B. *lucorum*, Bossert et al. (2016) already pointed out that the low genetic diversity of the species through Europe could be indicative of a rapid expansion from a glacial refugium. The divergence observed in the population of Sierra de Guadarrama with respect to European populations supports the hypothesis that the Iberian Peninsula could have acted as a glacial refugium during the Pleistocene glacial cycles. A high haplotype diversity would be expected if the peninsula acted as a refugium, therefore it would be necessary to sample more populations to finally confirm our hypothesis. Alternatively, the hypothesis of an expansion from Central Europe after the last glacial period should also be tested. Nevertheless, the differentiation found in the gene *cox1* in Sierra de Guadarrama coupled with the high inbreeding value obtained, highlight that populations of central Spain are important when evaluating the genetic diversity of *B. lucorum* as these population parameters are important factors to be measured in conservation strategies (Boettcher et al. 2010).

The lack of population structure observed with microsatellite data and geometric morphometrics is suggestive of ongoing gene flow between the populations of the Iberian Peninsula and those from Central Europe. The presence of two samples from Sierra de Guadarrama and the Pyrenees with shared haplotypes supports this view. Microsatellite data obtained for *B. lucorum* and other *Bombus* species have previously indicated that in general, there is gene flow between bumblebee populations on the mainland of Europe (Estoup et al. 1996; Moreira

et al. 2015; Potapov et al. 2018; Maebe et al. 2019) resulting in low levels of genetic differentiation despite of the geographic distance. Only significant geographical barriers, such as seas or mountain ranges seem to result in the reduction or interruption of gene flow what could be mirrored in the microsatellite *loci* variation (Estoup et al. 1996; Widmer & Schmid-Hempel 1999; Moreira et al. 2015). However, our data suggests that Pyrenees fail to act as a geographical barrier that interrupts gene flow between the northern and southern populations. It would be possible that a rapid expansion of *B. lucorum* from the Iberian Peninsula had caused a genetic homogeneity in microsatellite *loci* but given the high mutation rate of these markers it is more plausible that a minimum gene flow exists. Nevertheless, the population of Sierra de Guadarrama is the only one where a high inbreeding coefficient and a departure from Hardy–Weinberg equilibrium are observed. These results may reflect a higher degree of isolation or selective pressure in this population with respect to the northern populations analysed, probably affected by the distribution of this species: while in Europe *B. lucorum* is a common and abundant species, in the Iberian Peninsula is restricted to northern mountains (Ornosa & Ortiz-Sánchez 2004).

Although our work provides a first step in understanding the genetic structure and diversity of *B. lucorum* in the Iberian Peninsula, there is a need to sample other Iberian populations to better characterise the genetic and morphology of their populations. This will help to determine the intraspecific variability of this widespread and common species through Europe. Nonetheless, this study highlights the importance of including Iberian populations of bumblebees to determine and characterise their genetic diversity. Given the current situation of decline of insects in general (Sánchez-Bayo & Wyckhuys 2019) and pollinators in particular (Potts et al. 2010; Powney et al. 2019), our results must be taken into account when managing the conservation of bumblebee species. The observed absence of genetic structure suggests that neither geographical barriers such as

mountain ranges nor the European agricultural landscape are a barrier to gene flow. Although observed levels of genetic diversity are not as low as those observed in other species of the genus *Bombus* (Maebe et al. 2019), conservation and management plans should be developed to maintain or even increase current levels of genetic diversity. The connectivity of the current populations may be favoured to avoid the impact that changes in the landscape due to human, environmental or climate change pressure could produce limiting gene flow between populations of this species.

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Supplementary Material

Table S1. *Bombus lucorum* individuals used in this study. Populations considered in the study as *Pyr1* Pyrenees1, *Pyr2* Pyrenees2, *Pyr3* Pyrenees3, *Pyr4* Pyrenees4, *Gua* Sierra de Guadarrama, *Ard* Monts d'Ardèche, *Hfens* High Fens. Altitude (Alt.). Sex or caste (S./C.): *Q* Queen, *M* Male, *W* Worker. Mitochondrial *cox1* haplotype labelled as in Fig. 2. Microsatellite information availability (STR). Geometric morphometrics information availability (Mor.). The *hyphens* indicate missing data or non-analysed individual if they corresponded to sibling individuals or males, which were not included in that analysis.

Sample	Region	Country	Date	Alt.	GPS Lat.	GPS Lon.	S. / C.	Pop.	*cox1*	STR	Mor.
13_112	Huesca	Spain	10/07/2013	1720	42º38'12.6"N	0º0'35.0"W	W	Pyr1	VII	Yes	-
13_137	Huesca	Spain	11/07/2013	1950	42º38'5.4"N	0º2'18.2"W	Q	Pyr1	I	Yes	-
13_139	Huesca	Spain	11/07/2013	1950	42º38'5.4"N	0º2'18.2"W	W	Pyr1	I	Yes	-
13_153	Huesca	Spain	11/07/2013	1950	42º38'5.4"N	0º2'18.2"W	Q	Pyr1	I	Yes	-
13_156	Huesca	Spain	12/07/2013	1420	42º36'47.3"N	0º12'7.3"W	M	Pyr1	I	-	-
13_191	Madrid	Spain	20/07/2013	1323	40º45'14"N	4º3'50"W	M	Gua	III	-	-
13_194	Madrid	Spain	20/07/2013	1323	40º45'14"N	4º3'50"W	M	Gua	III	-	-
13_201	Madrid	Spain	20/07/2013	1323	40º45'14"N	4º3'50"W	M	Gua	IV	-	-
B14.257	Huesca	Spain	08/08/2014	1390	42º38'20.29"N	0º 1'57.11"W	W	Pyr1	VII	Yes	Yes
B14.258	Huesca	Spain	08/08/2014	1390	42º38'20.29"N	0º 1'57.11"W	W	Pyr1	VII	Yes	Yes
B14.260	Huesca	Spain	08/08/2014	1407	42º38'8.97"N	0º 1'40.22"W	M	Pyr1	I	-	-
B14.263	Huesca	Spain	08/08/2014	1665	42º38'7.00"N	0º 0'51.00"W	M	Pyr1	I	-	-
B14.266	Huesca	Spain	08/08/2014	1665	42º38'7.00"N	0º 0'51.00"W	M	Pyr1	VII	-	-
B14.269	Huesca	Spain	08/08/2014	1665	42º38'7.00"N	0º 0'51.00"W	W	Pyr1	I	Yes	Yes
B14.278	Huesca	Spain	08/08/2014	1649	42º38'6.69"N	0º 0'43.92"W	W	Pyr1	-	Yes	Yes
B14.283	Huesca	Spain	08/08/2014	1648	42º38'6.00"N	0º 0'39.00"W	M	Pyr1	VII	-	-
B14.295	Huesca	Spain	08/08/2014	1673	42º38'15.62"N	0º 0'5.95"W	Q	Pyr1	-	Yes	-
B14.317	Huesca	Spain	09/08/2014	1477	42º41'52.69"N	0º 7'18.82"W	W	Pyr1	IX	Yes	Yes
B14.347	Huesca	Spain	09/08/2014	1591	42º41'54.50"N	0º 7'46.04"W	M	Pyr1	I	-	-
B14.366	Huesca	Spain	09/08/2014	1381	42º42'2.00"N	0º 7'4.00"W	M	Pyr1	V	-	-
B14.392	Huesca	Spain	10/08/2014	914	42º37'49.19"N	0º18'58.17"W	M	Pyr1	I	-	-
B14.399	Huesca	Spain	11/08/2014	1053	42º44'24.90"N	0º47'4.33"W	M	Pyr1	I	-	-
B14.400	Huesca	Spain	11/08/2014	1053	42º44'24.90"N	0º47'4.33"W	W	Pyr1	VII	Yes	Yes
B14.401	Huesca	Spain	11/08/2014	1053	42º44'24.90"N	0º47'4.33"W	W	Pyr1	I	Yes	Yes
B14.402	Huesca	Spain	11/08/2014	1053	42º44'24.90"N	0º47'4.33"W	W	Pyr1	I	Yes	Yes
B14.403	Huesca	Spain	11/08/2014	1053	42º44'24.90"N	0º47'4.33"W	M	Pyr1	I	-	-
B14.404	Huesca	Spain	11/08/2014	1053	42º44'24.90"N	0º47'4.33"W	W	Pyr1	I	Yes	-
B14.414	Huesca	Spain	12/08/2014	1216	42º51'53.36"N	0º49'5.01"W	M	Pyr1	VII	-	-
14_136	Lérida	Spain	07/07/2014	1548	42º35'01"N	1º3'33"E	W	Pyr2	I	Yes	Yes
14_143	Lérida	Spain	08/07/2014	2035	42º35'15"N	1º0'4"E	W	Pyr2	I	Yes	Yes
14_165	Lérida	Spain	08/07/2014	2035	42º34'55"N	1º0'41"E	W	Pyr2	I	Yes	Yes
14_175	Lérida	Spain	08/07/2014	2152	42º35'21"N	0º59'16"E	W	Pyr2	VII	Yes	Yes
14_176	Lérida	Spain	08/07/2014	2152	42º35'21"N	0º59'16"E	W	Pyr2	I	Yes	Yes

14_178	Lérida	Spain	08/07/2014	2152	42º35'21"N	0º59'16"E	W	Pyr2	VII	Yes	Yes
14_179	Lérida	Spain	08/07/2014	2152	42º35'21"N	0º59'16"E	W	Pyr2	-	Yes	Yes
14_223	Lérida	Spain	10/07/2014	1369	42º34'52'N	01º07'33"E	W	Pyr2	X	Yes	-
14_238	Lérida	Spain	10/07/2014	1440	42º41'38'N	0º56'10"E	W	Pyr2	VII	Yes	-
14_340	Lérida	Spain	10/07/2014	1589	42º40'0'N	0º55'1"E	W	Pyr2	I	Yes	-
14_386	Madrid	Spain	29/07/2014	1797	40º47'31"N	4º3'35"W	M	Gua	-	Yes	-
14_388	Madrid	Spain	29/07/2014	1797	40º47'31"N	4º3'35"W	M	Gua	V	-	-
14_393	Madrid	Spain	29/07/2014	1797	40º47'31"N	4º3'35"W	M	Gua	V	-	-
14_396	Madrid	Spain	29/07/2014	1797	40º47'31"N	4º3'35"W	M	Gua	V	-	-
14_397	Madrid	Spain	29/07/2014	1797	40º47'31"N	4º3'35"W	W	Gua	V	Yes	Yes
14_410	Madrid	Spain	29/07/2014	1750	40º47'10"N	4º3'11"W	M	Gua	V	-	-
14_412	Madrid	Spain	29/07/2014	1750	40º47'10"N	4º3'11"W	M	Gua	V	-	-
14_415	Madrid	Spain	02/08/2014	1323	40º45'14"N	4º3'50"W	M	Gua	V	-	-
14_489	Madrid	Spain	08/08/2014	1830	40º49'23"N	3º57'42"W	M	Gua	V	-	-
14_495	Madrid	Spain	21/08/2014	1696	40º46'36"N	4º3'19"W	M	Gua	V	-	-
14_497	Madrid	Spain	21/08/2014	1696	40º46'36"N	4º3'19"W	M	Gua	V	-	-
14_511	Madrid	Spain	26/08/2014	1797	40º47'31"N	4º3'35"W	M	Gua	IV	-	-
14_518	Madrid	Spain	26/08/2014	1797	40º47'31"N	4º3'35"W	M	Gua	IV	-	-
14_519	Madrid	Spain	26/08/2014	1797	40º47'31"N	4º3'35"W	M	Gua	IV	-	-
14_530	Madrid	Spain	26/08/2014	1750	40º47'10"N	4º3'11"W	M	Gua	IV	-	-
B15.154	Lérida	Spain	02/09/2015	2174	42º35'20.4"N	0º59'13.2"E	W	Pyr2	I	Yes	Yes
B15.157	Lérida	Spain	02/09/2015	2174	42º35'20.4"N	0º59'13.2"E	W	Pyr2	VII	Yes	Yes
B15.164	Lérida	Spain	04/09/2015	1405	42º27'4.2"N	1º4'26"E	Q	Pyr2	VII	Yes	-
15_3	Madrid	Spain	01/05/2015	1260	40º45'14"N	4º3'50"W	Q	Gua	V	Yes	-
15_58	Gerona	Spain	8/07/2015	2175	42º25'36"N	2º15'52"E	W	Pyr3	-	Yes	-
15_70	Gerona	Spain	9/07/2015	1730	42º23'05" N	1º56'37"E	Q	Pyr3	I	Yes	-
15_73	Gerona	Spain	9/07/2015	1730	42º23'05" N	1º56'37"E	W	Pyr3	I	Yes	Yes
15_76	Gerona	Spain	9/07/2015	1730	42º23'05" N	1º56'37"E	W	Pyr3	I	Yes	Yes
15_81	Gerona	Spain	9/07/2015	1730	42º23'05" N	1º56'37"E	W	Pyr3	I	Yes	Yes
15_89	Gerona	Spain	9/07/2015	1730	42º23'05" N	1º56'37"E	W	Pyr3	I	Yes	Yes
15_96	Gerona	Spain	9/07/2015	1800	42º19'13" N	1º58'04"E	W	Pyr3	I	Yes	Yes
15_98	Gerona	Spain	9/07/2015	1800	42º23'05" N	1º58'04"E	W	Pyr3	I	Yes	Yes
15_100	Gerona	Spain	9/07/2015	1800	42º23'05" N	1º58'04"E	W	Pyr3	I	Yes	Yes
15_101	Gerona	Spain	9/07/2015	1800	42º23'05" N	1º58'04"E	W	Pyr3	I	Yes	Yes
15_102	Gerona	Spain	9/07/2015	1800	42º23'05" N	1º58'04"E	W	Pyr3	I	Yes	Yes
15_198	Madrid	Spain	20/07/2015	1797	40º47'31"N	4º3'35"W	M	Gua	V	-	-
15_200	Madrid	Spain	20/07/2015	1797	40º47'31"N	4º3'35"W	M	Gua	IV	-	-
15_205	Madrid	Spain	20/07/2015	1797	40º47'31"N	4º3'35"W	M	Gua	-	-	-
15_208	Madrid	Spain	20/07/2015	1797	40º47′31″N	4º3′35″W	W	Gua	-	Yes	-
15_212	Madrid	Spain	20/07/2015	1797	40º47′31″N	4º3′35″W	M	Gua	V	-	-
15_214	Madrid	Spain	20/07/2015	1797	40º47′31″N	4º3′35″W	M	Gua	V	-	-
15_220	Madrid	Spain	20/07/2015	1797	40º47′31″N	4º3′35″W	W	Gua	V	Yes	-
15_221	Madrid	Spain	20/07/2015	1797	40º47′31″N	4º3′35″W	W	Gua	V	Yes	-
15_224	Madrid	Spain	04/08/2015	1750	40º47′10″N	4º3′11″W	W	Gua	V	Yes	-

15_225	Madrid	Spain	04/08/2015	1750	40º47′10″N	4º3′11″W	M	Gua	V	Yes	-
15_230	Madrid	Spain	04/08/2015	1750	40º47′10″N	4º3′11″W	M	Gua	IV	-	-
15_232	Madrid	Spain	04/08/2015	1750	40º47′10″N	4º3′11″W	M	Gua	V	-	-
15_236	Madrid	Spain	04/08/2015	1750	40º47′10″N	4º3′11″W	M	Gua	-	-	-
15_242	Madrid	Spain	04/08/2015	1750	40º47'10"N	4º3'11"W	M	Gua	V	-	-
15_243	Madrid	Spain	04/08/2015	1750	40º47'10"N	4º3'11"W	M	Gua	I	-	-
15_245	Madrid	Spain	04/08/2015	1750	40º47'10"N	4º3'11"W	M	Gua	-	Yes	-
15_246	Madrid	Spain	04/08/2015	1750	40º47'10"N	4º3'11"W	M	Gua	IV	-	-
15_252	Madrid	Spain	20/07/2015	1797	40º47'31"N	4º3'35"W	W	Gua	V	Yes	Yes
15_253	Madrid	Spain	20/07/2015	1797	40º47'31"N	4º3'35"W	W	Gua	V	Yes	Yes
16_22	Madrid	Spain	26/08/2016	1797	40º47´31"N	4º3'35"W	W	Gua	-	Yes	Yes
17_14	Madrid	Spain	18/06/2017	1230	40º45'14"N	4º3'50"W	M	Gua	V	-	-
17_53	Palencia	Spain	21/06/2017	1355	43º02'29"N	4º27'10"W	W	-	VI	-	-
17_74	Madrid	Spain	25/06/2017	1230	40º45'14"N	4º3'50"W	W	Gua	V	Yes	Yes
17_85	Madrid	Spain	26/07/2017	1797	40º47'31"N	4º3'35"W	W	Gua	-	-	Yes
17_87	Madrid	Spain	26/07/2017	1797	40º47'31"N	4º3'35"W	M	Gua	V	-	-
17_88	Madrid	Spain	26/07/2017	1797	40º47'31"N	4º3'35"W	M	Gua	V	-	-
17_89	Madrid	Spain	26/07/2017	1797	40º47'31"N	4º3'35"W	W	Gua	V	Yes	Yes
17_90	Madrid	Spain	26/07/2017	1797	40º47'31"N	4º3'35"W	W	Gua	-	Yes	Yes
17_91	Madrid	Spain	26/07/2017	1797	40º47'31"N	4º3'35"W	M	Gua	V	-	-
17_97	Madrid	Spain	26/07/2017	1797	40º47'31"N	4º3'35"W	Q	Gua	V	Yes	-
17_106	Madrid	Spain	01/09/2017	1797	40º47'31"N	4º3'35"W	M	Gua	V	-	-
D1	Languedoc -Roussillon	France	03/07/2001	2130	42º38'38.1"N	02º12'22.2"E	W	Pyr4	VII	Yes	Yes
D2	Languedoc -Roussillon	France	03/07/2001	2150	42º38'38.1"N	02º12'25.9"E	W	Pyr4	I	Yes	Yes
D3	Languedoc -Roussillon	France	02/07/2001	2030	42º38'45.0"N	02º22'53.7"E	W	Pyr4	I	Yes	Yes
D4	Languedoc -Roussillon	France	02/07/2001	2030	42º38'45.0"N	02º22'53.7"E	W	Pyr4	XIII	Yes	Yes
D5	Languedoc -Roussillon	France	09/07/2001	1665	42º28'22.1"N	02º05'20.8"E	W	Pyr4	I	Yes	Yes
D6	Languedoc -Roussillon	France	05/07/2001	2160	42º38'27.4"N	02º12'55.2"E	W	Pyr4	I	Yes	Yes
D7	Languedoc -Roussillon	France	02/07/2001	2030	42º38'45.0"N	02º22'53.7"E	W	Pyr4	VII	Yes	Yes
D8	Languedoc -Roussillon	France	12/07/2001	2742	42º25'24.0"N	02º09'24.5"E	W	Pyr4	I	Yes	Yes
D9	Languedoc -Roussillon	France	08/07/2001	1817	42º28'35.9"N	02º06'17.2"E	W	Pyr4	VIII	Yes	Yes
D10	Languedoc -Roussillon	France	12/07/2001	2723	42º25'21.5"N	02º09'16.5"E	W	Pyr4	I	Yes	Yes
D11	Languedoc -Roussillon	France	12/07/2001	2723	42º25'21.5"N	02º09'16.5"E	W	Pyr4	I	Yes	Yes
D12	Languedoc -Roussillon	France	12/07/2001	2723	42º25'21.5"N	02º09'16.5"E	W	Pyr4	I	Yes	Yes

D13	Languedoc -Roussillon	France	12/07/2001	2723	42º25'21.5"N	02º09'16.5"E	W	Pyr4	I	Yes	Yes
D14	Languedoc -Roussillon	France	12/07/2001	2723	42º25'21.5"N	02º09'16.5"E	W	Pyr4	-	-	-
D15	Languedoc -Roussillon	France	09/07/2001	1665	42º28'22.1"N	02º05'20.8"E	W	Pyr4	I	Yes	Yes
D16	Languedoc -Roussillon	France	08/07/2001	1817	42º28'35.9"N	02º06'17.2"E	W	Pyr4	I	Yes	Yes
D17	Languedoc -Roussillon	France	08/07/2001	1817	42º28'35.9"N	02º06'17.2"E	W	Pyr4	VII	Yes	Yes
D18	Languedoc -Roussillon	France	04/07/2001	2070	42º38'37.7"N	02º12'57.3"E	W	Pyr4	-	Yes	Yes
D19	Languedoc -Roussillon	France	03/07/2001	2030	42º38'51.2"N	02º23'01.3"E	W	Pyr4	-	Yes	Yes
D20	Languedoc -Roussillon	France	08/07/2001	1817	42º28'35.9"N	02º06'17.2"E	W	Pyr4	VII	Yes	Yes
D23	Namur	Belgium	18/07/1991	200			W	-	-	-	-
D24	Namur	Belgium	21/07/1991	200			W	-	XIII	-	-
D25	Namur	Belgium	22/07/1991	200			W	-	XIII	-	-
D26	Liège	Belgium	21/07/1999	500	50º27'09"N	6º03'54"E	W	HFens	I	Yes	Yes
D28	Liège	Belgium	21/07/1999	500	50º27'09"N	6º03'54"E	W	HFens	I	Yes	Yes
D29	Liège	Belgium	26/07/1999	500	50º27'09"N	6º03'54"E	W	HFens	I	-	Yes
D30	Liège	Belgium	21/07/1999	500	50º27'09"N	6º03'54"E	W	HFens	I	-	Yes
D31	Liège	Belgium	27/07/1999	500	50º27'09"N	6º03'54"E	W	HFens	I	Yes	Yes
D32	Liège	Belgium	22/07/1999	500	50º27'09"N	6º03'54"E	W	HFens	I	Yes	Yes
D33	Liège	Belgium	21/07/1999	500	50º27'09"N	6º03'54"E	W	HFens	XIII	Yes	Yes
D34	Liège	Belgium	22/07/1999	500	50º27'09"N	6º03'54"E	W	HFens	I	Yes	Yes
D36	Liège	Belgium	12/07/1999	500	50º27'09"N	6º03'54"E	W	HFens	XIV	Yes	Yes
D37	Liège	Belgium	21/07/1999	500	50º27'09"N	6º03'54"E	W	HFens	I	Yes	Yes
D38	Liège	Belgium	06/07/1999	675	50º31'06"N	6º04'19"E	W	HFens	VII	Yes	Yes
D39	Liège	Belgium	27/07/1999	610	50º27'49"N	6º19'52"E	W	HFens	I	Yes	Yes
D40	Liège	Belgium	27/07/1999	610	50º27'49"N	6º19'52"E	W	HFens	-	Yes	Yes
D43	Ardèche	France	14/07/1986	1169			W	Ard	-	Yes	Yes
D44	Ardèche	France	14/07/1986	1169			W	Ard	-	Yes	Yes
D45	Ardèche	France	14/07/1986	1169			W	Ard	I	Yes	Yes
D46	Ardèche	France	14/07/1986	1169			W	Ard	-	Yes	Yes
D47	Ardèche	France	14/07/1986	1169			W	Ard	-	Yes	Yes
D48	Ardèche	France	14/07/1986	1169			W	Ard	-	Yes	Yes
D49	Ardèche	France	14/07/1986	1169			W	Ard	-	Yes	Yes
D50	Ardèche	France	14/07/1986	1169			W	Ard	-	Yes	Yes
D51	Ardèche	France	14/07/1986	1169			W	Ard	-	Yes	Yes

Table S2. GenBank accession numbers of *B. lucorum cox1* sequences from previous studies used in the phylogeographic study.

Sample	Country	Haplotype	Sample	Country	Haplotype
GU705906	Germany	XXIV	JN872600	Denmark	I
JQ843494	Switzerland	XXIV	JN872601	Ireland	I
GU705909	Germany	I	JN872602	Ireland	I
GU705910	Germany	I	JN872603	Ireland	I
JQ692957	Sweden	I	JN872605	Ireland	I
JQ843496	Latvia	I	JQ843530	Switzerland	I
JQ843499	Latvia	I	JQ843546	Sweden	I
JQ843500	Switzerland	I	JQ843493	Switzerland	I
JQ843504	Mongolia	I	JQ843497	Switzerland	I
JQ843505	Russia	I	JQ843502	Turkey	I
JQ843506	Russia	I	LN714024	Austria	I
JQ843507	Russia	I	JQ843492	Russia	I
JQ843509	Turkey	I	JQ843508	Russia	I
JQ843510	Turkey	I	JQ843517	Russia	I
JQ843511	Russia	I	JQ843518	Russia	I
JQ843513	Iceland	I	JQ843519	Russia	I
JQ843514	Russia	I	JQ843520	Russia	I
JQ843515	Russia	I	JQ843531	Switzerland	I
JQ843521	Russia	I	JQ843534	Switzerland	I
JQ843522	Russia	I	JQ843512	Mongolia	XXVII
JQ843523	Russia	I	JQ843542	Sweden	XXVI
JQ843524	China	I	JQ843516	China	XXVIII
JQ843525	Switzerland	I	JQ843545	UK	IX
JQ843528	Switzerland	I	LN714030	Austria	IX
JQ843529	Sweden	I	JN872588	Finland	VII
JQ843532	Switzerland	I	JN872589	Finland	VII
JQ843533	Switzerland	I	JQ843501	Latvia	VII

Sample	Country	Haplotype
JQ843535	Switzerland	I
JQ843536	China	I
JQ843537	Switzerland	I
JQ843538	China	I
JQ843539	Sweden	I
JQ843540	Sweden	I
JQ843541	Sweden	I
JQ843543	Sweden	I
JQ843544	UK	I
JQ843547	Sweden	I
JQ843548	Mongolia	I
LC123695	Japan	I
JN872590	Finland	I
JN872591	Finland	I
JN872592	Finland	I
JN872593	UK	I
JN872594	UK	I
JN872595	Ireland	I
JN872596	Denmark	I
JN872597	Denmark	I
JN872598	Denmark	I
JN872599	Denmark	I

Sample	Country	Haplotype
LN714036	Austria	VII
JQ843498	Switzerland	VII
LN714037	Austria	VII
LN714040	Austria	I
LN714039	Austria	XV
LN714025	Austria	XVI
LN714026	Austria	XVII
LN714027	Austria	XVIII
LN714038	Austria	II
LN714028	Austria	XIX
LN714035	Austria	XX
LN714034	Austria	XXI
LN714029	Austria	XXII
LN714033	Austria	XII
LN714031	Austria	X
LN714032	Austria	XI
JN872604	Ireland	XXV
KJ838784	Germany	XXIII
JQ843503	Latvia	I
JQ843526	Switzerland	I
JQ843527	Sweden	I

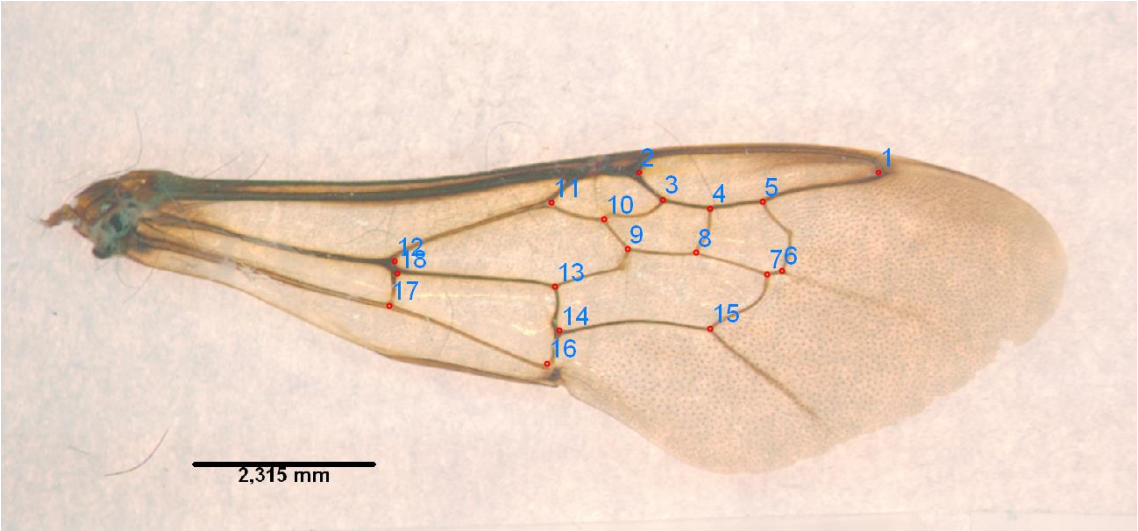


Fig. S1. *B. lucorum* worker wing with the eighteen landmarks plotted for geometric morphometrics analyses.

Table S3. *B. lucorum* intra-population and inter-population nucleotide divergence values based on mitochondrial variation. Only those populations with more than 12 individuals were selected. *Pyr1* Pyrenees1, *Pyr2* Pyrenees2, *Pyr3* Pyrenees3, *Pyr4* Pyrenees4, *Gua* Sierra de Guadarrama, *Hfens* High Fens.

	Intra-population divergence	Inter-population divergence				
		Gua	Pyr1	Pyr2	Pyr3	Pyr4
Gua	0.0010					
Pyr1	0.0014	0.0060				
Pyr2	0.0012	0.0065	0.0012			
Pyr3	0.0000	0.0056	0.0008	0.0008		
Pyr4	0.0010	0.0062	0.0012	0.0011	0.0006	
HFens	0.0009	0.0060	0.0012	0.0012	0.0004	0.0009

Fig. S2. Barplot from STRUCTURE showing proportion of membership of each pre-defined *B. lucorum* population in every cluster based on microsatellite data. The number of clusters shown is the optimal according to Structure Harvester (K = 2).

Table S4. Mahalanobis distances from Canonical Variance Analysis (CVA) based on geometric morphometrics data.

	Sierra de Guadarrama	Pyrenees	Monts d'Ardèche	High Fens
Sierra de Guadarrama	NA	<.0001*	0.0008*	0.0021*
Pyrenees	3.586*	NA	0.0080*	<.0001*
Monts d'Ardèche	3.900*	2.501*	NA	0.4411
High Fens	3.556*	2.397*	2.521	NA

Mahalanobis distance values are shown in the lower part of the matrix with their correspondent *p*-values for 10000 permutations in the upper part of the matrix. The *asterisks* indicate the significance at the 5% level after applying Bonferroni correction (*p*-value < 0.0083).

Table S5. Procrustres distances from Canonical Variance Analysis (CVA) based on geometric morphometrics of the wings.

	Sierra de Guadarrama	Pyrenees	Monts d'Ardèche	High Fens
Sierra de Guadarrama	NA	0.0636	0.3112	0.4007
Pyrenees	0.0090	NA	0.1360	0.0356
Monts d'Ardèche	0.0102	0.0076	NA	0.4859
High Fens	0.0090	0.0077	0.0080	NA

Procrustres distance values are shown in the lower part of the matrix with their correspondent *p*-values for 10000 permutations in the upper part of the matrix. No results were significant at the 5% level after applying Bonferroni correction (*p*-value < 0.0083).

Table S6. Discriminant Function Analysis (DFA) results from the leave-one-out cross-validation test. Percentage of individuals correctly classified within each population pair.

	Sierra de Guadarrama	Pyrenees	Monts d'Ardèche
Pyrenees	64.2%		
Monts d'Ardèche	58.8%	59.3%	
High Fens	76.2%	56.9%	54.5%

Table S7. ANOVA results for centroid size variation.

Effect	SS	MS	df	F	P
Population	7684.290924	2561.430308	3	0.22	0.8822
Individual	826740.068987	11644.226324	71		

Table S8. ANOVA results for shape variation.

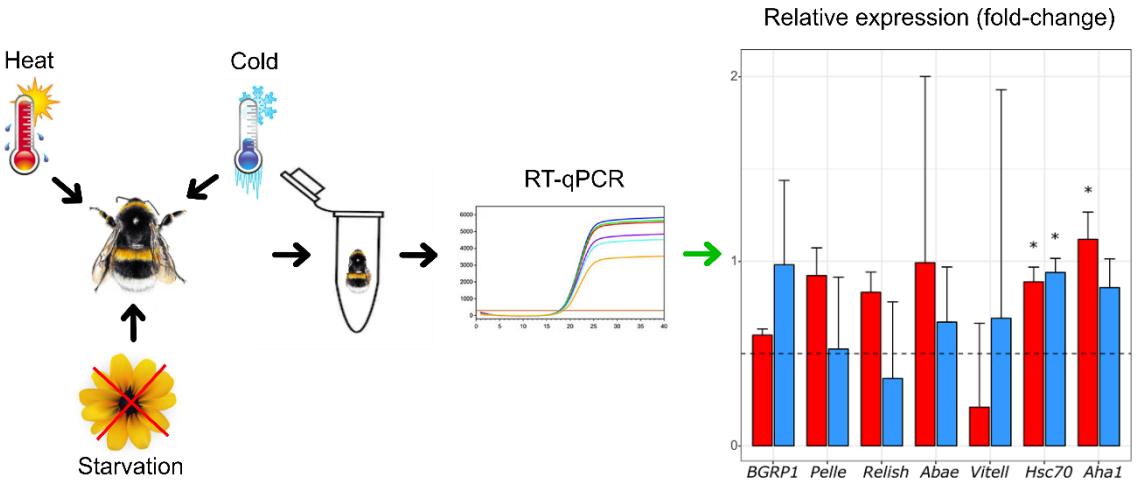
Effect	SS	MS	df	F	P
Population	0.00148192	0.0000154367	96	1,48	0,0022*
Individual	0.02376316	0.0000104591	2272		

The *asterisk* indicates the significance at the 5% level.

Part II

Chapter 3

The impact of temperature variation on immune gene expression in the bumblebee *Bombus terrestris*



Abstract

Body temperature changes can modify an insect's ability to fight infections by altering its immune activity. This work evaluated the impact of mild cold and heat thermal stress on the expression of different immune and heat shock genes in the species *Bombus terrestris*. Additionally, a thermal treatment was repeated under starvation to analyse a possible compromise of immune gene expression in favour of a thermal stress response when energetic resources are limited. Results pointed to a role of *Hsc70* and *Aha1* genes in tolerance to moderately high temperatures. Expression of the immune genes was not negatively affected by the cold or the heat treatments, and the receptor gene *BGRP1* was upregulated with cold, possibly indicating an increase in the cellular immune activity. Under starvation, the effect of the heat and the cold treatments caused a higher upregulation in all genes analysed, suggesting a synergistic effect of starvation and thermal stress on the activation of heat shock and immune genes expression.

Keywords: bumblebee, cold stress, heat stress, immunity, RT-qPCR

Introduction

Insects are ectothermic organisms whose basic physiological functions such as locomotion, growth and reproduction are strongly influenced by environmental temperature (Huey & Stevenson 1979). Changes in body temperature can affect the performance of insects, altering their capacity to fight infections (Thomas & Blanford 2003). It has been proposed that temperature can directly modify the immune response in different ways: altering thermosensitive cellular and enzymatic processes that drive immune activity (Somero 1995; Le Morvan et al. 1998), eliciting the activation of pathways and mechanisms that are shared between thermal and immune stresses (Sinclair et al. 2013) or compromising the immune response as a trade-off with the thermal response (Ferguson et al. 2018). Interaction between the immune and the thermal stress pathways (cross-talk) and activation of shared mechanisms (cross-tolerance) can confer an adaptive advantage if both stressors are encountered in nature simultaneously, e.g. an insect facing a pathogen and a cold temperature at the same time. Nonetheless, if the mechanisms activated by these two pathways are different and have a high energetic cost associated, protection against one of the stressors could be compromised with negative consequences (Sinclair et al. 2013).

Heat shock proteins (HSPs) are produced as a response mechanism for thermal stress. The transcription of this group of proteins was first found to increase dramatically under elevated temperatures (Ritossa 1962). However, they have also been found to increase under different stressful conditions such as cold, osmotic and oxidative stress, hypoxia, exposure to toxic substances or infections (Feder & Hofmann 1999). They help to protect other proteins from denaturation and assist in the refolding or degradation of aberrant ones. There are six major families according to their molecular mass and function: small HSP, HSP40, HSP60, HSP70, HSP90 and HSP100 (Fink 1999). In mammals, they have been

proposed to act as danger signals and immunoregulatory molecules (Pockley & Henderson 2017). In insects, there is also evidence supporting their role in the immune response (Wojda & Kowalski 2013; Merkling et al. 2015). For example, the heat shock transcription factor (Hsf) has been found to be involved in the antiviral response in *Drosophila* spp. (Merkling et al. 2015) and Paim et al. (2016) found that the knockdown of Hsp70/Hsc70 compromised the immune response in the hemipteran *Rhodnius prolixus*. HSP70 and HSP90 members are the most widely studied in insects under different stresses (Zhao & Jones 2012).

Among the HSP70 family, 70-kDa heat shock cognate protein (Hsc70) isoforms are constitutively expressed, highly conserved across organisms and involved in multiple cellular functions such as protein folding, intracellular transport, degradation and endocytosis (Stricher et al. 2013). On the other hand, Hsp90 isoforms can be constitutive or inducible, are also very conserved, and participate in protein maturation and degradation, signal transduction and protein homeostasis in different stress conditions (Taipale et al. 2010). In the bumblebee *Bombus terrestris*, previous studies have shown an upregulation of *Hsc70* and *Hsp90* genes in relation to diapause (Kim et al. 2008), and Riddell et al. (2011) found the gene that codifies the activator of the heat shock protein 90 ATPase *(Aha1)* to be upregulated upon infection when performing a subtractive suppression hybridization (SSH).

B. terrestris is the most common bumblebee species in Europe and an important commercial pollinator. Over the last 50 years, a decline in bumblebee populations has been documented and many stressors are thought to be implicated, including the recent spread of pathogens due to the transport of commercial bee species (Goulson et al. 2015). This event has stimulated an interest in characterising bumblebee immunity and its interaction with stressors such as protein limitation (Brunner et al. 2014), infection (Barribeau & Schmid-Hempel 2013), wounding (Erler et al. 2011) and exposure to pesticides (Walderdorff et al. 2018), and has

turned this species into a model for studying insect immunity. However, the effect of thermal stress on bumblebee immunity has not yet been characterised. As extreme weather events are predicted to increase with climate change and expected to have a large impact on bumblebee populations (Goulson et al. 2015), it seems necessary to understand how thermal stress may affect the bumblebee's ability to fight pathogens. Temperature shifts away from the thermal optima of the host are expected to increase their susceptibility to parasites, as the latter have shorter generation times and faster acclimation rates (Raffel et a. 2013; Cohen et al. 2017, Rohr et al. in review). Tobin et al. (2019) found that the infection outcomes of the parasite *Crithidia bombi* on *Bombus impatients* was constant across a range of moderate temperatures, while Palmer-Young et al. (2019) found that moderately high temperatures decreased infection even when they were optimal for the parasite growth in vitro. Therefore, the immune system of bumblebees could perform better under mild heat stress. Regarding cold, there are not previous studies that let us predict its effect on the immune system, but, considering that bumblebees are cold-adapted species, we expect them to maintain or even increase immune activity at low temperatures (Heinrich 1979).

The aim of this work is to provide a first insight into how mild thermal stress can affect bumblebee immunity and gain a better understanding of the interaction of these two factors on insect physiology. Our hypothesis is that thermal stress affects immune activity by increasing the expression of immune genes. To test this hypothesis, we analysed the expression of different immune and heat shock genes under mild thermal stress treatments in commercial nests of the species *B. terrestris*. We selected a hot (38 ºC) and a cold (9 ºC) temperature that bumblebees can face in their natural habitat but are far from their ideal temperature (28 ºC). The heat shock genes *Hsc70* and *Aha1* were selected as indicators of thermal stress, and immune genes codifying several components of the immune system were selected to obtain a general view of the immune activity. Expression was

measured at time points ranging from 2 to 48 h to capture the response of genes with distinct temporal expression patterns.

Materials and methods

Bumblebee thermal treatments

Three young colonies of *B. terrestris* were obtained from Agrobio S.L. (Almería, Spain) for gene expression analysis. Six groups of three callow workers per nest were collected and placed in microcolonies with pollen and 50% sugar/water *ad libitum* and kept in darkness at 28 ºC and 60% humidity. This microcolony-based set-up removed the thermoregulation effect of the colony and allowed us to measure the impact that environmental temperature would have upon foraging bumblebees. When workers were between 4 and 6 days of age, they were placed in an incubator and assigned to different temperature treatments: cold temperature (9 ºC), hot temperature (38 ºC) and control (28 ºC). For each colony and treatment, one group of three individuals was snap-frozen in liquid nitrogen after 2 h, 6 h, 12 h, 24 h and 48 h of treatment. They were always fed with pollen and sugar/water *ad libitum* except for the 6 h treatment that was replicated without feeding the bees to analyse the response under resource limitation (starvation) and test a possible trade-off between thermal stress and the immune activity. The cold treatment in starving bees (9 ºC) could not be tested in one of the nests, as it did not have enough callow workers. Individuals were kept at -80 ºC until they were analysed.

RNA extraction and cDNA synthesis

Workers were dissected and the abdomens and thorax kept to ensure the presence of both *hsp* and immune gene transcripts. Homogenization was performed with a TissueRuptor (Qiagen) in lysis RT solution (Invitrap kit,

Stratec), and each group of three individuals per colony and treatment was pooled. RNA extractions were performed following the manufacturer's instructions (Invitrap kit, Stratec). Isolated RNA was quantified with NanoDrop 1000 (Thermo Fisher Scientific®), diluted to a concentration of 200 ng/µl and treated with a TURBO DNA-free kit (Ambion, Life Technologies) to remove genomic DNA contamination. cDNA was synthesised using PrimeScript™ RT Reagent Kit (Takara Bio Inc.) and stored at −20 °C.

Quantitative PCR analysis

To select the optimal genes for normalisation, we analysed the stability of a set of reference genes: *arginine kinase (AK), elongation factor 1α (Ef1α), phospholipase A2 (PLA2), α-tubulin (TUB), β-actin (ACTB), ribosomal protein L13 (RPL13), ribosomal protein L23 (RPL23) and inositol 1,4,5-triphosphate (ITPR),* using the thorax and the abdomen of bumblebees in our experimental temperature conditions (38 °C, 28 °C and 9 °C).

Five genes from different classes were chosen to quantify the immune response: the receptor *BGRP1* involved in pathogen recognition, the signaling genes *Pelle* and *Relish* from the Toll and Imd pathways respectively, the antimicrobial peptide gene *Abaecin* and the gene *Vitellogenin* that codifies a protein with antimicrobial activity and is involved in metabolism, pathogen recognition and transgenerational immune priming (Barribeau & Schmid-Hempel 2013; Salmela et al. 2015; Park et al. 2018). The heat shock genes *Hsc70* and *Aha1* were analysed to quantify the thermal stress response. Primers used have been previously published **(Table S1)** except for *Hsc70* primers which were designed with Primer Express 3 software (Applied Biosystems). Efficiencies and specific amplifications for all genes were checked using serial dilutions of cDNA with the standard curve method. Quantitative PCR was performed with NZYSpeedy qPCR Green Master (NZYTech) with primers at 0.1 µM and following a program consisting

of an initial denaturation step at 95 °C for 10 min and 40 amplification cycles of denaturation at 95 °C for 15 s and annealing/elongation at 60 °C for 1 min. Non-template samples were used as negative controls and a final melt-curve step was added to check for non-specific amplifications. Every reaction was run in triplicate on a StepOnePlus™ Real-Time PCR system (Applied Biosystems).

Expression data analyses

Reference gene stability was checked with the 6 h treatment samples, following the procedures described above. Raw C_T data were obtained with StepOne Software v2.3, transformed to logarithmic scale and corrected using efficiencies. The most stable reference genes and the optimal number of needed genes were obtained with geNorm (Vandesompele et al. 2002) and NormFinder (Andersen et al. 2004) algorithms.

The selected reference genes were used as internal controls for the expression experiment with target genes. Raw data were obtained with the previous qPCR conditions and expression was analysed using the delta-delta-C_T ($\Delta\Delta C_T$)-method (Livak & Schmittgen 2001) considering efficiencies correction. Every pool of three individuals per nest was treated as a biological replicate, and three biological and three technical replicates were used. GraphPad Prism version 5.00 software (GraphPad Software, San Diego, California, USA, www.graphpad.com) was used for statistical analyses. An unpaired t-test was performed with deltaC_T mean values for every time point and gene to detect significant effects ($p < 0.05$) of the cold (9 °C) and heat (38 °C) treatments against the control (28 °C) treatment. Welch's correction was applied when variances were significantly ($p < 0.05$) different.

Results

Reference genes validation for thermal treatments

Three out of eight candidate reference genes were discarded from the validation test after showing unspecific amplifications or low efficiencies **(Table S1).** The five remaining genes (*PLA2, TUB, ACTB, RPL23* and *ITPR*) showed low and similar M values with both geNorm and NormFinder algorithms, indicating a high stability **(Table 1).** Since the optimal number of reference genes necessary for normalisation was two according to geNorm **(Fig. S1),** the genes *RPL23* and *TUB* were chosen for expression analyses.

Table 1. Stability values of reference genes (M) calculated with the algorithms geNorm and NormFinder. All genes were considered valid for normalisation as their stability values were under the cut-off value (M = 1.5) established by geNorm.

Algorithm	**Gene**				
	ACTB	***ITPR***	***PLA2***	***RPL23***	***TUB***
geNorm	0.240	0.464	0.326	0.272	0.270
NormFinder	0.177	0.082	0.095	0.144	0.128

Effect of thermal treatments on gene expression

Results from the analysed immune and heat shock gene expression dynamics are shown in **Fig. 1.** A general upregulation was observed under cold and hot temperatures with respect to the control treatment, except for the genes *Vitellogenin* and *Abaecin*, which were downregulated at several time points. Fold change values were usually below 0.5, but higher values were obtained for the genes *BGRP1*, *Abaecin*, *Hsc70* and *Aha1*. T-tests carried out with deltaC$_T$ values showed a significant change in expression in six out of the seven genes analysed: the cold treatment caused a significant upregulation in *BGRP1* (24 h log2 fold change = 1.02*), *Relish* (2 h log2 fold change = 0.48*), *Hsc70* (24 h log2 fold change = 0.40***) and a significant downregulation in *Abaecin* (6 h log2 fold change =

-0.79*), whereas the heat treatment caused a significant upregulation in *Pelle* (6 h log2 fold change = 0.44**), *Hsc70* (2 h log2 fold change = 1.81**; 6 h log2 fold change = 0.75*; 24 h log2 fold change = 0.31**) and *Aha1* (2 h log2 fold change = 1.01*; 24 h log2 fold change = 0.79*; 48 h log2 fold change = 0.94**). No clear expression patterns through time were observed except for *Hsc70,* which showed a peak of upregulation at 2 h followed by a decrease. The cold treatment apparently decreased the expression of *Vitellogenin* and *Aha1* progressively but no significant downregulation was obtained. A tendency for downregulation and upregulation of *Abaecin* was observed for the heat and cold treatments at 6 h, respectively, but there was a high variability between biological replicates.

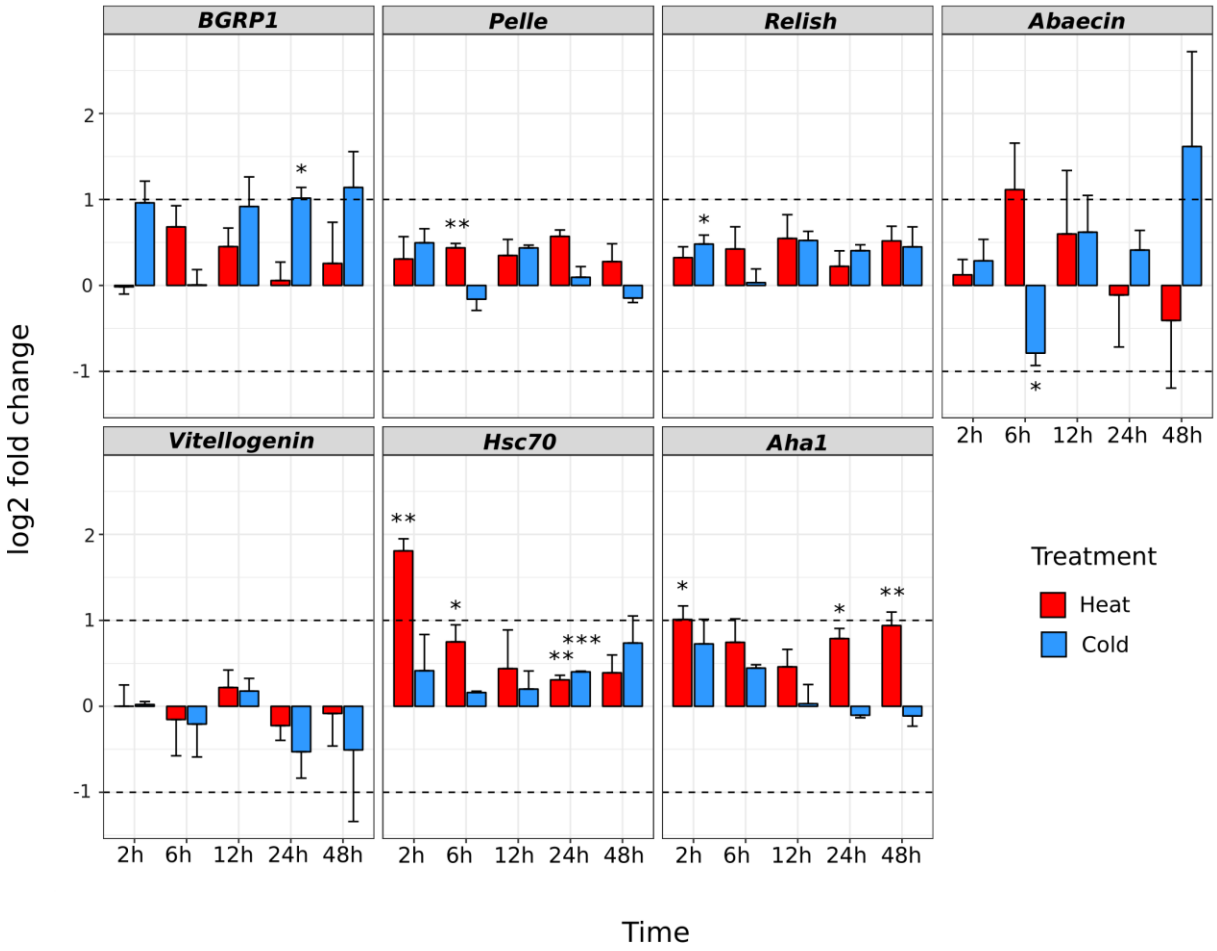


Fig. 1. Temporal expression of immune and heat shock genes. Log2 fold change values are shown for the cold (9 °C) and heat (38 °C) treatments with respect to the control temperature (28 °C). The x-axis indicates the time points at which the samples were taken. Dashed lines mark the values 1 and -1, corresponding to doubled and halved gene expression, respectively. *$p < 0.05$, **$p < 0.01$ ***$p < 0.001$.

Effect of thermal treatments on immune and heat shock genes under starvation

Results from the expression of immune and heat shock genes after 6 h of starvation and thermal treatments are shown in **Fig. 2.** Starvation caused a higher expression of all immune and heat shock genes measured when bumblebees were exposed to cold and hot temperatures. With respect to the treatment where bumblebees were fed *ad libitum*, *Pelle* upregulation was higher but not significant after the heat treatment, *Hsc70* was significant for both treatments showing higher upregulations (heat log2 fold change = 0.89*; cold log2 fold change = 0.94*),

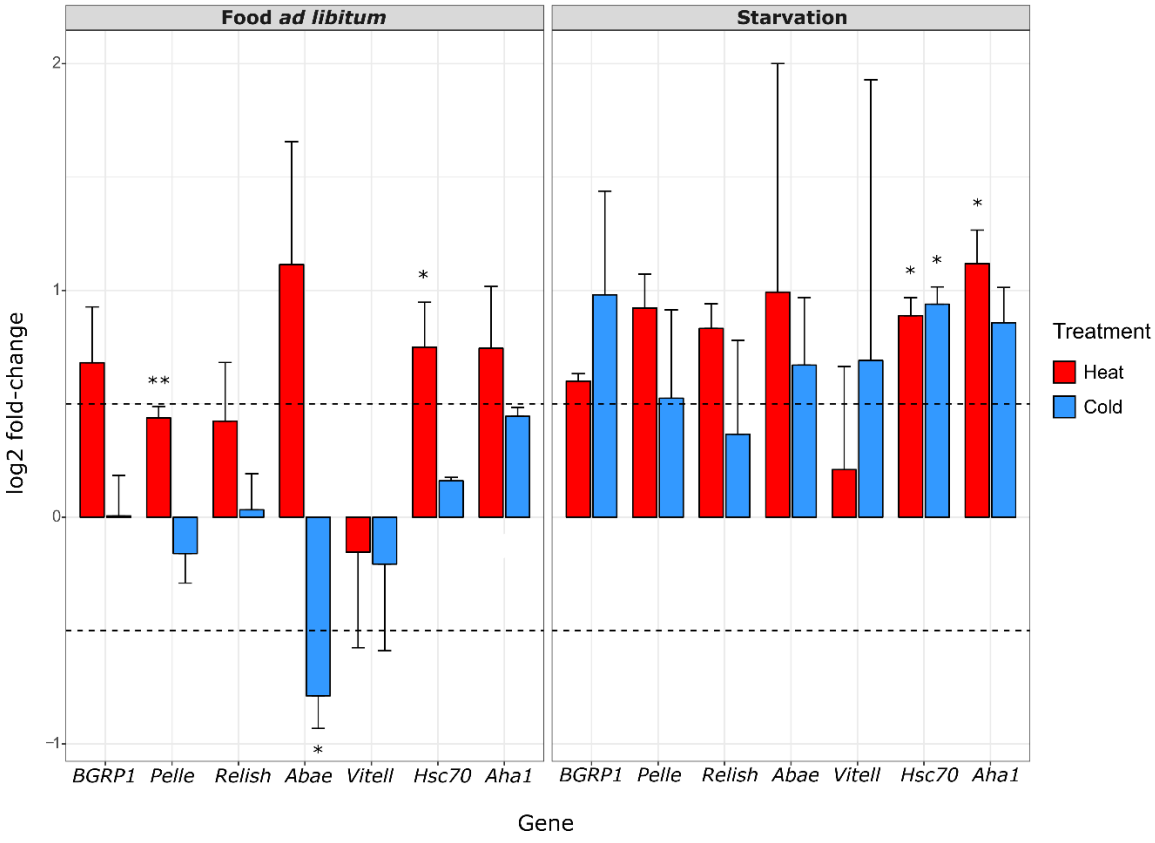


Fig. 2. Expression of immune and heat shock genes after 6 h of treatment in bumblebees fed *ad libitum* (right) or starved (left). Log2 fold change values are shown for the cold (9 °C) and heat (38 °C) treatments with respect to the control temperature (28 °C). The x-axis indicates the time points when the samples were taken. Only two biological replicates were obtained for the cold treatment under starvation. Dashed lines mark the values 1 and -1, corresponding to doubled and halved gene expression, respectively. *$p < 0.05$, **$p < 0.01$ ***$p < 0.001$.

and *Aha1* had a significantly higher upregulation with heat (log2 fold change = 1.12*). In general, the increase in expression was more notable with the cold treatment as the immune genes tended to be downregulated or did not change much when bumblebees were fed *ad libitum.*

Discussion

Effect of thermal treatments on heat shock gene expression

In our study, the heat treatment caused a significant upregulation of both *Hsc70* and *Aha1* at many time points, which also points to an increment in *Hsp90* activity. *Hsc70* and *Hsp90* have been shown to rise under high temperatures in different insects like the mirid bug *Apolygus lucorum* (Sun et al. 2014, 2016) and other hymenoptera species (Xu et al. 2010; Nguyen et al. 2016). We found the highest expression after 2 h with *Hsc70* declining later. The downregulation of heat shock proteins during acclimation to heat stress may be a mechanism to reduce the costs of a heat response in favour of fecundity and development when temperatures are high but not extreme (Hoffmann et al. 2003). Another explanation is that *Hsc70* could be replaced by other heat shock proteins involved in heat acclimation, as was observed by Luo et al. (2015), who analysed two *Hsc70* genes during heat shock recovery in the butterfly *Glanville fritillary* and found that one of them reduced its expression with time while the other did not. Even at the time points when the expression of heat-shock genes was maximum, the upregulation was much lower than that observed for *Hsp70* and *Hsp90* genes in other insects (Huang & Kang 2007; Chen et al. 2014). This probably occurred because we chose a temperature well bellow the critical thermal limits reported for ubiquitous bumblebee species: *B. lucorum* enters the heat stupor when maintained at 40 °C (Martinet et al. 2015), and *B. impatients* loses its postural control at 46 °C (Hamblin et al. 2017) and starts having muscular spasms at an average of 53 °C (Oyen & Dillon 2018). Possibly, expression of heat shock genes

is stronger at critical temperatures for survival, as it was observed in two *Liriomyza* species (Huang & Kang 2007).

The cold treatment caused a slight upregulation of *Hsc70* that was significant only after 24 h, and a non-significant increase of *Aha1* that was reduced during acclimation. It is possible that these heat shock proteins are not induced during cold stress but while insects are recovering as was observed in *Drosophila* (Colinet et al. 2010) and *Pyrrhocoris apterus* (Koštál & Tollarová-Borovanská 2009). This hypothesis might be supported by the increase of *Hsc70* and *Hsp90* expression in muscle observed in late and post diapause stages in *B. terrestris* (Kim et al. 2008). However, heat shock protein genes have been shown to be upregulated only in a certain range of cold temperatures and not when they are too extreme (Wang et al. 2012; Sun et al. 2014, 2016). Therefore, cold treatments with other temperatures might activate these genes in *B. terrestris.*

Effect of thermal treatments on immune gene expression

According to previous literature (Wojda 2017), an upregulation of immune genes in insects has been observed under thermal stress as a consequence of an interaction between immune and heat shock responses. However, McKinstry et al. (2017) found evidence for an antagonistic relationship between thermal stress and immune genes in the honey bee *Apis mellifera.*

In our study, the heat treatment caused a general upregulation of the receptor *BGRP1* and the signaling genes *Pelle* and *Relish* across all time points. However, it was very small and only significant in the case of *Pelle* at 6 h. This increase in transcription could be a consequence of a higher metabolic rate only (Somero 1995) or part of an enhanced immune response under heat stress as has been shown in other insects (Wojda & Jakubowicz 2007; Xu & James 2012; Wojda & Taszłow 2013). Other studies in *B. terrestris* have found signaling gene expression to be more stable compared with receptor or effector genes that had a strong

variation even between individuals (Erler et al. 2011; Barribeau & Schmid-Hempel 2013). In this study the elevated variance in *Abaecin* expression suggest a predominant effect of biological variation over thermal treatments. This maintenance of immune activity under moderately high temperatures could be explained by the ubiquitous distribution of *B. terrestris*, which may correlate with a higher resistance to heat than species restricted to colder climatic regions (Martinet et al. 2015).

The cold treatment also caused a small upregulation of *Pelle* and *Relish* at most time points, with a significant increase in *Relish* after 2 h. An alteration in metabolic rate causing this overexpression would be ruled out, because in that case, a downregulation would be expected with cold temperatures, suggesting a direct link between cold stress and an increase in their expression. On the other hand, the weak upregulation observed does not follow a clear pattern so it is also probable that it is being affected by other factors than cold temperature (e.g. biological variation). *BGRP1* remained high throughout the cold treatment and the increase was significant after 24 h, whereas *Abaecin* showed a significant downregulation after 6 h but progressively rose throughout the rest of the treatment. As a beta-1,3-glucan receptor, *BGRP1* is likely linked with the activation of the proPO system and the cellular response (Soltanian et al. 2009) in addition to the activation of the Toll pathway (Barribeau & Schmid-Hempel 2013), while *Abaecin* is an effector regulated by the Imd pathway (Schlüns & Crozier 2007). The upregulation of *BGRP1* in contrast to *Abaecin* is consistent with previous studies reporting a higher cellular response at low temperatures (Murdock et al. 2012; Franke & Fischer 2013) that may compensate for an impaired humoral activity to maintain some level of defences while conserving energy and investing in thermal tolerance during winter (Ferguson & Sinclair 2017; Ferguson et al. 2018). In bumblebees, an adaptive pressure to keep immune activity in the cold is expected due to the vertical transmission of bumblebee

pathogens through queens in diapause during winter (Imhoof & Schmid-Hempel 1999; Rutrecht & Brown 2008). Another possible explanation for an activation of the cellular immune response is that it could be simply induced by the tissue damage caused by thermal stress (Salehipour-Shirazi et al. 2017).

An upregulation of *Vitellogenin* was expected as the protein participates in protection against oxidative stress (Seehuus et al. 2006) and this gene has been shown to be upregulated with several stressors including heat (Bordier et al. 2017; Zhang et al. 2017) and cold (Zhang et al. 2017) in the honeybee. In opposition to these studies, we observed a relatively stable expression with a tendency for downregulation after 24 h with both the heat and cold treatments. Although the temperatures used in this study did not trigger the expression of *Vitellogenin,* it is likely that other temperatures might do it as it was observed in the case of the silkworm *Bombyx mori* (Paul & Keshan 2016).

Effect of thermal treatments on immune and heat shock genes in starved bees

The thermal treatment of 6 h was repeated with individuals under starvation to study how gene expression varies under resource limitation. If compensation were to occur, one would expect to see a decrease in the expression of immune genes in favour of energy saving and improved thermal tolerance, whereas if shared pathways or mechanisms exist between the thermal stress and the immune responses, one would expect to see a maintenance or increase in the expression of immune genes. We considered 6 hours of starvation to be enough to cause an energetic stress, since at this time some individuals had lost their postural control and showed an increased risk of death.

McKinstry et al. (2017) found evidence for a trade-off between the thermal and the immune responses in *A. mellifera*, congruent with the high costs of the activation of the immune response in *B. terrestris* (Moret & Schmid-Hempel,

2000). Contrarily, we found that all genes were more upregulated at 6 h in starving bees than in non-starving ones, especially with the cold treatment. Previous studies have related heat shock gene expression with starvation, including *Hsc70* and *Hsp90* (Wang et al. 2012; Paim et al. 2016). It is possible that stresses derived from temperature, starvation or even desiccation have a synergistic effect on activating immune activity. Several studies support this positive regulation: in *Drosophila*, starvation caused an upregulation of AMPs through the FOXO transcription factor (Becker et al. 2010) and heat-shock factor *(Hsf)*-deficient adults were hypersensitive to viral infection (Merkling et al. 2015); in *Rhodnius prolixus, Hsp70* knockdown compromised immune gene upregulation in addition to affecting tolerance to starvation (Paim et al. 2016). Remarkably, Riddell et al. (2011) found that *Aha1* upregulated when performing an SSH in *B. terrestris* infected with a natural parasite. Although this study could not confirm the results when performing a qPCR, it is possible that it constitutes a link between the immune response and thermal or even starvation stresses, similar to what has been observed for the *activator of hsp90* and other heat-shock protein genes in *Apis mellifera* (McMenamin et al. 2020) or the *Hsp90* gene in the frog *Quasipaa spinosa* (Miao-An et al. 2017).

To sum up, the heat but not the cold treatment caused a significant upregulation of heat shock genes *Hsc70* and *Aha1*, indicating a role of these proteins on tolerance to moderately high temperatures. Also, only the receptor gene *BGRP1* was clearly upregulated with cold, possibly reflecting an activation of immune cell activity. These results suggest that there is no negative effect of moderately high and low temperatures on *B. terrestris'* expression of immune-related genes. Further experimental infections would be necessary to elucidate if these temperatures do not affect the bumblebee's ability to fight pathogens. Finally, starvation produced a higher upregulation in all genes when bees were under heat and cold thermal treatments, suggesting a synergistic effect of both stressors.

This work provides a first insight into understanding the impact of temperature on the immune activity of *B. terrestris* and highlights the importance of studying the interaction of different factors. However, more studies are needed involving infection and the analysis of multiple immune components in species from different climatic regions to gain a better knowledge on how temperature affects the adaptability and survivability of bumblebees.

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Supplementary material

Table S1. Gene and primer details. E: primer amplification efficiencies. R^2: coefficient of determination from efficiencies calculations. An "X" indicates non-measured efficiencies after observing unspecific amplifications. An "(X)" indicates too low efficiencies. Primers with unspecific amplifications or too low efficiencies were discarded.

Gene	Putative function (pathway)	NCBI accession	Primers sequence	Product size (bp)	Reference	E (%)	R^2
BGRP1	Recognition receptor (Toll)	XM_003397996	Fw: AACGTGGAAGTCAAAGATGG Rv: GCGAACGATGACTTGGTATT	206	**1**	92.11	0.997
Pelle	Signaling molecule (Toll)	XM_003399470	Fw: TAAATCGACCTATGCAAGCC Rv: GGGTATAGCTGCTTCTGCTG	107	**1**	94.84	0.993
Relish	Signaling molecule (Imd)	XM_003399472	Fw: CAGCAGTAAAAATCCCCGAC Rv: CAGCACGAATAAGTGAACATA	156	**2**	89.44	0.998
Abaecin	Antimicrobial peptide	XM_003394653	Fw: GCCACAATATGTGGAATCCT Rv: ATGACCAGGGTTTGGTAATG	141	**1**	82.97	0.992
Vitellogenin	Metabolic, endocrine	XM_003402655	Fw: GTGACAAGCGAAGAGACTATTATG Rv: CCGTGTTATCTGGCGTGAC	154	**3**	94.05	0.989
Hsc70	Heat shock protein	EU124768	Fw: GCACCATTTCGGGTTTGAAT Rv: GCGCTCGGTGGTAGTTTTTT	95	This work	96.42	0.997
Aha1	Heat shock protein activator	XM_012317758	Fw: TAAGAAATGGCTAAGTGGGGTGAAG Rv: AGTTATTTACATTGGTGGCATCAGG	79	**4**	92.60	0.996

AK	Arginine kinase	AF492888	Fw: TGTCGGTATCTACGCGCCTG Rv: TTGGTGGATGCTTGTCAGTC	112	**5**	X	X
			Fw: CTGGACTCTGGTGTCGGTAT Rv: GTCTTTTGGTGGATGCTTGT	129	**1**	X	X
Ef1α	Elongation factor 1 α	AF492955	Fw: AGAATGGACAAACCCGTGAG Rv: CACAAATGCTACCGCAACAG	187	**5**	71.28 (X)	0.997
		XM_003401944	Fw: GCTGGTGACTCGAAGAACAATC Rv: GGGTGGTTCAACACAATAACCTG	74	**1**	X	X
PLA2	Phospholipase A2	FN391388	Fw: GGTCACACCGAAACCAGATT Rv: TCGCAACACTTCGTCATTTC	114	**5**	69.97 (X)	0.989
			Fw: TATCTTTCAATGCCCAGGAG Rv: GTCGTAACAAATGTCATGCG	157	**1**	101.56	0.996
TUB	α-Tubulin	FN391382	Fw: TGATCTTGCCAAGGTACAGC Rv: ACGAATGCACGTTTAGCGTA	117	**5**	103.13	0.977
ACTB	β-actin	FN391379	Fw: TGACGCAGATTATGTTTGAA Rv: AGCGTATAGCGAAAGTACAGC	77	**5**	92.74	0.999
RPL13	Ribosomal protein L13	FN391387	Fw: GGTTTAACCAGCCAGCTAGAAA Rv: CTTCACAGGTCTTGGTGCAA	83	**5**	X	X
RPL23	Ribosomal protein L23	XM_003400707	Fw: GGGAAAACCTGAACTTAGGAAAA Rv: ACCCTTTCATTTCTCCCTTGTTA	143	**6**	99.04	0.998

ITPR	Inositol 1,4,5-triphosphate	DQ_468668	Fw: TGCACGCAGACCAAGCGGAG Rv: ACGTCTTCCTTCGCGTCAAACGG	190	**7**	102.81	0.998

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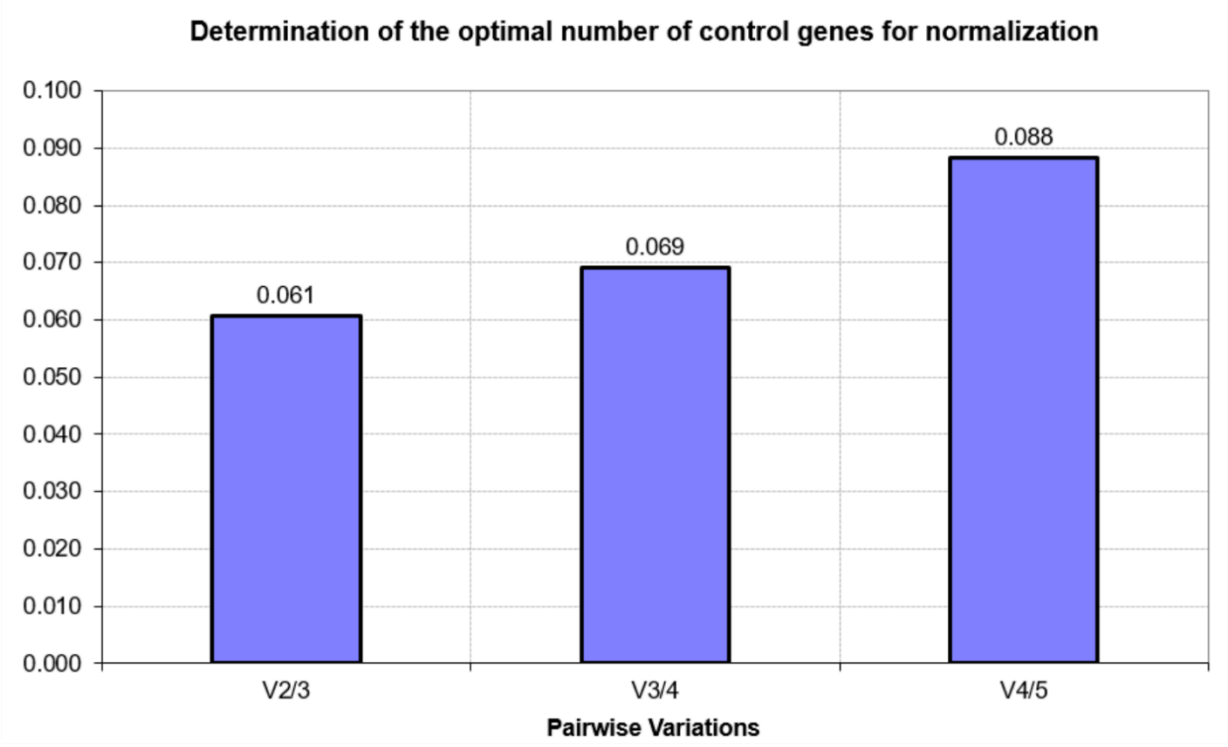
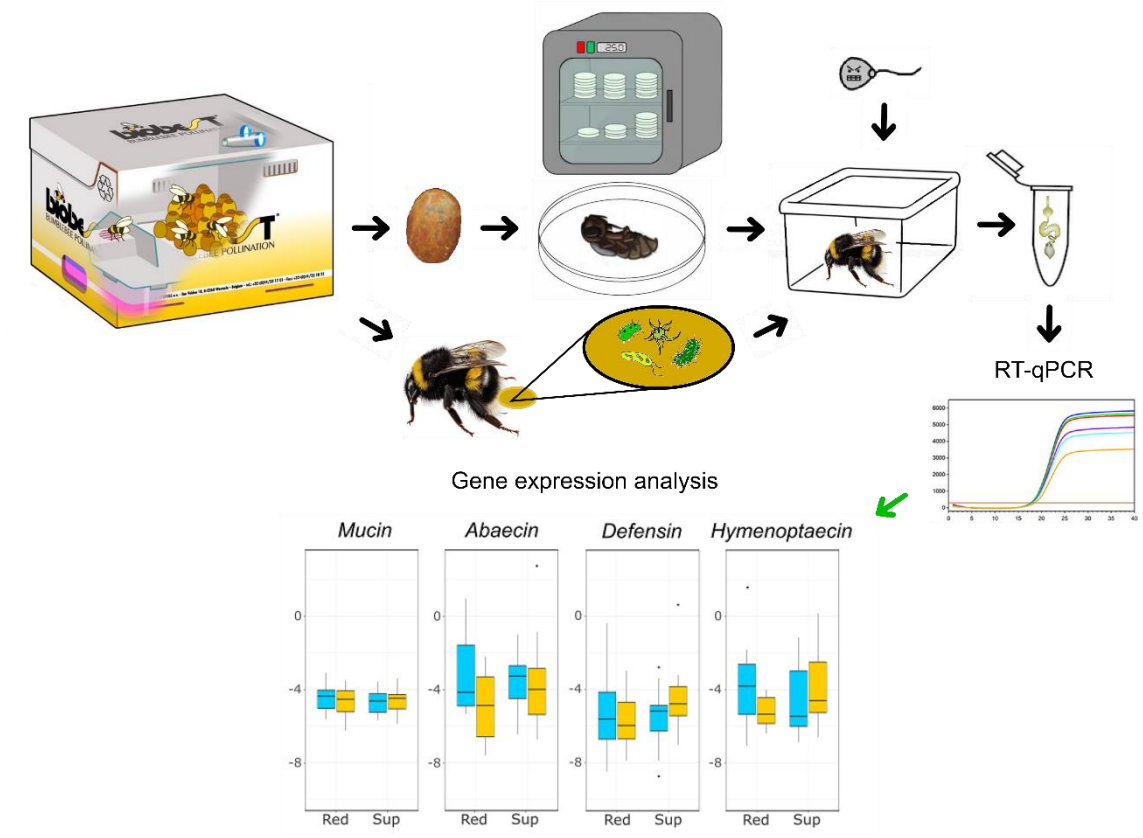


Fig. S1. Determination of the optimal number of reference genes for normalisation. The bar of V2/3 below the cut-off value (0.15) indicated that it was not necessary to add a third reference gene.

Chapter 4

The effect of microbiota on antimicrobial peptide and *Mucin-5AC* gene expression in *Bombus terrestris* upon infection with *Crithidia bombi*



Abstract

Gut microbiota in the bee genera *Bombus* and *Apis* confer protection against natural pathogens. In commercial bumblebee species *B. terrestris* and *B. impatiens*, the microbiota increases their resistance to the common and virulent trypanosomatid parasite *Crithidia bombi*. However, the mechanisms by which gut microorganisms protect the host are still unknown. Here, we test the hypothesis that microbiota can protect the host through stimulation of its immune response or protection of the gut epithelium. For this, we reduced the microbiota of workers and then exposed them to an infectious dose of *C. bombi*. We examined the expression of three antimicrobial peptide (AMP) genes and *Mucin-5AC*, a gene with a putative role in gut epithelium protection, after infection with *C. bombi* using qPCR. Although a protective effect against *C. bombi* was observed in bumblebees with supplemented microbiota, *Mucin-5AC* gene expression did not differ among microbiota treatments, suggesting that *Mucin-5AC* expression in the host is not involved in the mechanisms of protection. On the other hand, the presence of microbiota did not increase the basal AMP gene expression in the bumblebees but interacted with *C. bombi*, increasing AMP gene expression when the host was infected. Although this effect alone did not explain the protective effect observed, our results suggest that specific strains of gut microorganisms may stimulate host immune activity through interaction with the parasite in the gut, perhaps by making a less hospitable environment for *C. bombi* to establish.

Keywords: bumblebees; immunity; gut microbiota; trypanosomatid; *Bombus terrestris*; *mucin*

Introduction

Gut microbial communities play an important role in the health of a wide range of organisms (Flint et al. 2012; Engel & Moran 2013). Bees from the genera *Apis* and *Bombus* contain a simple and specific set of bacteria with some phylotypes found in both genera and absent in others or the environment (Martinson et al. 2011). These core phylotypes include the genera *Snodgrasella*, *Gilliamella* and *Lactobacillus,* and have been found in honeybees and bumblebees from North America, Europe, South Africa and Asia (Jeyaprakash et al. 2003; Martinson et al. 2011; Koch et al. 2013; Li et al. 2015; Lim et al. 2015). Additionally, a phylogenetic study found a significant correlation between the 16S rRNA gene bacterial tree of *Snodgrasella* and *Gilliamella* and the host tree (Koch et al. 2013). The same study found a weak correlation with geographic distance only in the case of *Gilliamella*. Together these data suggest that these bacterial partners have coevolved with their bee hosts to their mutual benefit (Martinson et al. 2011; Koch et al. 2013).

Several studies have provided evidence for the potential role of bee gut microbiota in the digestion of dietary components and protection against pathogens (Raymann & Moran 2018). In the honey bee *Apis mellifera*, diets supplemented with lactic acid bacteria from worker guts reduced larval mortality when infected with the pathogens *Paenibacillus larvae* and *Melissococcus plutonius* (Forsgren et al. 2010; Vásquez et al. 2012). In adults, having these bacteria is linked to lower infection load of the microsporidian parasite *Nosema ceranae* (Arredondo et al. 2018). Normal gut bacterial community can also serve to prepare the immune system as honeybee workers inoculated with *Gilliamella apicola* and *Snodgrasella alvi* were better able to clear a systemic *Escherichia coli* infection in the hemolymph (Kwong et al. 2017). In bumblebees, the gut microbiota is protective against the common trypanosomatid parasite *Crithidia bombi* (Koch & Schmid-Hempel 2011, 2012; Mockler et al. 2018), and a negative correlation was found between this parasite and the phylotypes *G. apicola* and *S.*

alvi in the field (Cariveau et al. 2014; Mockler et al. 2018). The simplicity of bee microbiota together with the well characterised response of the bumblebee *Bombus terrestris* to *C. bombi*, make this system a good model to understand the mechanisms behind gut bacterial protection of the host against natural infections.

There are two main ways in which bumblebee microbiota could protect the host from *C. bombi*: i) through direct interaction with the parasite by a variety of possible mechanisms as production of antimicrobial peptides, competition for resources (food or space in the bee gut), alteration of gut environmental conditions and promoting the growth of other protective bacteria; and ii) through stimulation of the host's immune system, increasing its resistance to *C. bombi* (Hamdi et al. 2011). In this sense, there is evidence pointing to a direct interaction between the gut microbiota and *C. bombi* cells as isolated phylotypes from *B. terrestris* have been shown to inhibit *C. bombi* when cultured *in vitro* (Praet et al. 2018) and *G. apicola* and *S. alvi* form a biofilm in the gut wall that might act as a barrier against the attachment of *C. bombi* (Engel et al. 2012; Martinson et al. 2012). Furthermore, a recent study found that *Lactobacillus bombicola*, a ubiquitous bacterial species in *Bombus* spp., acidifies the gut enough to inhibit the growth of *C. bombi* (Palmer-Young et al. 2019a). On the other hand, gut bacteria have been shown to activate the immune response in *A. mellifera* (Evans & López 2004; Janashia et al. 2016; Kwong et al. 2017) and influence immune activity in *B. terrestris* (Näpflin & Schmid-Hempel 2016).

The aim of this work was to better understand the mechanism of protection of *B. terrestris* gut microbiota against the parasite *C. bombi.* Therefore, we analysed the effect of the microbiota on the expression of immune-relevant genes in *B. terrestris* upon infection with *C. bombi.* We chose three AMP genes as representatives of the host immune response *(Abaecin*, *Defensin* and *Hymenoptaecin)* regulated by the two main immune pathways Imd and Toll (Schlüns & Crozier 2007; Lourenço et al. 2018) and involved in

the immune response to *C. bombi* (Riddell et al. 2009; Barribeau & Schmid-Hempel 2013; Deshwal & Mallon 2014). Additionally, we measured one gene putatively involved in gut mucus layer formation and the protection of gut epithelial cells *(Mucin-5AC)*. Our hypothesis is that gut bacteria protect their host through the stimulation of the immune system or protecting gut epithelium. Therefore, we expect to see increased expression of these genes in bumblebees treated with supplemented microbiota.

Materials and methods

Bumblebee treatments

Experiments were carried out with three commercial colonies of *B. terrestris* (Biobest). Colonies were kept at 28 ℃ and 60% relative humidity. In order to reduce the microbiota load, bumblebee pupae were extracted aseptically from cocoons and allowed to mature *in vitro* until eclosion. After eclosion, adult workers were maintained in semi-sterilised conditions in bleach-sterilised boxes with autoclaved litter and fed *ad libitum* with pollen previously heated at 85 ℃ for 30 min, and filtered sugar water (Koch & Schmid-Hempel 2011). We refer to these bees as microbiota reduced, as there is always potential for bacteria to establish. In fact, we checked the presence of the common *B. terrestris* endosymbiont *Snodgrasella alvi* by PCR and we found several samples to be positive **(Table S1).**

In order to restore the microbiota, 1-2 day-old bumblebees were starved for 3 h and half of them were fed with faeces collected from workers of their colony of origin and diluted in sugar water to 15 μl. The treatment was repeated two days later to ensure that they had ample opportunity to establish their colony's microbiota. Five days after the second microbiota treatment, all individuals were

starved for 4 h and half of each treatment group was exposed to a mixture of 20,000 cells from five different strains (4,000 cells per strain, internal strain codes 08.068, 08.076, 08.091, 08.161 and 08.175) of *C. bombi*. Cell concentration in the cultures was determined by counts with a haemocytometer. Four different treatments were established: I) bumblebees with supplemented microbiota exposed to *C. bombi*; II) bumblebees with supplemented microbiota but not exposed to *C. bombi;* III) bumblebees with reduced microbiota exposed to *C. bombi;* and IV) bumblebees with reduced microbiota and not exposed to *C. bombi.*

The bumblebees were sacrificed 18 h after the *C. bombi* treatment (N = 4-5 per treatment per colony) to analyse gene expression as the immune response to *C. bombi* at this time point remains strong and is well characterised (Barribeau & Schmid-Hempel 2013; Barribeau et al. 2014; Brunner et al. 2013, 2014). Between four and seven bumblebees of every treatment were sacrificed seven days after the *C. bombi* treatment, when the peak of the infection occurs (Schmid-Hempel & Schmid-Hempel 1993), to quantify the parasite load. The guts were extracted aseptically, snap-frozen in liquid nitrogen and kept at -80 ℃.

DNA and cDNA preparations

For *C. bombi* load quantification, guts were homogenised with sterilised plastic pestles and extractions were carried out with the ISOLATE II Genomic DNA Kit (Bioline) following manufacturer instructions and including a lysozyme incubation for Gram-positive bacteria extraction.

For expression analyses, guts were homogenised with zirkonium beads with a Qiagen TissueLyser II before extracting the RNA following the TRIzol protocol (Thermo Fisher Scientific Inc.). RNA was eluted in 30 µl of nuclease-free water and diluted to 1000 ng/µl before synthesising cDNA using the FIREScript RT cDNA synthesis Mix (Solis BioDyne).

qPCR reactions

C. bombi cells were quantified from DNA extractions by qPCR using the primers CriRTF2/CriRTR2 (Ulrich et al. 2011) and a program consisting of an initial denaturation step at 95 ℃ for 10 min, 40 amplification cycles of denaturation at 95 ℃ for 15 s and annealing/elongation at 60 ℃ for 1 min, and a final melt-curve step to check for non-specific amplification, with NZY qPCR Green Master Mix (NZYTech) on a StepOnePlus™ Real-Time PCR system (Applied Biosystems). Five DNA dilutions from 10^2 to 10^6 cells of *C. bombi* culture were used as standards. Raw C_T data was obtained with the software StepOnePlus™ Real-Time PCR System.

Expression of three AMP genes (*Abaecin, Defensin* and *Hymenoptaecin*), and one gene involved in gut wall protection (*Mucin-5AC*) was quantified through qPCR with three reference genes (*Arginine kinase - AK, Elongation factor 1 alpha - Ef1a and Phospholipase A2 – PLA*) with the primers listed in **Table 1.** The following program was used: 95 ℃ for 15 min, 40 cycles of an initial denaturation step at 95 ℃ for 10 min, annealing at 60 ℃ for 20 s and elongation at 72 ℃ for 20 s, with a final melt-curve step to check for non-specific amplification. Gene amplification efficiencies were calculated using a serial dilution pool of cDNA with the quantification standard curve method using HOT FIREPol EvaGreen qPCR Supermix (Solis BioDyne) on a LightCycler® 480 System. Raw C_T data was obtained with the program LightCycler 480 v 1.5.

qPCR data analyses

The stability of the reference genes was checked with geNorm (Vandesompele et al. 2002) and NormFinder (Andersen et al. 2004) algorithms in all samples.

Each bumblebee in each colony was treated as a biological replicate. Raw C_T

Table 1. Gene and primer details. E: primer efficiencies. R^2: coefficient of determination. Bp: base pairs.

Gene	Putative function (pathway)	NCBI accession	Primers	Product size (bp)	Primer reference	E (%)	R^2
Abaecin	Antimicrobial peptide	XM_003394653	Fw: GCCACAATATGTGGAATCCT Rv: ATGACCAGGGTTTGGTAATG	141	Barribeau et al. 2013	79.9	0.994
Defensin	Antimicrobial peptide	XM_003395924	Fw: GTCTGCCTTTGTCGCAAGAC Rv: GACATTAGTCGCGTCTTCTTCG	139	Barribeau et al. 2013	86.0	0.982
Hymenoptaecin	Antimicrobial peptide	XR_132450	Fw: TTCATCGTACTGGCTCTCTTCTG Rv: AGCCGTAGTATTCTTCCACAGC	85	Barribeau et al. 2013	88.9	0.983
Mucin-5AC	Glycoprotein protecting mucosa	XM_003399573	Fw: AGTGCCAACGGTGGAATAAGACC Rv: AAGCGTACGAGTTCGGTAGCAC	68	This study	83.0	0.990
AK	Arginine kinase	AF492888	Fw: CTGGACTCTGGTGTCGGTAT Rv: GTCTTTTGGTGGATGCTTGT	129	Barribeau et al. 2013	91.3	0.995
ef1α	Elongation factor 1 α	XM_003401944	Fw: GCTGGTGACTCGAAGAACAATC Rv: GGGTGGTTCAACACAATAACCTG	74	Barribeau et al. 2013	80.5	0.997
PLA2	Phospholipase A2	FN391388	Fw: TATCTTTCAATGCCCAGGAG Rv: GTCGTAACAAATGTCATGCG	157	Barribeau et al. 2013	90.3	0.993

values were corrected using efficiencies, and normalised against the mean of the three reference genes to obtain deltaC$_T$ values. DeltaC$_T$ values from every treatment were analysed with a MANOVA in base R (Team 2013) to detect significant effects of the microbiota treatment, *C. bombi* infection and colony on gene expression. The Yeo-Johnson transformation was applied to *Hymenoptaecin* data to meet the assumption of multivariate normality. If the MANOVA showed a significant effect of any factor or interaction, results were analysed for each gene individually with a three-way ANOVA. The number of *C. bombi* cells was calculated from deltaC$_T$ values through interpolation with a calibration curve. Results from every treatment were analysed with a two-way ANOVA in base R (Team 2013) to detect significant effects of the microbiota treatment and colony on parasite load.

Results

Effect of microbiota treatment on *Crithidia* infection intensity

Bumblebees with microbiota had less than half the *C. bombi* infection intensity than bumblebees without supplemented microbiota (reduced 398,744 ± 88,326 cells, supplemented 163,669 ± 45,066 cells, t = 2.370, df = 20.915, p = 0.027) **(Fig. 1, Table 2)**. The effect of the supplemented microbiota on reducing *C. bombi* infection was higher in colonies 2 and 3. However, there was no significant effect of the colonies on infection intensity nor a significant interaction of the colony with the microbiota treatment.

Table 2. Two-way ANOVA results for the effect of the microbiota treatment and the colony on *C. bombi* load 7 days after infection.

Factor	Df	SS	MS	F	p
Microbiota	**1**	**$4.278*10^{11}$**	**$4.278*10^{11}$**	**6.013**	**0.021**
Colony	2	$1.788*10^{11}$	$8.939*10^{10}$	1.256	0.302
Microbiota*Colony	2	$1.681*10^{11}$	$8.406*10^{10}$	1.181	0.323

Significant results at the 5 % confidence level are highlighted in boldface.

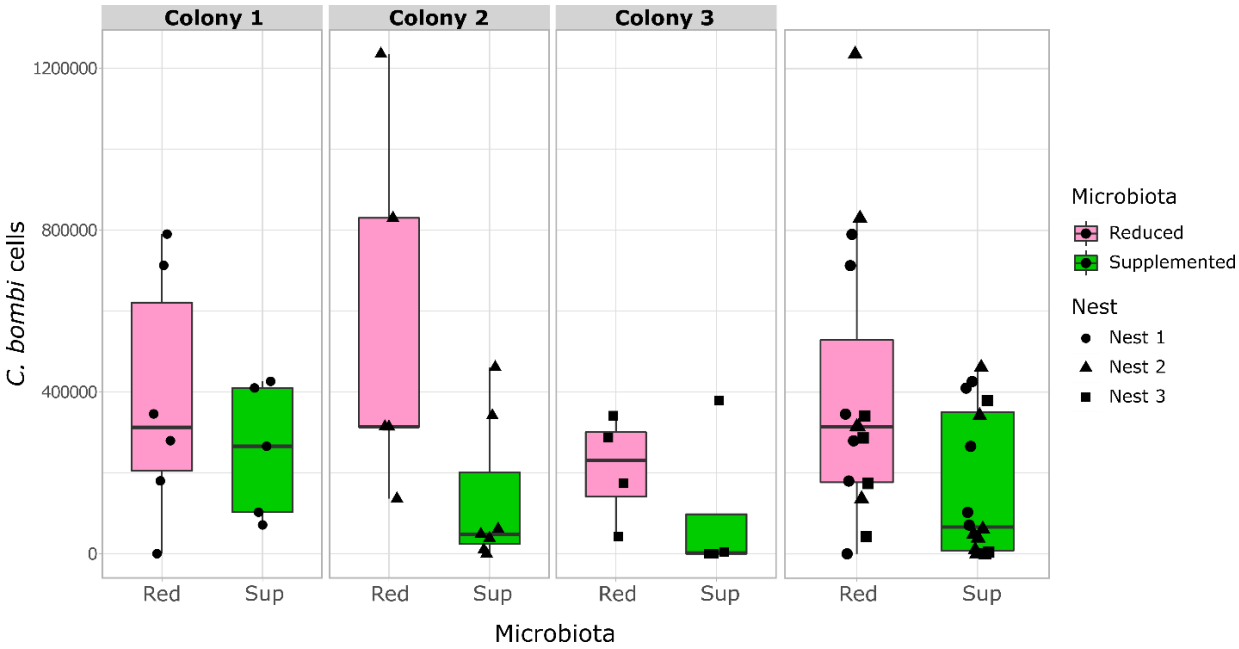


Fig. 1. Number of *C. bombi* cells present in the gut of bumblebees treated with supplemented microbiota (Supplemented) and bumblebees that received no supplemented microbiota (Reduced) 7 days after infection for three colonies (first three panels) and in aggregate (final panel).

Effect of microbiota and *Crithidia* infection on gene expression

Crithidia infection significantly decreased gene expression in comparison with non-infected individuals ($p = 0.028$) **(Fig. 2, Table 3).** The colony of origin also had a strong significant effect ($p < 0.001$), therefore univariate analyses were performed for all gene expression data **(Table S1).**

Microbiota and *Crithidia* treatments caused a high variability in AMP expression in bumblebees. In contrast, *Mucin-5AC* expression was less variable. We found a significant interaction between colony and microbiota treatment ($p = 0.039$) where one colony downregulated ($p = 0.029$) and another upregulated *Mucin-5AC* expression ($p = 0.045$) when the microbiota was supplemented**.** *Hymenoptaecin* expression varied among colonies ($p < 0.001$) and there was a significant interaction between microbiota treatment and *Crithidia* exposure ($p = 0.015$), driven by differences in colony 1 **(Table S1, Fig. 2)**. This interaction

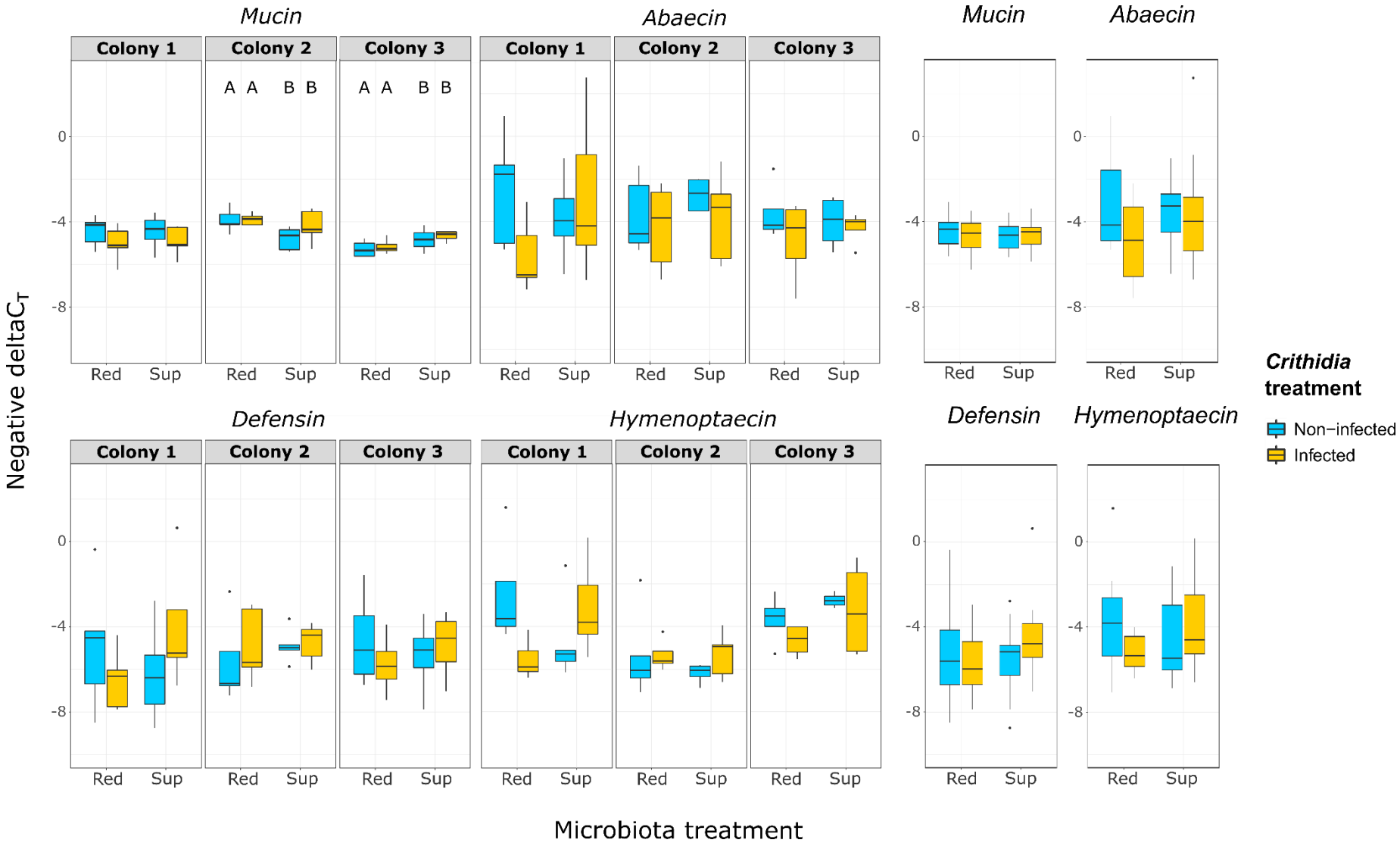


Fig. 2. Negative deltaC$_T$ values relative to average of invariant housekeeping genes in bees exposed to four treatment combinations of microbiota and *Crithidia* treatments. Higher values represent higher mRNA expression. Red: Reduced microbiota. Sup: Supplemented microbiota. Graphs on the right represent results for the three colonies together. Letters denote significantly different groups based on Tukey's honestly significant difference test.

was also observed in the same colony in the genes *Abaecin* and *Defensin* but it was less pronounced. Overall, the supplemented microbiota treatment caused a nominal increase in expression of the three AMPs upon *Crithidia* infection but it was not significant.

Table 3. MANOVA results for the full dataset of deltaC$_T$ values 18 h after infection with *C. bombi.* Yeo-Johnson transformation was applied for *Hymenoptaecin* data.

Factor	Df	Pillai	F	*p*-value
Microbiota	1	0.025	0.267	0.897
Crithidia	**1**	**0.227**	**3.010**	**0.028**
Colony	**2**	**0.638**	**4.925**	**<0.001**
Microbiota*_Crithidia_	1	0.143	1.713	0.165
Microbiota*Colony	2	0.219	1.292	0.258
_Crithidia_*Colony	2	0.201	1.173	0.324
Microbiota*_Crithidia_*Colony	2	0.162	0.926	0.499

Significant results at the 5 % confidence level are highlighted in boldface.

Discussion

Effect of a microbiota supplement on *C. bombi* infection

Bumblebees reared in semi-sterilised conditions had a higher resistance to *C. bombi* infection when they received a supplement of microbiota from their respective colonies. Bumblebees with supplemented microbiota had fewer *C. bombi* cells in their faeces 7 days after exposure, confirming previous studies of the beneficial effect of endogenous microbiota on *C. bombi* infection (Koch & Schmid-Hempel 2011, 2012; Mockler et al. 2018). These results also confirmed that although we could not rear completely germ-free bumblebees, differences in gut microbiota in our treatments were enough to cause different levels of protection.

Effect of supplemented microbiota on gene expression

In order to clarify the mechanisms of protection of bumblebee microbiota during *C. bombi* infection, we established four groups: with or without their microbiota and exposed or not to *C. bombi* in a factorial design. If the microbiota increases bumblebee resistance to infection through stimulation of its immune activity or through protection of its gut epithelium, we expected to see higher expression of the analysed genes.

Mucin-5AC putatively belongs to the group of the mucin proteins, glycoproteins that form the glycocalyx and the mucus layer associated with the peritrophic matrix in insects (Dias et al. 2018). The mucus layer acts as a barrier to protect the gut epithelium from pathogens and/or their toxins and provides binding sites for pathogens mimicking the glycocalyx (Ramsey et al. 2017; Erlandson et al. 2019). However, mucins may also provide a temporary location for parasite invasion (Abraham & Jacobs-Lorena 2004). In our study, the expression level of *Mucin-5AC* did not vary despite the microbiota and *C. bombi* treatments. Although previous studies have shown that peritrophic matrix synthesis in other insects is determined by gut microbiota (Erlandson et al. 2019), our results indicate that the expression level of the *Mucin-5AC* was independent of the present microbiota or alternatively, that the reduced microbiota from bumblebees reared in semi-sterilised conditions was enough to stimulate the constitutive level of this protein. *C. bombi* is expected to interact with the mucus layer as it attaches to the ileum with its flagellum to establish infection (Koch et al. 2019). We expected an effect of infection on *Mucin-5AC* expression as different expression levels have been linked to susceptibility to gut bacterial parasites in the insect *Diaphorina citri* (Ramsey et al. 2017) and in pigs (Quintana-Hayashi et al. 2015), but also to resistance to gut nematodes in mice (Hasnain et al. 2011). Given our results, it is possible that this mucin does not interact specifically with *Crithidia*. However, it is important to note that

synthesis of Mucin-5AC in pigs and mice occurred *de novo* in the gut upon infection (Hasnain et al. 2011, Quintana-Hayashi et al. 2015), whereas we obtained a constitutive expression. Therefore, we cannot discount an interaction with *Crithidi*a nor a translational regulation that seems to be common in mucin proteins (Johansson & Hansson 2016).

Crithidia infection caused a significant downregulation of AMPs contrary to previous studies that have shown an upregulation in these AMP genes at the same time after infection (Barribeau & Schmid-Hempel 2013; Brunner et al. 2013, 2014; Barribeau et al. 2014). However, there are specific interactions between host and parasite genotypes, including different immune response patterns (Riddell et al. 2009, 2011, 2014; Barribeau et al. 2014). Notably, some cases of downregulation of these AMPs have been shown on certain bumblebee colonies (Riddell et al. 2009; Barribeau et al. 2014) and *C. bombi* strains (Riddell et al. 2009; Barribeau & Schmid-Hempel 2013, Barribeau et al. 2014). However, here all individuals were exposed to the same inoculum of mixed genotypes of *C. bombi,* thus the suppressive effect on AMP expression represents an average across these parasite genotypes.

Microbiota supplement did not have a significant effect on AMP expression, but it showed a significant interaction with *Crithidia* infection on *Hymenoptaecin* expression. This interaction was observed in colony 1 and occurred at a minor level with *Abaecin* and *Defensin* expression. A controlled production of AMP in response to gut microbiota has been explained to occur as a mechanism to limit microbiota proliferation, avoiding dysbiosis and epithelial cell damage that would result from an excess of AMPs (Kajla et al. 2015; Lee et al. 2017). The increase in AMP production when the microbiota was reduced in non-infected individuals in colony 1 seems contradictory but could be caused by a dysbosis situation that might have caused the proliferation of potential pathogenic bacteria found as a different enterotype in some bumblebees (Li et al. 2015).

This situation would also explain why the immune response to *Crithidia* was negative in comparison to the non-infected group only in the reduced microbiota treatment and for this colony. In contrast, with non-infected bumblebees, when microbiota was supplemented, there was an increase in expression of the AMPs *Defensin* and *Hymenoptaecin* in infected individuals in colony 1. These results discount an effect of endogenous microbiota on the basal AMP production of the bumblebee as has been observed in mosquitos and honey bees (Ramirez et al. 2012; Janashia & Alaux 2016; Kwong et al. 2017) but points to the stimulation of immune activity when the host is infected. These results agree with those found by Näpflin & Schmid-Hempel (2016), when they performed microbiota transplants with different levels of protection against *Crithidia* and failed to find differences in the level of AMP expression. AMP stimulation in the host may only occur as a result of a tripartite interaction between the host, its microbiota and *Crithidia.*

Despite the significant interaction between the microbiota and *Crithidia* observed in colony 1, there was a protective level of the microbiota in all colonies. These results suggest that the microbiota must have other mechanisms of protection. One possibility is that intestinal microorganisms protect the host through the DUOX pathway, which has been suggested to be more involved than the Imd pathway in fighting gut infections in *Drosophila melanogaster* (Lee et al. 2017). This would be consistent with the results of Näpflin & Schmid-Hempel (2016), in which they found that microbiota transplants with different levels of protection against *Crithidia* had a significant effect on oxidative stress genes. In a different scenario, microbiota species might compete for resources with *Crithidia* or secrete metabolites that prevent its proliferation without affecting host immunity (Palmer-Young et al. 2019a). Nevertheless, results from Palmer-Young et al. (2019b) showed no evidence for a displacement of core bacteria families in *B. impatiens* upon *C. bombi* infection,

but instead for a displacement of Enterobacteriaceae and other non-core symbionts.

In conclusion, although we found a protective effect of supplemented microbiota against the parasite *C. bombi* in *B. terrestris*, expression of *Mucin-5AC* remained constant through the different treatments, suggesting that this protein is not involved in the microbiota mechanisms of protection. Additionally, we did not find a significant effect of microbiota on AMP expression, indicating that endogenous microbiota does not increase basal AMP production in *B. terrestris.* However, we found a significant interaction of the microbiota treatment with *Crithidia* infection in one colony, suggesting that specific strains of *B. terrestris* gut microorganisms can stimulate AMP production in a complex interaction between the host, the microbiota and the parasite. This could be one of the mechanisms of protection against *Crithidia* but does not explain alone the protective effect we found common to all colonies. We propose future studies focused on the ability of gut microorganisms to interact directly with *Crithidia* or alter ROS production in the host gut as the next step in understanding the protective mechanisms of *B. terrestris* microbiota against this parasite.

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Supplementary Material

Table S1. Three-way ANOVA results for the effect of the microbiota treatment, *C. bombi* infection and colony on expression of immune genes 18 h after infection. Yeo-Johnson transformation was applied to *Hymenoptaecin* data.

Gene	Factor	Df	SS	MS	F	*p*
	Microbiota	1	0.067	0.066	0.176	0.676
	Crithidia	1	0.012	0.012	0.033	0.857
	Colony	**2**	**5.838**	**2.919**	**7.710**	**0.001**
Mucin-5AC	Microbiota**Crithidia*	1	0.226	0.225	0.596	0.444
	Microbiota*Colony	**2**	**2.643**	**1.321**	**3.490**	**0.039**
	*Crithidia**Colony	2	1.825	0.912	2.410	0.101
	Microbiota**Crithidia**Colony	2	0.161	0.080	0.213	0.808
	Microbiota	1	4.05	4.054	0.962	0.332
	Crithidia	1	10.99	10.987	2.606	0.114
	Colony	2	3.34	1.671	0.396	0.675
Abaecin	Microbiota**Crithidia*	1	8.44	8.440	2.002	0.164
	Microbiota*Colony	2	1.17	0.587	0.139	0.870
	*Crithidia**Colony	2	0.26	0.130	0.031	0.970
	Microbiota**Crithidia**Colony	2	13.81	6.903	1.637	0.206
	Microbiota	1	2.13	2.130	0.538	0.467
	Crithidia	1	0.38	0.376	0.095	0.759
	Colony	2	1.16	0.582	0.147	0.864
Defensin	Microbiota**Crithidia*	1	9.21	9.207	2.325	0.134
	Microbiota*Colony	2	0.58	0.288	0.073	0.930
	*Crithidia**Colony	2	1.35	0.676	0.171	0.844
	Microbiota**Crithidia**Colony	2	12.03	6.016	1.519	0.230
	Microbiota	1	6.90	6.89	0.326	0.571
	Crithidia	1	8.60	8.61	0.407	0.526
	Colony	**2**	**473.7**	**236.8**	**11.20**	**<0.001**
Hymenoptaecin	**Microbiota*Crithidia**	**1**	**134.9**	**134.8**	**6.38**	**0.015**
	Microbiota*Colony	2	48.4	24.20	1.145	0.327
	*Crithidia**Colony	2	65.4	32.71	1.547	0.224
	Microbiota**Crithidia**Colony	2	12.41	6.206	2.490	0.075

Significant results at the 5 % confidence level are highlighted in boldface.

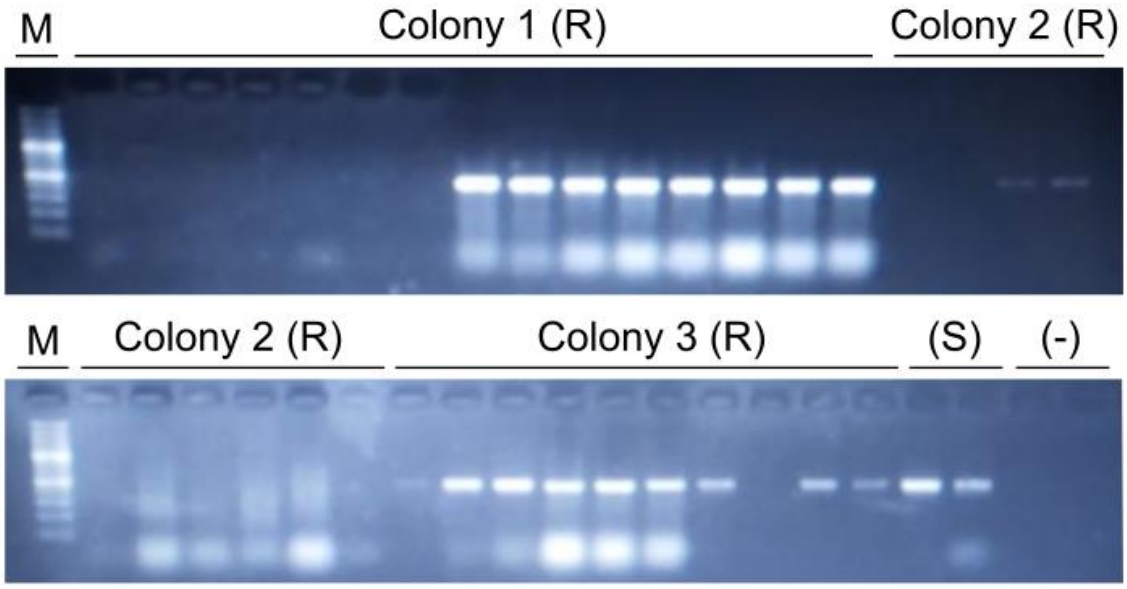

Fig. S1. PCR results from *Snodgrasella alvi* detection using the primers from Martinson et al. (2010) and following the same program. M: molecular weight marker (R): gut DNA extractions from bumblebees in the treatment with reduced microbiota (S) gut DNA extractions from bumblebees in the treatment with supplemented microbiota (-): negative control. *S. alvi* identification was confirmed by sequencing.

Conclusions

From this thesis, the following conclusions can be drawn:

- The cryptic species *Bombus magnus* is present in the Northern Plateau of the Iberian Peninsula, but it is very rare.
- The Iberian Peninsula acted as a glacial refuge for the populations of *B. lucorum* where they differentiated from the populations of the Pyrenees and Northern Europe, as can be seen in the variation of the mitochondrial *cox1* gene.
- There is a current gene flow among *B. lucorum* populations from the centre of the Iberian Peninsula and those from the north of the Pyrenees as indicated by microsatellite *loci* and geometric morphometrics analyses.
- A moderately high temperature (38 °C) causes the upregulation of the heat shock genes *Hsc70* and *Aha1* in the commercial bumblebee species *Bombus terrestris*.
- Expression of immune genes is maintained under moderately high and low temperatures (38 °C and 9 °C) in *B. terrestris*, although maintenance at 9 °C also causes the upregulation of the receptor gene *BGRP1*, related to cellular immune response.
- Starvation increased heat shock and immune gene expression with the thermal treatments at 38 °C and 9 °C, indicating a possible synergistic effect between nutritional and thermal stress conditions.
- The protective effect of *B. terrestris* microbiota against the gut parasite *Crithidia bombi* infection is not dependent on expression of the gene *Mucin-5AC*, which codifies a protein from the mucus layer in the host's gut.

- Endogenous gut microbiota does not protect *B. terrestris* against *C. bombi* by increasing its basal expression of the AMP genes *Abaecin*, *Defensin* and *Hymenoptaecin*. However, specific strains of gut microorganisms can interact with *C. bombi* and increase AMP expression during the infection. This mechanism can protect the host against the infection but does not explain alone resistance that the microbiota confers against the parasite.