TESIS DE DOCTORADO

# ELEVATIONAL GRADIENTS IN OAK DEFENCES AND HERBIVORY

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## Elevational gradients in oak defences and herbivory

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## Elevational gradients in oak defences and herbivory

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# RESUMEN

Las plantas y sus insectos herbívoros han coexistido durante más de 350 millones de años. Este largo período de convivencia ha dado lugar a un proceso de coevolución que puede asimilarse a una "carrera armamentista". A través de este proceso, las plantas han desarrollado un amplio arsenal de diferentes estrategias defensivas anti-herbivoros y los herbívoros han desarrollado a su vez adaptaciones a estas defensas. Estas dinámicas de coevolución han desencadenado tasas aceleradas de especiación tanto en insectos como en plantas. Los mecanismos de defensa de las plantas pueden clasificarse como mecanismos de tolerancia, definidos como la capacidad de las plantas para mitigar los efectos negativos en la eficacia biológica (*fitness*) de la planta causados por la herbivoría; o mecanismos de resistencia, que se definen como la capacidad de las plantas para reducir o evitar el daño por herbívoros al afectar a la fitness del insecto. Los rasgos defensivos se pueden clasificar también en defensas directas que incluyen rasgos químicos o físicos para disuadir a los herbívoros, reducir su consumo o disminuir su supervivencia; y defensas indirectas, que son rasgos químicos o físicos de las plantas que brindan refugio, recompensa o información sobre la presencia de herbívoros a los enemigos naturales de herbívoros (es decir, depredadores y parasitoides). Estas características defensivas pueden ser expresadas de forma constitutiva por las plantas (es decir, siempre presentes independientemente de que la planta esté bajo ataque o no) o pueden ser inducidas (es decir, sintetizadas por la planta solo después del daño por herbívoros).

La coevolución entre plantas e insectos herbívoros y la evolución de las defensas de las plantas han sido preguntas importantes en ecología. Se han propuesto varias teorías en las últimas décadas para explicar qué factores influyen en estos procesos. Inicialmente, varias teorías se centraron en la asignación de defensas desde la perspectiva de plantas individuales. Estas teorías predicen que, dado que las defensas de las plantas son costosas de producir, las plantas tienen

que elegir entre la asignación de recursos a las defensas y otras funciones como el crecimiento o la reproducción. Por ejemplo, la hipótesis de disponibilidad de recursos establece que las plantas que crecen en entornos con pocos recursos crecen más lentamente y asignan más recursos a las defensas, ya que el coste de reemplazar los tejidos dañados es mayor en entornos con recursos limitados. De manera similar, la teoría del balance de diferenciación del crecimiento predice una compensación entre los costes de producción de metabolitos secundarios en relación con la demanda de compuestos derivados de la fotosíntesis por el crecimiento. Finalmente, la teoría de defensa óptima predice que las plantas deberían asignar más defensas a los tejidos con mayor eficacia biológica para la misma (por ejemplo, tejidos reproductivos o meristemos apicales), así como a aquellos que reciben más ataques de herbívoros. Más recientemente, otro cuerpo de teorías ecológicas ha predicho cómo las características de las poblaciones de plantas (por ejemplo, densidad de plantas, distribución espacial) y comunidades (por ejemplo, diversidad de especies, composición) influyen en la asignación a defensas por las plantas. Por ejemplo, se ha demostrado que la densidad específica de la planta influye en la abundancia de insectos fitófagos y en los niveles de herbivoría y, en consecuencia, en las defensas de las plantas. Del mismo modo, la frecuencia de una especie dada en relación con la de los heteroespecíficos o la identidad de las plantas vecinas también puede influir en la herbivoría (y las defensas de las plantas) sobre las plantas focales a través de atracción o repelencia de los herbívoros compartidos (es decir, efectos de asociación). Se han invocado estos tipos de procesos para explicar los efectos de la diversidad y composición de especies de plantas en la herbivoría y las defensas de las plantas. Finalmente, en las últimas dos décadas, un nuevo cuerpo de teoría se ha centrado en patrones de mayor escala para investigar patrones de asignación de defensa intra- e inter- específicos. Los ejemplos de este último grupo de estudios incluyen análisis de defensas de plantas a lo largo de gradientes geográficos tales como gradientes latitudinales o de elevación.

Los gradientes de elevación son herramientas valiosas en ecología para estudiar los factores y mecanismos que rigen la fuerza de las interacciones entre especies. La variabilidad en factores bióticos (ej. herbívoros y depredadores) y abióticos (ej. condiciones climáticas y del suelo) a lo largo del gradiente da forma a clinas concomitantes en rasgos de especies, abundancia y diversidad. En particular, este enfoque ha sido útil para estudiar la interacción entre las plantas y los insectos herbívoros. El estudio de la variación en las interacciones y los rasgos de las especies a lo largo de gradientes ambientales proporciona una visión única del proceso mediante

el cual la herbivoría y el entorno abiótico conforman simultáneamente la variación espacial en las defensas de las plantas.

La teoría clásica sostiene que una mayor abundancia y diversidad de insectos herbívoros en torno a las condiciones climáticas más favorables y estables que se dan en elevaciones bajas conduce a una selección de herbivoría más fuerte en las defensas de las plantas. A pesar de la existencia de un respaldo empírico para estas predicciones, estudios recientes han puesto en duda la generalidad de este paradigma al no encontrar gradientes de elevación en herbivoría ni defensas de las plantas o al encontrar incluso patrones de defensas y herbivoría crecientes con elevación. A la luz de estos patrones opuestos, es necesario reevaluar los mecanismos ecológicos que dan forma a las interacciones planta-herbívoro a lo largo de gradientes ecológicos.

Existen ciertas brechas de conocimiento que impiden una comprensión completa de las interacciones planta-herbívoro a través de gradientes de elevación. La mayoría de estudios precedentes no han considerado los rasgos de la historia de vida de las plantas como fuentes importantes de variación subyacentes en los gradientes de elevación en las interacciones planta-herbívoro. Por ejemplo, las diferencias en la forma de crecimiento pueden determinar la fuerza del gradiente ya que se espera que las especies leñosas sean más fáciles de detectar por los herbívoros y, por lo tanto, es esperable que presenten mayor ataque que las plantas herbáceas. Estas diferencias deberían ser más notorias a bajas elevaciones donde la presión de herbivoría es mayor. Además, en el caso de las especies leñosas, los gradientes de elevación en las interacciones planta-herbívoro podrían depender del hábito de la hoja. Las plantas de hoja caduca a menudo exhiben tasas de crecimiento más altas que las plantas de hoja perenne. Al existir compromisos entre crecimiento y defensas, los árboles de hoja caduca estarán menos defendidos y, en consecuencia, más atacados por los herbívoros a bajas elevaciones. El estado ontogenética es otra fuente de variación en plantas que podría estar determinando los gradientes de elevación en las interacciones planta-herbívoro. Para las especies de vida larga, se espera que los niveles de defensa aumenten desde la plántula hasta las etapas posteriores del desarrollo adulto, una vez que haya un mayor conjunto de recursos disponibles para cumplir las funciones defensivas. Por lo tanto, podríamos esperar que los adultos al estar mejor defendidos exhiban un menor nivel de herbivoría que los juveniles, así como un gradiente de elevación en herbivoría más débil porque los niveles de defensa consistentemente altos amortiguarían o impedirían un

gradiente en herbivoría a pesar de los cambios reales en la presión de los herbívoros. con la elevacion.

Las inconsistencias encontradas en la literatura hasta previa también podrían deberse a que la mayoría de los estudios de elevación han observado la variación de rasgos defensivos individuales. Un asunto clave para completar el estudio de la variación de defensa de las plantas a lo largo de gradientes ecológicos es la inclusión de múltiples rasgos defensivos, incluidas las defensas directas e indirectas de las plantas, así como la distinción entre estrategias constitutivas e inducidas. Por lo tanto, estudiar múltiples rasgos es necesario para describir completamente el fenotipo defensivo de la planta, pero lo más importante es que también expone las diferencias o similitudes en los gradientes de diferentes rasgos defensivos, así como las correlaciones subyacentes. entre rasgos. Estas asociaciones de rasgos pueden ser positivas, lo que conduce a patrones de coexpresión, o negativas, que a menudo resultan de restricciones subyacentes en la asignación de recursos (es decir, compensaciones entre rasgos). La evidencia hasta ahora indica que los gradientes de elevación en la coexpresión de rasgos pueden surgir y son potencialmente importantes. Por ejemplo, cambios en la diversidad de herbívoros con elevación pueden dar como resultado distintos patrones de coexpresión de defensa de las plantas, en este sentido, comunidades más diversas de insectos características de elevaciones más bajas podrían seleccionar un repertorio más amplio de rasgos defensivos directos e indirectos expresados simultáneamente en comparación con elevaciones más altas. De manera alternativa, los cambios de elevación en la estructura trófica (por ejemplo, la proporción depredador: abundancia de herbívoros) podría modular la asignación a las defensas directas e indirectas, de modo que una mayor presión de depredadores a bajas elevaciones podría favorecer una mayor asignación de plantas a defensas directas e indirectas al mismo tiempo, mientras que las plantas de alta elevación asignarían más recursos únicamente a las defensas directas. En general, la consideración de múltiples rasgos y sus patrones de coexpresión a lo largo de los gradientes es un aspecto esencial para comprender cómo evolucionan las defensas de las plantas a lo largo de gradientes ecológicos.

Finalmente, otra consideración importante es que los estudios previos rara vez han llevado a cabo una evaluación explícita de los factores abióticos que se correlacionan con la elevación. La variabilidad clinal de factores abióticos, como las condiciones climáticas o la fertilidad del suelo, podría conducir a gradientes de elevación en las interacciones planta-herbívoro. Los factores abióticos pueden alterar la expresión de defensas por las planta a lo largo de los gradietes a través de cambios en la presión de herbívoros, así como directamente cuando los rasgos juegan un papel en la tolerancia abiótica o los niveles de expresión dependen de las condiciones ambientales, como la disponibilidad de recursos. Tener en cuenta estos factores abióticos es muy informativo, ya que pueden alterar los mecanismos directos e indirectos que influyen en la herbivoría a lo largo de los gradientes de elevación. La hipótesis de concentración de recursos predice que las especies de plantas que crecen en suelos pobres en nutrientes invierten más en defensas químicas en comparación con las especies que crecen en suelos ricos en nutrientes. Por lo tanto, se esperaría que la variación en las condiciones del suelo o el estrés abiótico a lo largo de los gradietnes de elevación influyan en las defensas y tales efectos pueden ser concurrentes a los efectos de la presión de herbívoros y dar lugar a resultados inesperados, por ejemplo, cuando la disponibilidad de nutrientes y la herbivoría exhiben gradientes de elevación opuestos.

El género *Quercus* es uno de los géneros de árboles más extendidos y con mayor diversidad de especies y ha colonizado una amplia gama de entornos diferentes. Este género incluye casi 600 especies de árboles y arbustos distribuidos desde el ecuador hasta latitudes del norte de Europa y América, y desde el nivel del mar hasta 4.000 m. A través de toda su distribución, las especies de *Quercus* son atacados por una gran comunidad de herbívoros de insectos generalistas y especialistas, principalmente masticadores de hojas, esqueletonizadores y minadores, que se alimentan de sus hojas y comprometen el fitness de los robles. En respuesta a los herbívoros, los robles poseen una amplia gama de defensas anti-herbívoro que incluyen defensas física (por ejemplo, contenido de fibra y tricomas), defensas química (por ejemplo, compuestos fenólicos), rasgos nutricionales (por ejemplo, nitrógeno, fósforo) y rasgos fenológicos (por ejemplo, longevidad de las hojas y tiempo de senescencia ). Dentro de los principales rasgos químicos de los robles se encuentran los compuestos fenólicos y los compuestos orgánicos volátiles. Los compuestos fenólicos se han estudiado principalmente como mediadores de la defensa directa en robles. Estos compuestos químicos tienen efecto repelente y tóxicos sobre los insectos herbivoros. Los compuestos fenólicos pueden clasificarse ampliamente en cuatro grupos: taninos condensados, taninos hidrolizables, flavonoides y ligninas. Además, las especies de roble también emiten una batería compleja de compuestos orgánicos volátiles para atraer enemigos naturales de los herbívoros (es decir, defensas indirectas). Los compuestos orgánicos volátiles son principalmente terpenoides (monoterpenos y sesquiterpenos), pero también pueden incluir algunos fenoles aromáticos, alcoholes y aldehídos. Tanto las defensas directas como las indirectas se expresan constitutivamente en robles, además se ha demostrado que son inducibles en respuesta al daño por herbívoros.

En esta tesis de doctorado he estudiado los gradientes de elevación en las defensas de las plantas y en herbivoría teniendo en cuenta los factores comúnmente olvidados comentados previamente para obtener una evaluación más sólida de los mecanismos y procesos que impulsan las interacciones planta-herbívoro y la evolución de los rasgos defensivos anti-herbivoría de las plantas. Para hacerlo, esta tesis incluye estudios bibliográficos y estudios experimentales en condiciones naturales de campo e invernadero. Para estos últimos seleccioné especies del género *Quercus* (comúnmente llamadas robles) como sistema modelo.

En el Capítulo I, realicé un análisis global de gradientes de elevación en herbivoría foliar. Para esto, recopilé datos mundiales de herbivoría en hojas para 1027 especies de plantas que cubren un gradiente de elevación de 2.755 m sobre el nivel del mar, incluyendo regiones tropicales y templadas. Las especies incluidas en el estudio presentaban diferencias en las formas de crecimiento (herbáceas versus especies leñosas) y en el hábito de hoja (caducifolias y perennes). Los objetivos específicos de este capítulo fueron: (i) evaluar un gradiente de elevación global en la herbivoría foliar; (ii) evaluar si los gradientes de elevación en la herbivoría son contingentes a la región climática (regiones templadas vs. tropicales), la forma de crecimiento (especies herbáceas versus leñosas) o el hábito de hoja (árboles caducifolios versus árboles de hoja perenne); y (iii) probar si los factores climáticos (temperatura y precipitación) tienen un efecto sobre la variación en herbivoría a lo largo del gradiente. No hubo evidencia de un efecto global de elevación en la herbivoría al considerar todas las especies juntas. Los gradientes de elevación en herbivoría fueron contingentes en forma de crecimiento; de acuerdo con la predicción de que las especies leñosas son más evidentes para los herbívoros y, por lo tanto, presentan un gradiente de elevación más fuerte que las especies herbáceas. Específicamente, el ataque por herbívoros aumentó a bajas elevaciones para las especies leñosas, pero no se encontró ningún efecto de elevación para las especies herbáceas. Además, dentro de las especies leñosas, el gradiente de elevación en la herbivoría fue dependiente del hábito de la hoja; Los árboles de hoja caduca presentaron niveles más altos de herbivoría a bajas elevaciones, pero no se encontró ningún efecto de elevación para los árboles de hoja perenne. Estos resultados concuerdan con el patrón esperado basado en la existencia de un compromiso entre crecimiento y defensas para árboles de crecimiento rápido. En contra de las predicciones, no hubo diferencias en los niveles de herbivoría entre las regiones templadas o tropicales. Finalmente, los factores climáticos (temperatura y precipitación) no tuvieron efecto en los gradientes de elevación observados.

En el Capítulo II, investigué el efecto combinado de la elevación y las etapas de desarrollo de la planta sobre las defensas de las plantas y el ataque de herbivoros. Para esto, muestreé ocho especies de roble en toda la Península Ibérica que juntas cubrían un gradiente de elevación de 1330 m. Las especies incluidas en el estudio fueron: *Quercus robur, Q. petraea, Q. suber, Q. ilex, Q. pubescens, Q. lusitanica, Q. canariensis* y *Q. coccifera*. Para cada una de estas especies, tomé muestras de tres poblaciones que correspondían a sitios de elevación baja, media y alta en función de la distribución de elevación de cada especie. Dentro de cada población, tomé muestras de hojas de árboles jóvenes y árboles adultos para estimar la herbivoría y cuantificar las defensas químicas de las hojas y los nutrientes. Los objetivos específicos de este estudio fueron (i) evaluar los gradientes de elevación en herbivoría foliar y en los rasgos de las plantas, (ii) evaluar si los gradientes de elevación dependían de la etapa ontogenética de la planta y (iii) evaluar si la variación en los niveles de herbivoría con la elevación y con la ontogenia se asoció con rasgos de la hoja. Se encontró un gradiente de elevación en las defensas de herbivoría y hojas, pero al contrario del patrón esperado, tanto la herbivoría como las defensas aumentaron hacia elevaciones más altas. En contra de las predicciones, la ontogenia no tuvo un efecto en los niveles de herbivoría, y los niveles defensivos fueron más altos para las plántulas. Sin embargo, la etapa ontogenética no determinó la variación en herbivoría o defensas a lo largo del gradiente.

En el Capítulo III, investigué la variación clinal interespecífica en múltiples estrategias defensivas y la expresión correlacionada entre diferentes rasgos defensivos. Además, investigué el efecto de los factores abióticos correlacionados con elevación (temperatura y precipitación) asociados con el nicho climático de cada especie en las defensas de las plantas como posibles factores subyacentes a los gradientes de elevación. Para este capítulo, realicé un experimento de invernadero con árboles jóvenes de 18 especies de robles cuya distribución natural abarca más de 2400 m de altura. Cuantifiqué la concentración de los niveles constitutivos de compuestos fenólicos en las hojas, así como su inducibilidad después del ataque de larvas de la polilla gitana generalista (*Lymantria dispar*, Lepidoptera). El análisis estadístico se realizó mediante un enfoque comparativo filogenético. Los objetivos específicos de este capítulo fueron: (i) evaluar los gradientes de elevación en las defensas constitutivas y su inducibilidad (es decir, la diferencia entre los niveles inducidos y constitutivos de las defensas) entre las especies de roble; (ii) evaluar las correlaciones positivas o negativas entre diferentes defensas y evaluar los gradientes de elevación en tales asociaciones; y (iii) probar si la temperatura o la precipitación determinan los gradientes de elevación en rasgos defensivos individuales o en la expresión correlacionada de defensas. Los resultados de este trabajo de investigación mostraron un gradiente de elevación para la inducibilidad de compuestos fenólicos, donde la inducibilidad aumentó para especies con rangos de elevación más altos. Contrariamente a lo esperado, no hubo efecto de elevación para las defensas constitutivas y, aunque encontramos una correlación positiva entre fenólicos constitutivos e inducidos, no hubo efecto de la elevación modulando la fuerza de dicha asociación. Finalmente, no hubo efecto de la temperatura o la precipitación sobre ninguno de los gradientes de elevación reportados.

En el capítulo IV, investigué la variación clinal en múltiples rasgos defensivos de la planta y la expresión correlacionada de defensas a nivel intraespecífico. También investigué el efecto de los factores climáticos correlacionados con la elevación (temperatura y precipitación) en los gradientes. Para este estudio, realicé un experimento de campo en 18 poblaciones naturales de *Quercus pyrenaica* abarcando un rango de elevación de 1300 m. Cuantifiqué las defensas foliares constitutivas directas (compuestos fenólicos) e indirectas (volátiles) y su inducibilidad en árboles juveniles después de ser atacados por el escarabajo especialista conocido como “ la pulga del roble” (*Altica quercetorum*, Coleoptera: Chrysomelidae). Los objetivos específicos del capítulo fueron: (i) evaluar los gradientes de elevación en defensas directas (compuestos fenólicos) e indirectas (compuestos orgánicos volátiles) constitutivas e inducidas; (ii) evaluar la existencia de patrones de correlación positiva o negativa entre diferentes defensas y patrones de variación en tales correlaciones con la elevación; y (iii) evaluar si la variación en factores climáticos (temperatura y precipitación) está asociada con variación concomitante en rasgos defensivos y patrones de correlación. Se encontró un gradiente de elevación para las defensas directas constitutivas, pero no se encontró ningún efecto de elevación para las defensas indirectas constitutivas o la inducibilidad de ninguno de los rasgos defensivos analizados. De acuerdo con las predicciones, los niveles constitutivos de compuestos fenólicos aumentaron hacia elevaciones más bajas. Además, hubo una expresión simultánea de fenoles constitutivos e inducidos y la asociación se correlacionó negativamente con la elevación. Esto significa que hubo un cambio en la asociación entre fenoles constitutivos e inducidos a lo largo del gradiente de elevación: de la coexpresión a bajas elevaciones se pasó a una correlación negativa a elevaciones altas. Finalmente, las condiciones climáticas explicaron los gradientes de elevación observados, ya que los gradientes de elevación en las defensas no cambiaron después de tener en cuenta la temperatura y la precipitación en los modelos estadísticos.

Los resultados de los diferentes capítulos evidenciaron que la dirección y la fuerza de los gradientes de elevación en los rasgos de las plantas pueden variar en respuesta a las diferencias en la escala espacial cubierta por el gradiente geográfico estudiado o debido a cambios en la escala taxonómica desde nivel multiespecífico a género o incluso nivel de especie. El valor de los estudios de defensas de plantas a lo largo de gradientes de elevación podría incrementar al realizar estudios mecanicistas que controlen la variación en los factores abióticos y la abundancia de artrópodos, ya que no es posible establecer un vínculo causal entre patrones y procesos a través de mecanismos presuntos sin realizar experimentos manipulativos. Por ejemplo, los estudios en jardines comunes y experimentos de trasplante recíproco utilizando diferentes genotipos de plantas podrían ayudar a separar el efecto de la adaptación local y la plasticidad fenotípica. Los estudios de campo futuros también deberían incluir una evaluación sólida de la comunidad de artrópodos que rodea a las especies de plantas de interés. La caracterización de la comunidad herbívora es necesaria para relacionar la abundancia de herbívoros con la presión de hervivoría sobre las plantas y para controlar las diferencias funcionales entre los grupos de herbívoros. Además, se necesita caracterizar la comunidad de depredadores y parasitoides para calcular la proporción de enemigos naturales y herbívoros y para comprender mejor el control por parte del tercer nivel trófico sobre los herbívoros.

En general, el trabajo de investigación presentado en esta tesis doctoral promueve una comprensión más sólida y holística de los factores que impulsan variación en la dirección y la fuerza de los gradientes de elevación en las interacciones planta-herbívoro. Nuestros hallazgos enfatizan el valor de considerar los rasgos de historia de vida de las plantas y las etapas de desarrollo como factores que dan forma a los gradientes elevacionales en las interacciones planta-herbívoro. Además, nuestros hallazgos también destacan la importancia de evaluar múltiples rasgos defensivos de la planta para lograr una comprensión más sólida de las fuentes de variación en la asignación a las defensas de la planta a lo largo de los gradientes de elevación.

# ABSTRACT

# ABSTRACT

Plants and insect herbivores have coexisted for more than 350 million of years. This long period of coexistence have given rise to a process of co-evolution that can be assimilated to an “arm’s race”. Through this process, plants have developed a wide arsenal of different anti-herbivory defensive strategies and herbivores have developed adaptations to these defences. These co-evolutionary dynamics have triggered accelerated rates of speciation in both, insects and plants. Plant defensive mechanisms can be classify as tolerance mechanisms, defined as the ability of plants to mitigate the negative fitness effects caused by herbivory; or resistance mechanisms, which are defined as the ability of plants to reduce or avoid herbivore damage by affecting the insect fitness. Defensive traits can be classified as well in direct defences that includes chemical or physical traits that deter herbivores, reduce their consumption or decrease their survival; and indirect defences, that are chemical or physical plant traits that provide shelter, reward, or information on herbivore presence to predators of herbivores (i.e. predators and parasitoids). These defensive traits can be constitutively expressed by plants (i.e. always present whether the plant is under attack or not) or can be induced (i.e. synthesized by the plant only after herbivore damage).

The co-evolution between plants and insect herbivores and the evolution of plant defences have been major inquiries in ecology. Several theories have been proposed in the last decades to explain which factors influence these processes. Initially, several theories focused on defence allocation from the perspective of individual plants. These theories predict that, since plant defences are costly to produce, plants have to choose between allocation to resources to defences and other functions like growth or reproduction. For instance, the Resource Availability Hypothesis states that plants growing in resource-poor environments grow more slowly and allocate more resource to defences, as the cost of replacing damaged tissues is higher

under resource-limited environments. Similarly, the Growth-Differentiation Balance Hypothesis predicts a trade-off between costs of secondary metabolite production relative to the demand for photosynthate by growth. Finally, the Optimal Defence Theory predicts that plants should allocate more defences to tissues of greater fitness value (e.g. reproductive tissues, apical meristems), as well as those that receive more attack by herbivores. More recently, another body of ecological theories has predicted how characteristics of plant populations (e.g. plant density, spatial distribution) and communities (e.g. species diversity, composition) influence allocation to plant defences. For example, conspecific plant density has been shown to influence the abundance of phytophagous insects and herbivory levels and consequently plant defences. Likewise, the frequency of a given species relative to that of heterospecifics or the identity of neighbouring plants can also influence herbivory (and plant defences) on focal plants via attraction or repellence of shared herbivores (i.e. associational effects). These types of processes have been invoked to explain the effects of plant species diversity and composition on herbivory and plant defences. Finally, over last two decades a new body of theory has focused on larger-scale patterns to investigate intra- and inter-specific patterns of defence allocation. Examples of this latter group of studies include analyses of plant defences along geographical clines such as latitudinal or elevational gradients.

Elevational gradients are powerful tools in ecology to study factors and mechanisms governing species interactions strength. The clinal variability in biotic (e.g. herbivory and predation) and abiotic factors (e.g. climate and soil conditions) along the gradient shapes concomitant clines in species traits, abundance and diversity. In particular, this approach have been useful to study the interaction between plants and their insect herbivores. The study of variation in interactions and species traits along environmental clines provides unique insight into the process by which herbivory and the abiotic environment concurrently shape spatial variation in plant defences.

Classical theory holds that higher abundance and diversity of insect herbivores toward more favourable and stable climatic conditions found at low elevations leads to stronger herbivory selection on plant defences. Despite the existence of empirical support for this predictions, the generality of this paradigm have been called into question by recent studies reporting no evidence of elevational gradients in herbivory and plant defences or even higher plant defences and herbivory with increasing elevation. In light of these contrasting patterns, there is a need of re-evaluation of the ecological mechanisms shaping plant-herbivore interactions along ecological gradients.

There are many knowledge gaps which prevent a complete understanding of plant-herbivore interactions across elevational gradients. Most previous studies have not considered plant life-history traits as important sources of variation underlying elevational gradients in plant-herbivore interactions. For example, differences in growth form may determine the strength of the gradient since woody species are expected to be easier to detect by herbivores and thus they should be more attack than herbaceous plants. These differences should be magnified at low elevations were herbivory pressure is higher. In addition, within woody species elevational gradients in plant-herbivore interactions could be contingent on leaf habit. Deciduous plants often exhibit higher growth rates than evergreen plants. As growth and defences trade-off, deciduous trees would be less defended and consequently more attacked by herbivores at low elevations. Ontogenetic stage is another source of plant–based variation which could underlie elevational gradients in plant-herbivore interactions. For long-lived species, defence levels are expected to build up from the sapling to later stages of adult development, once a greater pool of resources is available to fulfil defensive functions. Therefore, we could expect that better defended adults exhibit lower overall herbivory than juveniles, as well as a weaker elevational gradient in herbivory because the presence of consistently high defence levels along the cline would dampen or preclude a gradient in herbivory despite actual changes in herbivore pressure with elevation.

Inconsistencies from previous literature could also derive because most elevational studies have looked at variation in individual plant defensive traits. A key gap to fill in the study of plant defence variation along ecological gradients is the inclusion of multiple defensive traits, including plant direct and indirect defences, as well as the distinction between constitutive and induced strategies. Studying multiple traits is thus necessary to fully describe the plant defensive phenotype, but importantly it also exposes differences or similarities in trait clines, as well as underlying trait correlations. These trait associations may be positive leading to co-expression patterns, or negative which often result from underlying allocation constraints (i.e. trade-offs between traits). Evidence gathered thus far indicated that elevational gradients in trait co-expression can arise and are potentially important. For example, changes in herbivore diversity with elevation may result in distinct patterns of plant defence co-expression, whereby more diverse insect communities found at lower elevations could select for a broader repertoire of simultaneously expressed direct and/indirect defensive traits compared to higher elevations. Alternatively, elevational shifts in trophic structure (e.g. ratio of predator:herbivore abundance) might modulate allocation to direct and indirect defences, whereby greater predator pressure at low elevations could favour increased plant allocation to both direct and indirect defences whereas high-elevation plants allocate more to direct defences only. Overall, the consideration of multiple traits and their co-expression patterns along gradients is therefore an essential aspect for understanding how plant defences evolve along ecological clines.

Finally, another important consideration is that previous studies have rarely conducted explicit assessment of the abiotic correlates of elevation. Clinal variability of abiotic factors such as climatic conditions or soil fertility could drive elevational gradients in plant-herbivore interactions. Abiotic factors can alter plant defence expression along clines via changes in herbivore pressure, as well as directly when traits play a role in abiotic tolerance or expression levels are contingent on environmental conditions such as resource availability. Considering these abiotic factors is highly informative since they may alter both direct and indirect mechanisms influencing herbivory along elevational gradients. The Resource Concentration Hypothesis predicts that plant species growing in nutrient-poor soils invest more in chemical defences relative to species growing in nutrient-rich soils. Therefore, variation in soil conditions or abiotic stress along elevational clines would be expected to influence defences and such effects may be concurrent to effects of herbivore pressure and lead to unexpected outcomes, e.g., when nutrient availability and herbivory exhibit opposing elevational gradients

*Quercus* genus is one of the most widespread and species-rich tree genera and have colonized a wide array of different environments. This genus includes almost 600 species of trees and shrubs distributed from the equator to northern latitudes in Europe and America, and from the sea level up to 4,000 m. Through their entire distribution, *Quercus* species support a large community of generalist and specialists insect herbivores, mainly leaf chewers, skeletonizers and miners, that feed on their leaves and compromise the oaks fitness. In response to herbivores, oaks possess a wide array of anti-herbivore defences including physical (e.g. fiber content, and trichomes), chemical (e.g. phenolic compounds), nutritional (e.g. nitrogen, phosphorus) and phenological (e.g. leaf longevity and timing of senescence) traits. Within the main chemical traits of oaks are phenolic compounds and volatile organic compounds. Phenolic compounds have been primarily studied as the mediators of direct defence in oaks. These chemical compounds have repellent and toxic effects on insect herbivory. Phenolic compounds can be broadly classified in four groups: condensed tannins, hydrolysable tannins, flavonoids and lignins. In addition, oak species also emit a complex battery of volatile organic compounds to

attract natural enemies of herbivores (i.e. indirect defences). Volatile organic compounds are mainly terpenoids (monoterpenes and sesquiterpenes), but can also include some aromatic phenols, alcohols and aldehydes. Both direct and indirect defences are constitutively expressed in oaks and have been shown to be inducible in response to herbivore damage.

In this PhD dissertation, I studied elevational gradients in plant defences and herbivory accounting for the overlooked factors mention above in order to get a more robust assessment of the mechanisms and processes driving plant-herbivore interactions and the evolution of plant anti-herbivory defensive traits. For doing so, this thesis includes literature surveys and mechanism-based experiments under natural field and greenhouse conditions. I selected species of the *Quercus* genus (commonly called oaks) as a model system for these studies.

In the Chapter I, I performed a global analysis of elevational gradients in leaf herbivory. For this, I recorded worldwide data of leaf herbivory for 1027 plant species covering an elevational gradient of 2,755m and including tropical and temperate regions. The species included in the study presented differences in growth forms (herbaceous vs woody species) and leaf habit (deciduous vs evergreen). The specific objectives of this chapter were (i) to test for a global elevational gradient in leaf herbivory; (ii) to test whether elevational gradients in leaf herbivory are contingent in climatic region (temperate vs tropical regions), growth form (herbaceous vs woody species) or leaf habit (deciduous vs evergreen trees); and (iii) to test whether climatic factors (temperature and precipitation) have an effect on the clinal variation in herbivory. There was no evidence of a global effect of elevation on herbivory when considering all the species together. Elevational gradients in herbivory were contingent in growth form; according to the prediction that woody species are more apparent for herbivores and thus present a stronger elevational gradient that herbaceous species. Specifically, herbivory attack increased at low elevations for woody species but no effect of elevation was found for herbaceous species. Furthermore, within woody species, elevational gradient in herbivory was contingent on leaf habit; deciduous trees presented higher levels of herbivory at low elevations, but no effect of elevation was found for evergreen trees. These results agree with the expected pattern based on the existence of a trade-off between growth and defences for fast-growing trees. Counter to predictions, there were no differences in herbivory levels between temperate or tropical regions. Finally, the climatic correlates of elevation (temperature and precipitation) had no effect on the observed elevational gradients.

In the Chapter II, I investigated the combined effect of elevation and plant developmental stage on plant defences and herbivory attack. For this, I sampled eight oak species throughout the Iberian Peninsula that together covered an elevational gradient of 1330 m. The species included in the study were: *Quercus robur, Q. petraea, Q. suber, Q. ilex, Q. pubescens, Q. lusitanica, Q. canariensis,* and *Q. coccifera*. For each of these species, I sampled three populations that corresponded to low-, mid- and high-elevation sites based on the elevational distribution of each species. Within each population, I sampled leaves from saplings and adult trees to estimate herbivory and quantify leaf chemical defences and nutrients. The specific objectives of this study were (i) to test for elevational gradients in leaf herbivory and plant traits, (ii) to test whether the elevational gradients were contingent on plant ontogenetic stage, and (iii) to test whether elevational and ontogenetic variation in herbivory was associated with leaf traits. There was an elevational gradient in herbivory and leaf defences, but contrary to expected pattern both herbivory and defences increased toward higher elevations. Counter to predictions, ontogeny did not have an effect on the levels of herbivory, and defensive levels were higher for saplings. However, ontogenetic stage did not account for the clinal variation in herbivory or defences.

In the Chapter III, I investigated the inter-specific clinal variation in multiple defensive strategies and the correlated expression between different defensive traits. In addition, I investigated the effect of abiotic correlates (temperature and precipitation) associated with the climatic niche of each species on plant defences as possible factors underlying the elevational gradients. For this chapter, I performed a greenhouse experiment with saplings of 18 oak species which natural distribution spans together more than 2400 m in elevation. I quantified the concentration of constitutive levels of phenolic compounds in leaves as well as their inducibility after feeding by the generalist gypsy moth larvae (*Lymantria dispar*, Lepidoptera). The statistical analysis were performed using a phylogenetic comparative approach. The specific objectives of this chapter were: (i) to test for elevational gradients in constitutive defences and their inducibility (i.e., difference between induced and constitutive levels of defences) across oak species; (ii) to test for positive or negative correlations between different defences and test for elevational gradients in such associations; and (iii) to test whether temperature or precipitation account for elevational gradients in individual defensive traits or in the correlated expression of defences. The results of these research work showed an elevational gradient for the inducibility of phenolic compounds, where the inducibility increased for species with higher

elevational ranges. Contrary to expectations, there was no effect of elevation for constitutive defences and, although we found a positive correlation between constitutive and induced phenolics, there was no effect of elevation shaping the strength of such association. Finally, there was no effect of temperature or precipitation shaping any of the elevational gradients reported.

In the chapter IV, I investigated the clinal variation in multiple plant defensive traits and the correlated expression of defences at intraspecific level. I also investigated the effect of climatic correlates of elevation (temperature and precipitation) in the elevational gradients. For this study, I performed a field experiment in 18 natural populations of *Quercus pyrenaica* spanning an elevational range of 1300 m. I quantified constitutive direct (phenolic compounds) and indirect (volatiles, VOCs) leaf defences and their inducibility in saplings after feeding by the specialist oak flea beetle (*Altica quercetorum*, Coleoptera: Chrysomelidae). The specific objectives of the chapter were: (i) to test for elevational gradients in constitutive and induced direct (phenolic compounds) and indirect defences (volatile organic compounds); (ii) to test for patterns of positive or negative correlation between different defences and for patterns of clianl variation in such correlations; and (iii) to test whether climatic correlates of elevation (temperature and precipitation) are associated with concomitant clines in defensive traits and patterns of correlation. There was an elevational gradient for constitutive direct defences but no effect of elevation was found for constitutive indirect defences or the inducibility of any of the defensive traits analysed. According to predictions, constitutive levels of phenolic compounds increased toward lower elevations. Furthermore, there was a simultaneous expression of constitutive and induced phenolics and the association was negatively correlated with elevation. This means that there was a shift in the association between constitutive and induced phenolics along the elevation gradient, from co-expression at low elevations to a negative correlation at high elevations. Finally, climatic conditions did not underlie the observed elevational gradients, as elevational gradients in defences did not change after accounting for temperature and precipitation in the models.

The results of the different chapters evidenced that the direction and the strength of the elevational gradients in plant traits may vary in response to differences in the spatial scale covered by the geographic gradient studied or due to changes in the taxonomic scale from multispecific level to genus or even species level. The robustness of the study of plant defences along elevational gradients would increase by performing mechanistic studies controlling for

variation in abiotic factors and arthropod abundance, since it is not possible to establish a causal link between patterns and processes via presumed mechanisms without performing manipulative experiments. For example, common gardens and reciprocal transplant experiments studies using different plant genotypes could help teasing apart the effect of local adaptation and phenotypic plasticity. Future field studies should also include a robust assessment of the arthropod community surrounding the plant species of interest. The characterization of the herbivore community is needed to relate the abundance of herbivores to herbivory pressure on plants and to control for functional differences across herbivore guilds. In addition, characterizing the community of predators and parasitoids is needed to calculate the ratio of these natural herbivores to herbivores and to get a better understand of top-down control exerted on herbivores by their natural enemies.

Overall, the research work presented in this PhD dissertation builds towards a more robust and holistic understanding of the drivers of variation in the direction and strength of elevational gradients in plant-herbivore interactions. Our findings emphasize the value of considering plant life-history traits of plants and developmental stages as factors shaping elevational gradients in plant-herbivory interactions. Moreover, our findings also highlight the importance of assessing multiple plant defensive traits to achieve a more robust understanding of sources of variation in the allocation to plant defences along elevational gradients.

# GENERAL INTRODUCTION


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# GENERAL INTRODUCTION

## EVOLUTIONARY ECOLOGY IN PLANT DEFENCES AGAINST INSECT HERBIVORES

Plants and insect herbivores have coexisted and interacted for approximately 350 million of years (Labandeira 2007). In that long period, plants and insect herbivores have engaged in a co-evolutionary process which can be assimilated to an “arm’s race” (Ehrlich and Raven 1964). Plants as autotrophic organisms are the target of all herbivores that feed on their tissues moving the energy to the rest of the tropic web and compromising plant fitness. In this way, herbivores act as selective forces enhancing the evolution of plant defences. As a result, plants have developed a wide array of potent anti-herbivore defences (Schoonhoven et al. 2005) and herbivores, in turn, have developed adaptations to these plant defences (Glauser et al. 2011, Strauss et al. 2013). These co-evolutionary dynamics between insect herbivores and plants have triggered accelerated rates of speciation in both groups (Futuyma and Agrawal 2009).

Plant defensive mechanisms can be broadly classify into tolerance traits and traits that confer resistance to herbivores (Núñez-Farfán et al. 2007). Plant tolerance is defined as the ability of plants to mitigate the negative fitness effects caused by herbivory (Strauss and Agrawal 1999, Stowe et al. 2000). Plant tolerance mechanisms include re-growth capacity, overcompensation in reproduction, increased photosynthetic rates and changes in nutrient or biomass allocation between tissues (Agrawal et al. 1999, Moreira et al. 2012, Robert et al. 2014). Plant resistance, on the other hand, is defined as the ability of plants to reduce or avoid herbivore damage (Rausher 2001, Carmona et al. 2011). Defence traits can be classified as direct (Agrawal 2007) and indirect (Heil 2008) defences. Plant direct defences are conferred by chemical (e.g. phenolics, terpenoids, alkaloids) and physical (e.g. thorns, spines, trichomes, leaf toughness) traits that deter herbivores, reduce their consumption or decrease their survival (Agrawal 2007, Carmona et al. 2011, Mithöfer and Boland 2012). Indirect defences, on the other hand, involve physical (e.g. domatia, extrafloral nectar) and chemical (e.g. volatile organic compounds) traits that provide shelter, reward, or information on herbivore presence to predators of herbivores (i.e. predators and parasitoids) (Dicke and Baldwin 2010, Kessler and Heil 2011, Turlings and Erb 2018, Pearse et al. 2020). These natural enemies suppress herbivores and, in turn, indirectly increase plant biomass and reproduction (Hairston et al. 1960, Schmitz et al. 2000, Romero and Koricheva 2011). Both direct and indirect defences can be always present in the plants (i.e. constitutive defences) or synthesized only after herbivore damage (i.e. induced defences) (Karban and Baldwin 1997, Dicke and van Loon 2000).

Understanding the tremendous variation in plant defences among species, among individual plants within species, and even among types of tissue within individual plants has been a central topic of research during the last five decades (Marquis 1992, McCall and Fordyce 2010). Investment in anti-herbivore defences is crucial for plants to reduce the adverse effect of herbivory that otherwise might compromise plant survival.

From the perspective of individual plants, several ecological theories have been developed to explain patterns of defence allocation. These theories are based on the premise that, because plant defences are costly to produce, plants frequently face trade-offs between allocation to defenses and other functions (e.g. growth, reproduction) which underlie variation in plant defence allocation (Stamp 2003). For instance, the Resource Availability Hypothesis posits that plants growing in resource-poor environments grow more slowly and allocate more to defences (Coley et al. 1985, Endara and Coley 2011). This is based on the existence of a growth-defence trade-off, and on the fact that herbivore-damaged tissues are more costly to replace under low resource availability (Coley and Kursar 2014). Similarly, the Growth-Differentiation Balance Hypothesis also predicts that there is a physiological trade-off between growth and secondary metabolism (Herms and Mattson 1992). In particular, as resource availability increases, carbon is allocated to rapidly dividing meristems (i.e. for growth demands) at the expense of secondary metabolism (Herms and Mattson 1992). Finally, the Optimal Defence Theory has been and influential framework to explain within-plant defence allocation patterns (reviewed by McCall and Fordyce 2010). It predicts that because defences are costly to produce, plants allocate more defences to tissues of greater fitness value (e.g. reproductive tissues, apical meristems), as well as those that receive more attack by herbivores (McKey 1979, Zangerl and Bazzaz 1992, Zangerl and Rutledge 1996).

More recently, research on ecological factors affecting plant-herbivore interactions has focused on understanding how features of plant populations (e.g. plant density, spatial distribution) and communities (e.g. species diversity, composition) influence allocation to plant defence (reviewed by Agrawal et al. 2006). For example, conspecific plant density has been shown to increase the abundance of phytophagous insects and herbivory levels (Hambäck et al. 2014, Kim and Underwood 2015). Likewise, the frequency of a given species relative to that of heterospecifics or the identity of neighbouring plants can also influence herbivory on focal plants via attraction or repellence of shared herbivores (i.e. associational effects; reviewed by Barbosa et al. 2009). These types of processes have been addressed to explain the effects of plant species diversity and composition on patterns of plant defence allocation (reviewed by Moreira et al. 2016).

Finally, over the last two decades an increasing number of studies have attempted to address broader-scale (biogeographic) patterns in defences within-species as well as across species using phylogenetic methods (Körner 2007, Schemske et al. 2009). Examples of this latter group of studies include work on defences along climatic or latitudinal gradients (Moles et al. 2011), elevational gradients (Rasmann et al. 2014a, Moreira et al. 2018a), as well as continental-scale comparisons (Moreira et al. 2018b).

## ECOLOGICAL GRADIENTS IN HERBIVORY AND DEFENCES

Ecological gradients are powerful tools for understanding how the environment shapes species traits and the communities in which species are embedded (Dobzhansky 1950, Schemske et al. 2009). Ecological gradients have been shown to shape plant communities, populations and traits through variation in both abiotic (e.g. climate) and biotic (species interactions) factors (Schemske et al. 2009, Rasmann et al. 2014b). In this regard, changing environmental conditions along latitudinal and elevational gradients are thought to have selected for concomitant gradients in species traits, abundance, and diversity (Schemske et al. 2009, Rasmann et al. 2014a). First evidences documenting higher species diversity and speciation in the tropics (Darwin 1859, Wallace 1878, Dobzhansky 1950) gave rise to the main theory in the evolution of plant-insect interactions. Theory holds that biotic interactions should be stronger toward warmer and more stable climatic conditions found near the equator and the sea level

(Dobzhansky 1950, Janzen 1970, Connell 1971), and this should had leaded to stronger selection on plant defences towards low latitudes (Schemske et al. 2009, Rasmann and Agrawal 2011, Marquis et al. 2012) and elevations (Scheidel and Bruelheide 2001, Zehnder et al. 2009, Rodríguez-Castañeda et al. 2010, Garibaldi et al. 2011, Pellissier et al. 2012, Pellissier et al. 2014, Rasmann et al. 2014c, Rasmann et al. 2014a, Rasmann et al. 2014b, Pellissier et al. 2016). The study of variation in interactions and species traits along environmental clines provides unique insight into the process by which herbivory and the abiotic environment concurrently shape spatial variation in plant defences.

Latitudinal studies have traditionally received a lot of attention by ecologist pursuing to disentangle the selective forces driving variation in species traits. Similarly, though comparatively less studied, elevational gradients in biotic or abiotic forcing may also drive concomitant variation in species traits. Elevational variation in abiotic and biotic factors recapitulates latitudinal variation over much smaller geographical scales and results in abrupt climatic or biotic shifts over relatively short distances. In spite of these advantages, research on elevational gradients still reports inconsistencies in the strength and direction of elevational gradients in herbivory and plant defences. Two recent reviews led by my PhD advisors have shown mixed evidence for the predicted patterns (Rasmann et al. 2014a, Moreira et al. 2018a). In particular, plant defences and herbivory increased toward lower elevations in only 48% and in 60% of the reported studies, respectively (Moreira et al. 2018a). Therefore, ecological theory needs a re-evaluation of the mechanisms driving geographical variation in plant-herbivory interactions.

## POTENTIAL SOURCES OF CONTROVERSY SURROUNDING ELEVATIONAL PATTERNS IN HERBIVORY AND DEFENCE

### Elevational gradients in plant-herbivore interactions could be contingent on plant life-history traits and developmental stage

The radiation of plant species into different environments has promoted the evolution of a great diversity of life-history traits (Díaz et al. 2016). However, this variability is not accounted for in previous work about elevational gradients in plant-herbivore interactions. One of these traits is growth form (e.g. non-woody vs. woody species), which may alter expected outcomes if one considers that woody species are longer-lived, larger in size and could therefore be thought of as resources that are more predictable and easier to detect by herbivores relative to smaller and ephemeral non-woody (e.g. herbaceous) species ("Plant Apparency Hypothesis"; (Feeny 1976). Accordingly, recent work has shown that woody plants usually suffer a higher level of herbivory than do non-woody species (Turcotte et al. 2014a). We would therefore predict that elevational gradients in herbivory should be more frequent and stronger for woody species relative to non-woody species.

Additionally, differences in life history strategies associated with resource use and acquisition may influence plant defence investment, and therefore, patterns of herbivory (Lim et al. 2015). Evergreen species typically exhibit a more conservative resource-use strategy and slower growth rates (Givnish 2002, Wright et al. 2004), whereas deciduous species exhibit an exploitative resource-use strategy and higher growth rates (Reich et al. 1998, Poorter and Garnier 2007). If growth and defence trade off, as often shown across many plant taxonomic groups (Coley et al. 1985, Endara and Coley 2011), then evergreen species would be expected to grow less and be more highly defended, and therefore exhibit weaker elevational gradients in herbivory relative to their less-defended and faster growing deciduous species.

Finally, differences among developmental stages are another overlooked aspect possibly underlying variation in elevational gradients in plant-herbivore interactions. One reason to expect differences is that plant defence levels are known to vary with development (Barton and Koricheva 2010, Barton and Boege 2017). For long-lived species, defence levels are expected to build up from the sapling to later stages of adult development, once a greater pool of resources is available to fulfil defensive functions (Boege and Marquis 2005, Barton and Koricheva 2010). Under this scenario, one prediction could be that better defended adults exhibit lower overall herbivory than juveniles, as well as a weaker elevational gradient in herbivory because the presence of consistently high defence levels along the cline would dampen or preclude a gradient in herbivory despite actual changes in herbivore pressure with elevation. Another reason to expect ontogenetic variation in elevational gradients is that, as explained above, variation in herbivory and plant defence levels could be a function of the risk of being attacked by herbivores (i.e. "Plant Apparency Hypothesis"; (Feeny 1976). In this sense, adult plants are more conspicuous than juveniles and should thus exhibit higher herbivory (Moreira et al. 2017). Under this scenario, we would expect more heavily attacked adults to be more defended, as well as exhibit a stronger (i.e. steeper) elevational cline in herbivory because changes in herbivore abundance and diversity (i.e. pressure) with elevation would translate into concomitant changes in herbivory when plants are more apparent. A stronger cline in herbivory would then result in a concomitantly stronger cline in defences for adults relative to saplings.

Taken together, it is important to stress the need to assess the influence of plant life-history traits (growth form, leaf habit) and developmental stages (ontogeny) on elevational gradients in herbivory and its underlying mechanisms, as these are likely important sources of plant-based variation dictating elevational gradients in plant-herbivore interactions.

### Plants deploy multiple defensive strategies that are usually not simultaneously considered in studies about plant-herbivore interactions along elevational gradients

Plants have developed a wide arsenal of defence traits whose expression respond to variation in environmental conditions and are prone to show clinal variation along elevation. Theory on elevational gradients in plant defence and herbivory assumes that levels of constitutive plant secondary metabolites or physical defences are strongly associated with herbivore preference or performance. However, other defensive strategies such as tolerance (e.g., re-growth capacity or overcompensation in reproduction, (Carmona et al. 2011), induced defences (Karban 2011), and traits associated with indirect defence (Agrawal 2011) might also exhibit elevational gradients but have rarely been tested. Further, different defensive strategies may co-vary along an elevational gradient leading to trait associations that can be negative, as a result of underlying allocation constraints (i.e. trade-offs between traits; Agrawal and Hastings 2019), or positive leading to co-expression patterns (i.e. defence syndromes, Agrawal and Fishbein 2006, Defossez et al. 2018).

In some cases there may be trade-offs between defensive strategies depending on the costs and benefits of each under different ecological contexts (Agrawal 2000). For example, constitutive and induced defences frequently trade off (Koricheva et al. 2004, Kempel et al. 2011, Rasmann et al. 2011, Moreira et al. 2014), and shifts in the relative allocation to each strategy may occur along environmental gradients. We would expect that environments with low herbivore pressure (e.g. high elevations) should select for low constitutive defences and high levels of induction because the costs of continuously producing constitutive defences are high when damage is low (i.e., costs of constitutive defences outweigh their benefits; (Zangerl and Rutledge 1996). In contrast, environments with high herbivore pressure (low elevations) should select for high constitutive but low induced defences because the benefits of reduced fitness losses to herbivory outweigh the costs of continuous production of constitutive defences (Zangerl and Rutledge 1996, Moreira et al. 2014, Rasmann et al. 2014c). In addition, plants are embedded in multi-trophic communities that may vary along ecological clines, such that changes in the ratio of herbivore to predator abundance might incur shifts in the relative allocation to traits associated to direct vs. indirect defence (Rasmann et al. 2011, Pellissier et al. 2016). For instance, predator diversity and abundance are thought to generally be greater at lower elevations (Hodkinson 2005, Rasmann et al. 2014a), such that low-elevation plants would be expected to rely and invest relatively more in traits associated with predator attraction (i.e. indirect defences) than their high-elevation counterparts (see Godschalx et al. 2019 and Kergunteuil et al. 2019a). Finally, trade-offs between tolerance and resistance have also been reported in several systems (Fineblum and Rausher 1995, Agrawal et al. 1999) and may lead to co-variation in these defensive strategies along elevational gradients.

Recent work has demonstrated that trade-offs between defensive strategies is not the only scenario of co-variation of plant defensive mechanisms. Studies have shown that, in order to cope with a wide range of herbivore species, plants simultaneously deploy an arsenal of defensive traits including plant chemical and physical defences, indirect defences and tolerance, and that this may result in positive correlations between defensive strategies (Agrawal and Fishbein 2006). For instance, Pellissier et al. (2016) found that direct and indirect defences were positively correlated and that the simultaneously expression of these two strategies was stronger at lower elevations. The authors argue that higher herbivore abundance and species richness at lower elevations has selected for multiple defences at these sites such that synergistic or complementary interactions between defensive strategies provides the most effective means of resistance (Pellissier et al. 2016).

Overall, plant defence is inherently multivariate. A holistic approach of plant multivariate defensive phenotypes is needed to understanding how extrinsic pressures and intrinsic constraints simultaneously shape defensive traits.

## Abiotic factors concurrently influence plants, herbivores and their interactions along elevational clines

Recent studies have demonstrated that abiotic correlates of elevation may concurrently influence levels of herbivory and plant defences independent of one another (e.g., Pellissier et al. 2014, Abdala-Roberts et al. 2016, Pellissier et al. 2016). For example, Abdala-Roberts et al. (2016) recently found that climatic conditions (temperature) influenced elevational variation in insect herbivory independently of chemical defences in the dominant oak *Q. robur*. In addition, using a temperature-controlled experiment Pellissier et al. (2014) demonstrated that herbivore pressure and temperature concurrently accounted for most of the elevational variation in defence investment in the herbaceous plant *Plantago lanceolata*. Similarly, soil characteristics may also be important drivers of plant defences but their influence remains largely ignored in elevational studies. The Resource Concentration Hypothesis predicts that plant species growing in nutrient-poor soils invest more in chemical defences relative to species growing in nutrient-rich soils (Coley et al. 1985, Endara and Coley 2011). Therefore, variation in soil conditions or abiotic stress along elevational clines would be expected to influence defences and such effects may be concurrent to effects of herbivore pressure and lead to unexpected outcomes, e.g., when nutrient availability and herbivory exhibit opposing elevational gradients (Abdala-Roberts et al. 2016, De Long et al. 2016, Pellissier et al. 2016). In a recent study, Pellissier et al. (2016) found that although soil fertility influenced plant chemical defences in Cardamine species (plants adapted to nutrient-poor soils were better defended), soil conditions only partially explained elevational gradients in defences. In another study, De Long et al. (2016) reported that soil nitrogen availability drove elevational gradients in plant community-level chemical defences in a subartic tundra heath.  These studies, however, are exceptions when in reality most work has failed to account for abiotic correlates, therefore hampering our understanding of elevational co-variation in plant defences and herbivory.

## OAKS AS A MODEL SYSTEM

*Quercus* (Fagaceae) genus (commonly called oaks) includes close to 600 species of shrubs and trees that have colonized a huge range of variable environments throughout the Northern Hemisphere (Manos and Stanford 2001, Hipp et al. 2020). Oaks are dominant species in many temperate regions, ranging from dense forest to open grasslands and savannas. Paleoecological data indicate that the genus originated in America where highest levels of species richness are currently found at middle latitudes (Mexico), and subsequently spread to the Old World (Nixon

1993, Manos et al. 1999). *Quercus* species are nowadays distributed from the equator (e.g. Colombian and Indonesia) to northern latitudes in Europe and America, and from the sea level up to 4,000 m (e.g. Yunnan province in China) (Camus 1936, de Beaulieu and Lamant 2010).

The genus *Quercus* thus represents one of the most widespread and species-rich tree genera, and this makes them a highly suitable model to test for clinal variation in plant defences and herbivore pressure (Figure 1).

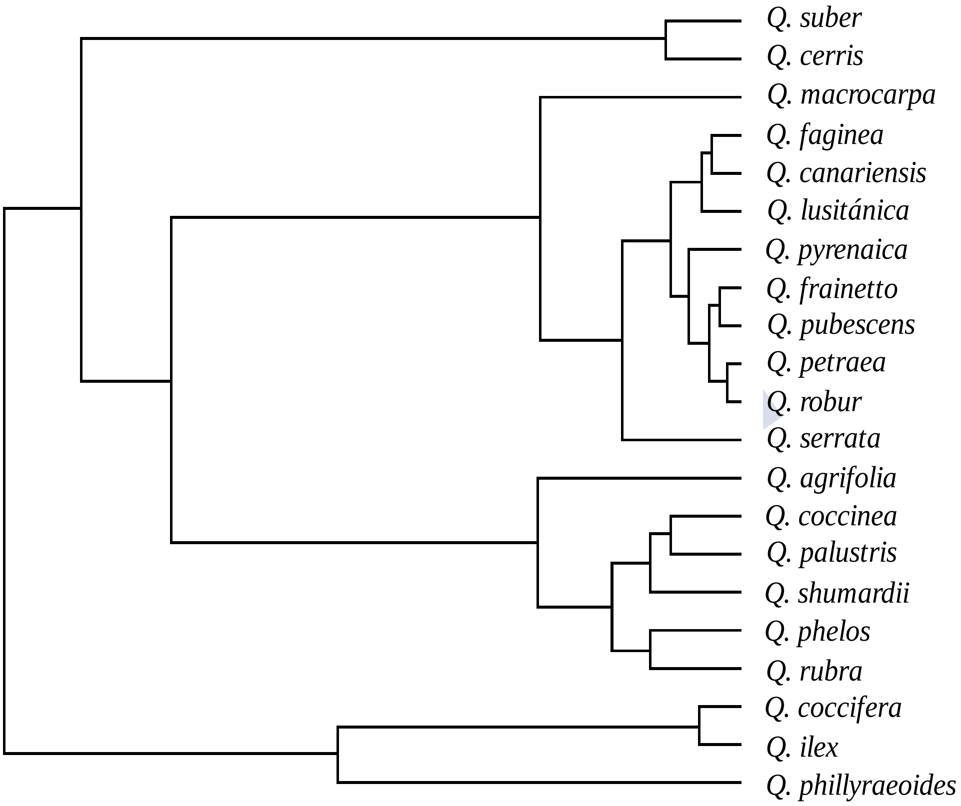

**Figure 1.** Phylogenetic tree of the studied *Quercus* species based on Single Nucleotide Polymorphism matrices by ddRAD sequencing.

Through their distribution range, *Quercus* species are attacked by a wide range of vertebrate and invertebrate herbivores that compromises plant fitness (survival, growth, and reproduction). *Quercus* species support a large community of generalist and specialists insect herbivores, mainly leaf chewers, skeletonizers and miners (Roslin and Salminen 2008, Pearse and Hipp 2009, Tack et al. 2010, Moreira et al. 2018b). Some of the main species of insect herbivores that attack oaks in Europe are the gypsy moth *Lymantria dispar* (Lepidoptera), the oak processionary *Thaumetopoea processionea* (Lepidoptera), the sycamore *Acronicta aceris* (Lepidoptera), the green oak tortrix *Tortrix viridana* (Lepidoptera) and the oak flea *Altica quercetorum* (Coleoptera) (Figure 2).

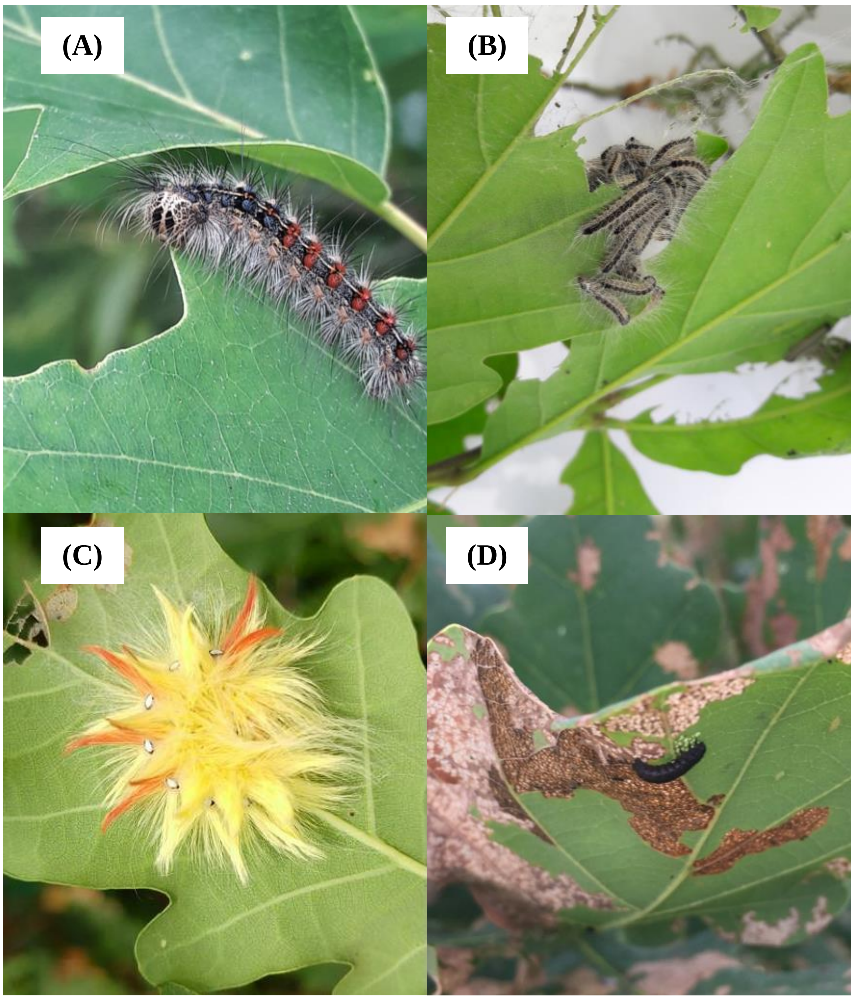

**Figure 2**. (A) The gypsy moth *Lymantria dispar* (Photo credit: Thomas Damestoy), (B) the oak processionary *Thaumetopoea processionea* (Photo credit: Thomas Damestoy), (C) the sycamore *Acronicta aceris* (Photo credit: Bastien Castagneyrol), (D) the oak flea *Altica quercetorum* (Photo credit: Javier García).

In response to herbivory, oaks make use of a broad array of anti-herbivore defences, most of which have been studied for leaves, including physical traits such as toughness, fiber content, and trichomes (Pearse and Hipp 2009, Moreira and Pearse 2017, Abdala-Roberts et al. 2018), chemical traits such as phenolic compounds (e.g. Lill and Marquis 2001, Forkner et al. 2004, Pearse and Hipp 2012, Moreira et al. 2018b), nutritional traits (e.g. nitrogen, phosphorus;

(Abdala-Roberts et al. 2018) and phenological traits including leaf longevity and timing of senescence (Pearse and Karban 2013).

Phenolic compounds have been primarily studied as the mediators of direct defence in oaks (Feeny 1970, Roslin and Salminen 2008, Pearse and Hipp 2012). These chemical compounds have repellent and toxic effects on insect herbivory (Damestoy et al. 2019). Phenolic compounds can be broadly classified in four groups: condensed tannins, hydrolysable tannins, flavonoids and lignins. In addition, oak species also emit a complex blend of volatile organic compounds to attract natural enemies of herbivores (i.e. indirect defences;Ghirardo et al. 2012, Pearse et al. 2013). Volatile organic compounds are mainly terpenoids (monoterpenes and sesquiterpenes), but can also include some aromatic phenols, alcohols and aldehydes (Dudareva et al. 2004). Both direct and indirect defences are constitutively expressed in oaks and have been shown to be inducible in response to herbivore damage.

## OBJECTIVES

The main goal of my PhD dissertation was to identify the underlying factors and processes behind elevational patterns in insect herbivory and plant defences as an attempt to shed light into the evolutionary mechanisms driving the evolution of plant defences against herbivores. To address this goal, I performed both literature surveys and several mechanism-based experiments under natural field and greenhouse conditions.

In the first chapter, I analysed levels of insect leaf herbivory recorded for 1027 plant species spanning an elevation gradient of 2,755 m and found in both tropical and temperate regions. These species had different growth forms (non-woody vs. woody species), as well as contrasting leaf habit (deciduous vs. evergreen) in the case of woody species. The specific goals of this chapter were to address whether there is an elevational gradient in leaf herbivory worldwide, whether elevational cline in leaf herbivory is dependent upon climatic region, growth form or leaf habit, and whether such elevational gradients in herbivory are associated with climatic factors.

In the second chapter, I conducted a field test of elevational gradients in insect leaf herbivory and leaf defensive and nutritional traits for saplings and mature trees of eight oak species. The specific goals of this chapter were to address whether elevational gradients in insect leaf herbivory and plant defensive and nutritional traits are contingent on plant ontogenetic stage, and whether elevational and ontogenetic variation in herbivory is associated with variation in leaf traits.

In the third chapter, I performed a greenhouse experiment using one-year-old plants belonging to 18 oak species that naturally grow along an elevational gradient of more than 2400 m, and quantified the concentration of constitutive chemical defences in leaves as well as their induced levels after damage inflicted by larvae of the generalist caterpillars of the gypsy moth (*Lymantria dispar*). The specific goals of this chapter were to address whether there are elevational gradients in constitutive and induced chemical defences, whether there are elevational gradients in the correlated expression of chemical defensive traits, either in the form of negative (trade-offs) or positive associations encompassing multiple defensive strategies, and whether climatic correlates of elevation explain elevational clines in individual defensive strategies and their correlated expression.

Finally, in the fourth chapter I sampled 18 populations of the oak species *Quercus pyrenaica* distributed along a 1300 m-elevational gradient and quantified constitutive direct (phenolic compounds) and indirect (volatiles) defences in leaves and their inducibility after in situ experimental manipulation of feeding by the specialist oak flea beetle (*Altica quercetorum*). The specific goals of this chapter were to address whether there are elevational gradients in constitutive and induced direct and indirect defences, whether there are emergent patterns of correlated expression between direct and indirect defences, whether these co-expression patterns exhibit elevational clines, and whether climatic correlates of elevation are associated with elevational clines in individual defensive traits and their correlated expression patterns.

The results of the work presented in these four chapters have already been published in peer-reviewed scientific journals, or are currently being considered for publication. The resulting research papers are listed below:

**Galmán, A.**, Abdala-Roberts, L., Wartalska, P., Covelo, F., Röder, G., Szenteczki, M., Moreira, X., Rasmann, S. (2020). Elevational gradients in constitutive and induced oak defences based on individual traits and their correlated expression patterns. Oikos (accepted pending minor revisions).

**Galmán, A**., Petry, W. K., Abdala-Roberts, L., Butrón, A., de la Fuente, M., Francisco, M., Kergunteuil, A., Rasmann, S., Moreira, X., 2019. Inducibility of chemical defences in young oak trees is stronger in species with high elevational ranges. Tree Physiology 39, 606-614.

**Galmán, A**., Abdala-Roberts, L., Covelo, F., Rasmann, S., Moreira, X., 2019. Parallel increases in insect herbivory and defenses with increasing elevation for both saplings and adult trees of oak (*Quercus*) species. American Journal of Botany 106, 1558-1565.

**Galmán, A**., Abdala-Roberts, L., Zhang, S., Berny-Mier y Terán, J. C., Rasmann, S., Moreira, X., 2018. A global analysis of elevational gradients in leaf herbivory and its underlying drivers: effects of plant growth form, leaf habit, and climatic correlates. Journal of Ecology 106, 413-421.

In addition, during my PhD I also participated as a coauthor in the following publications:

Valdés-Correcher E, Bourdin A, González-Martínez SC, Moreira X, **Galmán A**, Brachi B, Hampe A, Castagneyrol B (2020) Leaf chemical defences and insect herbivory in pedunculate oak (*Quercus robur*): accounting for canopy position unravels marked genetic relatedness effects. Annals of Botany, in press.

Moreira X, Abdala-Roberts L, **Galmán A**, Bartlow AW, Berny-Mier y Teran JC, Carrari E, Covelo F, de la Fuente M, Ferrenberg S, Fyllas NM, Hoshika Y, Lee SR, Marquis RJ, Nakamura M, Nell CS, Pesendorfer MB, Steele MA, Vázquez-González C, Zhang S, Rasmann S (2020) Ontogenetic consistency in oak defence syndromes. Journal of Ecology, in press.

Moreira X, Abdala-Roberts L, Bruun HH, Covelo F, De Frenne P, **Galmán A**, Gaytán A, Jaatinen R, Pulkkinen P, ten Hoppen JPJG, Timmermans BGH, Tack AJM,

Castagneyrol B (2020) Latitudinal variation in seed predation correlates with latitudinal variation in seed defensive and nutritional traits in a widespread oak species. Annals of Botany 125:881-890.

Castagneyrol, B., Valdés-Correcher, E., Bourdin, A., Barbaro, L., Bouriaud, O., Branco, M., Centenaro, G., Csóka, G., Duduman, M.-L., Dulaurent, A.-M., Eötvös, C. B., Faticov, M., Ferrante, M., Fürjes-Mikó, Á., **Galmán, A**., Gossner, M. M., Harvey, D., Howe, A. G., Kaennel-Dobbertin, M., Koricheva, J., Löveï, G. L., Lupaştean, D., Milanović, S., Mrazova, A., Opgennoorth, L., Pitkänen, J.-M., Popović, M., Roslin, T. V., Scherer-Lorenzen, M., Sam, K., Tahadlová, M., Thomas, R., Tack, A. J. M., 2020. Can school children support ecological research? Lessons from the oak bodyguard citizen science project. Citizen Science: Theory and Practice 5:< 10.

Moreira X, Castagneyrol B, de la Mata R, Fyllas NM, **Galmán A**, García-Verdugo C, Larrinaga AR, Abdala-Roberts L (2019) Effects of insularity on insect leaf herbivory and chemical defences in a Mediterranean oak species. Journal of Biogeography 46:1226–1233.

Moreira X, **Galmán A**, Francisco M, Castagneyrol B, Abdala-Roberts L (2018) Host plant frequency and secondary metabolites are concurrently associated with insect herbivory in a dominant riparian tree. Biology Letters 14:20180281

Abdala-Roberts L, **Galmán A**, Petry WK, Covelo F, de la Fuente M, Glauser G, Moreira X (2018) Interspecific variation in leaf functional and defensive traits in oak species and its underlying climatic drivers. PLoS ONE 13:e0202548.

Moreira X, Abdala-Roberts L, **Galmán A**, Francisco M, de la Fuente M, Butrón A, Rasmann S (2018) Assessing the influence of biogeographical region and phylogenetic history on chemical defences and herbivory in *Quercus* species. Phytochemistry 153:64-73.

# CHAPTER I

# A global analysis of elevational gradients in leaf herbivory and its underlying drivers: effects of plant growth form, leaf habit, and climatic correlates

That give rise to the publication Galmán, A., Abdala-Roberts, L., Zhang, S., Berny-Mier y Terán, J. C., Rasmann, S., Moreira, X., 2018. A global analysis of elevational gradients in leaf herbivory and its underlying drivers: effects of plant growth form, leaf habit, and climatic correlates. Journal of Ecology 106, 413-421.


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# CHAPTER I:

# A global analysis of elevational gradients in leaf herbivory and its underlying drivers: effects of plant growth form, leaf habit, and climatic correlates

## ABSTRACT

Research on elevational gradients in species interactions holds that herbivore pressure increases towards warmer and more stable climates found at lower elevations. However, the generality of this expectation has been questioned by recent studies reporting no evidence of expected trends or even positive associations between elevation and herbivory, presumably due to uncontrolled biotic and abiotic factors influencing such relationships. Using a worldwide data set of insect leaf herbivory including 1027 plant species and spanning an elevation gradient of 2,755 m, we tested whether elevational gradients in herbivory were contingent on whether species were found at tropical vs. temperate latitudes, plant growth form (non-woody vs. woody species), and leaf habit (deciduous vs. evergreen woody species). In addition, we tested the influence of climatic correlates of elevation presumably underlying such elevational gradients. Although there was no evidence of an overall relationship between elevation and damage, we found that elevational gradients in herbivory were contingent on species growth form whereby herbivory increased towards lower elevations for woody species, but no gradient was observed for non-woody species. We further found that elevational gradients in herbivory were contingent on leaf habit as herbivory increased towards lower elevations for deciduous species, but no gradient was present for evergreen species. Elevational variation in damage was not contingent upon latitudinal region. Finally, analyses of climatic factors indicated that although a significant association between temperature and herbivory for woody species was detected, the elevational gradient in leaf herbivory remained largely unchanged after accounting for temperature and precipitation, suggesting that climate does not fully account for the gradient. This study delivers the first global assessment of elevational gradients in leaf herbivory and emphasizes the role of plant (e.g., life history or defensive) traits and climatic factors in shaping elevational gradients in herbivory.

**Key-words**: deciduous, elevation, evergreen, herbivory, non-woody species, precipitation, temperate regions, temperature, tropical regions, woody species.

## INTRODUCTION

Biotic (e.g., herbivory, predation) and abiotic (e.g., climate, soil conditions) factors often co-vary in predictable ways along environmental gradients, and this variation in turn shapes concomitant clines in species abundance, traits, and diversity (Linhart and Grant 1996, Schemske et al. 2009, Woods et al. 2012). Environmental gradients occurring along latitude or elevation have been especially useful for understanding spatial variation in the global imbalance of plant and herbivore species richness and plant-herbivore interactions. Theory predicts that higher rates of herbivory under warmer and more stable climates, found at low latitudes or low elevations, have led to selection for more potent levels of plant defences (Johnson and Rasmann 2011, Moles et al. 2011, Rasmann et al. 2014a) and ultimately higher rates of plant (and herbivore) diversification (Schemske et al. 2009, Coley and Kursar 2014).

Although elevational gradients in species interactions have historically received less attention by ecologists relative to latitudinal gradients, recent work has shown that elevation is a good proxy of clinal variation in species interactions and community attributes (Rodríguez-Castañeda et al. 2010, Pellissier et al. 2012, Pellissier et al. 2014, Rasmann et al. 2014a, Pellissier et al. 2016). Further, the study of elevational gradients provides several advantages over latitudinal gradients. For instance, elevational gradients occur over much smaller spatial scales and are thus less prone to confounding effects in broad-scale latitudinal studies such as dispersal limitation and species turnover (Körner 2007, Rasmann et al. 2014a). In addition, climatic variation found along elevational gradients occurs separately from variation in day length, and regional inter-annual variation in climate affects the whole gradient simultaneously.

Elevational gradients therefore provide an ideal opportunity for understanding how clinal variation in abiotic factors drives variation in species interactions.

Studies conducted thus far have reported inconsistent associations between elevation and herbivory, ranging from the expected negative relationship (higher herbivory at lower elevations), to no association or even positive relationships (reviewed by Rasmann et al. 2014a). The variability in sign and magnitude of such associations can be explained by several factors (see Anstett et al. 2016a for latitudinal gradients). One of these is the often cited, but seldom evaluated, influence of abiotic forcing, particularly climate, which may influence elevational gradients in herbivory in at least two ways. First, broad-scale differences in climatic conditions between tropical and temperate latitudes may condition the outcome of elevational gradients in herbivory because, for a similar elevational range, the magnitude of change in climatic conditions when moving from low- to high-elevation is greater in tropical than in temperate regions, leading to more pronounced concomitant clines in herbivore pressure in the tropics (Janzen 1967). Therefore, the lower end of elevational gradients is under greater pressure in the tropics than in temperate regions such that not only mean herbivory is greater in the tropics, but the gradient in herbivore pressure (and selection on defences) may be steeper when moving from high to low elevations. However, thus far studies on elevational gradients in herbivory have been restricted to either tropical or temperate regions (Schemske et al. 2009, Rasmann et al. 2014a), which could explain some of the inconsistency in the magnitude of these relationships. Second, and independently of latitudinal variation in climate, elevation per se has traditionally been used as a proxy for multiple co-varying abiotic factors whose independent effects have not been explicitly measured or teased apart (Körner 2007). Recent studies have demonstrated that addressing the simultaneous effects of these abiotic correlates (e.g., temperature and precipitation) is necessary to understand how local climate conditions set the template for how plant-herbivore interactions unfold along elevational gradients (Moreira et al. 2014, Pellissier et al. 2014, Abdala-Roberts et al. 2016, De Long et al. 2016, Pellissier et al. 2016).

Another important aspect which could explain inconsistencies in elevational gradients in herbivory, is that most studies have not considered whether such gradients are contingent upon plant life-history strategies. For instance, differences in growth form (e.g., woody vs. herbaceous) might influence the strength of elevational patterns considering that woody species are larger and longer-lived and this presumably makes them easier to detect by herbivores as well as more predictable resources relative to smaller and ephemeral non-woody species (Feeny 1976, Strauss et al. 2015). In accordance with these expectations, recent work has shown that woody plants suffer higher amounts of herbivory globally compared to non-woody species (Turcotte et al. 2014a). Under an elevational context, woody plants would not only suffer from greater mean herbivory across the entire gradient, but would presumably also be more attacked on the lower end of the gradient, therefore leading to a steeper increase in herbivory with decreasing elevation relative to herbaceous species. Likewise, differences in life-history strategies associated with resource use and acquisition might also influence plant defence investment and herbivory along elevational gradients (Lim et al. 2015, Moreira and Pearse 2017). In the case of woody species, leaf habit is an important predictor of differences in resource use; evergreen species typically exhibit a more conservative resource-use strategy and this translates into slower growth rates (Givnish 2002, Wright et al. 2004), whereas deciduous species exhibit an exploitative resource-use strategy and higher growth rates (Reich et al. 1998, Poorter and Garnier 2007). Assuming that growth and defences trade off, as they often do across many plant taxonomic groups (Coley et al. 1985, Endara and Coley 2011), evergreen species should grow less and be more highly defended whereas faster-growing deciduous species should be less defended. Under an elevational context, deciduous species would not only exhibit greater mean herbivory across the entire gradient but would also be more attacked on the lower end of the gradient than evergreen species leading to a steeper increase in herbivory with decreasing elevation.

To test the above predictions on latitudinal variation in the steepness of elevational gradients in herbivory and the contingency of such gradients on plant life-history traits, we analysed published data on leaf herbivory recorded for 1027 plant species spanning an elevation gradient of 2,755 m (from 40ºS to 64ºN in latitude). Specifically, we asked the following: (i) is there an overall elevational gradient in leaf herbivory? (ii) Is any such elevational gradient dependent upon latitudinal region (tropical vs. temperate), growth form or leaf habit? And (iii) are elevational gradients in leaf herbivory associated with climatic factors, namely temperature and precipitation? By addressing these questions, this study delivers a novel global assessment of elevational clines in plant-herbivore interactions and sheds light into biotic and abiotic controls over such associations.

## MATERIAL AND METHODS

### Literature search, selection criteria and data acquisition

Herbivory data were obtained from two published data sets (Turcotte et al. 2014b, Kozlov et al. 2015), as well as by searching the Web of Science and Google Scholar using the keywords "herbivory", "leaf area loss", "leaf damage", "defoliation", "folivory" and "plant-herbivory interaction" (Zhang et al. 2016). The access dates for this literature search spanned 1952 to 30 November 2014. Following (Zhang et al. 2016), the criteria used for data acquisition were as follows: (i) In order to be coherent with the two original data sets (Turcotte et al. 2014b, Kozlov et al. 2015), studies used were restricted to those where herbivory was measured as the percentage of leaf area consumed; (ii) only data representing most of the damage experienced during leaf lifetime was used, i.e., herbivory in mature leaves, herbivory at the end of the growing season and data summed damage of individual leaves over one or more months; (iii) in the case of experimental studies, only data from control treatments was considered; (iv) if herbivory was measured in a time series, the mean value of the of the time points was used; and (v) only data from studies performed under field conditions was considered. We previously analysed latitudinal gradients in leaf herbivory based upon this data set and these results are reported in Zhang et al. 2016.

Means, standard errors/deviations and sample sizes were extracted from texts, tables or figures (Zhang et al. 2016). For data expressed in figures, the UTHSCSA Image Tool (University of Texas, USA) was used to obtain exact values (Zhang et al. 2016). Elevation values (m above sea level) were obtained based on information on the latitude and longitude coordinates of each site (Zhang et al. 2016). We excluded studies with unclear or imprecise information on the coordinates of the sampling site. The data set also included information on the mean annual temperature (°C) and mean annual precipitation (mm). These climatic variables were taken from original literature or from web searches of the study sites. In cases where this information was not available, climatic data were extracted from the CRU CL 2.0 data set which is a set of global climate grids with 10 min spatial resolution (New et al. 1999). In addition, we also categorized the plant species as temperate or tropical based on geographic coordinates of sampling points, as well as by growth form (non-woody or woody species) and by leaf habit (evergreen vs. deciduous) in the case of woody species. In the first case, we adopted the commonly accepted latitudinal boundaries of climate zones, defining tropical regions as those below 23.5º (north and south, i.e., between the tropics of Cancer and Capricorn) and temperate regions as those from 23.5º to 66.5º (north and south, i.e., between the tropics and the polar circles). We excluded a few data points corresponding to polar regions (above 66.5ºN) that were included in Zhang et al. (2016). Overall, the data set included 1847 leaf herbivory data points for a total of 1027 plant species distributed from 0 to 2,755 m of elevation above sea level. These plant species were divided in 484 species growing in temperate regions and 543 species growing in tropical regions. Likewise, 353 species were non-woody and 674 were woody, of which 294 were deciduous and 380 were evergreen.

### Data analyses

For all statistical analyses, we logit-transformed leaf herbivory data and in those cases where a species was sampled at multiple space or time points we used species-level means for herbivory as well as the mean value of elevation, temperature and precipitation across the sampling points (Lim et al. 2015). We also conducted analyses at the population-level (using the species as a random factor) and found similar results compared with analyses at species-level (Table S1 in Appendix A). We note, however, that a high number of plant species in the data set had a single data point and in those cases where there were multiple data points per species these were strongly skewed towards a handful of species with high population-level replication. Because this may introduce potential biases in the results (i.e., some species contributing much more to the overall pattern), we opted for the species-level over the population-level analyses.

We analysed elevational gradients in leaf herbivory in the following way. First, to test for an overall elevational gradient in leaf herbivory we performed an ordinary least-squares linear regression using the full data set where elevation predicted leaf herbivory. Second, to analyse differences in elevational gradients depending on latitudinal region and plant life-history traits we performed an ANCOVA model using the full data set where leaf herbivory was predicted by elevation, latitudinal region (tropical vs. temperate), plant growth form (non-woody vs. woody), and all two- and three-way interactions among these factors. The two-way interactions between elevation and latitudinal region and elevation and growth form tested for differences in the magnitude or direction of elevational patterns in herbivory between tropical vs. temperate regions and between non-woody vs. woody species, respectively. In those cases where the two-way interactions were significant, we ran univariate regression with elevation as predictor separately for each level of the factor (growth form or latitudinal region) that interacted with elevation. We did not include latitude per se as a continuous predictor in this ANCOVA model because a previous study using a subset of our data set (Zhang et al. 2016) treated latitude in such a way and we preferred not to repeat the same analysis. In addition, our hypothesis was based on differences across broad latitudinal zones, and treating this predictor as a categorical predictor provided an explicit test of this latitudinal effect. Similarly, although previous results by Zhang et al. (2016) indicated contrasting latitudinal patterns in herbivory between hemispheres, we did not include this factor in the statistical model because we had no a priori expectation or candidate mechanisms to explain the effects of hemisphere on elevational variation in leaf herbivory. Third, the previous ANCOVA indicated a significant elevational gradient only for woody species (significant growth form by elevation interaction) and to investigate the mechanistic underpinning this finding we ran a model using only woody species where herbivory was predicted by elevation, leaf habit and their interaction. The interaction term tested for differences in elevational gradients between evergreen vs. deciduous woody species. Likewise, if the interaction was significant we ran univariate regressions separately for deciduous and evergreen species. To further describe the results from the ANCOVAs, in those cases where a factor interacted with elevation we ran univariate regressions between elevation and herbivory separately for each level of that factor.

If the overall elevational gradient was significant in the univariate regression model using the full data set, we assessed whether such gradient was underlined by climatic factors by running a multiple regression that included elevation, mean annual temperature, and annual precipitation. Similarly, in those cases where elevation interacted significantly with a given factor (from ANCOVA models described above) we ran univariate regressions separately for each level of the factor we included (in a multiple regression) temperature and precipitation. If the effect of elevation in the univariate regression turned non-significant after accounting for climatic variables, we interpreted this as evidence that climate underlies the elevational gradient in herbivory. Because collinearity between predictors may influence this analysis, we previously explored the associations between elevation and climatic variables. Results from univariate regressions indicated that associations for elevation vs. temperature and elevation vs. precipitation were weak ($R^2$ = 0.05 and $R^2$ = 0.02, respectively). Although this suggested a moderate influence of collinearity, we nonetheless decided to explore a more conservative approach where the residuals from a model where temperature and precipitation predicted herbivory were regressed onto elevation. Findings from this analysis did not change the interpretation of the results relative to the multiple regression with climatic predictors. Based on these findings, and considering that the multiple regression has the advantage over the model using residuals of providing an explicit evaluation of the individual effects of climatic variables underlying the elevational gradient, we opted for the multiple regression approach.

In all of the above statistical models we conducted phylogenetic least squares regressions (PGLS) to account for the influence of evolutionary relationships among species (Martins and Hansen 1997). The species-level phylogeny was obtained from a super tree using Phylomatic (v.3.0, stored tree “20120829”;(Webb and Donoghue 2005). Divergence times were calculated in the trees with the BLADJ algorithm in Phylocom (Webb et al. 2008). We ran all the statistical analyses in R (version 3.1.1; (R Core Team 2014) and the PGLS analyses were conducted using the phylolm package (Ho and Ane 2014) and the phylogenetic models for the error term were selected based on the AIC values.

It is important to note that the dataset analysed by Turcotte et al. (2014b) included information about the method used to quantify leaf herbivory (single time point measurements vs. multiple measurements on single leaves to measure rates or cumulative damage). However, we lacked this information for species from the other data sets (e.g., Kozlov et al. 2015) so we were not able to account for this factor our statistical analyses.

## RESULTS

### Elevational gradients in leaf herbivory

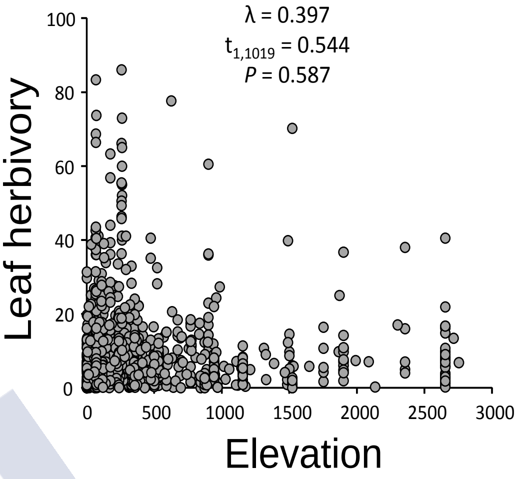

**Figure 1**. Elevation as a predictor of leaf herbivory (percentage of leaf area consumed) in 674 woody and 353 non-woody species. Shown are Student's t-test values (t) with associated degrees of freedom and P-values based upon logit- transformed data after controlling for phylogeny using a phylogenetic least squares regression (PGLS) are shown. Phylogenetic correlation parameter (λ) was estimated using maximum likelihood. Circles represent plant species means.

Using the full data set (all species included), we found no significant association between leaf herbivory and elevation (Fig. 1; Table 1). Results from the ANCOVA using the full data set including categorical factors (latitudinal region, growth form) and their interactions with elevation indicated a significant effect of growth form (percentage of leaf area consumed: 10.74 ± 0.46 for woody species vs. 8.20 ± 0.67 for non-woody species), and a marginally significant effect of latitudinal region (percentage of leaf area consumed: 9.61 ± 0.0.62 for tropical regions vs. 9.33 ± 0.52 for temperate regions) (Table 1). More importantly, there was a significant elevation × growth form interaction indicating that elevational gradients in herbivory were contingent on whether species were non-woody or woody (Table 1). Subsidiary univariate regressions indicated an elevational gradient in leaf herbivory for woody species, in which herbivory increased towards lower elevations (Fig. 2b), but not for non-woody species (Fig. 2a). In contrast, the elevation × latitudinal region interaction was not significant, indicating that the effects of elevation on herbivory were not contingent upon latitude (Table 1). In fact, we found no evidence of elevational gradients in herbivory for either temperate (Fig. 2c) or tropical (Fig. 2d) species. Results from this model indicated that the three-way elevation × latitudinal region × growth form interaction on leaf herbivory was not significant (Table 1).

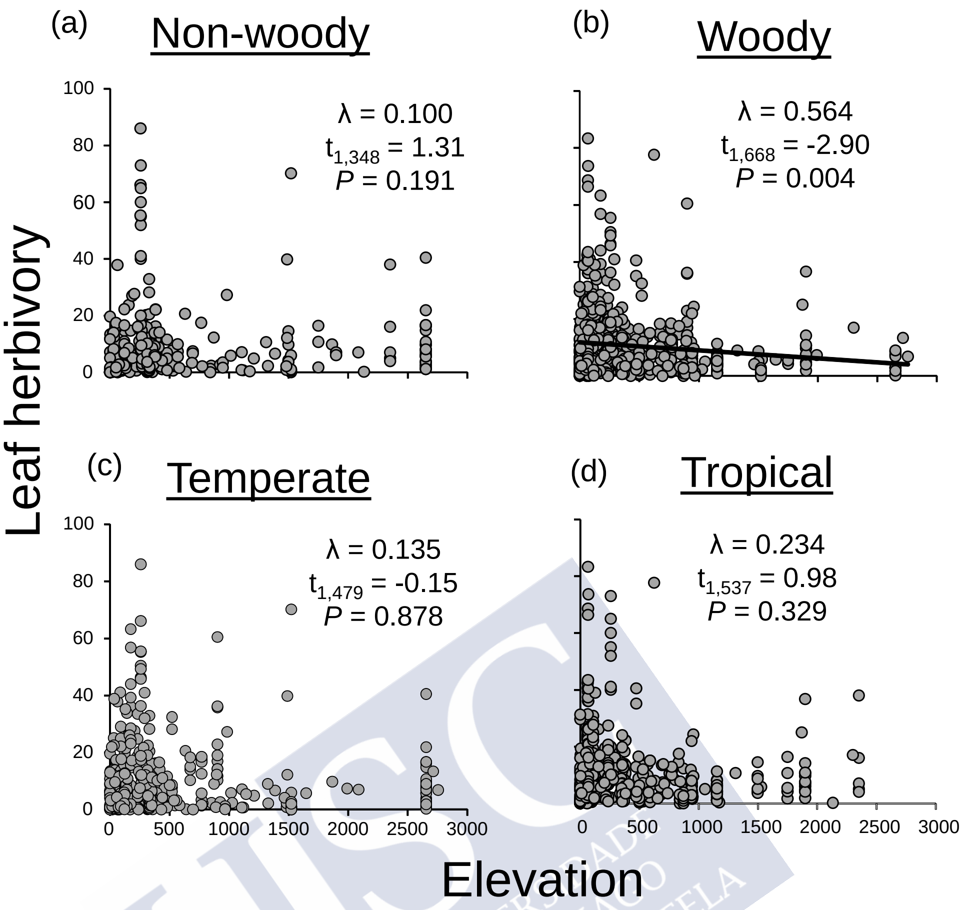

**Figure 2**. Elevation as a predictor of leaf herbivory (measured as the percentage of leaf area consumed) in (a) non-woody species (n = 353 species), (b) woody species (n = 674 species), (c) temperate regions (236 species), and (d) tropical regions (n = 438 species). Shown are Student's t-tests (t) with associated degrees of freedom and P-values based upon logit-transformed data after controlling for phylogeny using a phylogenetic least squares regression (PGLS) are shown. Phylogenetic correlation parameters (λ) were estimated using maximum likelihood. Circles represent plant species means.

**Table 1**. Effects of elevation, latitudinal region (tropical vs. temperate), growth form (non-woody vs. woody) and their interactions on leaf herbivory (measured as the percentage of leaf area consumed) in 353 non-woody and 674 woody species. Degrees of freedom, student's t-tests (t) and *P*-values for an analysis based upon logit-transformed data after controlling for phylogeny using a phylogenetic least squares regression (PGLS) are shown. Significant ($P < 0.05$) *P*-values are typed in bold.

| Variable | $DF_{num,den}$ | t-value | *P*-value |
|---|---|---|---|
| Elevation | 1, 1013 | 1.31 | 0.191 |
| Latitudinal region | 1, 1013 | -1.88 | 0.060 |
| Growth form | 1, 1013 | 3.86 | **<0.001** |
| Elevation × Latitudinal region | 1, 1013 | 1.58 | 0.114 |
| Elevation × Growth form | 1, 1013 | -1.97 | **0.049** |
| Elevation × Latitudinal region × Growth form | 1, 1013 | -0.65 | 0.513 |

Finally, the ANCOVA for woody species indicated a non-significant effect of leaf habit and a significant elevation × leaf habit interaction on leaf herbivory (Table 2). Univariate regressions for each category of leaf habit showed a significant elevational gradient for deciduous species where herbivory increased towards lower elevations (Fig. 3a), but no significant pattern for evergreen species (Fig. 3b).

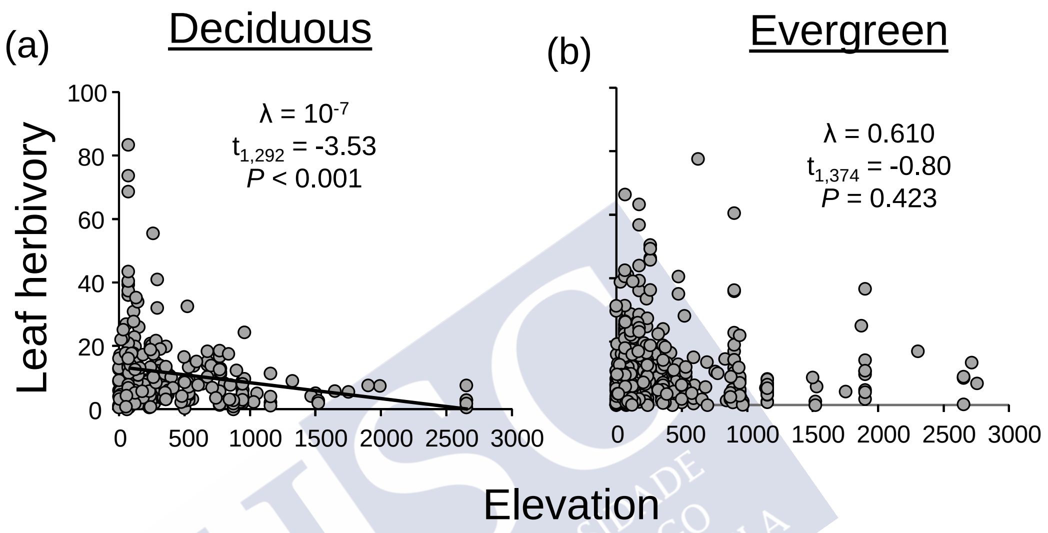

**Figure 3**. Elevation as a predictor of leaf herbivory (measured as the percentage of leaf area consumed) in (a) deciduous (n = 294 species) and (b) evergreen (n = 380 species) woody species. Shown are Student's t-tests (t) with associated degrees of freedom and *P-values* using logit-transformed data after controlling for phylogeny using a phylogenetic least squares regression (PGLS) are shown. Phylogenetic correlation parameters (λ) were estimated using maximum likelihood. Circles represent plant species means.

**Table 2**. Effects of elevation, leaf habit (evergreen vs. deciduous) and their interaction on leaf herbivory (measured as the percentage of leaf area consumed) in 674 woody species. Degrees of freedom, student's t-tests (t) and P-values for an analysis based upon logit-transformed data after controlling for phylogeny using a phylogenetic least squares regression (PGLS) are shown. Significant (*P* < 0.05) *P*-values are typed in bold.

| Variable | $DF_{num,den}$ | t-value | *P*-value |
|---|---|---|---|
| Elevation | 1, 666 | -3.40 | **<0.001** |
| Leaf habit | 1, 666 | -1.23 | 0.221 |
| Elevation × Leaf habit | 1, 666 | 1.97 | **0.049** |

### Climatic correlates of elevational gradients in leaf herbivory

After accounting for both climatic variables (i.e., temperature, precipitation), the multiple regression model for woody species only indicated that mean annual temperature (but not precipitation) was significantly and positively associated with leaf herbivory (i.e., species growing in warmer sites exhibited higher rates of leaf herbivore damage Table 3a). However, the effect of elevation on herbivory remained significant after including climatic predictors (Table 3a), suggesting that temperature did not fully account for the elevational gradient for woody species.

In the case of deciduous woody species, for which a significant elevational gradient was also observed, the multiple regression with climatic variables indicated that mean annual temperature and annual precipitation were not significantly associated with leaf herbivory (Table 3b). In addition, the effect of elevation on herbivory remained significant after including climatic predictors (Table 3b), suggesting that the climatic correlates of elevation did not explain the observed gradient for deciduous species.

**Table 3.** Multiple regression showing the effects of elevation and climate (mean annual temperature and annual precipitation) on leaf herbivory (measured as the percentage of leaf area consumed) in (a) woody species (n = 674 species) and (b) deciduous woody species (n = 674 species) and (b) deciduous woody species (n = 294 species). Degrees of freedom, student's t-tests (*t*) and *P-values* for an analysis based upon logit-transformed data after controlling for phylogeny using a phylogenetic least squares regression (PGLS) are shown. Significant ($P < 0.05$) *P-values* are typed in bold.

| | $DF_{num,den}$ | t-value | *P*-value |
|---|---|---|---|
| a) Woody species | | | |
| Elevation | 1, 664 | -2.21 | 0.027 |
| Temperature | 1, 664 | 2.41 | 0.016 |
| Precipitation | 1, 664 | 0.01 | 0.995 |
| b) Deciduous woody species | | | |
| Elevation | 1, 288 | -3.22 | 0.001 |
| Temperature | 1, 288 | 1.42 | 0.157 |
| Precipitation | 1, 288 | -1.53 | 0.127 |

## DISCUSSION

This study represents the first global assessment of elevational gradients in leaf herbivory across tropical and temperate regions, and further explores the mechanistic underpinnings of such gradients by evaluating the influence of plant life-history traits and climatic factors. Following predictions, we found that elevational gradients in leaf herbivory were contingent on species growth form whereby herbivory on woody species increased towards lower elevations, but no gradient was observed for non-woody species. Further, elevational gradients within woody species were contingent on leaf habit whereby herbivory increased towards lower elevations for deciduous woody species, but no gradient was observed for evergreen woody species. However, contrary to expectations, elevational variation in herbivory was not contingent on latitudinal region (i.e., tropical vs. temperate), and although an association between temperature and herbivory was detected (in the case of woody species), elevational gradient in leaf herbivory remained largely unchanged after accounting for temperature and precipitation, suggesting that climate does not fully account for the gradient.

With respect to latitudinal region, our expectation was that the magnitude of change in climatic conditions from low- to high-elevation sites would be greater in tropical relative to temperate regions, and that this would set the template for steeper elevational gradients in herbivory in the tropics. Supporting this prediction, Fernandes and Price (1988) reported a steeper elevational gradient in the number of galling species feeding on plants in the tropics in comparison with their temperate counterparts. Counter to this study, we found that the elevational gradient in leaf herbivory was not steeper in tropical relative to temperate zones. Despite the null influence of latitudinal region on elevational gradients in herbivory, we consider that examining the interactive effects of latitude and elevation on geographic variation in herbivory represents an important consideration for future research. Controlled experiments using groups of related species where herbivory was measured on “phytometer” plants or common gardens planted at low and high elevations, and with this design being replicated at different latitudes (and at the same elevational range), would advance our understanding of ecological and evolutionary patterns of herbivory.

Following predictions, our results showed that elevational gradients in leaf herbivory were only present for woody species. Our prediction was that woody species represent a resource with more predictable availability and are easier to locate resource by herbivores relative to short-lived, herbaceous species(Feeny 1976, Strauss et al. 2015) and that this would presumably lead to a more pronounced elevational clines herbivory due to a steeper increase in damage towards lower elevations. In one of the few available studies, (Fernandes and Price 1988) found partial support for our prediction as they reported an elevational gradient of a decreasing number of galling insects with elevation for shrubs, whereas no gradient was found for the number of galls on herbaceous species. Unfortunately, this study is to our knowledge one of the few (if not the only) previous study to test for effects of plant growth form on elevational gradients of herbivory, such that it is yet not possible to reach any type of generalization. Future work is needed to test for the influence of plant traits, population and community attributes along elevational gradients by integrating theoretical frameworks in community ecology (e.g., Resource Concentration Hypothesis, Plant Apparency Theory) and plant-centered evolutionary ecology (e.g., Resource Availability Hypothesis) to explain gradients in plant-herbivore interactions. In addition, testing for the individual effects of different plant traits while controlling for others (e.g., choosing pairs of species with contrasting traits or performing phylogenetically controlled experiments) will be key in order to achieve a mechanistic and predictive understanding of the role of bottom-up variation in shaping elevational gradients in herbivory

Our results further indicated that heterogeneity in elevational gradients of herbivory within woody species, as only deciduous species exhibited a significant (negative) cline in leaf herbivory. Leaf habit is a powerful correlate of resource use and acquisition strategies in plants (e.g., Givnish 2002, Wright et al. 2004), where evergreen species are often associated with slow growth and more conservative resource use strategies, which, if growth and defences trade off, is expected to lead to higher investment in defences. In addition, evergreen species have to produce leaf defences against herbivores year round. In contrast, deciduous species tend to be faster-growing and would be expected to invest less in defences if growth and defences trade off, and additionally do not require to invest in leaf defences against herbivory during winter; e.g., (Reich et al. 1998, Poorter and Garnier 2007, Pringle et al. 2011, Pearse and Karban 2013, Bai et al. 2015). Accordingly, we predicted that less defended deciduous species would exhibit a steeper increase in herbivory at low elevation relative to better-defended evergreen species. Supporting our findings, (Rasmann et al. 2014b) observed steeper elevational gradients in leaf damage for seedlings of deciduous tree seedlings in comparison with those of evergreen species (mainly conifers) along an elevation gradient in the French Alps. However, not all evidence is supportive, as a related study by Lim et al. (2015) found that leaf herbivory increased toward the equator for evergreen species, but no association between herbivory and latitude for deciduous species. These authors argue that steeper latitudinal gradients in herbivory are expected for evergreen species because insect damage on these species is low or close to absent during the winter months in temperate zones, whereas herbivory in the tropics remains more consistent throughout the year presumably leading to higher cumulative herbivory year round (Lim et al. 2015). In contrast, deciduous species are only attacked during the growing season and do not suffer herbivory during the winter in both temperate and tropical zones, which would lead to a weaker year round differences in herbivore pressure across tropical and temperate latitudes in these species. Likewise, longer leaf life span in evergreen species increases exposure to damage throughout the year, presumably leading to greater investment in plant defences, and this would thus increase the chance of detecting latitudinal gradients relative to short-lived leaves in deciduous species. Although these arguments could also apply for explaining elevational gradients in herbivory for different plant life forms, our results do not seem to fit with these explanations. Further studies are necessary to test these competing hypotheses by measuring year round herbivory rates (total amount of herbivory throughout the year), as well as relate these herbivory patterns with measurements of plant growth rate, leaf senescence, and defences.

Although climatic factors associated with elevation are considered important drivers of herbivore abundance, diversity and damage (Pellissier et al. 2014, Abdala-Roberts et al. 2016), our results indicated that they did not fully explain elevational gradients in leaf herbivory for woody species or woody deciduous species. Particularly, we found a positive association between temperature and herbivory for woody species (the only group for which herbivory was significantly associated with climatic variables) which is expected given that this variable exerts strong controls over herbivore abundance and feeding rates (Körner 2007). However, including temperature in the statistical model did not eliminate the effect of elevation on herbivory, suggesting that other abiotic factors (e.g., other climatic variables or soil characteristics) should be considered for better understanding abiotic control over elevational gradients in herbivory. For example, Abdala-Roberts et al. (2016) recently found that temperature-related climatic variables strongly influenced elevational gradients of insect herbivory in the dominant oak *Q. robur*. Similarly, in a manipulative experiment Pellissier et al. (2014) demonstrated that temperature partially accounted for elevational variation in herbivore pressure in the herbaceous plant *Plantago lanceolata*. At the same time, however, and in agreement with our results, evidence from other investigations has shown that elevational gradients in abiotic factors are not always associated with geographic patterns of herbivory, as shown here. For example, Pellissier et al. (2016) found that neither temperature nor precipitation influenced herbivory in *Cardamine* species growing along an elevational gradient. Although correlative approaches have contributed to understanding the concurrent effects of abiotic factors on geographic variation in plant traits (Abdala-Roberts et al. 2016),experimental manipulations of candidate climatic drivers are ultimately needed to disentangle the effects of climatic factors on plant-herbivore systems across elevational gradients (Pellissier et al. 2014).

It is important to also note that soil characteristics may influence plant defences and herbivory, but their influence remains largely ignored in elevational studies, including our present work. Within this context, the Resource Availability Hypothesis predicts that plant species growing in nutrient-poor soils invest more in chemical defences relative to species growing in nutrient-rich soils (Coley et al. 1985, Endara and Coley 2011). Shifts in soil resource availability along elevational clines could therefore influence plant defences and herbivory, and in some cases lead to unexpected outcomes depending on whether key nutrients decrease or increase with elevation (Abdala-Roberts et al. 2016, De Long et al. 2016, Pellissier et al. 2016). For example, in a recent study De Long et al. (2016) reported that soil nitrogen availability drove elevational gradients in plant community-level chemical defences in a subartic tundra heath, and that such effects presumably exert an important bottom-up control on herbivory. In addition, high resource availability is associated with faster life histories characterized by higher leaf nutrient concentrations and this may in turn result in high rates of herbivory. Further studies measuring and manipulating soil resources as well as climatic factors are needed to tease a part the direct and indirect effects of abiotic factors on plant defences and herbivory.

Overall, this study builds towards a better understanding of plant traits and climatic factors shaping elevational gradients in herbivory. However, a typical limitation of global datasets and purely correlative studies such as ours is that it is not possible to establish a strong link between pattern and process via a presumed mechanism without performing manipulative experiments. In addition, there are potentially important geographic and taxonomic biases in data bases such as that used here. For example, certain taxonomic groups in some geographic regions may dominate the data sets (e.g., *Inga* spp. in tropical regions and *Quercus* or *Pinus* spp. in temperate regions), such that results are driven by certain taxonomic groups that are overrepresented. As sampling effort increases (both spatially and taxonomically), databases such as this will become less influenced by potential biases and we will gain a more complete understanding of overall elevational gradients in herbivory and its underlying drivers. At the same time, the robustness of individual studies would increase by including replicated designs across multiple sites at a given latitude or elevation, as well as sampling schemes that spanned both tropical and temperate zones. It would be particularly useful that such studies: (i) measure plant physical and chemical defences, (ii) compare patterns by specialist and generalist herbivores and associate these with plant traits, (iii) use pairs of plant species with contrasting traits under a phylogenetically controlled setting, and (v) manipulate climatic and soil variables. Addressing these aspects will bring a deeper understanding of the relative importance and interactive effects of plant traits, abiotic factors, and variation in herbivore communities on elevational gradients in plant defence and herbivory.

# CHAPTER II

# Parallel increases in insect herbivory and defenses with increasing elevation for both saplings and adult trees of oak (*Quercus*) species

That give rise to the publication Galmán, A., Abdala-Roberts, L., Covelo, F., Rasmann, S., Moreira, X., 2019. Parallel increases in insect herbivory and defenses with increasing elevation for both saplings and adult trees of oak (*Quercus*) species. American Journal of Botany 106, 1558-1565.


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# CHAPTER II:

## Parallel increases in insect herbivory and defenses with increasing elevation for both saplings and adult trees of oak (*Quercus*) species

### ABSTRACT

Herbivory is predicted to increase toward warmer and more stable climates found at lower elevations, and this should select for higher plant defenses towards these environents. Still, a number of recent studies have reported either no evidence of such gradients or reverse patterns. One source of inconsistency may be that plant ontogenetic variation is usually not accounted for and may influence levels of plant defenses and herbivory. We tested for elevational gradients in insect leaf herbivory and leaf traits potentially associated with herbivore resistance across eight oak (*Quercus*, Fagaceae) species and compared these patterns for saplings and adult trees. To this end, we surveyed insect leaf herbivory and leaf traits (phenolic compounds, toughness and nutrients) in naturally occurring populations of each oak species at low-, mid- or high-elevation sites throughout the Iberian Peninsula.

Leaf herbivory and chemical defenses (lignins) were higher at mid- and high-elevation sites than at low-elevation sites. In addition, leaf chemical defenses (lignins and condensed tannins) were higher for saplings than adult trees, whereas herbivory did not significantly differ between ontogenetic stages. Overall, elevational variation in herbivory and plant chemical defenses were consistent across ontogenetic stages, suggesting that elevational gradients were not contingent upon ontogeny, and herbivory and leaf traits were not associated across elevations. These findings suggest disassociated patterns of elevational variation in herbivory and leaf traits, which, in turn, are independent of plant ontogenetic stage.

**Key words:** elevational gradients; Iberian Peninsula; nutrients; phenolic compounds; plant–herbivore interactions; plant ontogeny; *Quercus*.

## INTRODUCTION

Understanding the drivers of spatial variation in species interactions represents a major challenge in ecology (Linhart and Grant 1996). A useful approach to this end has been to study interactions along environmental (i.e., along latitude or elevation) gradients (Schemske et al. 2009, Rasmann et al. 2014a). In particular, gradient-based analyses have been important for studying interactions between plants and their herbivores. Theory holds that herbivory (defined as the consumption of all or a part of a plant) increases toward warmer and more stable climates characteristic of lower elevations, and this increase has selected for greater investment in plant defenses at low relative to high elevations (e.g.,Scheidel and Bruelheide 2001, Zehnder et al. 2009, Garibaldi et al. 2011, Pellissier et al. 2012, Pellissier et al. 2016). However, the generality of this prediction has been called into question by a number of studies reporting no evidence of elevational gradients in herbivory and plant defenses or higher plant defense and herbivory with increasing elevation (reviewed by Rasmann et al. 2014a, Moreira et al. 2018a).

Mixed support for the predicted elevational gradients in herbivory and plant defenses can be explained in several ways. Most studies (ca. 65%) have assessed elevational gradients for only one or a few plant defensive traits, mostly chemical defenses (reviewed by Moreira et al. 2018a), without necessarily establishing clear criteria for the selection of target traits or their role in herbivore resistance (Rasmann et al. 2014a). As a result, assessments of plant defensive phenotypes have usually been incomplete or have failed to target ecologically important traits for the studied system. In this sense, other plant traits associated with plant quality to herbivores, such as physical features (e.g., toughness) or macronutrients (Mattson 1980, Agrawal 2007) could also influence herbivores, but so far they have been overlooked (Moreira et al. 2018a). A more comprehensive assessment of multivariate defensive phenotypes that includes secondary metabolites, physical defenses, and traits related to the nutritional value for herbivores will likely improve our understanding of the ecological mechanisms and evolutionary consequences of elevational clines in plant–herbivore interactions.

Another important factor to consider when studying plant–herbivore interactions along ecological gradients is plant developmental stage (Galmán et al. 2018). For long-lived plants, defense levels are expected to build up from the sapling to the adult stage, following an increase in available resources to be diverted to defensive functions (Boege and Marquis 2005, Barton and Koricheva 2010, Barton and Boege 2017). If adult plants are better defended than saplings, we would expect less difference in herbivory between life stages at high elevations because herbivore pressure is already low and being more defended has less impact than at low elevation (Galmán et al. 2018). The resulting elevational change in herbivory should thus be weaker for adults than saplings (Galmán et al. 2018). Alternatively, adults may be more detectable and therefore more attacked than saplings (Castagneyrol et al. 2013, Moreira et al. 2017). Here we would also expect differences in herbivory (and, concomitantly, defenses) between adults and saplings to be weaker at high elevation where detectability has less impact because herbivore pressure is already low. However, the resulting elevational change in herbivory and defenses would be expected to be stronger for adults than saplings, because increased detectability leads to a more pronounced increase in herbivory (and thus defenses) with increasing herbivore pressure at low elevations. Studies that explore how gradients in defense and herbivory depend on plant ontogeny are needed to test these predictions and in so doing increase our understanding of clinal variation in plant–herbivore interactions.

In this study, we conducted a field test of elevational gradients in insect leaf herbivory and leaf defensive and nutritional traits for saplings and adult trees of eight oak (*Quercus*, Fagaceae) species. Specifically, we asked: (1) Are there elevational gradients in insect leaf herbivory and in plant defenses and nutritional traits? (2) Are any such elevational gradients contingent on plant ontogenetic stage? (3) Is elevational and ontogenetic variation in herbivory associated with variation in leaf traits? To this end, we surveyed herbivory and leaf traits in naturally occurring populations of each oak species found at low-, mid- and high-elevation sites in the Iberian Peninsula. For each population, we quantified leaf damage by chewing insects, specific leaf area (a physical trait correlated with toughness), and the concentration of phenolic compounds (putative defenses in oaks), and phosphorus and nitrogen (proxies of nutritional content) in saplings and adult oak trees. Overall, this study builds toward a better understanding of the ecological mechanisms behind elevational gradients in plant–herbivore interactions.

## MATERIALS AND METHODS

### Natural history

To address whether elevation and plant ontogenetic stage affected insect leaf herbivory and leaf traits, we sampled eight oak species throughout the Iberian Peninsula which collectively encompass an elevational gradient of 1330 m, namely: *Quercus robur, Q. petraea, Q. suber, Q. ilex*, *Q. pubescens*, *Q. lusitanica*, *Q. canariensis*, and *Q. coccifera* (Table 1). At the studied sites, leaf burst for these *Quercus* species usually occurs in April and leaves turn brown and drop off in October. The studied oaks are attacked by several insect herbivores, mainly leaf chewers such as *Tortrix viridana* (Lepidoptera: Tortricidae), *Lymantria dispar* (Lepidoptera: Lymantridae), and *Malacosoma neustria* (Lepidoptera: Lasiocampidae) (Abdala-Roberts et al. 2016). Leaf miners and gall formers were less common at the study sites (<5% of the leaves; A. Galmán, personal observation).

**Table 1.** Latitude, longitude (decimal degrees), and elevation (m a.s.l.) of populations sampled for each oak (Quercus) species. These populations were sampled following the optimal elevational ranges (low, mid and high elevations) of each oak species. Optimal elevational range of each oak species was estimated from literature.

| **Plant species** | **Latitude** | | | **Longitude** | | | **Elevation (m)** | | | **Elevation range (m)** |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Low** | **Mid** | **High** | **Low** | **Mid** | **High** | **Low** | **Mid** | **High** | |
| *Q. canariensis* | 36.35928 | 37.31829 | 36.53187 | -5.6445 | -8.5427 | -5.5872 | 120 | 400 | 600 | 0-600 |
| *Q. coccifera* | 42.68305 | 42.01907 | 42.01002 | -1.7951 | 1.0294 | 1.0184 | 400 | 800 | 990 | 0-1000 |
| *Q. ilex* | 42.3882 | 42.41805 | 42.36947 | -7.1851 | -6.6602 | -6.6385 | 300 | 680 | 1046 | 0-1400 |
| *Q. lusitanica* | 42.87298 | 37.35346 | 37.31002 | -9.1065 | -8.47320 | -8.5287 | 124 | 350 | 790 | 0-600 |
| *Q. petraea* | 42.84838 | 42.86471 | 42.81339 | -6.9194 | -6.8604 | -6.9016 | 829 | 1130 | 1450 | 600-1800 |
| *Q. pubescens* | 42.05505 | 42.70484 | 42.72323 | 0.9514 | -0.7890 | -0.3079 | 600 | 830 | 1140 | 500-1500 |
| *Q. robur* | 42.45307 | 42.46818 | 42.47016 | -8.5828 | -8.4995 | -8.3487 | 120 | 347 | 743 | 0-1000 |
| *Q. suber* | 42.39777 | 42.34394 | 37.31362 | -8.1384 | -8.6772 | -8.5363 | 224 | 430 | 650 | 0-800 |

### Field sampling and leaf herbivory measurements

At the end of the growing season, from late September to mid October 2017, we surveyed three populations of each species. Populations spanned virtually the entire elevational range of the studied oaks, and corresponded to low-, mid- and high-elevation sites based on the elevational range reported for each species (Table 1). Each site included at least 15 adult oak trees for which we randomly selected five adult (reproductive) trees and five saplings (<1 m tall). In total, we sampled 240 trees corresponding to 8 oak species × 3 elevations × 2 ontogenetic stages × 5 individuals. Most leaf damage was caused by insect leaf chewers, and we did not find signs of vertebrate leaf herbivory for either saplings or adult trees (A. Galmán, personal observation).

For each adult tree, we randomly selected two low-hanging branches (2–3 m from the ground) and collected 25 leaves per branch. For each leaf, we visually estimated the percentage of leaf area removed by insect leaf chewers using the following scale: 0 = undamaged; 1 = 1–5% damaged; 2 = 6–10% damaged; 3 = 11–25% damaged; 4 = 26–50% damaged; 5 = 51–75% damaged; 6 = >75% damaged) ("leaf herbivory" hereafter; Moreira et al., 2019). Values were averaged across all leaves to obtain a mean value per branch, and then averaged across branches to obtain a single mean per tree for statistical analyses. For saplings, we collected 15 leaves throughout the plant canopy and visually estimated the percentage of leaf area removed by insect leaf chewers (as above) for all leaves per plant. We then averaged values across leaves to obtain a single mean value per sapling for statistical analyses. All sampled leaves were transported to the laboratory where they were scored by the same person (A. Galmán) to avoid biases in estimating leaf damage. Our method could have underestimated leaf herbivory by mammals (e.g., deer), although direct and indirect evidence of mammal presence suggest they were rare at most of the studied sites.

We also collected four fully expanded leaves per branch for adult individuals and six fully expanded leaves near the apical meristem for saplings to quantify plant traits (see ahead). We selected leaves with little or no evidence of herbivory to reduce variation in defenses or nutrients caused by site-specific induction (Moreira et al. 2019). We sampled completely expanded leaves, of roughly the same age (position on branch, color and consistency) and location in the canopy. Leaves were oven-dried for 48 h at 40°C, ground with liquid nitrogen, and stored at room temperature.

### Quantification of specific leaf area

We estimated specific leaf area (SLA) for each leaf by dividing the surface area of a 9.5-mm disk by its dry mass. We selected mature leaves in a similar position along the branch, and only measured one leaf per plant as previous trials demonstrated relatively low leaf-to-leaf variation within individuals. SLA is correlated with leaf toughness(Mattson 1980, Hanley et al. 2007) and was therefore taken as a proxy of leaf physical or structural resistance against herbivory.

### Quantification of phenolic compounds

Phenolic compounds have been reported to confer resistance against insect herbivores in oaks (Feeny 1970, Roslin and Salminen 2008, Moreira et al. 2018b Moreira et al. 2018c); therefore, they provide a suitable proxy of chemical defenses. Phenolic compounds were extracted from 20 mg of dry leaf tissue with 1 mL of 70% methanol in an ultrasonic bath for 15 min, followed by centrifugation (Moreira et al. 2014). We then transferred the extracts to chromatographic vials.

For the chromatographic analyses, we used an ultra-high-performance liquid chromatograph (UHPLC Nexera LC-30AD; Shimadzu Corp., Kyoto, Japan) equipped with a Nexera SIL-30AC injector and one SPD-M20A UV/VIS photodiode array detector. Compound separation was carried out on a Kinetex 2.6 µm C18 82-102 Å, LC Column 100 × 4.6 mm (Phenomenex, Torrance, CA, USA), protected with a C18 guard cartridge. The flow rate was 0.4 mL min$^{-1}$, and the oven temperature was set at 25°C. The mobile phase consisted of two solvents: water-formic acid (0.05%) (A) and acetonitrile-formic acid (0.05%) (B), starting with 5% B and using a gradient to obtain 30% B at 4 min, 60% B at 10 min, 80% B at 13 min and 100 % B at 15 min. The injection volume was 10 µL.

For phenolic compound identification, we used an ultra-performance liquid chromatograph coupled with electrospray ionization quadrupole (Thermo Dionex Ultimate 3000 LC; Thermo Fisher Scientific, Waltham, MA, USA) time-of-flight mass spectrometer (UPLC-Q-TOF-MS/MS; Bruker Compact, Bruker Corp., Billerica, MA, USA). We identified four groups of phenolic compounds: flavonoids, ellagitannins and gallic acid derivatives (“hydrolyzable tannins” hereafter), proanthocyanidins (“condensed tannins” hereafter) and hydroxycinnamic acid precursors to lignins (“lignins” hereafter). We quantified flavonoids as rutin equivalents, condensed tannins as catechin equivalents, hydrolyzable tannins as gallic acid equivalents, and lignins as ferulic acid equivalents (Moreira et al. 2018b, Galmán et al. 2019). We quantified these phenolic compounds by external calibration using calibration curves at 0.25, 0.5, 1, 2 and

5 µg $mL^{-1}$. We expressed phenolic compound concentrations as milligrams per gram dried tissue.

### Quantification of nutrients

Phosphorus and nitrogen in leaves represent good proxies of leaf nutrient status since both of these macronutrients are strongly associated with levels of leaf herbivory across many plant taxa (Mattson 1980, Huberty and Denno 2006), and previous work has shown positive correlations between leaf herbivory and these nutrients in oaks (Forkner and Hunter 2000, Eatough Jones et al. 2008, Moreira et al. 2018b). To quantify these nutrients, we digested approximately 0.1 g of ground dried leaf material in a mixture of selenous sulphuric acid and hydrogen peroxide (Moreira et al. 2012). Diluted aliquots of the digestion were analyzed by colorimetry to quantify nitrogen (indophenol blue method) and phosphorus (molybdenum blue method) concentration using a Biorad 650 microplate reader (Bio-Rad Laboratories, Philadelphia, PA, USA) at 650 nm and 700 nm, respectively (Walinga et al. 1995). We expressed nitrogen and phosphorus concentrations as milligrams per gram dried tissue.

### Statistical analyses

#### *Effects of elevation and ontogeny on leaf herbivory and traits*

We ran general linear mixed models (GLMMs) using data at the plant level testing for effects of elevation (low, mid, and high), plant ontogeny (saplings vs. adult trees), and their interaction (all fixed factors) on insect leaf herbivory, chemical defenses, nutrients, and SLA. In the case of chemical defenses and nutrients, we ran separate models for each group of phenolic compounds and type of nutrient. The elevation by ontogeny interaction tested whether the magnitude of change in herbivory or plant traits with elevation differed between adults and saplings. For each model, we also included the effect of oak species, the oak species × elevation interaction, and the oak species × elevation × ontogeny interaction as random factors in order to analyze the main effects of elevation and ontogeny with the appropriate error terms.

### *Leaf traits associated with effects of elevation and ontogeny on herbivory*

Based on the previous analyses, if elevation and/or ontogeny showed significant effects on leaf herbivory, we ran the model again (with the same main fixed effects and their interaction), while additionally including leaf traits as covariates potentially associated with effects on leaf herbivory ("mechanistic model"; Abdala-Roberts et al. 2016, Moreira et al. 2017). We expected that if leaf traits account for a large fraction of the total variance when measuring the effect of elevation and plant ontogeny on herbivory, then a significant main effect in the initial model should turn nonsignificant after including leaf traits as covariates. We caution that our assessment of traits associated with herbivory is by no means exhaustive (i.e., other unmeasured traits could be equally or more important) and that these analyses do not test for causality. Rather, the analyses can identify candidate traits that should be addressed more robustly in subsequent work.

We performed all statistical analyses in R v 3.4.1 using the lme4 package (R Core Team 2019). We log-transformed all data to achieve normality of the residuals. We report least-squares means and standard errors in the original scale as descriptive statistics.

## RESULTS

### Effects of elevation and ontogeny on leaf herbivory and traits

There was a significant effect of elevation but no effect of oak ontogeny on insect leaf herbivory (Table 2, Fig. 1). Contrary to expectations, mean herbivory damage was 23% higher for individuals at high elevations than for those at low elevations, whereas there was no significant difference in damage between high- and mid-elevation or between mid- and low-elevation (low elevation: 1.91 ± 0.21, mid-elevation: 2.12 ± 0.21, high elevation: 2.34 ± 0.21; Fig. 1). In addition, there was no elevation by ontogeny interaction on herbivory (Table 2, Fig. 1), indicating that the effect of elevation was similar for adults and saplings.

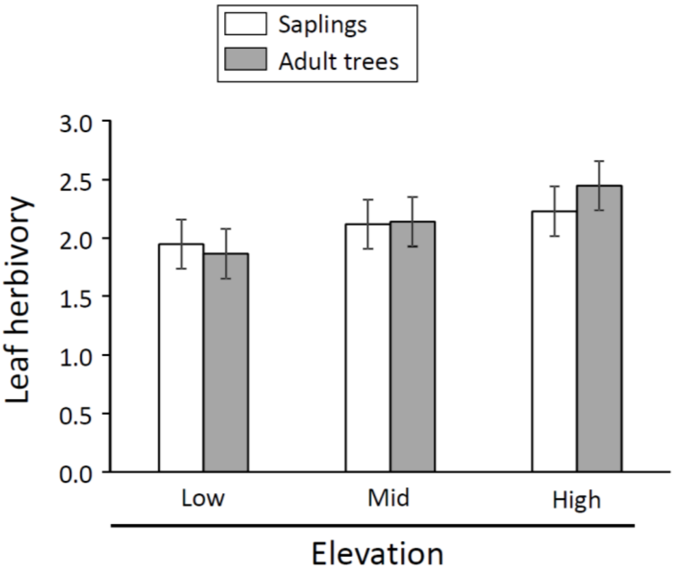

**Figure 1.** Leaf herbivory by insect chewers in adult reproductive trees (gray bars) and saplings (white bars) of eight oak (*Quercus*) species growing at low, mid and high elevations. Leaf chewer damage was visually estimated using an ordinal scale based on percent leaf area removed. Bars are least square means ± standard error. Results of the linear mixed models are presented in Table 2.

The mean concentration of lignins was significantly higher for individuals found at high and mid elevations relative to those at low elevations (40% and 30% respectively; Table 2, Fig. 2d). There was no significant difference between high and mid elevations (low elevation: 1.80 ± 0.36 mg $g^{-1}$ d.w., mid-elevation: 2.54 ± 0.36 mg $g^{-1}$ d.w., high elevation: 2.36 ± 0.36 mg $g^{-1}$ d.w.; Fig. 2d). In contrast, there was no significant effect of elevation on leaf condensed or hydrolyzable tannins, flavonoids, phosphorus, nitrogen or SLA (Table 2, Figs. 2a–c, 3a–c). Mean concentration of condensed tannins and lignins was 14% and 18% higher (respectively) for saplings than for adult trees (condensed tannins: saplings = 9.20 ± 1.19 mg $g^{-1}$ d.w.; adult trees = 8.11 ± 1.19 mg $g^{-1}$ d.w.; lignins: saplings = 2.42 ± 0.32 mg $g^{-1}$ d.w.; adult trees = 2.05 ± 0.32 mg $g^{-1}$ d.w; Fig. 2a, 2d). In contrast, there was no significant effect of plant ontogeny on leaf hydrolyzable tannins, flavonoids, phosphorus, nitrogen or SLA (Table 2, Figs. 2b-c, 3a-c). Finally, there was no significant elevation by ontogeny interaction for any of the leaf traits measured (Table 2, Figs. 2, 3).

A scheme summarizing the effects of elevation and plant ontogeny on leaf herbivory and traits is shown in the Figure S1 (see Appendix B).

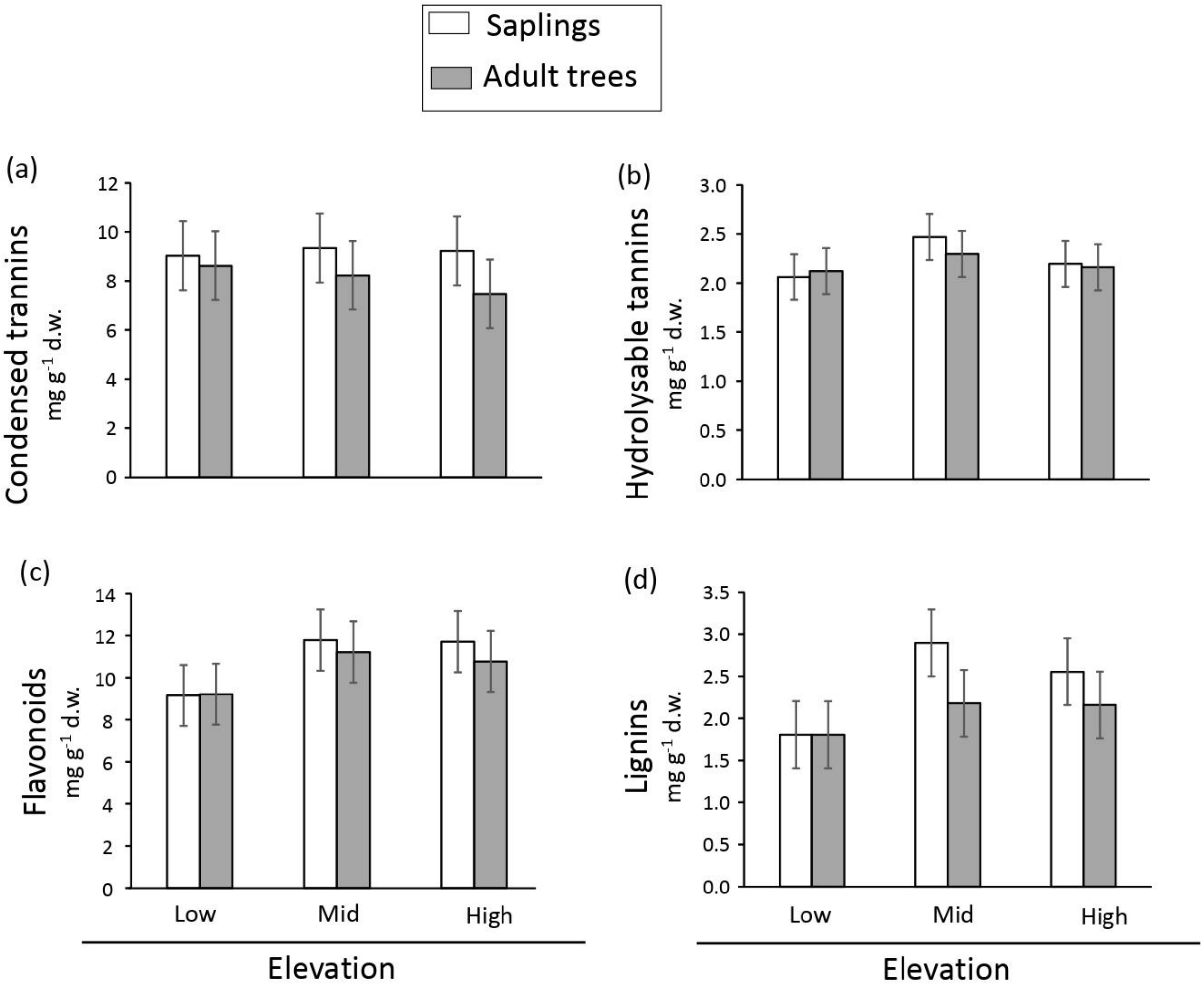

**Figure 2.** Concentration of leaf (a) condensed tannins, (b) hydrolyzable tannins, (c) flavonoids, and (d) lignins in adult reproductive trees (gray bars) and saplings (white bars) of eight oak (*Quercus*) species growing at low, mid and high elevations. Bars are least square means ± standard error. Results of the linear mixed models are presented in Table 2.

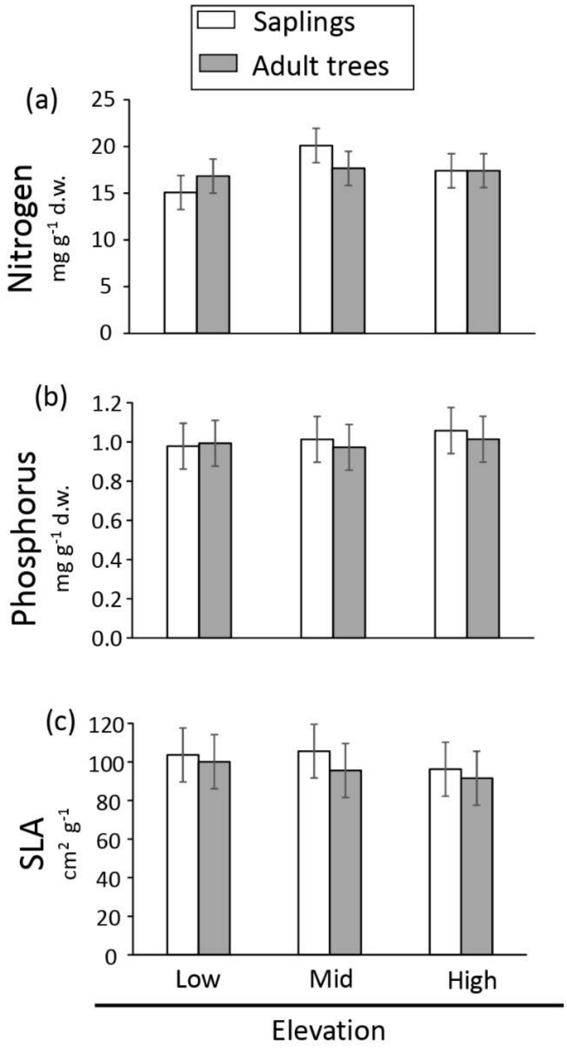

**Figure 3.** Concentration of leaf (a) nitrogen and (b) phosphorus, and (c) specific leaf area (SLA) in adult reproductive trees (gray bars) and saplings (white bars) of eight oak (*Quercus*) species growing at low, mid and high elevations. Bars are least square means ± standard error. Results of the linear mixed models are presented in Table 2.

### Leaf traits underlying elevation and ontogenetic effects on herbivory

The significant effect of elevation on leaf herbivory observed in the initial model (Table 2) remained significant after including leaf traits as covariates (Table 3). This result suggests that the included leaf chemical and structural traits did not account for the observed increase in leaf herbivory from low to high elevations. None of the covariates had a significant effect on herbivory (Table 3).

**Table 2.** Summary of results from linear mixed models testing for the effects of elevation (low, mid and high), ontogeny (saplings vs. adult trees) and their interaction on leaf herbivory by chewing insects (% damage), concentration of leaf chemical defenses (condensed and hydrolyzable tannins, flavonoids and lignins) and nutrients (nitrogen and phosphorus), and specific leaf area (SLA) in eight oak (*Quercus*) species. We also included the effect of oak species, the oak species × elevation interaction, and the oak species × elevation × ontogeny interaction as random factors. *F*-values with degrees of freedom (numerator, denominator) and associated significance levels (*P*-values) are shown. Significant *P*-values ($P < 0.05$) are in bold.

| | Elevation (E) | | Ontogeny (O) | | E × O | |
|---|---|---|---|---|---|---|
| Variable | $F_{2,\,14}$ | $P$ | $F_{1,\,21}$ | $P$ | $F_{2,\,21}$ | $P$ |
| Insect leaf herbivory | 3.95 | **0.044** | 0.29 | 0.595 | 0.82 | 0.453 |
| Condensed tannins | 0.37 | 0.698 | 4.88 | **0.038** | 0.61 | 0.552 |
| Hydrolyzable tannins | 1.55 | 0.246 | 0.08 | 0.776 | 0.20 | 0.823 |
| Flavonoids | 1.42 | 0.274 | 0.15 | 0.700 | 0.47 | 0.629 |
| Lignins | 4.50 | **0.031** | 5.69 | **0.027** | 1.82 | 0.189 |
| Nitrogen | 1.99 | 0.173 | 0.09 | 0.768 | 2.76 | 0.086 |
| Phosphorus | 0.19 | 0.829 | 0.53 | 0.476 | 0.37 | 0.697 |
| SLA | 0.67 | 0.528 | 2.60 | 0.122 | 0.41 | 0.667 |

**Table 3.** Summary of results from linear mixed models testing for the effects of elevation (low, mid and high elevations), ontogeny (saplings vs. adult trees) and their interaction on leaf herbivory by chewing insects (% damage) in eight oak (*Quercus*) species. We included chemical (condensed and hydrolyzable tannins, flavonoids, lignins) and structural (specific leaf area) defenses and nutritional (nitrogen and phosphorus) traits as covariates to test whether elevational effects on insect leaf herbivory were determined by these leaf traits. We also included the effect of oak species, the oak species × elevation interaction, and the oak species × elevation × ontogeny interaction as random factors. *F*-values, degrees of freedom (numerator, denominator) and associated significance levels (*P*-values) are shown. Significant *P*-values ($P < 0.05$) are in bold.

| | Insect leaf herbivory | | |
|---|---|---|---|
| Variable | $F$ | $\mathrm{df}_{\mathrm{num,den}}$ | $P$ |
| Elevation | 4.97 | 2, 14 | **0.023** |
| Ontogeny | 0.03 | 1, 21 | 0.855 |
| Elevation × ontogeny | 0.90 | 2, 21 | 0.422 |
| Condensed tannins | 0.57 | 1, 184 | 0.451 |
| Hydrolyzable tannins | 0.04 | 1, 184 | 0.843 |
| Flavonoids | 3.83 | 1, 184 | 0.052 |
| Lignins | 0.48 | 1, 184 | 0.489 |
| Nitrogen | 0.31 | 1, 184 | 0.582 |
| Phosphorus | 0.40 | 1, 184 | 0.530 |
| Specific leaf area | 0.06 | 1, 184 | 0.804 |

## DISCUSSION

The present study documented elevational gradients for insect leaf herbivory and lignin concentration across the eight studied oak species in the Iberian Peninsula. Unexpectedly, both leaf herbivore damage and chemical defenses (lignins) increased toward higher elevations. In addition, elevational variation in herbivory was not associated with variation in any of the leaf traits measured. Also contrary to expectations, we found that the concentrations of lignins and condensed tannins were higher in saplings than in adults, with no ontogenetic difference in herbivory. Together, these results suggest independent elevational patterns in herbivory and oak defenses and that these elevational patterns are independent of plant ontogenetic stage.

Counter to classic predictions on elevational gradients in plant defense and herbivory, our results showed that insect leaf herbivory and leaf chemical defenses (lignins) increased toward higher elevations. In our recent review of clinal studies of herbivory (Moreira et al. 2018a), we reported that 30% of the studies found higher herbivory with increasing elevation, and 40% found higher plant defenses with increasing elevation, highlighting that reverse gradients in plant-herbivore interactions are common. In the case of herbivores, lower predator pressure at higher elevations could release herbivores from top-down control and result in greater herbivory at higher elevations (Moreira et al. 2018a) Alternatively, an increase in herbivore diet breadth with increasing elevation (Rasmann et al. 2014a) could also lead to greater herbivory by generalist species or guilds of insect herbivores at high elevations. In the case of defenses, plant defensive traits respond not only to herbivore pressure, but could also be adaptive for tolerance to climatic conditions and resource availability (Abdala-Roberts et al. 2016, Moreira et al. 2018c). Accordingly, more stressful environments (e.g., at higher elevations) are commonly associated with slow-growing species with high tissue value (i.e., resource availability hypothesis; (Coley et al. 1985, Endara and Coley 2011). Consequently, plant species adapted to more stressful environments would be expected to increase allocation to plant defenses because the cost of replacing tissues consumed by herbivores is higher when resources are low.

Our results also showed that elevational variation in herbivory was not explained by leaf traits across the studied oak species, suggesting that other (unmeasured) plant traits (e.g., indirect defenses, induced defenses) or abiotic factors (e.g., temperature, precipitation, soil conditions) could play an important role in generating variation in herbivory pressure. We similarly found in a previous study that insect leaf herbivory and the concentration of phenolic compounds in pedunculate oak (*Q. robur*), one of the species included in the present study, increased toward higher elevations, but phenolics did not underlie elevational variation in herbivory (Abdala-Roberts et al. 2016). In addition, Pellissier et al. (2016) reported increasing herbivory but decreasing chemical defenses (glucosinolates) at low elevations across 16 *Cardamine* species, but again these chemical defenses did not explain the elevational gradient in herbivory. Together, our present work and these studies indicate decoupled elevational gradients in plant defenses and herbivory but at the same time (given contrasting gradients) suggest different mechanisms underlying elevational variation in herbivory and plant defenses across systems. Patterns might be reconciled by conducting measurements at multiple time points because most studies use single time-point measurements and thus provide a limited or inaccurate assessment of plant defense levels and herbivore pressure (Rosado-Sánchez et al. 2018). In addition, combining these observational results with reciprocal transplant experiments and bioassays of herbivore performance on leaf tissue from different populations is necessary to causally link targeted plant traits and herbivory, a necessary task to elucidate the ecological mechanisms and evolutionary implications of elevational changes in plant-herbivore interactions (Moreira et al. 2018a).

Surprisingly, we found no effect of plant ontogeny on insect leaf herbivory, contradicting the prediction that plants that are easier to locate (i.e., adult trees in our case) exhibit higher herbivory (Feeny 1976, Strauss et al. 2015). Although adult trees would presumably be more easily located by herbivores and offer large amounts of leaf tissue, most of the leaves are mature with relatively low nutritional value (thicker and tougher) that are not used by insect herbivores (Barton and Koricheva 2010). Thus, the amount of herbivory on adults might be lower despite their increased detectability and ultimately diminish the difference in herbivory between life stages. On the other hand, we also found that the concentration of leaf chemical defenses (condensed tannins and lignins) was greater for saplings than for adult trees, a pattern that counters the prediction that defense levels build up from the sapling to the adult stage (Boege and Marquis 2005, Barton and Koricheva 2010). Supporting our findings, we recently found that saplings of *Q. robur* also exhibited greater levels of chemical defenses (lignins and hydrolyzable tannins) than adult trees (Moreira et al. 2017). One explanation for these findings could be that saplings can rapidly increase their photosynthetic capacity (Huijser and Schmid 2011) and in turn allocate more resources to defense (relative to their total energetic budget) than adult trees. Alternatively, the sapling stage is crucial for subsequent plant survival and establishment in trees, such that growing tissues should be better defended in saplings than in adult trees (i.e., optimal defense theory; McCall and Fordyce 2010).

Contrary to expectations, we found that differences in insect leaf herbivory and plant defenses across elevations were consistent for adults and saplings. These results do not support that differences in allocation to defenses or in detectability lead to weaker differences in herbivory and plant defenses between adults and saplings at high elevations. Alternatively, it is possible that if adults are both better defended and more easily detected, the proposed mechanisms cancelled each other out. Further work explicitly testing and separating them is necessary to determine whether they are actually occurring and to what degree they interact. For example, the influence of tree defense levels on elevational clines in herbivory could be assessed in common gardens with plants of two early ontogenetic stages (e.g., seedlings and saplings) across an elevational gradient. At each elevation, defense induction elicitors (e.g., jasmonic acid) could be used to both reduce and increase ontogenetic differences in defense levels. This experimental design would allow to test whether differences in overall defense levels (combination of constitutive and induced) shape ontogenetic differences in herbivory and if this effect varies with elevation. Experiments involving adults would be unfeasible for long-lived trees, but this type of scheme including all ontogenetic stages (from seedlings to adults) could be applied to herbs and shrubs.

### Limitations and future work

Previous work with oaks has demonstrated that leaf phenology is an important factor driving leaf herbivory (Pearse and Karban 2013, Pearse et al. 2015a, Pearse et al. 2015b). Notably, early leaf phenology increased leaf miner and chewer damage in valley oak (*Q. lobata*) trees (Pearse et al. 2015a, Pearse et al. 2015b) and leaf miner damage among 55 oak species in a common garden (Pearse and Karban 2013). In the present study, we sampled leaves at the end of the growing season across the entire elevational gradient to obtain an estimate of cumulative damage before leaf drop, thus reducing the influence of elevational differences in the phenology of plant defenses and herbivory, which could have come into play by sampling in the early or mid-season. If phenology had played a decisive role, we would have expected sites with earlier phenology found at lower elevations to exhibit higher levels of herbivory, but we instead found the opposite pattern. Unfortunately, it is not possible to separate the effects of total growing season length from those of season onset, and only by conducting further measurements at multiple time points during the season can the influence of different phenological variables be assessed.

Finally, it is also important to note that, because allocation to plant defenses and growth can vary among years, having only two ontogenetic categories may not be enough to understand plant ontogenetic effects on herbivory and defenses on centennial trees. Accordingly, further work should consider a more careful subdivision involving multiple ontogenetic stages as well as multi-annual measurements to test for the consistency in allocation patterns to growth and defense.

# CHAPTER III

# Inducibility of chemical defences in young oak trees is stronger in species with high elevational ranges

That give rise to the publication Galmán, A., Petry, W. K., Abdala-Roberts, L., Butrón, A., de la Fuente, M., Francisco, M., Kergunteuil, A., Rasmann, S., Moreira, X., 2019. Inducibility of chemical defences in young oak trees is stronger in species with high elevational ranges. Tree Physiology 39, 606-614.


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# CHAPTER III:

# Inducibility of chemical defences in young oak trees is stronger in species with high elevational ranges

## ABSTRACT

Elevational gradients have been highly useful for understanding the underlying forces driving variation in plant traits and plant-insect herbivore interactions. A commong view has been that greater herbivory under warmer and less variable climatic conditions found at low elevations has resulted in stronger herbivore selection on plant defences. However, this prediction has been called into question by conflicting empirical evidence, and could be explained by number of causes such as an incomplete assessment of defensive strategies (ignoring other axes of defence such as defence inducibility) or unaccounted variation in abiotic factors along elevational clines. We conducted a greenhouse experiment testing for inter-specific variation in constitutive leaf chemical defences (phenolic compounds) and their inducibility in response to feeding by gypsy moth larvae (*Lymantria dispar*, Lepidoptera) using saplings of 18 oak (*Quercus*, Fagaceae) species. These species vary in their elevational distribution and together span >2,400 m in elevation, therefore allowing us to test for among-species elevational clines in defences based on the elevational range of each species. In addition, we further tested for elevational gradients in the correlated expression of constitutive defences and their inducibility and for associations between defences and climatic factors potentially underlying elevational gradients in defences. Our results showed that oak species with high elevational ranges exhibited a greater inducibility of phenolic compounds (hydrolysable tannins), but this gradient was not accounted for by climatic predictors. In contrast, constitutive defences and the correlated expression of constitutive phenolics and their inducibility did not exhibit elevational clines. Overall, this study builds towards a more robust and integrative understanding of how multivariate plant defensive phenotypes vary along ecological gradients, and their underlying abiotic drivers.

**Keywords**: climate, defences, elevation, herbivory, phenolic compounds, *Quercus*,

## INTRODUCTION

Understanding the factors and mechanisms governing species interaction strength represents a major challenge in ecology (Linhart and Grant 1996). A powerful approach in this regard has been to study species interactions along environmental or geographical (i.e., latitudinal or elevational) gradients (Schemske et al. 2009). This gradient-based approach has been particularly useful for studying interactions between plants and their insect herbivores, providing important insights and new theory in recent years (Anstett et al. 2016b, Moreira et al. 2018a). With respect to elevation, it has been proposed that greater insect herbivory under warmer and less variable climates at low elevations is accompanied by stronger herbivore selection on plant defences (Galmán et al. 2018, Moreira et al. 2018a). In turn, heightened herbivore pressure at low elevations has resulted in greater plant investment in defences compared to high elevations (Rasmann et al. 2014b). Yet, recent work has questioned these predictions, therefore opening a new debate on the underlying drivers of observed patterns. A review by Moreira et al. (2018a) reported that plant defences and herbivore damage decrease with elevation in 48% and 60% of the cases (respectively), whereas insect herbivore abundance and species richness decrease with elevation in 48% of the cases. Overall patterns thus indicate that the predicted association between elevation and plant defences is not to be universal and exhibits inherent variability due to unaccounted factors.

Variation among studies in the strength and direction of elevational gradients in herbivory and plant defences could be explained by differences in how plant defences are quantified and the type of traits measured. First, studies about elevational gradients often assume that levels of constitutive plant secondary metabolites or physical defences are strongly associated with elevational variation in herbivore preference or performance (Rasmann et al. 2014a). However, other defensive strategies such as induced defences (i.e., those that are only expressed after herbivore damage) are rarely considered in elevational studies but could nonetheless also co-vary with elevation (e.g., Pellissier et al. 2016, Defossez et al. 2018). Second, there may be negative (e.g. trade-offs) or positive correlations between defensive strategies depending on the costs and benefits of each under different ecological contexts (Agrawal 2000), such that two or more defensive strategies may co-vary along an elevational gradient. Ignoring correlations between defensive strategies may lead to erroneous conclusions about the nature and presence of elevational clines (Pellissier et al. 2016, Defossez et al. 2018). In particular, trade-offs between constitutive and induced defences have often been observed (Kempel et al. 2011, Rasmann et al. 2011, Moreira et al. 2014), and shifts in the relative allocation to each strategy are likely to occur along environmental gradients (Moreira et al. 2014). Environments with low herbivore pressure (presumably at high elevations under the prevailing view) might select for low constitutive defences and high induction because the costs of continuously producing constitutive defences are high when damage is low and not persistent (i.e., costs of constitutive defences outweigh their benefits; (Zangerl and Rutledge 1996). In contrast, environments with high herbivore pressure (at low elevations) would select for high constitutive but low induced defences because the fitness benefits of reduced losses to herbivory outweigh the costs of continuous production of constitutive defences (Zangerl and Rutledge 1996, Moreira et al. 2014). Overall, not accounting for multiple axes of defensive strategies or addressing plant defence from a multi-variate perspective has likely contributed to inconsistent patterns or erroneous conclusions about the nature of elevational gradients in plant defensive investment.

Another important consideration is that most studies have considered ‘elevation’ as a proxy for multiple co-varying abiotic (e.g., climatic) factors without a detailed analysis of these drivers (Körner 2007). Recent studies have demonstrated that an explicit consideration of climatic variables can shed light into how the abiotic environment shapes elevational clines of herbivory and plant defences (Pellissier et al. 2014, Abdala-Roberts et al. 2016, Pellissier et al. 2016). For example, Abdala-Roberts et al. (2016) found that climatic conditions (temperature) influenced elevational variation in insect herbivory independently of chemical defences in English oak *Quercus robur*, a pattern that may arise when defences are influenced by resource availability or when these compounds play a role in abiotic tolerance. Similarly, Pellissier et al. (2016) found that temperature and precipitation strongly affected both herbivory and chemical defences across *Cardamine* species growing along an elevational gradient. Empirical evidence and theory thus argue for the dual consideration of climatic factors and herbivory, as, their concurrent and potentially interactive influences have likely shaped the evolution of plant defence traits along elevational gradients.

In this study we tested for elevational gradients in defensive traits in oak (*Quercus,* Fagaceae) species. We approached this by studying how individual defensive strategies vary with species elevational ranges, as well as whether the simultaneous or correlated expression of different defensive strategies also exhibits such clinal variation. Using a phylogenetic comparative approach we addressed the following questions: (1) Are there elevational gradient in constitutive chemical defences and their inducibility (i.e., difference between induced and constitutive levels of defences) across oak species? (2) Are there elevational gradients in the correlated expression (negative or positive) of constitutive chemical defences and their inducibility? And (3) do climatic factors account for elevational clines in individual defensive traits and strategies or their correlated expression? To address these questions, we quantified the concentration of constitutive levels of phenolic compounds in leaves as well as their inducibility after feeding by the generalist gypsy moth larvae (*Lymantria dispar,* Lepidoptera) in greenhouse experiment with 18 oak species collectively spanning >2,400 m in elevation. We further investigated the effects of climatic factors (temperature and precipitation) associated with the climatic niche of the different oak species on plant defences. Overall, this study builds towards a more robust and integrative understanding of variation in plant multi-variate defensive phenotypes vary along ecological gradients and their underlying abiotic drivers.

## MATERIALS AND METHODS

### Natural history

We used 18 species of the genus Quercus, of which 11 have a Palearctic distribution range, namely: Quercus robur, Q. faginea, Q. petraea, Q. suber, Q. ilex, Q. pubescens, Q. frainetto, Q. phillyraeoides, Q. cerris, Q. serrata and Q. pyrenaica, and seven have a Nearctic distribution, namely: Q. agrifolia, Q. macrocarpa, Q. coccinea, Q. rubra, Q. shumardii, Q. palustris and Q. phellos. These oak species are widespread along the northern hemisphere and encompass a 2,423 m elevational gradient (in average, from -3 for Q. cerris to up 2,420 meters above sea level for Q. serrata) (Manos et al. 1999, Hipp et al. 2018).

*Quercus* species have developed a broad spectrum of defence traits against phytophagous insects, including chemical defences such as phenolic compounds (Feeny 1970, Roslin and Salminen 2008, Pearse and Hipp 2012, Abdala-Roberts et al. 2016, Abdala-Roberts et al. 2018, Moreira et al. 2018b). These chemical defences are the most important chemical barrier against herbivory in oak trees as they function as digestibility reducers in insects (Pearse and Hipp 2012). They are present at high constitutive levels in all tissues, but can also be induced upon damage (Pearse and Hipp 2009, Mizumachi et al. 2012).

*Quercus* species are attacked by a large community of insect herbivores including generalist and specialist species, mainly leaf chewers and miners (Roslin and Salminen 2008, Pearse and Hipp 2009, Tack et al. 2010, Moreira et al. 2017, Moreira et al. 2018b). Among these herbivores, the generalist gypsy moth (*Lymantria dispar*, Lepidoptera) is one of the most destructive pests of broad-leaf and conifer trees in Europe and, following introduction, in North America (Milanovic et al. 2014). Larvae of this species feed at night on leaves, and reach densities that can cause extensive damage on a large proportion of an individual tree's leaves (Miller and Hanson 1989). Although co-evolution of oaks and the gypsy moth is thought to have mainly occurred in the Palearctic region, this phytophagous insect also feeds on many species of Nearctic *Quercus* (Elkinton and Liebhold 1990). Moreover, this insect could be also viewed as a good candidate to test inducibility of quantitative defences because a previous study using our dataset found that oaks from the two biogeographic regions were similar in their inducibility of chemical defences following gypsy moth feeding (Moreira et al. 2018c).

### Plant growth, greenhouse conditions and treatments

In March 2017, in a glasshouse, we individually planted one-year-old plants (purchased from Planfor nursery, Uchacq-et-Parentis, France) in 4-L pots containing peat. All oak stocks belong to native provenances, but saplings of each species used in our experiment came from multiple source populations of unknown location (and thus elevation). Despite this, we expected that among-species variation due to adaptation to specific elevational ranges would be greater than within-species variation and followed a similar approach to previous studies for which variation for each species was based on single-population estimates or seed source locations were unknown (e.g., Rasmann and Agrawal 2011, Pearse and Hipp 2012, Moreira et al. 2014). Plants were grown under controlled conditions: minimum of 12 h of light per day, temperature b etween 10°C at night and 25°C during the day and were watered daily. After two months of acclimatization, we randomly assigned half of the plants of each species to one of two herbivory treatments: (1) damaged plants by gypsy moth larvae and (2) undamaged control plants. The experimental design followed a randomized split-plot design replicated across six blocks, with herbivory treatment applied at the whole factor and oak spec ies as the split factor. This design comprised six blocks × 18 oak species × two herbivory treatments (control and infestation by gypsy moth larvae), for a total of 216 plants.

We placed a fourth-instar larva on fully expanded leaves of each plant assigned to the infestation treatment (Figure 1). Larvae fed on the plants for four days and were then removed and we estimated the percent of total leaf area damaged at the whole-plant level by visually assigning each plant to one of the following categories: : 0 = undamaged; 1 = 1-10% damaged; 2 =11-20% damaged; 3 = 21-30% damaged; 4 = 31-40% damaged; 5 = 41-50% damaged; 6 = 51-60% damaged; 7 = 61-70% damaged; 8 = 71-80% damaged; 9 = 81-90% damaged; 10 = 91-100% damaged (Moreira et al. 2018c). To avoid biases in our herbivory estimates, the same person (XM) scored all the leaves. After this estimation, we collected three random leaves per plant for subsequent quantification of phenolic compounds. Leaves were oven-dried for 48 h at 40°C, ground with liquid nitrogen, and stored at room temperature.

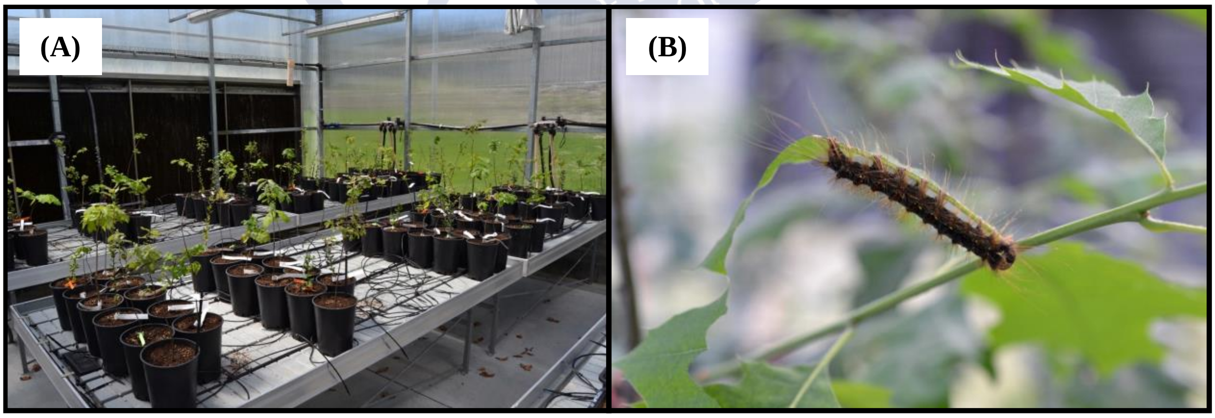

**Figure 1**. (A) Experimental design in the greenhouse and (B) *Lymantria dispar* (Lepidoptera) larvae feeding on a *Quercus* species.

### Chemical analyses

Phenolic compounds were extracted from 20 mg of dry plant tissue with 1 mL of 70% methanol in an ultrasonic bath for 15 min, followed by centrifugation and subsequent dilution of the methanolic extract (Moreira et al. 2014). We diluted these methanolic extracts (1:5 vol:vol) with the extraction solvent and transferred them to chromatographic vials. We did not use an internal standard because previous trials with a subset of our samples using resorcinol (Sigma-Aldrich, #398047) as internal standard indicated that recovery rates were >90% in all cases. To perform the chromatographic analyses we used Ultra-High-Performance Liquid-Chromatograph (UHPLC Nexera LC-30AD; Shimadzu) equipped with a Nexera SIL-30AC injector and one SPD-M20A UV/VIS photodiode array detector. The compound separation was carried out on a Kinetex™ 2.6 µm C18 82-102 Å, LC Column 100 × 4.6 mm, protected with a C18 guard cartridge. The flow rate was 0.4 mL min$^{-1}$ and the oven temperature was set at 25 ºC. The mobile phase consisted of two solvents: water-formic acid (0.05%) (A) and acetonitrile-formic acid (0.05%) (B), starting with 5% B and using a gradient to obtain 30% B at 4 min, 60% B at 10 min, 80% B at 13 min and 100 % B at 15 min. The injection volume was 30 µL. We previously ran some of our samples with an ultrahigh-pressure liquid chromatography-quadrupole-time-of-flight mass spectrometry (UHPLC-QTOF-MS) to identify the phenolic compounds. We quantified flavonoids as rutin equivalents, condensed tannins as catechin equivalents, hydrolysable tannins as gallic acid equivalents, and lignins as ferulic acid equivalents (Moreira et al. 2018b). We achieved the quantification of these phenolic compounds by external calibration using calibration curves at 0.25, 0.5, 1, 2 and 5 µg mL$^{-1}$. We quantified all compounds using a wavelength of 330 nm. We expressed phenolic compound concentrations in mg g$^{-1}$ tissue on a dry weight basis.

### Species distribution range and niche characterization

For each of the 18 oak species, we constructed a species distribution model and extracted climatic data from the estimated species range. We obtained georeferenced species presence data from the Global Biodiversity Information Facility database (GBIF; https://www.gbif.org/). We used these occurrence data to construct a Maximum Entropy Species Distribution Model (SDM) for each oak species using the MaxEnt software (Phillips et al. 2006) as implemented via the DISMO package (Hijmans et al. 2010) for R v. 3.4.1 (R Core Team 2019). We randomly assigned each species presence record to one of five equal partitions for k-fold cross-validation of the model fit for each species. Four of these partitions were used to train the models with 30-arcsecond resolution climate data from 19 BIOCLIM variables from the WorldClim database (Hijmans et al. 2005) as predictors (Table S1 in Appendix C). The fifth data partition was used to assess the model fit using the area under the receiver-operator curve (AUC). For each oak species, this procedure was repeated such that each species presence data partition was used once as the validation dataset. We discretized the SDM by taking all areas with an occurrence probability ≥75% as the species range.

We extracted the elevation of each cell within the discretized species range using digital elevation data at 30 arc-second resolution from the NASA Shuttle Radar Topographic Mission (SRTM) database (http://www.cgiar-csi.org). We supplemented the elevational range for well-sampled species where we could get a better estimate from the literature (see Fig. S1 in the Appendix C). We also estimated the latitudinal range of each oak species. However, latitudinal range did not significantly affect any plant defence (analyses not shown) and was thus removed from the statistical analyses.

## Statistical analysis

### *Summarizing constitutive and induced defences and climatic variables*

In order to analyse all defensive traits without inflating type I error through multiple individual tests, species-level (i.e., species means) variation in the chemical defensive compounds studied (flavonoids, lignins, condensed tannins, and hydrolysable tannins) was summarized by means of two independent principal component analyses (PCA) using Proc FACTOR (rotation = varimax) in SAS 9.4 (SAS System, Cary, NC), one for constitutive defences and another for inducibility of defences (Moreira et al. 2014, Moreira et al. 2015). In each case, constitutive defences and their inducibility were summarized based on the first two principal components. The first two axes of the PCA explained 71% of the variance for constitutive defensive traits, with PC1 being positively associated with constitutive concentration of lignins and hydrolysable tannins, and PC2 being positively associated with constitutive concentration of flavonoids. Similarly, the first two axes of PCA explained 73% of the variance in inducibility of defences, with PC1 being positively associated with the inducibility of condensed tannins, and PC2 being positively associated with the inducibility of hydrolysable tannins. The standardized *z*-scores of the first two principal components from each analysis were subsequently used in univariate and multiple regression analyses testing for elevational gradients in plant defensive strategies (see ahead).

Similarly, we summarized the 19 climatic variables by conducting two independent PCAs using Proc FACTOR (rotation = varimax) in SAS 9.4, one for temperature variables and one for precipitation variables (Moreira et al. 2014, Moreira et al. 2015). In each case, climatic variables were summarized with the first principal component. The first principal component explained 52% of the variance for the 11 temperature variables across oak species ("temperature" hereafter), and was positively related to mean temperature of coldest month, mean temperature of driest quarter and mean temperature of coldest quarter and negatively related to temperature seasonality and temperature annual range. Similarly, the first principal component explained 55% of the variance for the eight precipitation variables across oak species ("precipitation" hereafter), and was positively related to annual precipitation, precipitation of the wettest month, precipitation of the wettest quarter and precipitation of the warmest quarter. The standardized z-scores of the first principal component of each analysis were then used to test for the effects of climate in the multiple regressions ahead.

### *Testing for elevational gradients in oak defensive strategies and their underlying climatic drivers*

First, to assess the presence of elevational gradients in constitutive chemical defences and in their inducibility, we individually regressed the oak species' PC scores for (i) constitutive defences and (ii) defence inducibility onto the mean of the species elevational range. In the case of inducibility, we included leaf damage score as a covariate [so that the inducibility of each species was compared on the same scale (per-unit leaf loss) in light of significant leaf consumption differences among oak species ($F_{16,88} = 5.52$, $P < 0.001$)]. Second, to investigate the influence of climatic correlates of elevational variation in plant defensive traits, we performed multiple regressions including elevation and climatic variables (temperature PC1 z-scores, precipitation PC1 z-scores) as predictors of constitutive chemical defences and their inducibility. If any such significant effect of species elevational range in the former regression models not accounting for climatic variables turned non-significant and the slope estimator for the elevation effect exhibited a substantial change (i.e., reduction) after accounting for climatic factors, this would suggest that climate underlies the elevational gradient in defences.

For all the previous analyses, we accounted for phylogenetic non-independence among oak species by performing phylogenetically-corrected generalized least square analyses (pGLS) with the PGLS function in the CAPER package for R (Orme 2013). These pGLS were based on a phylogenetic tree of *Quercus* species using Single Nucleotide Polymorphism matrices by ddRAD sequencing (Moreira et al. 2018c).

### *Testing for elevational gradients in the correlated expression of oak defensive strategies and their underlying climatic drivers*

We tested for a shared structure between constitutive chemical defences and their inducibility, which would represent a correlated expression of both defensive strategies, using a coinertia analysis (Pellissier et al. 2016, Defossez et al. 2018). In other words, here we tested whether the matrices of constitutive chemical defences and their inducibility were correlated across species (Pellissier et al. 2016). These analyses were performed using the ADE4 package for R (DRAY ET AL. 2003, DRAY AND DUFOUR 2007), with the significance of the shared variance assessed using a Monte-Carlo test (10,000 simulations).

If the coinertia analysis was significant, we performed species-level univariate regressions to test for effects of species elevational range on the coinertia axis. Next, and following the same approach as in the previous section testing for effects climate on trait PCs, we performed multiple regression analyses to test for effects of both species elevational range and climate on the coinertia axis. If this significant effect of species elevational range in the univariate regressions turned non-significant after accounting for climatic variables, we interpreted this as evidence that climate underlies the elevational gradient in the correlated expression of constitutive defences and their inducibility.

## RESULTS

### Species variation in constitutive chemical defences and their inducibility

Constitutive defensive traits exhibited 1.7-fold (flavonoids) to 33.0-fold (hydrolysable tannins) variation among species, whereas the inducibility of these defensive traits exhibited 4.4-fold (condensed tannins) to 188-fold (hydrolysable tannins) variation (Table 1).

**Table 1.** Descriptive statistics (least square means ± standard error and species range) showing variation among oak (*Quercus*) species in constitutive leaf phenolic compounds (condensed tannins, hydrolysable tannins, lignins, and flavonoids, in mg $g^{-1}$ dry weight) and their inducibility after feeding by the generalist gypsy moth larvae (*Lymantria dispar*, Lepidoptera).

| | Mean ± SE | Range |
|---|---|---|
| **Constitutive phenolics** | | |
| Condensed tannins | 2.07 ± 0.21 | 0.30 – 4.22 |
| Hydrolysable tannins | 55.80 ± 15.47 | 8.57 – 283.31 |
| Lignins | 4.63 ± 0.85 | 0.00 – 11.38 |
| Flavonoids | 11.04 ± 0.36 | 8.93 – 14.73 |
| **Inducibility of phenolics** | | |
| Condensed tannins | -0.37 ± 0.18 | -1.92 – 1.46 |
| Hydrolysable tannins | -15.34 ± 9.39 | -162.63 – 24.34 |
| Lignins | -0.81 ± 0.49 | -4.52 – 4.36 |
| Flavonoids | -0.83 ± 0.23 | -2.66 – 0.92 |

### Elevational gradients in oak defensive strategies and underlying climatic drivers

We found evidence for an elevational gradient in oak defences for defence inducibility, but not for constitutive defences. We found no significant associations between either PC of constitutive defences and species elevational range (Table 2, Fig. 2a, 2b), and the subsequent model including climatic variables indicated that neither temperature nor precipitation significantly predicted constitutive defences (Table 3). On the other hand, there was a significant positive association between PC2 of inducibility and species elevational range (Table 2), i.e. oak species with high elevational ranges exhibited a greater inducibility of hydrolysable tannins (Fig. 2d). The effect of species elevational range turned marginally significant ($P = 0.062$) after accounting for temperature and precipitation (Table 3), though this represented a slight change in the significance value and the estimator of the elevation effect remained similar after including climatic factors (Table 2 vs. 3), which suggests that climate did not play an important role in mediating the elevational gradient in defences. There was no significant association between species elevational range and PC1 of inducibility (i.e., inducibility of condensed tannins) (Table 2; Fig. 2c), though the subsequent model with climatic variables indicated that both temperature and precipitation were (marginally) significantly and positively correlated with this PC1 of inducibility (Table 3).

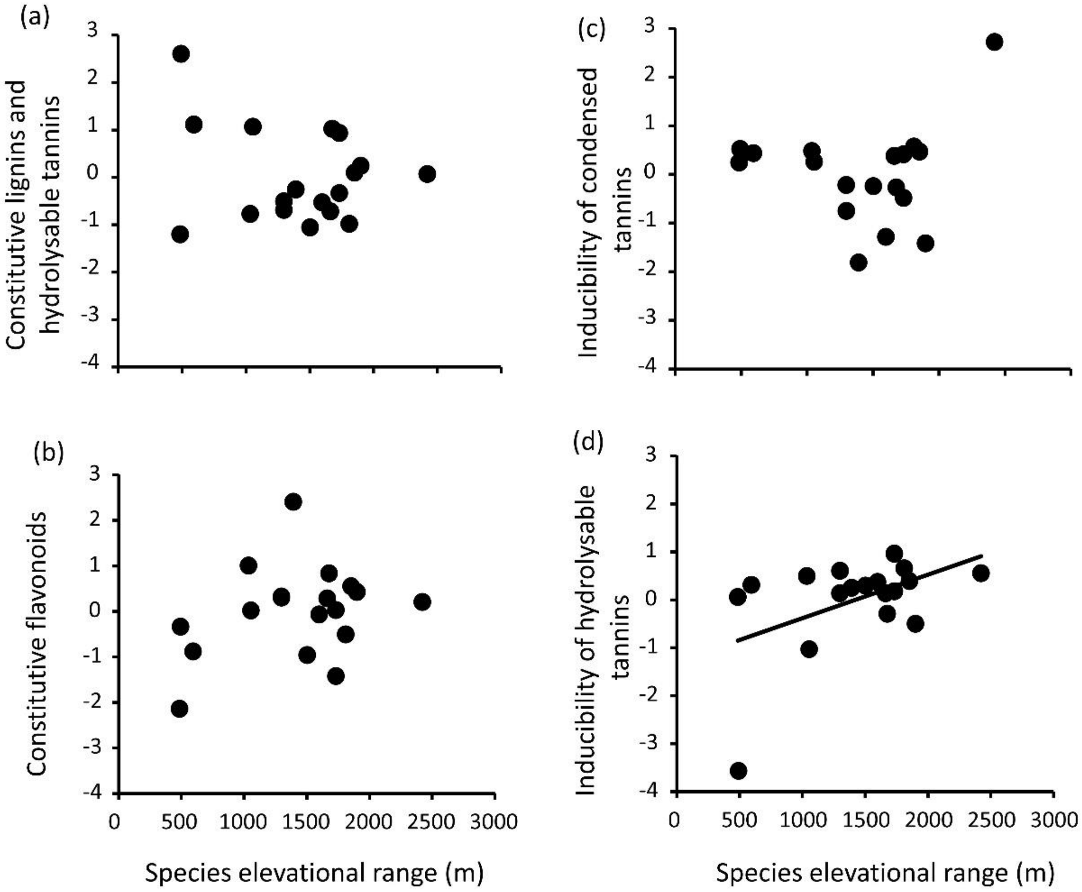

**Figure 2.** Correlations between species elevational range and (a) constitutive lignins and hydrolysable tannins, (b) constitutive flavonoids, (c) inducibility of condensed tannins and (d) inducibility of hydrolysable tannins in leaves of one-year-old trees from 18 *Quercus* species. Constitutive phenolics and their inducibility were grouped based upon a principal components analysis so the y axis shows the standardized *z*-scores of the first two principal components from each analysis. Black solid line represents a significant correlation (pGLS; $P < 0.05$; see Table 2).

### Elevational gradients in the correlated expression of oak defensive strategies and underlying climatic drivers

The co-inertia analysis showed a marginally significant correlated expression of constitutive chemical defences and their inducibility (P = 0.058). However, we found no significant association between the correlated expression of constitutive chemical defences and their inducibility and species elevational range (Table 2, Fig. 3), and the subsequent model including climatic variables indicated that neither temperature nor precipitation significantly predicted this correlated expression (Table 3).

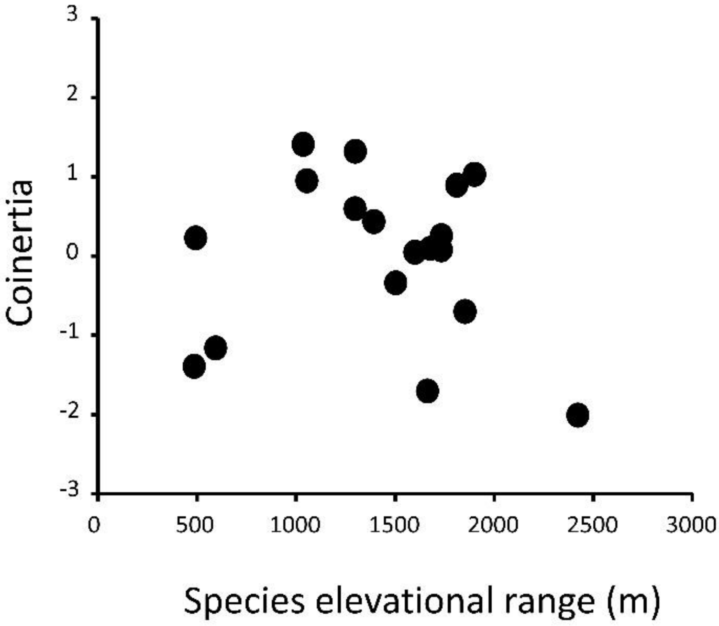

**Figure 3.** Correlation between species elevational range and the correlated expression of constitutive defences and their inducibility (coinertia axis) in one-year-old trees from 18 *Quercus* species.

**Table 2**. Effects of species elevational range on constitutive leaf chemical defences, their inducibility (based upon a principal components analyses summarizing a suite of phenolic compounds) and the correlated expression of constitutive defences and their inducibility (coinertia) in one-year-old trees from 18 Quercus species. Leaf damage score by gypsy moth (Lymantria dispar) was used as a covariate in the inducibility models. Degrees of freedom, student's t-tests (t) and P-values are shown. We accounted for phylogenetic non-independence among Quercus species by performing phylogenetic correct generalized least square analyses (pGLS). Significant coefficients (P < 0.05) are in bold.

| | | Elevational range | | Leaf damage | |
|---|---|---|---|---|---|
| Response variable | DF | *t* | *P*-value | *t* | *P*-value |
| Constitutive lignins and hydrolysable tannins | 1,16 | -1.02 | 0.321 | | |
| Constitutive flavonoids | 1,16 | 0.32 | 0.757 | | |
| Inducibility of condensed tannins | 1,15 | 1.33 | 0.205 | 2.17 | **0.048** |
| Inducibility of hydrolysable tannins | 1,15 | 2.21 | **0.044** | -2.08 | 0.057 |
| Coinertia | 1,16 | -0.38 | 0.710 | | |

**Table 3.** Multiple regressions showing the effects of species elevational range and climate (based upon a principal components analysis summarizing a suite of variables associated to precipitation or temperature) on constitutive leaf chemical defences, their inducibility (based upon a principal components analysis summarizing a suite of phenolic compounds) and correlated expression of constitutive defences and their inducibility (coinertia) in one-year-old trees from 18 *Quercus* species. Leaf damage score by gypsy moth (*Lymantria dispar*) was used as a covariate in the inducibility models. We accounted for phylogenetic non-independence among *Quercus* species by performing phylogenetic correct generalized least square analyses (pGLS). Degrees of freedom, student's t-tests (*t*) and *P*-values are shown. Significant coefficients (*P* < 0.05) are in bold. Marginally-significant coefficients (0.05 < *P* < 0.10) are in italics.

| | | Elevational range | | Leaf damage | | Temperature | | Precipitation | |
|---|---|---|---|---|---|---|---|---|---|
| Response variable | DF | *t* | *P*-value | *t* | *P*-value | *t* | *P*-value | *t* | *P*-value |
| Constitutive lignins and hydrolysable tannins | 1,14 | -1.26 | 0.227 | | | 0.99 | 0.337 | -0.66 | 0.517 |
| Constitutive flavonoids | 1,14 | 0.65 | 0.528 | | | -0.35 | 0.733 | 1.18 | 0.258 |
| Inducibility of condensed tannins | 1,13 | 0.37 | 0.721 | 1.71 | 0.113 | 3.83 | **0.002** | 1.93 | *0.078* |
| Inducibility of hydrolysable tannins | 1,13 | 2.05 | *0.062* | -1.84 | *0.091* | -0.39 | 0.707 | -0.22 | 0.830 |
| Coinertia | 1,14 | 0.10 | 0.924 | | | -1.79 | 0.096 | 0.69 | 0.501 |

## DISCUSSION

By studying multiple defensive traits and strategies and their underlying climatic drivers, this work provides several important insights into the nature of elevational gradients in oak defence. First, we found evidence for an elevational gradient in the inducibility of leaf chemical defences, but not in constitutive chemical defences nor in the correlated expression of constitutive chemical defences and their inducibility. In the former case, the inducibility of leaf hydrolysable tannins was stronger in species with high elevational ranges. Second, the observed elevational gradient in the inducibility of chemical defences remained mostly unchanged after accounting for temperature and precipitation variables, suggesting that climate does not underlie such gradient. Overall, our study provides a deeper understanding of the underlying factors and processes behind elevational patterns in plant defences and, in doing so, builds towards a more robust understanding of biogeographical patterns of plant-herbivore interactions.

It is often assumed that plant-herbivore interactions are stronger towards warmer and more stable climates found at lower elevations, and this selects for higher plant constitutive defences at low relative to high elevations (Hegland et al. 2009, Rasmann et al. 2014b). In contrast to this commonly held view, we found no evidence for elevational gradients in constitutive leaf phenolics across the studied oak species. One plausible explanation for our findings might be that, in order to increase seedling establishment, young oak trees should maximize the production of constitutive defensive traits regardless of their elevation. Constitutive defences are the first barrier to herbivory and are considered more costly to produce than induced defences (Franceschi et al. 2005), so that they should be maximized during the earliest and most vulnerable stages of plant and leaf growth (Boege and Marquis 2005). Although leaf phenolics are thought to be a primary defence in oaks, our results do not preclude elevational gradients in other types of defences including chemical (e.g., terpenes) and physical (e.g., toughness, trichomes) defences, as well as leaf nutrients (e.g., nitrogen, phosphorus).

Our study is one of the first showing an elevational gradient in defence inducibility. We found that oak species with high elevational ranges exhibited a greater inducibility of hydrolysable tannins. This result agrees with Rasmann et al. (2014c) who found that *Vicia faba* plants growing at high elevations displayed increased inducibility of volatile organic compounds, an indirect defence that facilitates natural enemy recruitment. In contrast, Pellissier et al. (2016)

found that inducibility of glucosinolates (a chemical anti-herbivore defence) in *Cardamine* species increased towards lower elevations, whereas Moreira et al. (2014) found no elevational gradients in the inducibility of chemical defences (resin and phenolics) in pine species. Multiple ecological factors may explain these discrepancies among studies, including variation in the strength of trade-offs between defensive strategies shaping the relative strength and direction in constitutive vs. induced defence gradients. In oaks, our results indicate that these two defensive strategies did not trade-off but rather they are expressed simultaneously along the elevational gradients. These findings suggest that resource allocation constraints, or negative co-variance between constitutive and induced defences, are not influencing elevational gradients in phenolic compounds across the studied oak species.

The realization that plant defensive phenotypes are multivariate in nature has clarified how biotic and abiotic factors shape the concurrent expression of multiple defensive traits and mechanisms. However, few studies to date have tested for elevational gradients in the correlated expression of different defensive traits or strategies (Pellissier et al. 2016, Defossez et al. 2018). In one of these studies, Pellissier et al. (2016) found that direct and indirect defences in *Cardamine* species were positively correlated and that their co-expression was stronger at lower elevations. The authors argued that higher herbivore pressure at lower elevations has selected for multiple defences such that synergistic or complementary interactions between defensive strategies provides the most effective means of resistance (Pellissier et al. 2016). In our case, we found positively correlated expression of constitutive chemical defences and their inducibility (i.e., no trade-offs) in oak species, however this correlated expression was not significantly associated with species elevational range (Rasmann and Agrawal 2011). This finding is surprising because we expect that trees should have increased the correlated expression of both strategies at low elevation where herbivore diversity and levels of herbivory would presumably be higher (Moreira et al. 2018a). A robust characterization of oak-associated herbivore communities and herbivory levels across elevations would help to clarify the drivers of this pattern.

Recent studies have demonstrated that climatic correlates of elevation may shape elevational gradients in herbivory and plant defences (e.g., Moreira et al. 2014, Pellissier et al. 2014, Abdala-Roberts et al. 2016). However, we found that temperature and precipitation did not explain the observed elevational gradient in the inducibility of chemical defences. In particular, our results showed that species elevational range still (marginally) predicted plant defence allocation after accounting for these climatic factors, suggesting the gradient was not underlain by the climatic conditions that often change most dramatically over elevation—temperature and precipitation. Other abiotic factors such as soil characteristics may be important drivers of elevational gradients in plant defences. For instance, Pellissier et al. (2016) found that soil fertility partially explained elevational gradients in plant chemical defences in *Cardamine* species, whereby plant species adapted to nutrient-poor soils were better defended. In another study, De Long et al. (2016) reported that soil nitrogen availability drove elevational gradients in plant community-level chemical defences in a subartic tundra heath, whereby plant species growing in soils with low nitrogen concentration had higher defence levels. Overall, although correlative approaches have contributed to our understanding the concurrent effects of abiotic factors, these studies—including our own work (Moreira et al. 2014, Pellissier et al. 2016)—are inherently limited in assigning causality. Experimental manipulations of candidate abiotic drivers are ultimately needed to disentangle their effects on plant-herbivore systems (Pellissier et al. 2014).

In conclusion, our findings emphasize the value of measuring multiple types of defensive traits and strategies along ecological gradients as a mean for understanding the drivers of plant biotic resistance. The observed patterns of variation across studied oak species suggest that the presence and magnitude of elevational gradients in plant defences are contingent upon the defensive strategy under study: we find that inducibility of defences varies with species elevational range, but not constitutive defences. Surprisingly, climatic factors appeared not to underlie the elevational gradient in induced defences, suggesting other sources of abiotic (or biotic) variation are at work and should be investigated.

# CHAPTER IV

# Elevational gradients in constitutive and induced oak defences based on individual traits and their correlated expression patterns

That give rise to the publication Galmán, A., Abdala-Roberts, L., Wartalska, P., Covelo, F., Röder, G., Szenteczki, M., Moreira, X., Rasmann, S. (2020). Elevational gradients in constitutive and induced oak defences based on individual traits and their correlated expression patterns. Oikos (accepted pending minor revisions).


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# CHAPTER IV:

# Elevational gradients in constitutive and induced oak defences based on individual traits and their correlated expression patterns

## ABSTRACT

Elevational gradients are useful ecological settings for revealing the biotic and abiotic drivers of plant trait variation and plant-insect interactions. However, few studies to date have correlated the expression of multiple plant defensive traits and their associated abiotic drivers. This is a crucial task, given the multivariate nature of plant defence and its contingency on abiotic forcing. To address this knowledge gap, we studied elevational variation in direct (phenolic compounds) and indirect (volatile organic compounds) constitutive defences and their inducibility after feeding by a specialist beetle (*Altica quercetorum*) in saplings of 18 wild populations of *Quercus pyrenaica*. We tested for: (1) clines in each defensive trait individually, (2) their patterns of correlated expression, and (3) associations between any clines and climatic correlates. We found that constitutive direct defences (lignins and hydrolysable tannins) decreased with increasing elevation, whereas we observed no elevational pattern for indirect defences (VOCs). Additionally, we found no elevational clines for the inducibility of either direct or indirect defensive traits. However, our analysis of trait correlated expression patterns indicated a significant association between induced and constitutive phenolics, which shifted from co-expression at low elevations to a negative correlation at high elevations. Climatic correlates of elevation do not appear to explain these patterns in direct defences or trait co-expression patterns. Taken together, our findings represent a first step in moving beyond elevational clines in individual plant defences, toward revealing novel evidence for elevational shifts in the nature of correlated expression of plant defences.

Keywords: *Altica quercetorum*, defence syndromes, northwestern Spain, phenolics, *Quercus pyrenaica*, volatile organic compounds

## INTRODUCTION

Ecological gradients are potent tools for understanding how the environment shapes species traits, communities, and ecosystem function (Dobzhansky 1950, Schemske et al. 2009). Changing environmental conditions along large-scale ecological gradients, such as along elevation, are characterized by variation in biotic (e.g. species abundances or composition) and abiotic (e.g. temperature, irradiance) factors, which shape clinal variation in species interactions and traits (Schemske et al. 2009, Rasmann et al. 2014a). Within this context, higher abundance and diversity of insect herbivores (leading to greater herbivore pressure) toward warmer and more stable climatic conditions, such as those found at lower elevations, is expected to select for greater investment in plant defences (Scheidel and Bruelheide 2001, Rasmann et al. 2014b, Pellissier et al. 2016, Galmán et al. 2018, Moreira et al. 2018b). Despite multiple layers of empirical support for this prediction in early studies (Zehnder et al. 2009, Garibaldi et al. 2011, Pellissier et al. 2012), recent studies have defied this paradigm by reporting either no evidence for decreasing interaction intensity with elevation, greater herbivory and defences at intermediate elevations, or even inverse gradients where plant defences and herbivory increase with elevation (reviewed in Rasmann et al. 2014a, Moreira et al. 2018a). Consequently, there is a need to resolve inconsistencies along elevation gradients of plant-herbivore interactions, in order to identify common generative mechanisms.

To date, most elevational studies have looked at variation in single plant defensive traits (Moreira et al. 2018a). A key gap to fill in the study of plant defence variation along ecological gradients is therefore the integration of multiple defensive traits. These include direct (traits that deter or reduce herbivore feeding) and indirect (traits that recruit natural enemies of herbivores) defences, as well as a separation of constitutive (basal levels in the absence of attack) and induced (increased levels in response to attack) trait expression levels (Karban 2011). Studying multiple traits and variation in their expression levels is therefore necessary to better describe plant defensive responses, but importantly it may also expose larger patterns in trait clines, revealing underlying trait correlations. These trait associations may be positive, leading to co-expression patterns (Pellissier et al. 2016), or negative, which often result from underlying allocation constraints (i.e. trade-offs between traits; Agrawal and Hastings 2019).

Notably, recent research indicates that elevational gradients in trait co-expression can arise and are presumably ecologically important (Defossez et al. 2018, Kergunteuil et al. 2018). Changes in herbivore abundance or diversity with elevation may result in distinct patterns of plant defence co-expression, whereby more diverse and functionally complex insect communities found at lower elevations could select for a broader repertoire of simultaneously expressed direct or indirect defensive traits compared to higher elevations (Pellissier et al. 2016). Alternatively, elevational shifts in trophic structure (e.g. ratio of predator to herbivore abundance; Godschalx et al. 2019) or complexity (three- vs. two-trophic level chains; Abdala-Roberts et al. 2019) might modulate differential patterns of allocation to direct and indirect defences. Greater herbivore and predator pressure at low elevations would favour increased plant allocation to both direct and indirect defences, whereas reduced predator to herbivore abundances or predator-less environments at high elevations would favour only direct plant defences (Rasmann et al. 2014a, Moreira et al. 2018a, Godschalx et al. 2019). Overall, the consideration of multiple traits and their co-expression patterns is therefore an essential aspect for understanding how plant defences evolve along environmental clines.

Another important gap is that, despite the implicit importance of abiotic variation in driving changes in species traits and interactions along elevation, studies have usually not conducted formal evaluations of these environmental correlates. Abiotic factors can alter plant defence expression along clines by affecting herbivore abundance or diversity (and thus pressure), as well as when traits play a role in abiotic tolerance or their expression levels are contingent on environmental conditions such as resource availability via production costs (Johnson 2011, Abdala-Roberts et al. 2016, Moreira et al. 2018b). In the latter case, abiotic factors can impose costs on the expression of multiple traits, particularly when there are allocation constraints between defensive traits which can shape their co-expression along elevational gradients (Rasmann et al. 2014a, Moreira et al. 2018a). For instance, traits may be co-expressed under benign conditions but not when plants are under abiotic stress (e.g. due to allocation constraints); the former case is expected for low elevations, while the latter is expected in more constraining environments found at high elevations (Moreira et al. 2018a). Likewise, increasing abiotic stress toward higher elevations may also exacerbate negative correlations between traits.

For example, harsher conditions at high elevations should select for increased constitutive defences but reduced inducibility, as the cost of replacing consumed plant tissues is higher under limiting conditions (Coley et al. 1985, Endara and Coley 2011). This pattern has been supported by a few recent elevational studies (Moreira et al. 2014, Pellissier et al. 2016). Overall, these studies show that the explicit evaluation of the abiotic variables is essential for understanding how climate shapes elevational clines in the expression of multiple plant defence traits.

In this study, we tested for elevational gradients in constitutive direct and indirect chemical defences and their inducibility (i.e. differences between induced and constitutive levels of defences), as well as in the simultaneous expression of these defensive traits, of the Pyrenean oak (*Quercus pyrenaica*, Fagaceae). We also investigated whether climatic correlates of elevation were associated with any such clinal patterns in plant defences. Specifically, we asked: (i) are there elevational gradients in constitutive and induced direct and indirect defences? (ii) Are there emergent patterns of correlated trait expression, and do these exhibit elevational clines? And (iii) are climatic correlates of elevation (namely, precipitation and temperature) associated with concomitant clines in single defensive traits and their correlated expression patterns? To address these questions, we sampled 18 populations of *Q. pyrenaica* distributed along a 1300 m-elevational gradient and quantified constitutive direct (phenolic compounds) and indirect (volatiles, VOCs) defences in leaves and their inducibility after *in situ* manipulation of feeding by the specialist oak flea beetle (*Altica quercetorum*, Coleoptera: Chrysomelidae). Taken together, these results help to fill key gaps in our understanding of abiotic drivers of elevational clines in plant defence co-expression.

## MATERIALS AND METHODS

### Natural history

The Pyrenean oak *Quercus pyrenaica* is a deciduous tree native to southwestern Europe and northwestern Africa, which grows from ca. 400 m up to 1800 m above sea level. A number of specialist and generalist insect herbivores attack *Q. pyrenaica* across its distribution range, many of which are leaf chewers and skeletonizers. Among these, the specialist oak flea beetle *A. quercetorum* is one of the most destructive pests of oak trees in southwestern Europe

(Mijuskovic 1980, Fernandez de Ana et al. 1996), and is also an important herbivore associated with *Q. pyrenaica*. This species is a foliar skeletonizer, which reaches peak abundances on *Q. pyrenaica* at the end of the summer season (mid to late August).

*Q. pyrenaica* produces secondary metabolites in leaves, which are potentially important direct defensive barriers against herbivores. Among these, phenolic compounds have been shown to be toxic for a broad range of phytophagous insects (Feeny 1970, Pearse and Hipp 2012, Moreira et al. 2018b). Oak species have also been shown to produce volatile organic compounds (VOCs) which attract natural enemies of herbivores (i.e. indirect defences; (Ghirardo et al. 2012, Pearse et al. 2013). *Quercus pyrenaica* also produce and emit VOCs (data from this study, but see also (Ramilo et al. 2017), although their defensive role has not yet been demonstrated. Both direct and indirect defences in oaks have been shown to be inducible in response to herbivore damage (Moreira et al. 2018c, Galmán et al. 2019).

### Field sampling and induction of plant defences

At the end of the growing season (late August to early September), before leaf senescence, we sampled 18 *Q. pyrenaica* populations spanning a 1300 m elevational gradient (from 370 to 1614 m, Table S1 in the Appendix D) in the mountains of Ancares (Galicia, North-western Spain). The sampled gradient covers most of the elevational gradient experienced by this oak species throughout its distribution range. Each population included at least 10 adult oak trees, and within each one we selected six saplings (mean height = 70.5 ± 18.6 cm) with little to no evidence of herbivory, separated by a minimum of 2 m. We sampled plants with little evidence of leaf herbivory so that leaf defence levels should largely represent constitutive levels of defence expression, thus also serving as baseline to later measure inducible levels.

After selecting saplings, we randomly assigned half of them at each site to one of two treatments: (i) herbivore induction with *A. quercertorum* larvae or (ii) no induction (control). We obtained *A. quercertorum* larvae from a population of *Q. robur* situated 200 km away from our study area. For both control and induced saplings, we selected a branch with three or four fully expanded leaves. Although herbivory levels were absent or low in most cases for selected leaves, we estimated percent leaf area removed (mainly by miners and leaf chewers) prior to treatment application to account for pre-existing herbivory, which may influence defence levels due to site-specific induction. For this, we used the following scale: 0 = undamaged; 1 = 1–5% damaged; 2 = 6–10% damaged; 3 = 11–25% damaged; 4 = 26–50% damaged; 5 = 51–75% damaged; 6 = >75% damaged. For each beetle-induced sapling, we placed one third-instar larva on each leaf, and individually covered all leaves with a nylon bag to prevent herbivore dispersal or predation. For control saplings, we also covered the chosen leaves with a nylon bag but without adding the larvae to control for the effect of bagging. Larvae were allowed to feed for two days, after which they were removed (with the nylon bags) and percent leaf area removed by the larvae was visually estimated, using the previously described scale. Prior baseline damage and the damage caused by the beetle larvae are clearly distinguishable, as the latter produces a characteristic leaf skeletonization pattern, whereas previous damage was caused mainly by leaf chewers and miners. Shortly after removing the beetle larvae we collected leaf VOCs following Rasmann et al. (2011). Briefly, to collect VOCs, we bagged the branches of the treatment leaves with a 1 L Nalophan bag, and we trapped VOCs on a charcoal filter (ANASORB CSC; SKC, Eighty-Four, PA, USA) for 3 h at a rate of 250 mL min$^{-1}$. After VOC collection, we stored all traps at -80 ºC until chromatographic analyses. Right after VOCs collection, we collected the same leaves to quantify leaf phenolic compounds (see ahead). Leaves were oven-dried for 48 h at 40°C.

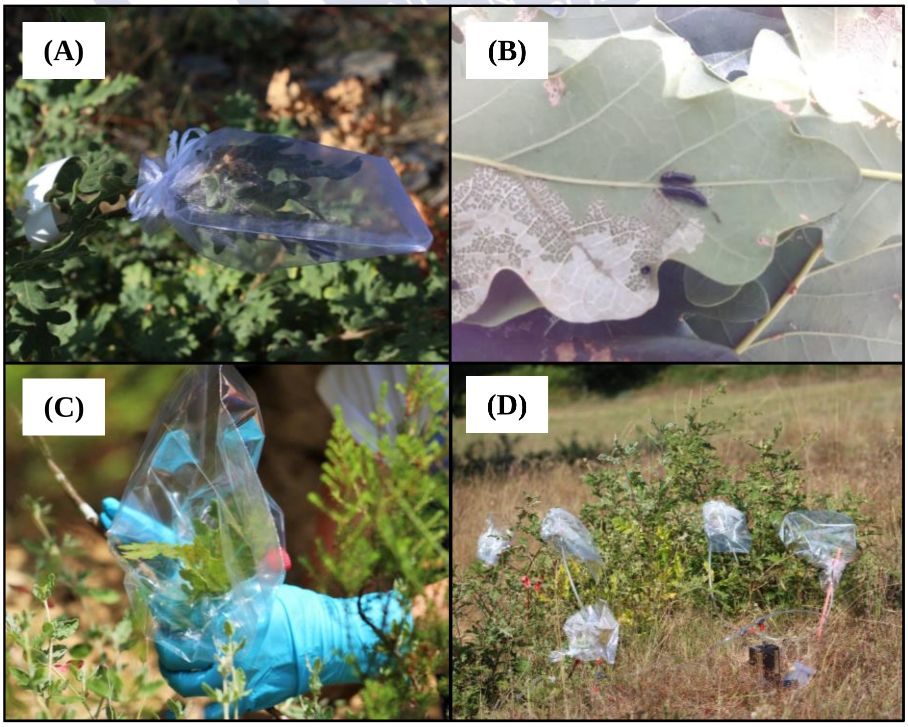

**Image 1**. (A) Leaves covered with a nylon bag, (B) leaves damaged by Altica quercetorum, (C) branch bagged with a Nalophan bag, (D) volatile sampling.

### Characterization and quantification of volatile organic compounds

To perform chromatographic analyses of leaf VOCs, we first added 5 µL of Naphthalene (20 ng/ml) as an internal standard (Pellissier et al. 2016) to the charcoal traps, and then eluted their contents with 400 µL of dichloromethane (CAS#75-09-2, Merck, Dietikon, Switzerland). We then injected 2 µL of the extract for each sample into an Agilent 6890 Gas Chromatograph (GC) (Agilent, Santa Clara, CA, USA) coupled with a 5973 Mass Selective Detector fitted with a 30 m × 0.25 mm × 0.25 mm film thickness HP-5MS fused silica column (Agilent, Santa Clara, California, United States). We operated the GC in splitless mode with helium as the carrier gas (flow rate 0.9 mL min$^{-1}$). The GC oven temperature program was: 1 min hold at 40 °C, and then 10 °C min-1 ramp to 240 °C. We identified volatile terpenes using Kovats retention index from published work (Table S2 in the Appendix D the NIST Standard Reference Database 1A v17, and by comparison with commercial standards when available. We identified two groups of VOCs: monoterpenes and sesquiterpenes (Table S2) for subsequent analyses. Finally, we quantified individual VOCs as a proportion to the internal standards.

### Characterization and quantification of phenolic compounds

We extracted phenolic compounds from 20 mg of dry leaf tissue with 1 mL of 70% methanol in an ultrasonic bath for 15 min, followed by centrifugation (Galmán et al. 2019b). We then transferred the extracts to chromatographic vials. To perform the chromatographic analyses, we used Ultra-High-Performance Liquid-Chromatograph (UHPLC Nexera LC-30AD; Shimadzu Corporation, Kyoto, Japan) equipped with a Nexera SIL-30AC injector and one SPD-M20A UV/VIS photodiode array detector. The compound separation was carried out on a Kinetex™ 2.6 µm C18 82-102 Å, LC Column 100 × 4.6 mm (Phenomenex, Torrance, CA, USA), protected with a C18 guard cartridge. The flow rate was 0.4 mL min-1 and the oven temperature was set at 25 ºC. The mobile phase consisted of two solvents: water-formic acid (0.05%) (A) and acetonitrile-formic acid (0.05%) (B), starting with 5% B and using a gradient to obtain 30% B at 4 min, 60% B at 10 min, 80% B at 13 min and 100 % B at 15 min. The injection volume was 15 µL.

For phenolic compound identification, we used an ultra-performance liquid chromatography coupled with electrospray ionization quadrupole (Thermo Dionex Ultimate 3000 LC; Thermo Fisher Scientific, Waltham, MA, USA) time-of-flight mass spectrometry

(UPLC-Q-TOF-MS/MS; Bruker Compact™, Bruker Corporation, Billerica, MA, USA). We identified four groups of phenolic compounds: flavonoids, ellagitannins and gallic acid derivates ("hydrolysable tannins" hereafter), proanthocyanidins ("condensed tannins" hereafter) and hydroxycinnamic acid precursors to lignins ("lignins" hereafter). We quantified flavonoids as rutin equivalents, condensed tannins as catechin equivalents, hydrolysable tannins as gallic acid equivalents, and lignins as ferulic acid equivalents (Galmán et al. 2019, Moreira et al. 2020). We achieved the quantification of these phenolic compounds by external calibration using calibration curves at 0.25, 0.5, 1, 2 and 5 µg $mL^{-1}$. We expressed phenolic compound concentrations in mg $g^{-1}$ tissue on a dry weight basis.

### Climatic variables

To estimate climatic conditions at sampling site, we extracted a subset of eight climatic variables from the WorldClim database (Hijmans et al. 2005) at a resolution of 30-arc-second for the populations coordinates. Specifically, we selected BIO1 (annual mean temperature, °C), BIO4 (temperature seasonality, expressed as the standard deviation of temperature among months × 100), BIO5 (maximum temperature of the warmest month, °C), BIO6 (minimum temperature of the coldest month, °C), BIO12 (annual precipitation, mm), BIO13 (precipitation of the wettest month, mm), BIO14 (precipitation of the driest month, mm), BIO15 (precipitation seasonality, expressed as standard deviation of precipitation across months) (Abdala-Roberts et al. 2016). We summarized these variables by conducting a principal component analyses (PCA) using the prcomp function in R ver. 3.4.1 (R Core Team 2019). The first principal component ("PC climate" hereafter) explained 89% of the variance in the climatic variables across populations and was positively associated with annual mean temperature, maximum temperature of the warmest month, and minimum temperature of the coldest month but negatively associated with annual precipitation, precipitation of the wettest month, and precipitation of the driest month (Fig. S1 in the Appendix D).

### Statistical analyses

#### *Elevational gradients in constitutive and induced defences and their climatic correlates*

First, to investigate whether there were elevational gradients in constitutive direct and indirect defences and their inducibility, we performed population-level multiple regressions (i.e. using population means) using the *lm* function in the *lme4* package in R ver. 3.4.1 (R Core Team 2019). Specifically, we tested for the effect of elevation on constitutive phenolics and VOCs (i.e. using only control plants), as well as their inducibility, estimated as the mean level for induced plants minus the mean level for control plants at each site. For both constitutive and induced levels, we ran separate models for each group of phenolic compounds (condensed tannins, hydrolysable tannins, flavonoids and lignins) and VOCs (monoterpenes and sesquiterpenes), as well as for total phenolics and total VOCs. In each of these models, we also included leaf herbivory to account for variation in natural herbivory and the amount of experimental damage caused by larvae. For control saplings, leaf herbivory corresponded to damage before establishing herbivory treatments (as bagging prevented any further damage during the experiment). For herbivore-induced saplings, herbivory represented damage prior to establishing the experiment plus the damage caused by *A. quercetorum* larvae due to the induction experiment.

We also ran linear mixed models testing for the effect of elevation, herbivore treatment, their interaction, and leaf herbivory (fixed factors) on direct and indirect defences using data at individual tree level. Each of these models included population as a random factor. Results were very similar to those found using population means (Table S3 in the Appendix D), and we therefore only report results for population-level analyses in the main manuscript to be consistent with the test of climatic correlates which was only possible at the population level (detailed below).

To investigate the influence of climatic correlates of elevational variation on constitutive direct and indirect defences and their inducibility, in those cases where elevation had a significant effect on a trait (or totals for a given trait type), we re-ran the above multivariate regressions. We kept elevation and herbivory as predictors, but now also included the first principal component of the PCA of climatic variables (PC climate, see above) as predictor. If a significant effect of elevation in the former regression model for a trait (or totals) turned non-significant after accounting for climatic variation, this would suggest that climate accounts for (i.e. underlies) the elevational gradient in such trait.

### *Elevational gradients in trait co-expression patterns*

We tested for correlated trait expression patterns by means of series co-inertia analyses using population means, using the *ade*4 package in R (Dray et al. 2003, Dray and Dufour 2007). These analyses tested whether matrices of constitutive and induced direct and indirect defences using individual compound values were correlated across oak populations (Pellissier et al. 2016, Defossez et al. 2018). First, we assessed whether there was correlated expression between constitutive and induced trait levels, separately for each type of trait (direct and indirect). Specifically, we tested for correlated expression between constitutive and induced phenolics (coinertia test 1) and between constitutive and induced VOCs (coinertia 2). Second, we assessed whether there was correlated expression between direct and indirect defences, separately for constitutive and induced levels. In this case, we tested for correlated expression between constitutive phenolics and constitutive VOCs (coinertia 3), and between induced phenolics and induced VOCs (coinertia 4). When one of the above coinertia tests was significant we proceeded to run a multiple regression with elevation and herbivory as predictors separately for each of the first two axes of the coinertia test. Then, if the effect of elevation was significant for a given axis, we followed the same approach as above and re-ran the model including PC climate, to test whether the pattern of trait correlated expression for a given axis of a coinertia test was underlain by climatic variation.

## RESULTS

### Elevational gradients in defences and their climatic correlates

We found significant effects of elevation on constitutive levels of some groups of phenolic compounds, but not for others or total phenolics (Table 1, Fig. 2, Fig. S2). Specifically, we found significant negative effects of elevation on constitutive lignins and hydrolysable tannins, whereas we observed no effect of elevation for flavonoids or condensed tannins (Table 1, Fig. 2). For indirect defences, we found no significant effect of elevation on the emissions of monoterpenes, sesquiterpenes or total VOCs (Table 1, Fig. 2, Fig. S2). In addition, we found no significant effect of elevation on the inducibility for total phenolics, total VOCs or any of the groups of phenolic compounds or VOCS when analysed separately (Table 1, Fig. 3, Fig. S2).

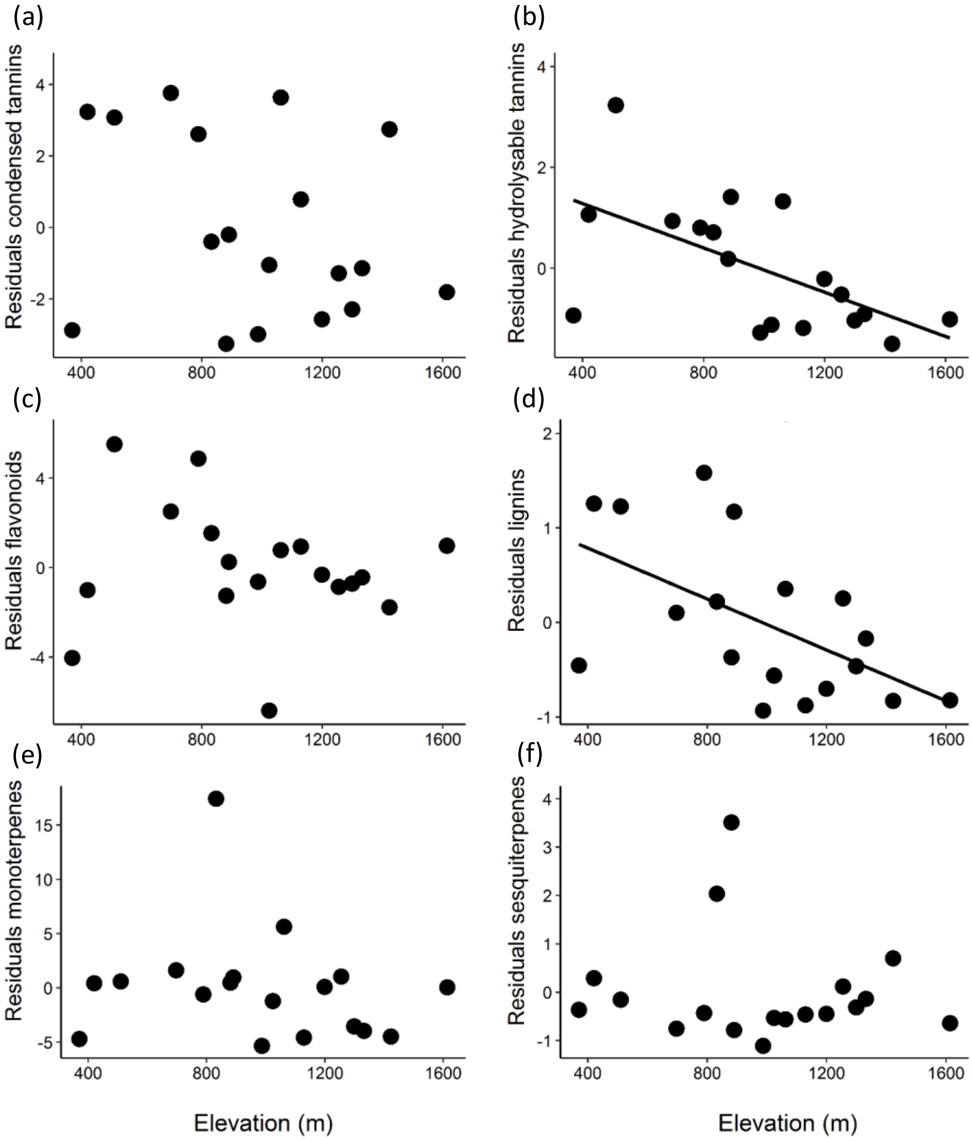

**Figure 2.** Partial residual plots from the multiple regressions, showing the relationships between elevation and constitutive (a) condensed tannins, (b) hydrolysable tannins, (c) flavonoids, (d) monoterpenes and (e) sesquiterpenes in leaves of saplings from *Quercus pyrenaica*. Dots represent *Q. pyrenaica* populations (N = 18). Predicted relationships come from simple linear regressions and the slope of each relationship is the partial correlation coefficient ($r^2$) for the effect of the predictor (X axis) on a given plant trait (Y axis) ($r^2$ values are reported in Table 1). Black solid line represents a significant correlation ($P < 0.05$).

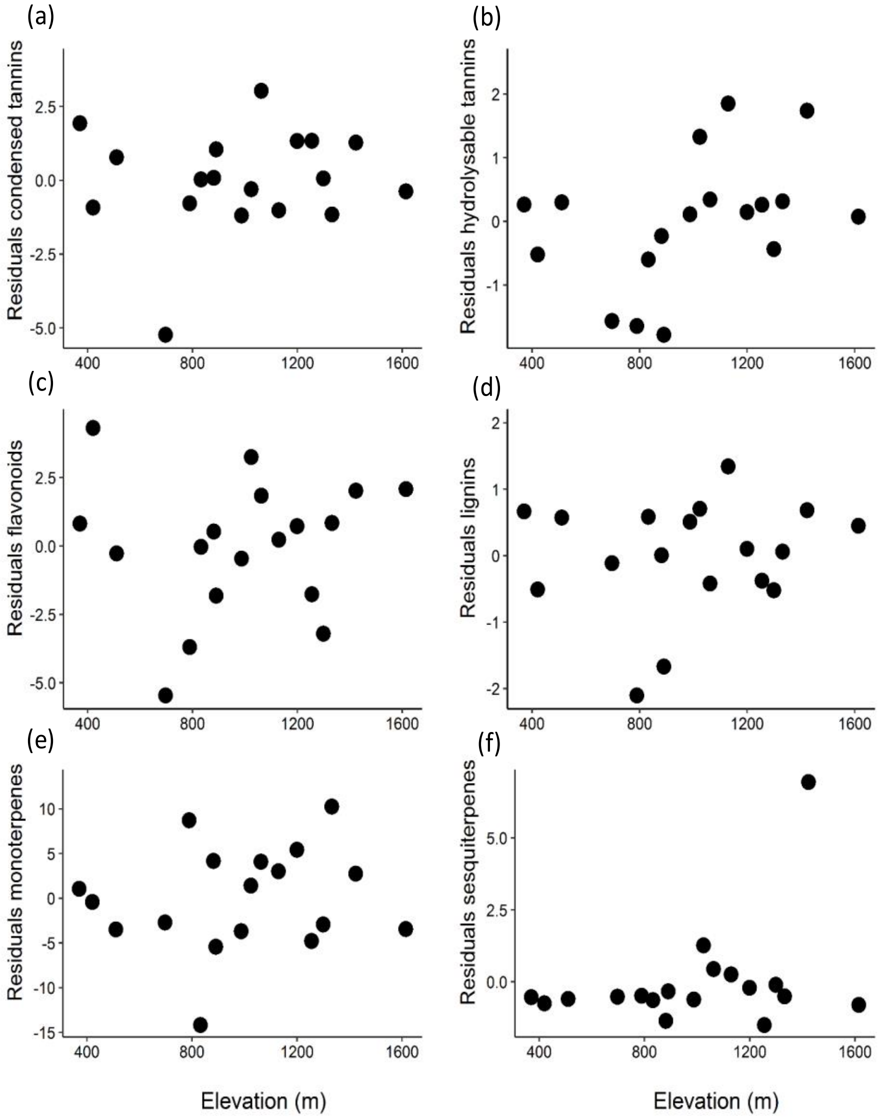

**Figure 3.** Partial residual plots from the multiple regressions, showing the relationships between elevation and inducibility of (a) condensed tannins, (b) hydrolysable tannins, (c) flavonoids, (d) monoterpenes and (e) sesquiterpenes in leaves of saplings from *Quercus pyrenaica*. Dots represent *Q. pyrenaica* populations (N = 18). Predicted relationships come from simple linear regressions and the slope of each relationship is the partial correlation coefficient ($r^2$) for the effect of the predictor (X axis) on a given plant trait (Y axis) ($r^2$ values are reported in Table 1). Black solid line represents a significant correlation ($P < 0.05$).

**Table 1.** Multiple regressions showing the effects of elevation and leaf herbivory on the concentration of constitutive direct and indirect defences and their inducibility, as well as the correlated expression between constitutive and induced phenolics (coinertia 1), in leaves of saplings from 18 *Quercus pyrenaica* populations. β = slope estimator, $r^2$ = partial correlation coefficient. Significant *P*-values ($P < 0.05$) are in bold.

| | Elevation | | | Leaf herbivory | | |
|---|---|---|---|---|---|---|
| | *β* | $r^2$ | *P-value* | *β* | $r^2$ | *P-value* |
| **Constitutive defences** | | | | | | |
| Condensed tannins | -0.29 | 0.08 | 0.262 | -0.22 | 0.05 | 0.388 |
| Hydrolysable tannins | -0.60 | 0.35 | **0.012** | -0.59 | 0.34 | **0.012** |
| Lignins | -0.57 | 0.32 | **0.018** | -0.46 | 0.21 | 0.061 |
| Flavonoids | -0.14 | 0.02 | 0.594 | -0.63 | 0.40 | **0.007** |
| Monoterpenes | -0.17 | 0.03 | 0.517 | -0.11 | 0.01 | 0.685 |
| Sesquiterpenes | -0.09 | 0.01 | 0.725 | -0.27 | 0.08 | 0.286 |
| Total phenolics | -0.38 | 0.14 | 0.134 | -0.56 | 0.31 | **0.019** |
| Total VOCs | -0.16 | 0.03 | 0.532 | -0.09 | 0.01 | 0.717 |
| **Inducibility of defences** | | | | | | |
| Condensed tannins | 0.12 | 0.01 | 0.654 | -0.08 | 0.01 | 0.751 |
| Hydrolysable tannins | 0.39 | 0.15 | 0.119 | 0.01 | <0.01 | 0.978 |
| Lignins | 0.14 | 0.02 | 0.596 | 0.04 | 0.01 | 0.889 |
| Flavonoids | 0.08 | 0.01 | 0.771 | 0.01 | <0.01 | 0.963 |
| Monoterpenes | 0.15 | 0.02 | 0.565 | -0.40 | 0.16 | 0.114 |
| Sesquiterpenes | 0.34 | 0.12 | 0.181 | 0.04 | 0.01 | 0.891 |
| Total phenolics | 0.20 | 0.04 | 0.452 | -0.02 | <0.01 | 0.948 |
| Total VOCs | 0.25 | 0.06 | 0.329 | -0.35 | 0.12 | 0.166 |
| **Correlated expression of constitutive and induced defences** | | | | | | |
| Coinertia 1 | -0.48 | 0.23 | **0.049** | 0.32 | 0.10 | 0.213 |

Our assessment of climatic effects underlying the above significant elevational gradients in plant defences indicated that the effect of elevation on the concentration of constitutive hydrolysable tannins and lignins remained significant after accounting for climate in the models (Table 2), suggesting that climate did not underlie elevational gradients observed for these traits.

### Elevational gradients in the correlated expression of defensive traits

Results from the coinertia analyses indicated a significant association between the correlated expression of constitutive and induced phenolics (coinertia 1, Table 3). We extracted the first two axes from this analysis, whereby both axes were associated with a mixture of constitutive and induced levels of different compounds (see Fig. S3 in the Appendix D). Positive values of both axes indicate co-expression of mixtures of constitutive and induced phenolic compounds, whereas negative values indicate negative correlations between mixtures of constitutive and induced compound levels. In contrast, the other coinertia tests indicated no significant association between the correlated expression of constitutive and induced VOCs (coinertia 2), constitutive phenolics and constitutive VOCs (coinertia 3), or induced phenolics and induced VOCs (coinertia 4) (Table 3).

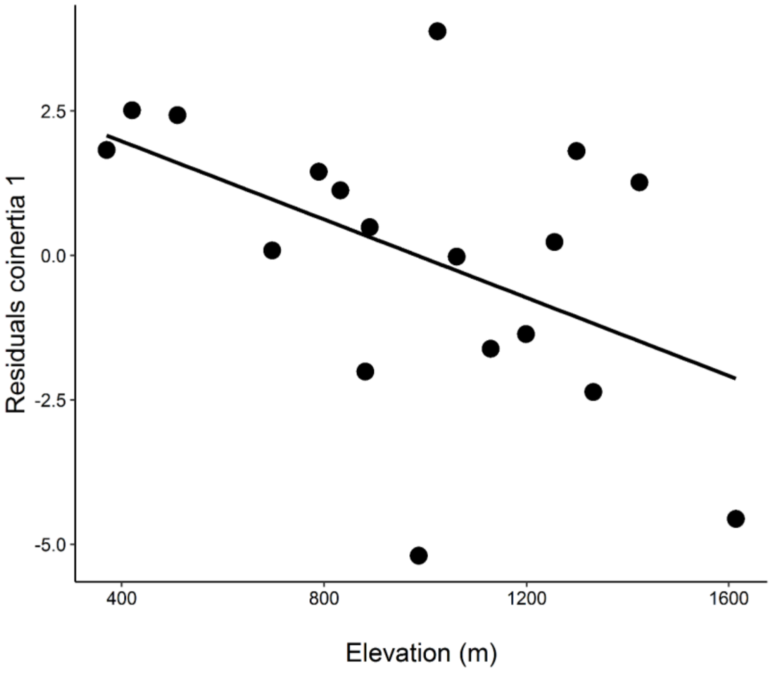

**Figure 4.** Correlation between elevation and the correlated expression of constitutive and induced phenolics (second axis of the coinertia analysis 1; see *Methods*) in leaves of saplings from *Quercus pyrenaica*. Dots represent *Q. pyrenaica* populations (N = 18). Predicted relationship comes from simple linear regression and the slope of each relationship is the partial correlation coefficient ($r^2$) for the effect of the predictor (X axis) on a given plant trait (Y axis) ($r^2$ values are reported in Table 1). Black solid line represents a significant correlation ($P < 0.05$).

Given the above results, we tested for and found a significant negative effect of elevation on the correlated expression of constitutive and induced phenolics for one of the axis of the coinertia 1 (Table 1). This indicates that mixtures of constitutive and induced phenolics were co-expressed at low elevations, whereas at high elevations mixtures of induced and constitutive phenolics were negatively correlated (Fig. 4). The effect of elevation on values for this axis 2 remained significant after accounting for climate in the model (Table 2), suggesting that climatic factors did not underlie the elevational gradient of correlated expression in constitutive and induced phenolics depicted by this axis.

**Table 2.** Multiple regressions showing the effects of elevation, leaf herbivory and climate (based on a principal component analysis summarizing a subset of bioclimatic variables) on constitutive hydrolysable tannins and lignins and the correlated expression between constitutive and induced phenolics (coinertia 1) in leaves of saplings from 18 *Quercus pyrenaica* populations. β = slope estimator, $r^2$ = partial correlation coefficient. Significant *P*-values ($P < 0.05$) are in bold.

| | Elevation | | | Leaf herbivory | | | PC climate | | |
|---|---|---|---|---|---|---|---|---|---|
| | *β* | $r^2$ | *P*-value | *β* | $r^2$ | *P*-value | *β* | $r^2$ | *P*-value |
| C.hydrolysable tannins | -0.003 | 0.38 | **0.010** | -1.668 | 0.37 | **0.012** | -0.274 | 0.06 | 0.374 |
| C.lignins | -0.002 | 0.40 | **0.008** | -0.808 | 0.26 | **0.045** | -0.266 | 0.12 | 0.183 |
| Coinertia 1 | -0.546 | 0.30 | **0.030** | 0.307 | 0.09 | 0.247 | -0.292 | 0.09 | 0.271 |

**Table 3.** Results of the coinertia analyses between constitutive and induced phenolics (coinertia 1), constitutive and induced volatiles (VOCs) (coinertia 2), constitutive phenolics and constitutive VOCs (coinertia 3) and induced phenolics and induced VOCs (coinertia 4). Significant *P*-values ($P < 0.05$) are in bold.

| Coinertia | Defence (coinertia) axis | $P_{Coinertia}$ |
|---|---|---|
| 1 | Constitutive phenolics - Induced phenolics | **0.003** |
| 2 | Constitutive VOCs - Induced VOCs | 0.112 |
| 3 | Constitutive phenolics - Constitutive VOCs | 0.666 |
| 4 | Induced phenolics - Induced VOCs | 0.663 |

## DISCUSSION

Our study found partial evidence for elevational gradients in direct defences in *Q. pyrenaica*, but no elevational variation for indirect defences, or the inducibility of either group of traits. Specifically, we found that constitutive lignins and hydrolysable tannins decreased with increasing elevation. In addition, the coinertia analysis indicated an association between constitutive and induced phenolics. Importantly, this correlated expression pattern was negatively associated with elevation, indicating clinal variation in multi-variate defence expression patterns. Finally, neither single traits nor their correlated expression appeared to be driven by climatic variables.

We found a pattern whereby constitutive lignins and hydrolysable tannins decreased with elevation. However, we found no evidence of an elevational cline in constitutive VOCs for *Q. pyrenaica*, suggesting an absence of elevational clines in predator pressure, or that predator pressure was not strong enough to shape concomitant variation in volatile emissions. Our results for phenolics agree with previous studies showing that constitutive defences decreased with increasing elevation, likely because constitutive defences should be favored at low elevations where herbivore pressure is higher and more consistent (Pellissier et al. 2012, Pellissier et al. 2014, Rasmann et al. 2014c). Our previous studies on *Pinus* spp. and *Cardamine* spp., found the inverse pattern, whereby constitutive defences have been shown to increase with elevation, possibly because the cost of tissue loss and replacement is greater in more limiting environments found at higher elevations (Moreira et al. 2014, Pellissier et al. 2016). Overall, it therefore appears that two mechanisms can lead to contrasting patterns of allocation to direct defences along elevational gradients, one likely driven by low resource availability at high elevations selecting for increased constitutive expression increase (Coley et al. 1985), and the other pattern driven by herbivore pressure favoring greater investment to constitutive defence expression at low elevations. Further work along elevational gradients that explicitly manipulates the effects of resource availability, herbivore and predator pressure, and measures the costs of defence expression is necessary to tease apart these mechanisms across a greater number of plant taxa.

Contrary to expectations, we found no evidence for elevational gradients in either direct or indirect induced defences. This pattern contrasts with the few available studies showing either greater (Rasmann et al. 2014c, Buckley et al. 2019, Galmán et al. 2019) or lower (Moreira et al. 2014) inducibility of plant defences at higher elevations. Again, there appears to be two mechanisms leading to contrasting gradients in these studies. On the one hand, inconsistent or unpredictable herbivore or predator pressure at high elevations would favor inducibility as a cost-saving strategy (Rasmann et al. 2014c). On the other, increasing resource limitation at high elevations would favor constitutive over induced defences due to higher intrinsic costs of tissue replacement (Moreira et al. 2014, Buckley et al. 2019). Neither of these mechanisms appears to be at work for the studied populations of *Q. pyrenaica*. Furthermore, our results contrast with our previous research reporting increasing inducibility with elevation across 18 oak species (including *Q. pyrenaica*) (Galmán et al. 2019), and underscores differential intra- vs. inter-specific clines in plant defences (Moreira et al. 2018a). Intra-specific patterns found for *Q. pyrenaica* therefore suggest that constitutive (direct) defences are the primary means of defence in response to changing biotic and abiotic pressures with elevation.

A few previous studies reporting on contrasting elevational clines in constitutive vs. induced defences have invoked allocation constraints leading to trade-offs as a likely underlying mechanism (reviewed by Moreira et al. 2018a). Likewise, recent studies have also reported on shifts in the co-expression of direct and indirect defences along elevation gradients, presumably resulting from changes in selective forces favoring single vs. simultaneously expressed traits which in turn shape the unfolding of defensive syndromes along elevational gradients (e.g. Pellissier et al. 2016, Defossez et al. 2018). Our coinertia analysis explicitly addressed these trait correlations and uniquely revealed an elevational change in the pattern of correlated expression of induced and constitutive phenolics, whereby these were co-expression at low elevations but negatively correlated at high elevations. Thus, rather than a trade-off in the expression of induced vs. constitutive defence levels that leads to contrasting patterns of expression at low vs. high elevation as reported in previous studies (see Moreira et al. 2018a), we found a shift in the nature of the association between induced and constitutive chemical defences. This finding contrasts with our previous work showing no evidence of an elevational gradient in the correlated expression of induced and constitutive direct defences across 18 oak species (Galmán et al. 2019), again pointing at differences when analyzing intra- vs. inter-specific patterns of correlated expression of plant traits. The only other study we are aware of that addresses these patterns is work by (Pellissier et al. 2016) with *Cardamine* spp. which found increasing co-expression of induced direct and indirect defences towards low elevations, whereas at high elevations constitutive defences were favored. Although comparisons should be made with caution as this study also assessed correlations across (rather than within) species, it is worth noting that their patterns of correlated expression involved both direct and indirect defensive traits, whereas our study revealed associations involving only direct defences (phenolics) for *Q. pyrenaica*. Combined, these results suggest similarities in patterns of clinal variation in constitutive and induced defences, but also differences in the degree of involvement of indirect defences. The latter may presumably come to play in correlated expression patterns for plants or communities that rely more heavily on natural enemies for indirect defence (e.g. Rasmann et al. 2014c), which appears not to be the case for *Q. pyrenaica*.

Previous research has demonstrated that abiotic factors are important drivers of plant defence allocation along elevation gradients (e.g. Moreira et al. 2014, Pellissier et al. 2014, Kergunteuil et al. 2019b). Specifically, these studies found that climatic variables (e.g. temperature and precipitation) correlate with single defensive traits (reviewed by Moreira et al. 2018a) as well as patterns of correlated trait expression (Pellissier et al. 2016, Galmán et al. 2019). Our findings indicated that the observed elevational clines in constitutive levels of phenolic compounds (lignins and hydrolysable tannins), and the correlated expression of constitutive and induced phenolic compounds remained significant after accounting for climatic variables. This suggests that abiotic forcing via climate did not underlie the observed elevational patterns of direct chemical defences in *Q. pyrenaica*. However, these findings are not unique, as evidence from other studies also suggests that climate only partially accounts for elevational gradients in plant defences (Pellissier et al. 2016, Galmán et al. 2018, Galmán et al. 2019). Instead, other abiotic factors such as soil conditions could underlie variation in plant defence across elevations (De Long et al. 2016, Pellissier et al. 2016). For instance, Pellissier et al. (2016) found that soil fertility positively correlates with constitutive glucosinolates in plants of *Cardamine* plant species growing at higher elevations. In another study, De Long et al. (2016) reported that soil nitrogen availability drove elevational gradients in plant community-level chemical defences in a subartic tundra heath. Although correlative approaches such our current study are fruitful for understanding the concurrent effects of abiotic factors (Moreira et al. 2014, Abdala-Roberts et al. 2016, Pellissier et al. 2016, Galmán et al. 2019), they also carry several limitations (see discussion in Abdala-Roberts et al. 2016). Experimental manipulations of candidate abiotic drivers are needed to disentangle their effects on plant-herbivore systems (Pellissier et al. 2014). Ultimately, phenological changes in leaf chemical composition may occur. Depending on the season, the consequences of leaf herbivore for the plant are not the same and the plant may shift between the different defensive strategies. Further studies should therefore investigate the differences in phenology of plant defences across elevation by sampling at the early or mid-season.

Our results emphasize the importance of assessing elevational gradients in multiple rather than single plant defensive traits to achieve a more holistic understanding of clinal variation in plant defence expression. By doing so, we reveal a unique elevational pattern of correlated expression of constitutive and induced chemical defences in *Q. pyrenaica*. It is possible that increased herbivore pressure and/or resources at low elevations favors the simultaneous expression of these strategies, and that increasing resource limitation at high elevations prevents co-expression and leads to trade-offs. Further manipulations of herbivore pressure and resource availability are necessary to disentangle these mechanisms. Additionally, research on the genetic variations underlying patterns of trait-correlated expression is needed to assess the evolutionary implications of our results. Overall, our findings agree with recent evidence from studies pointing at contrasting patterns of correlated expression in plant defensive traits along elevational gradients (Pellissier et al. 2016, Defossez et al. 2018), indicating an emerging component of multi-variate plant defence that should be included in theory of clinal variation in plant defences (Moreira et al. 2018a). Further assessments of indirect defences in *Q. pyrenaica* are also warranted before arriving at conclusions about their importance. Given the pervasive influence of abiotic correlates of elevation on community trophic complexity, our approach should be strongly pursued in future elevational studies measuring multiple defenses.

# GENERAL DISCUSSION

# GENERAL DISCUSSION

## ELEVATIONAL GRADIENTS IN PLANT-HERBIVORE INTERACTIONS ARE CONTINGENT ON PLANT LIFE-HISTORY TRAITS BUT NOT ON PLANT ONTOGENY

In this PhD dissertation, we showed that elevational gradients in leaf herbivory were contingent on plant growth form and leaf habit, but no effect of plant ontogenetic stage was found.

In the Chapter I, we performed a global assessment of elevational gradients in leaf herbivory where we found that clinal variation in leaf herbivory was contingent on growth form and leaf habit. In particular, woody species experienced greater herbivory at low comparing to high elevations, while no elevational gradient was found for herbaceous species. In addition, within woody species, we found that deciduous trees experienced greater herbivory at low comparing to high elevations, but no effect of elevation was found for evergreen species. These findings support the general idea that stronger pressure of herbivores towards low elevations should be more evident for more apparent woody species (Apparency Hypothesis; Feeny 1976, Strauss et al. 2015). Within such woody species, elevational clines should be more pronounced for fast-growing deciduous trees based on a presumably trade-off between growth and defences (Endara and Coley 2011), according to which, deciduous trees would grow faster and be less defended than evergreen trees. Our study is within the few, if not the only one, addressing the effect of growth form and leaf habit on clinal variation in herbivory (but see Fernandes and Price 1988, Rasmann et al. 2014b, Lim et al. 2015).The value of our findings could be reinforced by performing a complementary global analysis assessing the variability of plant defences along geographic gradients in order to explore a causative relation between levels of herbivory and levels of plant defences.

The global pattern of variation in leaf herbivory reported for woody species was not reproducible at smaller scale when studying natural populations of eight species of *Quercus* (as shown in the Chapter II). In this case, we found that leaf herbivory increased toward higher elevations. Our results revealed that ontogeny did not account for the variability in herbivory along the gradient. Contrary to predictions that adult trees should be more detectable for herbivores (Feeny 1976, Strauss et al. 2015), we found no effect of plant ontogeny on herbivory, and elevational differences were similar for adult trees and saplings. In the Chapter II, we also detected an elevational gradient in oak defences where, counter to predictions, chemical defences were higher at high elevations. Although we documented increased levels of chemical defences for saplings comparing to adult trees, ontogenetic stage did not account for the clinal variation on the studied defences along the gradient. Counter to expected patterns, chemical defences increased at higher elevations for both saplings and adult trees. Greater herbivory and plant defences at higher elevations has been commonly observed in previous literature (Moreira et al. 2018a), suggesting that a generalised clinal patterns in defences or herbivory may not suit the different study systems. The direction and the strength of the gradient may vary in response to differences in the spatial scale covered by the geographic gradient studied or due to changes in the taxonomic scale from multispecific level to genus or even species level. Further ontogenetic assessments including the seedling stage as well as several intermediate ontogenetic stages are needed to more robustly test how plant ontogenetic stage shapes the interactions between plants and their herbivores (see Quintero et al. 2013).

## PLANTS DEPLOY MULTIPLE DEFENSIVE STRATEGIES ALONG ELEVATIONAL GRADIENTS

In this PhD dissertation, we showed that the direction and strength of the elevational gradient in oak defences vary depending on the defensive strategy studied. In addition, the patterns were also different when studying individual traits or the co-expression patterns between different traits. These findings evidence the existence of shifts in the allocation to different defences by plants depending on the environmental conditions. Contrary to predictions, in the Chapter III we found no effect of elevation in constitutive direct defences (phenolic compounds) for the 18 oak species sampled. However, in the Chapter IV we found the predicted pattern of clinal variation when studying the same defences at intraspecific level using 18 populations of *Quercus pyrenaica*. In particular, levels of constitutive phenolic increased toward lower elevations, in line with the prediction that constitutive defences should be favoured at low elevations where herbivore pressure is higher and more consistent (Pellissier et al. 2012, Pellissier et al. 2014, Rasmann et al. 2014c). Similarly, we found differences in the patterns of expression of induced phenolic compounds between both studies. In line with the expectation that species with higher elevational ranges favour cost-saving strategies, in the Chapter III we found a positive association between inducibility of phenolic compounds and elevation for the 18 oak species used. However, in the Chapter IV no clinal variation was found for the inducibility of the same compounds in the 18 populations of *Q. pyrenaica* sampled, evidencing again that fundamental physiological and ecological differences exist when studying intra- versus inter-specific patterns (Agrawal et al. 2010).

In the Chapter IV, we also addressed the patterns of clinal variation in indirect defences, a trait commonly overlook along geographic gradients (see Godschalx et al. 2019). Specifically, we explored the role of the third trophic level over herbivore pressure by measuring the emission of constitutive and induced leaf volatile organic compounds in the surrounding headspace. We expected that plants at low elevations would invest more in indirect defences to attract natural enemies of insect herbivores, since this strategy should be more efficient in environments with high abundance of predators and parasitoids (Rasmann et al. 2014a). Counter to predictions, we found no effect of elevation on the production of volatile organic compounds at any level of expression (i.e. constitutive and induced). Again, evidences of elevational gradient in indirect defences are scarce and the reported patterns are mixed, varying from positive (Pellissier et al. 2016) to negative (Dostálek et al. 2016) associations with elevation. Without an explicitly assessment of predator pressure (see Kergunteuil et al. 2019a), we cannot determine if an elevational cline in predator pressure would shape concomitant variation in volatile emission.

One of the more remarkable findings of this work is the evidence for a simultaneous expression between defensive strategies along elevation gradients. In both the Chapter III (interspecific level) and the Chapter IV (intraspecific level), we found a positive co-expression between constitutive and induced phenolic compounds, in accordance with previous findings by Rasmann and Agrawal (2011) when studying cardenolides in several species of milkweed. This result contrast with the general expectation that constitutive and induced defences should trade off based on resource allocation constrains (Agrawal 2000, Moreira et al. 2014). However, the correlated expression was significantly associated with elevation only when studying intraspecific variation in oak defences in the Chapter IV. Specifically, we found that the simultaneous expression of constitutive and induced phenolic was favoured at low elevations, meanwhile, at high elevation we observed a shift in the nature of the association, in which the strategies appeared to be negatively correlated. The differences in the associations between the coexpression of defences and elevation reported when comparing both chapters (III and IV) again points at differences when analysing intra- vs. inter-specific patterns of expression of plant traits.

Overall, the observed patterns suggest that two mechanisms mediate patterns of allocation to defences along elevational gradients: one is based on the differential pressure of biotic factors (e.g. herbivore pressure) along the elevation gradient, and the other is based on variability in resource availability along the gradient. Low elevation environments with more available resources, as well as more herbivory pressure, favour allocation to constitutive defences as well as stronger co-expression between constitutive and induced defences, since resources are not limiting the allocation to multiple defences that are favourable for plant fitness (Rasmann et al. 2014a). By contrast, at high elevation environments resources tend to be more limited, forcing plants to deal with allocation constrains (Coley et al. 1985). Moreover, given the uncertainty of herbivory attack or the low efficiency of predators, plants would select for inducibility of direct defences over constitutive, ultimately leading to form trade-offs between these two defensive strategies. That said, inconsistencies in such pattern exist, particularly when studying different taxonomic levels, and different systems. Contrasting taxonomical scales have been pointed out before as main sources of conflicting patterns (Anstett et al. 2016b, Moreira et al. 2018d). However, few studies have simultaneously addressed intra- and interspecific clinal variation in allocation to plant defences (but see Anstett et al. 2016b, Descombes et al. 2017). In addition, comparisons of different study systems should be taken with caution. For instance, the methodologies involved in chapters III and IV are conceptually different. For the Chapter III, we estimated the elevational range of the global distribution of each species and we extracted climatic data from the estimated species range. By contrast, in the chapter IV we studied an elevation gradient at a more local scale (i.e. within Galicia, Spain) and we measured the climatic conditions directly in situ. Second, while the experiment in the chapter III was performed in a greenhouse, where all trees grew in the same controlled conditions with no limitation of resources, the experiment in the chapter IV was performed using plants growing in natural populations, and thus likely plants were exposed to a degree of uncontrolled variation in the environmental conditions. Based on these different approaches, we cannot differentiate whether our findings respond to differences in genetic plasticity or different adaptability to their environment. It is not possible to establish a causal link via presumed mechanisms without performing manipulative experiments. For example, mechanistic studies controlling for levels of herbivory and different defensive traits under greenhouse conditions or common gardens with reciprocal transplants using different using different plant genotype could help teasing apart local adaptation, to genetic differences, to phenotypic plasticity. Future field studies should also include a robust assessment of the arthropod community surrounding the focal plant species. The characterization of the herbivore community is needed to know to which extent herbivory pressure on plants in linked to herbivore abundance, and the ratio of predators to herbivores. On the one hand, different herbivores species, and their functional type therein, can enhance different defence pathways in plants (Rodriguez-Saona et al. 2010), leading to shifts in defensive traits. For instance, the effect of specialist and generalist herbivores has different consequences on plant defence evolution (Ali and Agrawal 2012, Moreira et al. 2018d). Our experiments manipulating herbivory were performed with larvae of two species: the generalist *Lymantria dispar* and the specialist *Altica quercetorum*. Further studies should perform experiments including more species to control for functional differences across herbivore guilds. For instance, herbivore diet breadth commonly increase with increasing elevation (Rasmann et al. 2014a), and this could lead to greater herbivory by generalist species of insect herbivores at high elevations, in turn likely selecting for broad-range defences in high elevation plants. On the other hand, the top-down control exerted by predators and parasitoids could release plants from herbivory pressure and shift plants to favour indirect strategies over direct defence strategies at sites where natural enemies of herbivores are highly present.

## ABIOTIC FACTORS DO NOT INFLUENCE PLANTS, HERBIVORES AND THEIR INTERACTIONS ALONG ELEVATIONAL CLINES

All the research presented in this PhD dissertation tested for the effect of climate on plant-herbivore interactions along elevational gradients. Surprisingly, neither temperature nor precipitation explained any of the patterns of clinal variation reported in herbivory or plant defences. Although we found an effect of temperature on herbivory in the chapter I and in the inducibility of phenolics in the chapter III, the effect of elevation remained unchanged after accounting for temperature and precipitation in the statistical models. This suggests that there might likely be other abiotic factors (e.g. soil characteristics) influencing the interactions between plant and insect herbivores along elevational gradients that were not included in our studies (De Long et al. 2016, Pellissier et al. 2016). It is commonly assumed that climatic conditions affect herbivore population sizes and feeding season length and this shapes herbivore pressure and herbivory selection on plant defences (Anstett et al. 2016b). However, climate could also influence plant defence allocation directly, independently from herbivory (Moreira et al. 2018b). Experiments manipulating climatic variables are needed to better understand the effect of climate shaping plant-herbivore interactions. For instance, the robustness of our results could be confirmed in future studies that use common gardens across multiple elevations, and containing plants from natural populations, as well as plants from populations at different elevations. Reciprocal transplant experiments such as these ones would enhance our understanding of the adaptive differentiation in plant defences against herbivores mediated by both biotic and abiotic factors along elevational clines (Cheplick 2015).

# CONCLUSIONS


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# CONCLUSIONS

**I.** In the Chapter I, the global analysis revealed that elevational gradients in leaf herbivory were contingent on growth form. Woody species experienced higher levels of herbivory toward lower elevations, but no gradient was observed for non-woody species.

**II.** Within woody species, I found that elevational gradients in leaf herbivory were contingent on leaf habit. Deciduous species exhibited higher levels of herbivory toward lower elevations, but no gradient was present for evergreen species.

**III.** The global analysis also revealed that elevational variation in herbivore damage was not contingent upon latitudinal region (i.e. tropical vs. temperate).

**IV.** In the Chapter II, I found an elevational gradient in herbivory and plant defences in eight oak species where both leaf herbivory and phenolic compounds increased toward higher elevations. There was an effect of ontogeny for defensive levels (saplings had higher levels of phenolics than adults), but not for herbivory. However, ontogenetic stage did not account for clinal variation in leaf herbivory or chemical defences.

**V.** Patterns of clinal variation of direct defences were different at intra- vs interspecific level. In the Chapter III (greenhouse experiment), I found no effect of elevation on constitutive phenolics in saplings of 18 oak species, but in the Chapter IV (field experiment) I found that constitutive phenolic compounds in saplings of *Quercus pyrenaica* populations increased toward lower elevations. By contrast, while I found that inducibility of phenolic compounds in saplings of 18 oak species increased toward higher elevations (Chapter III) I found no effect of elevation on induced phenolics in saplings of *Q. pyrenaica* populations (Chapter IV).

**VI.** In the Chapter IV, I found that constitutive and induced levels of indirect defences (emission of volatile organic compounds) did not show any pattern of clinal variation with elevation.

**VII.** In the Chapter IV, I found that constitutive and induced phenolics in saplings of *Quercus pyrenaica* populations showed a positive correlated expression and such association revealed a shift across elevation. In particular, constitutive and induced phenolics were co-expressed at low elevations but negatively correlated at high elevations.

**VIII.** In all chapters, I found that climatic correlates of elevation (temperature and precipitation) did not drive clinal variation in herbivory or plant defences.

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# APPENDIX


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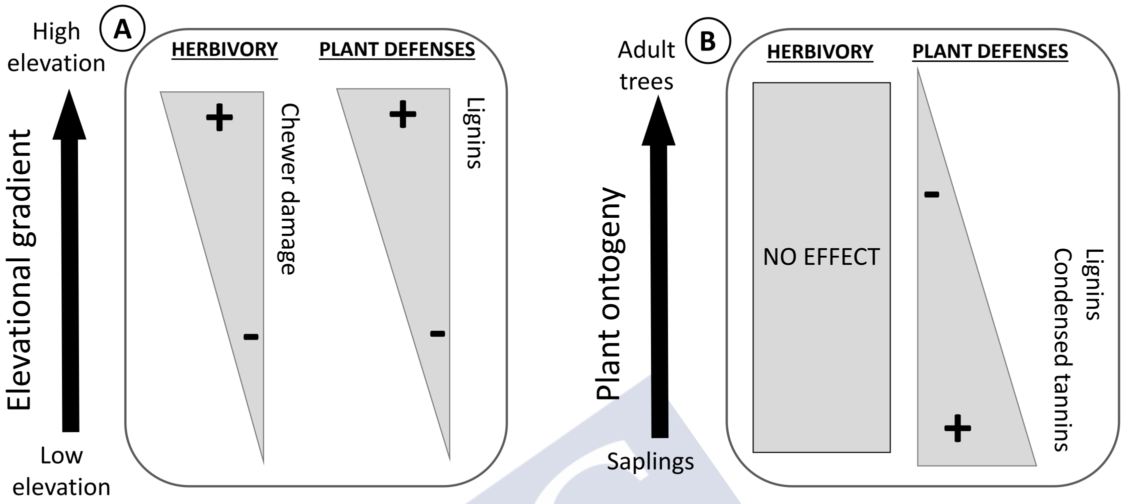
# APPENDIX

## APPENDIX A

**Table S1.** Effects of elevation, latitudinal region (tropical vs. temperate), growth form (non-woody vs. woody) and their interactions on leaf herbivory (measured as the percentage of leaf area consumed) at population-level. We included the effect of plant species as a random factor to account for repeated measures taken from the same species. Degrees of freedom, F-values and *P*-values for an analysis based upon logit-transformed data are shown. Significant ($P < 0.05$) P-values are typed in bold.

| Variable | $DF_{num,den}$ | F-value | *P*-value |
|---|---|---|---|
| Elevation | 1, 815 | 0.21 | 0.651 |
| Latitudinal region | 1, 815 | 0.02 | 0.880 |
| Growth form | 1, 815 | 20.12 | **<0.001** |
| Elevation × Latitudinal region | 1, 815 | 0.16 | 0.687 |
| Elevation × Growth form | 1, 815 | 8.08 | **0.005** |
| Elevation × Latitudinal region × Growth form | 1, 815 | 6.58 | **0.011** |

## APPENDIX B



**Appendix S1.** Scheme summarizing the effects of (A) elevation and (B) plant ontogeny on leaf herbivory by chewing insects and plant traits associated with herbivore resistance in eight oak (*Quercus*) species. (A) Both leaf herbivore damage by insect chewers and chemical defenses (lignins) increased towards higher elevations. (B) Leaf chemical defenses (lignins and condensed tannins) were higher in saplings than in adults, and no ontogenetic difference in herbivory.

## APPENDIX C

**Table S1.** Description of 19 BIOCLIM variables from the WorldClim database used to estimate climate niche of each oak species

| Variable | Description | Units |
|---|---|---|
| BIO1 | Annual Mean Temperature | ºC |
| BIO2 | Mean Diurnal Range (Mean of monthly (max temp - min temp) | ºC |
| BIO3 | Isothermality (BIO2/BIO7) (* 100) | [dimensionless] |
| BIO4 | Temperature Seasonality (standard deviation *100) | [dimensionless] |
| BIO5 | Max Temperature of Warmest Month | ºC |
| BIO6 | Min Temperature of Coldest Month | ºC |
| BIO7 | Temperature Annual Range (BIO5-BIO6) | ºC |
| BIO8 | Mean Temperature of Wettest Quarter | ºC |
| BIO9 | Mean Temperature of Driest Quarter | ºC |
| BIO10 | Mean Temperature of Warmest Quarter | ºC |
| BIO11 | Mean Temperature of Coldest Quarter | ºC |
| BIO12 | Annual Precipitation | mm |
| BIO13 | Precipitation of Wettest Month | mm |
| BIO14 | Precipitation of Driest Month | mm |
| BIO15 | Precipitation Seasonality (Coefficient of Variation) | [dimensionless] |
| BIO16 | Precipitation of Wettest Quarter | mm |
| BIO17 | Precipitation of Driest Quarter | mm |
| BIO18 | Precipitation of Warmest Quarter | mm |
| BIO19 | Precipitation of Coldest Quarter | mm |

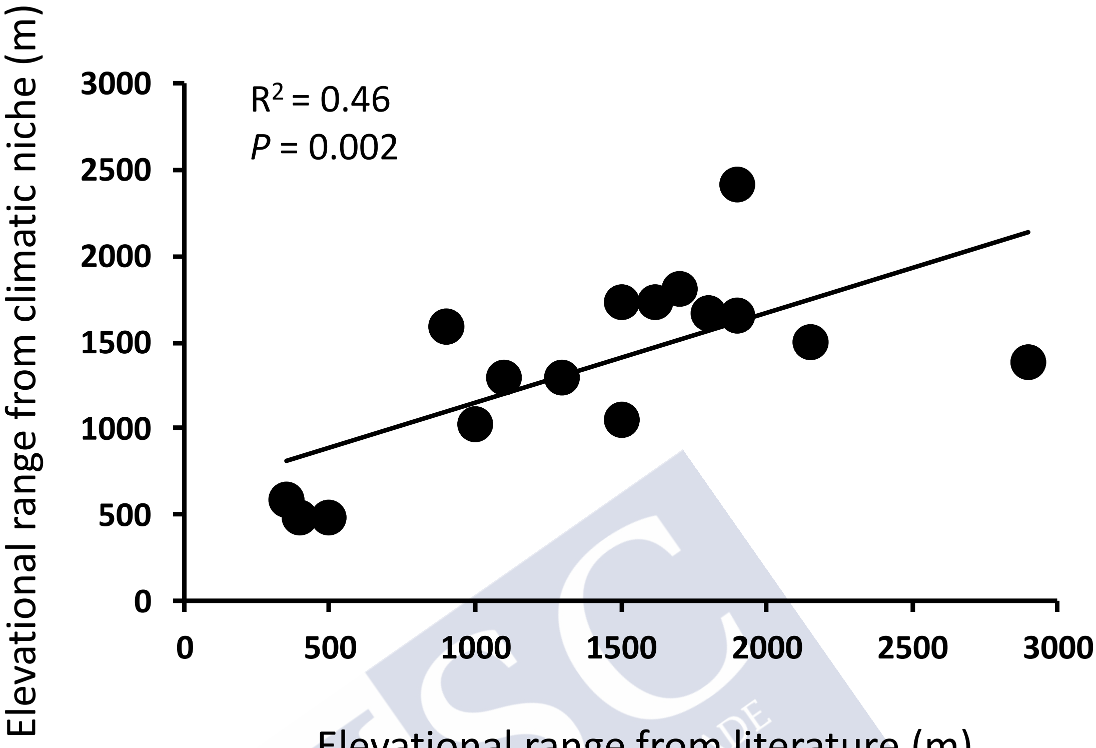


**Figure S1.** Correlation between species elevational range estimated from the models with 30-arcsecond resolution climate data from 19 BIOCLIM variables from the WorldClim database (Y axis) and the elevational range estimated from literature (X axis) for each oak (*Quercus*) species. R-squared coefficients ($R^2$) and *P*-values are shown. Dots represent species means (N = 18 oak species).

## APPENDIX D

**Table S1.** Elevation (m a.s.l), latitude and longitude (decimal degrees), annual mean temperature (ºC) and annual precipitation (mm) of the *Quercus pyrenaica* populations sampled.

| Population | Elevation | Latitude | Longitude | Temperature | Precipitation |
|---|---|---|---|---|---|
| 1 | 370 | 42.883 | -7.072 | 12.8 | 840 |
| 2 | 420 | 42.882 | -7.072 | 12.8 | 840 |
| 3 | 510 | 42.893 | -7.103 | 11.7 | 919 |
| 4 | 697 | 42.866 | -7.059 | 11.5 | 921 |
| 5 | 789 | 42.843 | -7.056 | 11.1 | 944 |
| 6 | 832 | 42.844 | -7.054 | 11.1 | 944 |
| 7 | 881 | 42.836 | -7.023 | 10.2 | 995 |
| 8 | 890 | 42.856 | -6.891 | 10.0 | 965 |
| 9 | 987 | 42.866 | -6.890 | 10.2 | 948 |
| 10 | 1024 | 42.841 | -6.760 | 9.7 | 945 |
| 11 | 1062 | 42.842 | -6.758 | 8.2 | 1065 |
| 12 | 1129 | 42.843 | -6.758 | 8.2 | 1065 |
| 13 | 1199 | 42.847 | -6.757 | 8.2 | 1065 |
| 14 | 1255 | 42.826 | -6.953 | 8.2 | 1132 |
| 15 | 1299 | 42.828 | -6.953 | 8.2 | 1132 |
| 16 | 1332 | 42.828 | -6.955 | 8.2 | 1132 |
| 17 | 1423 | 42.814 | -6.908 | 7.2 | 1196 |
| 18 | 1614 | 42.819 | -6.905 | 7.2 | 1197 |

**Table S2.** Summary of results from linear mixed models testing for the effects of elevation, treatment (control vs. induced) and their interaction on concentration of direct defences (condensed and hydrolysable tannins, flavonoids and lignins and total phenolics) and indirect defences (monoterpenes, sesquiterpenes and total VOCs) in *Quercus pyrenaica* saplings. We also included the effect of oak populations and oak population × treatment interaction as random factors. F-values with degrees of freedom (numerator, denominator) and associated significance levels (P-values) are shown. Significant *P*-values ($P < 0.05$) are in bold.

| | Elevation (E) | | Treatment (T) | | E × T | |
|---|---|---|---|---|---|---|
| | $F_{1,31}$ | $P$ | $F_{1,31}$ | $P$ | $F_{1,31}$ | $P$ |
| Condensed tannins | 1.81 | 0.189 | 12.26 | **0.001** | 1.35 | 0.255 |
| Hydrolysable tannins | 5.86 | **0.021** | 33.67 | **<0.001** | 7.72 | **0.009** |
| Flavonoids | 0.038 | 0.846 | 10.32 | **0.003** | 0.05 | 0.826 |
| Lignins | 4.42 | **0.044** | 24.24 | **<0.001** | 4.17 | **0.049** |
| Monoterpenes | 0.24 | 0.62 | 4.19 | **0.043** | 1.34 | 0.251 |
| Sesquiterpenes | 0.01 | 0.931 | 2.28 | 0.142 | 0.02 | 0.883 |
| Total phenolics | 1.88 | 0.179 | 23.19 | **<0.001** | 1.90 | 0.178 |
| Total VOCs | 0.01 | 0.925 | 5.07 | **0.032** | 0.67 | 0.413 |

**Table S3.** Identification and classification of the 79 volatile organic compounds found in the 18 populations of *Quercus pyrenaica*. Compounds were identified by comparing the retention times (RT; min) and mass spectra in the chromatogram to those in NIST mass spectral library and based on the comparison of the calculated Kovats ($KI_c$) Index with those published in the literature for the same column type (Adams 2007). MT = monoterpenes, ST = sesquiterpenes, FAD = fatty acid desaturase.

| **Compound** | **Class** | **RT (min)** | **$KI_c$** |
|---|---|---|---|
| 3-Penten-2-ol | other | 3.76 | 403 |
| 3-Penten-2-ol | other | 3.85 | 408 |
| 2-Pentene -4-ol | other | 3.99 | 415 |
| Butane 2-ethoxy-2-methyl- | other | 4.46 | 442 |
| Pentane 2,3,4-trimethyl- | other | 4.85 | 463 |
| Pentane 2,3,3-trimethyl- | other | 4.96 | 469 |
| Pentane 3-ethyl-2-methyl- | other | 5.12 | 478 |
| Heptane 4-methyl- | other | 5.28 | 487 |
| Hexane,2,2,4trimethyl | other | 5.66 | 523 |
| Iso glutamine | other | 5.79 | 543 |
| 1-Isopropoxy-2 | other | 5.97 | 570 |
| Hexane 3-ethyl- | other | 6.11 | 591 |
| (E)3-Hexenol | FAD | 6.19 | 605 |
| pyrrolidinyloxy | other | 6.26 | 620 |
| 2-Pentanol 4-methyl | other | 6.37 | 642 |
| Heptane2,3-dimethyl | other | 6.53 | 676 |
| Heptane2,4-dimethy | other | 6.75 | 705 |
| octylphenol | other | 6.84 | 709 |
| 1,8-Nonadien-3-ol | other | 7.13 | 723 |
| 2,4-Dimethyl-1-heptene | other | 7.32 | 732 |
| (z)2-Hexenol | FAD | 7.81 | 755 |
| Heptane2,2,4,trimethyl | other | 8.28 | 777 |
| Heptane2,2,4,trimethyl | other | 8.43 | 784 |
| Ciclopropano,1,1,2-trimethyl | other | 8.56 | 790 |
| Hexenal <(3Z)-> | FAD | 8.63 | 793 |
| Hexenal <(2E)-> | FAD | 8.93 | 843 |
| Cyclopentene,1,2,3,3,4-pentamethyl | other | 9.05 | 877 |
| Cyclopentene,1,2,3,3,4-pentamethyl | other | 9.05 | 877 |
| Cyclopentanone 2-(1 methylpropyl)- | other | 9.68 | 917 |
| Tricyclene | MT | 9.75 | 919 |
| Thujene | MT | 10.02 | 927 |
| Cyclohexane | other | 10.1 | 930 |
| Pinene-a | MT | 10.2 | 933 |
| Cyclohexanol <3-methyl> | other | 10.38 | 938 |
| Other | other | 10.44 | 940 |
| Fenchene <α-> | MT | 10.59 | 945 |
| Camphene | MT | 10.67 | 947 |

| | | | |
|---|---|---|---|
| Thiazole <2-ethoxy-> | Other | 10.8 | 951 |
| Geranyl linalool <(Z,Z)-> | MT | 11.13 | 961 |
| Sabinene | MT | 11.5 | 973 |
| Pinene <β-> | MT | 11.58 | 975 |
| Myrcene | MT | 12.01 | 989 |
| Linalool oxide <dehydroxy trans-> | MT | 12.08 | 991 |
| Octanal | other | 12.37 | 1000 |
| Phellandrene <α-> | MT | 12.47 | 1003 |
| Carene <δ-3-> | MT | 12.65 | 1008 |
| Terpinene <α-> | MT | 12.82 | 1014 |
| Limonene | MT | 13.06 | 1021 |
| B-phellandrene | MT | 13.17 | 1025 |
| Cineole <1,8-> | MT | 13.3 | 1029 |
| Cineole <1,8-> | MT | 13.37 | 1031 |
| Other | Other | 13.48 | 1035 |
| Ocimene <(Z)-β-> | MT | 13.63 | 1039 |
| Ocimene <(E)-β-> | MT | 13.82 | 1045 |
| Ocimene <(E)-β-> | MT | 13.95 | 1049 |
| Terpinene <γ-> | MT | 14.22 | 1058 |
| Cymenene <ρ-> | MT | 14.9 | 1065 |
| Terpinolene | MT | 15.05 | 1079 |
| Linalool | MT | 15.54 | 1084 |
| Nonanal | other | 15.69 | 1100 |
| Dimethyl nona triene (DMNT) | MT | 16.06 | 1105 |
| Terpineol <trans-β-> | MT | 17.32 | 1117 |
| Terpineol <α-> | MT | 18.31 | 1159 |
| Cyclocitral <β-> | MT | 18.71 | 1183 |
| Linalool acetate | MT | 20.13 | 1192 |
| Undecanal | other | 21.55 | 1206 |
| Other | Other | 21.78 | 1256 |
| Other | Other | 22.17 | 1307 |
| Cubebene <α-> | ST | 22.75 | 1316 |
| Cubebene <α-> | ST | 22.81 | 1330 |
| Copaene <α-> | ST | 23.47 | 1352 |
| Cubebene <β->//Cyperene | ST | 23.73 | 1355 |
| Bergamotene <α-trans-> | ST | 24.26 | 1379 |
| Caryophyllene <(E)-> | ST | 24.64 | 1389 |
| Copaene <β-> | ST | 24.86 | 1410 |
| Curcumene <ar-> | ST | 25.79 | 1425 |
| Gurjunene <γ-> | ST | 25.87 | 1434 |
| Muurolene <α-> | ST | 26.17 | 1471 |
| Farnesene <(E,E)-α-> | ST | 26.75 | 1474 |
| Cadinene <δ-> | ST | 27.2 | 1486 |
| Humulene epoxide | ST | 29.18 | 1510 |

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