Determinants of plant and herbivore interactions along elevation gradients

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DETERMINANTS OF PLANT AND HERBIVORE INTERACTIONS ALONG ELEVATION GRADIENTS

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SUMMARY

Environmental gradients structure species assemblages globally, directly through abiotic filtering or indirectly via changes in biotic interactions. In particular, elevation gradients in mountain systems are associated to climatic shifts over very short geographic distances. Changes in abiotic conditions are further related with a shift in species composition, abundance, and their functional traits. Climatic drivers together with species taxonomic or functional turnover along elevation could potentially modify how species interact with each other with consequences on ecosystem stability and functioning. Nevertheless, the study of species interaction networks along environmental gradients was limited by the time necessary to detect species interactions across many species and at multiples sites. In this thesis, we investigate how plant and herbivore communities change along elevation gradients, and the consequences of those changes for the structuration of their interactions. Combining field surveys in Swiss alpine grasslands with the development of molecular and statistical analytical tools dedicated to network analyses, the main research axes of this thesis consist in (i) investigating the responses of above- and belowground herbivores communities and their food plants to elevation through the quantification of community metrics and functional indices; (ii) studying the variation of structural properties of plant–herbivore networks along elevation, and (iii) identifying the rules of species interactions and whether those are constant or changing along elevation.

In chapter 1, we investigated how assemblages of soil nematodes and orthopteran herbivores change along elevation gradients as regard to their taxonomic and functional composition. Our results reveal that orthopteran communities show a decline in species richness and abundance, while nematodes show opposite relationships. These findings suggest that soil biotas might be buffered against harsher aboveground climatic conditions in alpine environments and could be governed by ecological determinants that differ from those acting at the surface. This chapter highlights the need for further investigations of the belowground compartment to reach a more complete understanding of how abiotic parameters influence biodiversity across ecosystem compartments.

In chapter 2, we contribute to a review of the major methodological approaches and their challenges associated with the comparison of ecological networks along environmental

gradients. We discuss the inherent biases in network comparison and provide a concise description of the most up-to-date analytical tools to address these difficulties. We further propose new analytical tools to compare the structure and the rules of ecological networks along environmental gradients. More generally, this chapter serves as a theoretical and methodological basis for the development of chapters 3 and 4.

In chapter 3, we explore how the structural properties of plant–orthoptera bipartite networks vary along elevation. We reconstruct ecological networks by applying a DNA metabarcoding sequencing approach to insect feces. The results show a decline of network specialization along elevation. This pattern was associated with increased resilience to species extinction in high-elevation alpine meadows when comparing the signal to null expectations. Our results suggest that network structural properties are associated to climate variation along elevation, possibly as a result of change in environmental predictability and functional traits.

In chapter 4, we investigate the ecological rules that shape plant–orthoptera networks. We infer rules of species interaction assembly by defining *a priori* hypotheses related to plant phylogeny, resources abundance and trait-based processes including a trait matching between herbivore mandibular strength and leaf toughness. These analyses reveal that the ecological rules show different explanatory power to explain species interaction along the elevation and across biogeographical regions. Overall, these results indicate that there is spatial variation in the ecological rules governing species interactions.

Collectively, the chapters of this thesis support the broad hypothesis that abiotic constraints shape plant–herbivore systems from taxonomic and functional composition down to the mechanisms structuring their interactions. In addition, this work shows that climatic shifts along elevation have a contrasting influence on above *vs*. belowground herbivores communities. We further found evidence that network structure and the ecological rules of species interaction are not necessarily conserved at the landscape scale. Through investigation of network resilience to species extinction, this work has further implications for conservation practices that are oriented toward the preservation of the multiple facets of natural systems. This thesis emphasizes that it is essential to study diversity patterns with regard to species interactions in order to aim at a global comprehension of the organisation of life under environmental changes.

RÉSUMÉ

Les gradients environnementaux structurent les assemblages d'espèces à l'échelle mondiale directement par des changements de conditions abiotiques et indirectement en influençant les interactions biotiques. Les gradients d'altitude dans les systèmes alpins sont associés, en particulier, à la modification des conditions climatiques sur de très courtes distances géographiques. Ces changements abiotiques sont notamment responsables d'une modification de la composition des espèces, de leur abondance et de leurs caractéristiques fonctionnelles. Les variations climatiques ainsi que les changements taxonomiques ou fonctionnels des espèces le long de l'altitude peuvent potentiellement modifier la façon dont ces dernières interagissent entre elles, avec des conséquences sur la stabilité et le fonctionnement des écosystèmes. Pourtant, l'étude des réseaux écologiques le long des gradients environnementaux a longtemps été limitée par le temps nécessaire à la détection à large échelle des interactions entre les espèces. Dans le cadre de cette thèse, nous étudions comment les communautés de plantes et d'herbivores changent le long de l'altitude et quelles sont les conséquences de ces modifications sur la structure des réseaux écologiques que ces communautés forment. En combinant des données de terrain collectées dans les Alpes suisses avec le développement d'outils d'analyse moléculaire et statistique dédiés aux analyses de réseaux, les principaux axes de recherche de cette thèse consistent à i) quantifier les réponses à l'altitude des plantes et des communautés d'herbivores vivant à la surface et sous le sol en mesurant des métriques des communautés et des indices fonctionnels ; ii) étudier la variation des propriétés structurelles des réseaux plantes–herbivores le long de l'altitude et iii) étudier les règles des interactions entre espèces et déterminer si celles-ci sont constantes ou changent le long de l'altitude.

Dans le chapitre 1, nous étudions comment les communautés d'herbivores de surface, les orthoptères, et les nématodes du sol changent le long de gradients d'altitude au niveau de leur composition taxonomique et fonctionnelle. Ces résultats révèlent que le long de l'altitude, la richesse et l'abondance des communautés d'orthoptères diminuent, tandis que les nématodes présentent des relations opposées. Nos analyses suggèrent également que les organismes du sol pourraient être mieux protégés contre les conditions climatiques de surface plus rigoureuses en haute altitude et qu'ils seraient donc régis par des déterminants écologiques différents de ceux des biotes de surface. Ce chapitre souligne la nécessité d'approfondir les recherches sur les

communautés du sol pour parvenir à une meilleure compréhension de la manière dont les paramètres abiotiques influencent la biodiversité dans les différents compartiments écosystémiques.

Dans le chapitre 2, nous conduisons une revue des principales approches méthodologiques de la comparaison des réseaux écologiques le long des gradients environnementaux. Nous discutons des défis et des biais inhérents à la comparaison de réseaux écologiques et nous présentons les outils d'analyse les plus récents permettant de résoudre ces difficultés. Nous proposons également de nouveaux outils d'analyse pour comparer la structure et les règles des réseaux d'interactions le long de gradients environnementaux. Ce chapitre sert de base théorique et méthodologique à l'élaboration des chapitres 3 et 4.

Dans le chapitre 3, nous étudions la variation des propriétés structurelles des réseaux bipartites plantes–orthoptères le long de l'altitude. Nous reconstruisons les réseaux en appliquant une approche de *barcoding* de l'ADN de plantes sur des échantillons de fèces d'insectes. Grâce à cette méthode, nous avons pu mettre en évidence une diminution de la spécialisation des réseaux écologiques le long de l'altitude. Nous avons également montré que cette variation était associée à une plus grande résilience des réseaux à l'extinction des espèces en haute altitude lorsque le signal est comparé à un modèle nul. Nos résultats suggèrent que les propriétés structurelles des réseaux sont associées aux variations climatiques le long de l'altitude, potentiellement en raison d'un changement de la prévisibilité environnementale et des caractéristiques fonctionnelles des espèces.

Dans le chapitre 4, nous nous intéressons aux règles écologiques qui structurent les réseaux plantes–orthoptères. Nous étudions ces règles d'assemblage entre espèces en définissant des hypothèses *a priori*, liées à la phylogénie des plantes, à l'abondance des ressources et aux traits fonctionnels, tels que la relation entre la force mandibulaire des herbivores et la résistance physique des feuilles. Ces analyses révèlent que ces règles écologiques n'ont pas le même pouvoir explicatif pour structurer les interactions entre espèces le long de l'altitude ou entre régions biogéographiques. En définitive, ces résultats indiquent qu'il existe une variation spatiale dans les règles écologiques structurant les interactions entre espèces.

RÉSUMÉ

Dans l'ensemble, les résultats de cette thèse confirment l'idée que des contraintes abiotiques façonnent les systèmes plantes–herbivores ; de leur composition taxonomique et fonctionnelle jusqu'aux mécanismes qui structurent leurs interactions. Du reste, ce travail révèle que les variations climatiques le long de l'altitude influencent différemment les communautés d'herbivores souterraines de celles en surface. Nous avons également démontré que la structure des réseaux et les règles d'interactions entre espèces ne sont pas forcément conservées à l'échelle du paysage. En investiguant la résilience des réseaux à l'extinction d'espèces, ce travail s'inscrit également dans le cadre de mesures de conservation orientées vers la préservation des multiples facettes des systèmes naturels. Cette thèse renforce l'idée qu'il est essentiel d'étudier la diversité biologique sous l'angle des interactions entre espèces, pour permettre une compréhension globale de l'organisation de la biodiversité sous l'effet des changements environnementaux.

"L’homme peut avoir vécu une vie grise dans un domaine de terres obscures et d’arbres noirs, les événements les plus importants ont pu passer, alignés, anonymes, et dépourvus de couleur, cela ne compte pas. Car à la minute de la grâce, soudain le chant d’un criquet enchante l’oreille, l’odeur de la terre charme les narines et la lumière tamisée par un arbre régénère l’œil. Alors l’homme devient source et il est intarissable."

A l’est d’Eden - John Steinbeck

GENERAL INTRODUCTION

Environmental gradients structure the distribution of a highly diversified fauna and flora across the globe (Doebeli & Dieckmann 2003; Rundle & Nosil 2005). The processes that shape the diversity of organisms along environmental clines are broadly associated to a combination of biotic and abiotic factors (Rosenzweig 1992; Gaston 2000) and can be of ecological and evolutionary nature (Endler 1977). For instance, divergent selection caused by contrasting ecological factors along environmental clines has been shown to foster the diversification of organisms by promoting local adaptation (Doebeli & Dieckmann 2003; Gueuning *et al*. 2017). Environmental gradients thus act as filters to determine range expansion, promote speciation and ultimately shape the composition of natural communities. Turnover in species assemblages have been documented along multiple gradients including salinity (Telesh *et al*. 2013), nutrients (Declerck *et al*. 2007) , acidity (Bardhan *et al*. 2012), moisture (Zelnik & Čarni 2008), land use (Culman *et al*. 2010) or temperature (Mayhew *et al*. 2012). These abiotic factors shape the local composition of communities along environmental gradients (Doebeli & Dieckmann 2003) and biogeographic patterns at regional to global scales (e.g. latitudinal diversity gradients, Stehli *et al*. 1969). In particular, the process of orogeny generates steep climatic gradients over very narrow geographic distance (Dewey & Bird 1970) and form a variety of ecological niches that were gradually occupied by species over evolutionary times (Hoorn *et al*. 2013; Antonelli *et al*. 2018). In turn, biotas show very organized assemblage structure that is further shaped by present ecological interactions that vary along elevation gradients (Tylianakis & Morris 2017).

The biodiversity patterns along elevation

The pioneering work of Alexander von Humboldt on gradients of elevation resulted in a conceptual model of altitudinal zonation (Fig. 1), where specific organisms occupy specific elevation belts (von Humboldt & Bonpland 1807). This key concept is still used to explain why mountain regions and more specifically elevation gradients are associated to exceptionally high levels of biodiversity (Sergio & Pedrini 2010). Multiple abiotic parameters (e.g. shifts in atmospheric pressure and chemistry, precipitations, wind velocity, solar radiation, slopes exposure and degree) have been associated to change in elevation (Körner 2007). Yet, temperature is expected to represent the dominant driver of species turnover along elevation in mountain regions (Rahbek 1995; Barry 2008; Mccain & Grytnes 2010). Surface temperature is generally recognize to promote biodiversity in warmer environments by stimulating

metabolic and diversification rates (Clarke & Fraser 2004, Allen et al. 2006, Hatfield and Prueger 2015) resulting in a decline of species richness towards upland environments (Rahbek 1995; Grytnes & McCain 2013).

The study of the ecological signatures of elevation on biotas is strongly biased toward aboveground organisms (Rahbek 1995), while the shift in soil community composition along elevation remain largely unknown (Bardgett & van der Putten 2014). The rare investigations on soil fauna found contrasting if not opposite elevational patterns to those found at the surface (Bryant *et al*. 2008; Fierer *et al*. 2011; Pellissier *et al*. 2014; Kergunteuil *et al*. 2016), suggesting that aboveground abiotic conditions might be buffered in the soil compartment (Beyens *et al*. 2009). Therefore, the respective response of above- and belowground species communities to elevation may result in different species turnover rates (Bardgett & van der Putten 2014) and ultimately influence ecosystem processes given their common role in mediating nutrient and energy cycles.

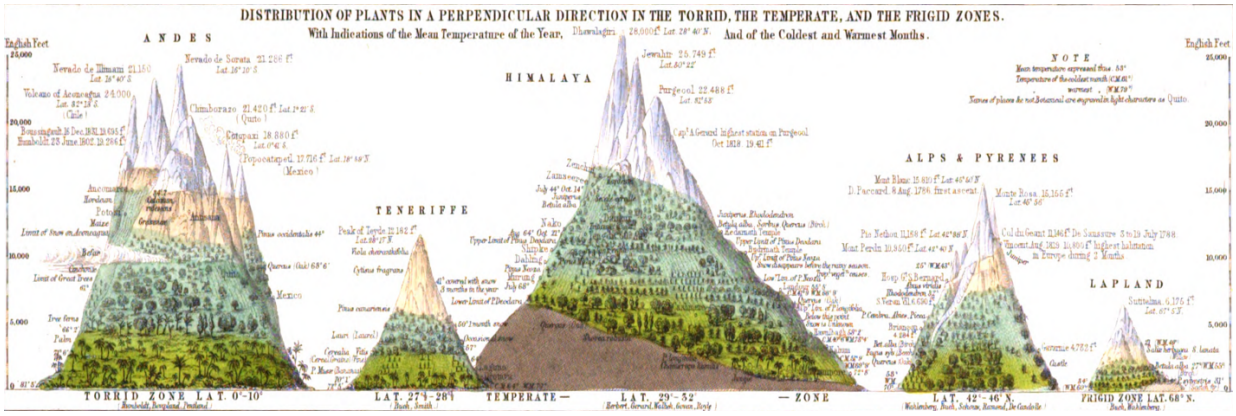

Figure 1 Clinal layering of plant distribution along elevation in perpendicular direction of latitudinal zones, drawn by Johnston and published in "*The physical atlas of natural phenomena*", edition of 1850 (image from Wikimedia commons public domain collection).

Beside measures of species richness and abundance, species turnover along elevation can be studied together with morphological or physiological trait measurements (Hodkinson 2005; Read *et al*. 2014; Halbritter *et al*. 2018). Species traits are expected to be linked to functional mechanisms that are significant for species persistence in their environment (Violle *et al*. 2007). As a corollary, species turnover along elevation are expected to be associated with important changes in the species functional attributes (Lenfant 1973; Callis-Duehl *et al*. 2017; Descombes *et al*. 2017; Wong *et al*. 2019). The mechanism of environmental filtering proposes

that the local environment selects a subset of the functional traits allowing the species survival from the regional functional species pool (Lenfant 1973; Hodkinson 2005; Moreira *et al*. 2018). Decrease in the length of the growing season (Körner 2003; Hodkinson 2005), increased risk of frost damages (Agrawal *et al*. 2004) and lower levels of energy are generally associated with increasing elevation (Wright 1983; Evans *et al*. 2005). Upland biotas present specific physiological adaptations to high elevation environments, including for instance shorter stature and tougher leaves in plant (Billings 1974; Körner *et al*. 1989), darker coloration and decrease voltinism in insect (Sømme & Block 1991) or difference in lung morphometry in mammals (Lenfant 1973). Moreover, species are not isolated entities, but interact in the form of ecological networks to determine the dynamic of natural communities (Proulx *et al*. 2005). Therefore, by influencing taxonomic and functional facets, environmental gradients are also expected to alter the realization and the persistence of interactions between species which may in turn affect species performance (Welti & Joern 2015; Tylianakis & Morris 2017).

The ecological rules of species interaction

Networks have recently been integrated in ecological theories to inform on the processes that determine the coexistence of species in a given environment (Delmas *et al*. 2019). Specifically, the study of ecological networks can unravel how the environmental constraints on species interaction determine the non-random structuration of species assembly (Jordán & Scheuring 2004). Important advances in the study of ecological networks, in particular for mutualistic interaction type (Bascompte 2010), drastically improve our comprehension of species assemblages and ecosystem functioning (Borrett *et al*. 2014; Harvey *et al*. 2017). Studying the mechanisms structuring interaction networks is challenging as the ecological rules determining network architecture are entangled and of multiple origins (Dormann *et al*. 2017). The structure of ecological networks can be determined by several mechanisms, associated to species traits, phylogenetic affiliations and demographic properties (Vázquez *et al*. 2007; Bascompte & Stouffer 2009; Laigle *et al*. 2018). In particular, evolutionary inheritance reflected by the species phylogeny are expected to be a major determinant of species interactions (Rohr & Bascompte 2014). Functional traits, selected with a sufficient *a priori* knowledge of the study organism and the type of interaction, were also shown to be associated to the structure of ecological networks (Laigle *et al*. 2018). In this context, trait matching constraints are shown to be fundamental to the realization of interactions resulting from coevolutionary mechanisms that strengthen the links between species (Janzen

1980; Martín González *et al*. 2015; Brousseau *et al*. 2018). For marine systems, Gravel *et al*. (2013) developed a trait-matching framework integrating the relationship between the body size of fish predator and prey species to infer interactions and food web structure. Trait-matching rules have also been extensively used in plant–pollinator systems where the correlation between size measurements of the plant resource (e.g. corolla depth, fruit size) and feeding appendage of the pollinator (e.g. bird beak length, insect proboscis length) allows to explain pairwise interactions (Garibaldi *et al*. 2015; Pichler *et al*. 2019). In antagonistic relationships, trait matching has spurred the arms race, giving rise to a plethora of chemical, mechanical and behavioural innovations, optimized for food acquisition or defence against natural enemies (Becklin 2008; Cagnolo *et al*. 2011). In contrast, the role of abundance-based processes in determining interactions is not always assessed despite being recognized as important determinant of species interactions (Vázquez *et al*. 2007; Carnicer *et al*. 2009; Canard *et al*. 2014). These eco-evolutionary drivers are expected to be intertwined to determine species interactions and should be studied together to uncover the complexity of the mechanisms ruling ecological networks (Canard *et al*. 2014; Spaniol *et al*. 2019). In spite of considerable progress in network ecology, important efforts are required to aim at unifying theories on the assembly mechanism shaping ecological networks through space and time (Bascompte 2009; Thebault & Fontaine 2010; Baiser *et al*. 2019). Addressing this challenge relies on further development of analytical tools but foremost on the extensive collection of network metadata.

Documenting species interactions networks

The first ecological networks were assembled by observing interactions directly on site (Fig. 2a, Summerhayes & Elton 1923; Anderson & Wright 1952) or assumed based on co-occurrence between species when interaction could not be accurately documented (Hardy 1924). Co-occurrences generally poorly reflect empirical interactions as they can be explained by similar ecological preference and not necessarily by the presence of an interaction (Freilich *et al*. 2018). In contrast, the identification of true interactions within local networks provides a better picture of the realized interactions under local environmental conditions, but can be extremely resource-intensive and expensive. A variety of approaches have been used to sample ecological networks. For species interactions that cannot be directly observed on the field and for which physical traces of interaction are difficult to sample, video cameras-system and automated detection (Maglianesi *et al*. 2014; Weinstein & Graham 2017) can be useful to

document interactions, but are incompatible with several interaction types (Roslin *et al*. 2019). Similarly, the compilation of literature-reference data enables a large-scale reconstruction of tetrapods food webs (O'Connor *et al*. 2019), but this approach lack spatial and taxonomic completeness for several groups of organisms (Poisot *et al*. 2020). Diet analyses through visual analysis of feces, stomach or gut samples – have been used to compile broad lists of consumed items (Hyslop 1980; Green 1987). However, the manual analysis of diet composition is time-consuming and does not allow accurate quantification of the ingested amount of food as diet components might not be equally digested (Rindorf & Lewy 2020). For insect herbivores, Mulkernand et al. (1958) proposed a method to visually analyze the plant material present in feces samples. Cells of food plant are colored with analine blue and their morphological characteristics are compared under a microscope to type reference slides of the plant collected at the study site. The extensive collection of interactions in space and time is hindered by the efforts required in visual analyses and in the building of the reference slide collection. Prey items present in the digestive track or feces has also been successfully quantified using stable isotopes and fatty acids profiles but at a coarse resolution (Traugott *et al*. 2013). Methodological limitations have been recently alleviated been the use of genetic tools, accelerating the collection of species interaction (Vacher *et al*. 2016).

The development of high throughput DNA sequencing methods has revitalized network ecology by offering new possibilities to extensively and efficiently quantify species interactions (Fig. 2b; Clare 2014; Pornon *et al*. 2016; Nielsen *et al*. 2018). Widely adopted by network ecologists, DNA metabarcoding method relies on the amplification of one or several genetic markers to identify the content of feces, pollen or gut samples. Deagle et al. (2007) were among the first to apply a DNA metabarcoding protocol to study the diet of the macaroni penguins in the Indian Ocean. The method was further extended to reconstruct ecological networks of all types, including mutualist and antagonist interactions (Richardson *et al*. 2015; Toju & Baba 2018); terrestrial (Lopes *et al*. 2015) and aquatic systems (Casey *et al*. 2019); carnivores (Shehzad *et al*. 2012) and herbivores trophic relationships (García-Robledo *et al*. 2013).

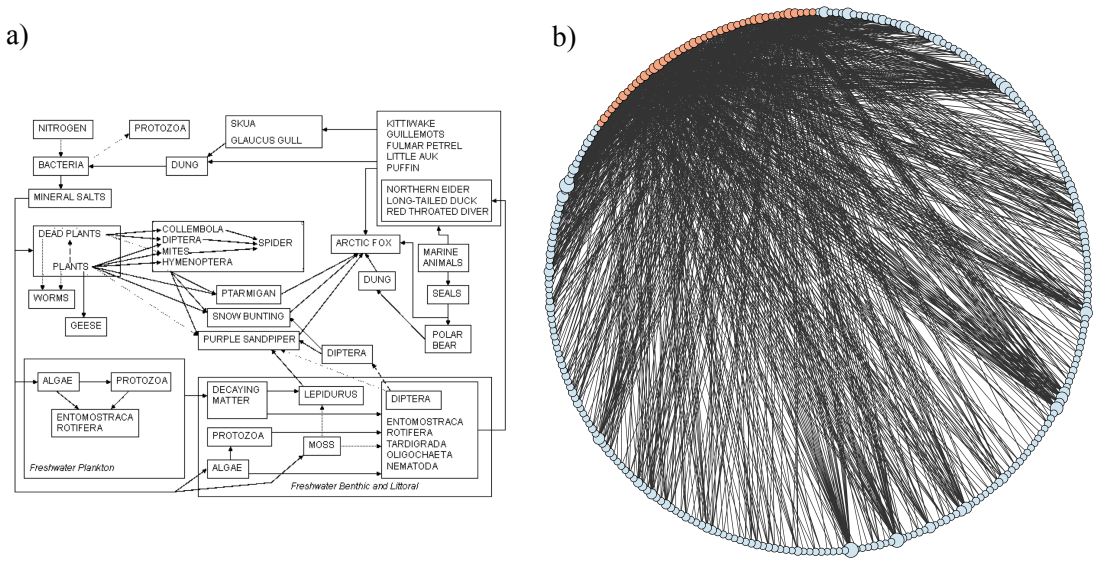

Figure 2 Representation of earliest and the most recent ecological networks reconstructed. Elton (1927) pioneered the food web representation of species relationships between multiple trophic groups (**a**) from field observations on Spitsbergen and Bear Islands (Summerhayes & Elton 1923, image from Wikimedia commons public domain collection). Today, the reconstruction of multiple networks at a high taxonomic resolution is feasible by using high thoughput DNA sequencing techniques. A single Illumina sequencing lane can allow for thousands of interactions between two trophic levels (**b**, with primary producers in light blue, consumers in orange).

The first challenge associated to the use of DNA metabarcoding in ecological network studies related to quality of the DNA present in environmental samples. In particular, DNA in feces samples is usually highly fragmented which preclude the PCR amplification of long and phylogenetically more informative genetic markers (Joly *et al*. 2014). The marker should thus be of a short length while providing the highest possible taxonomic resolution and the universality to capture species belonging to distant phylogenetic groups (Trivedi *et al*. 2018). For plant, the selection of a genetic marker has been long debated (Valentini *et al*. 2009; Hollingsworth 2011). Early on, the ITS2 plant marker was presented as an appropriate plant barcode, combining the ability to capture most Streptophyta taxa with a high taxonomic resolution (Hollingsworth *et al*. 2011; Li *et al*. 2011; García-Robledo *et al*. 2013; Moorhouse-Gann *et al*. 2018). The second challenge of the application of DNA metabarcoding methods in network ecology lies in the difficulty to reproduce the wet lab procedure from protocols described in scientific publications. Similarly, bioinformatic pipelines are not always easily reproducible and adaptable to others systems (Coissac *et al*. 2012). This could be solved by

consistently sharing well detailed and user–friendly protocols, which would also serve to agree on standardized practices for the generation and processing of bioinformatic data (Deagle *et al*. 2019). Maintaining efforts to build online high-quality DNA barcode reference databases will also facilitate the accurate identification of species interactions (Coissac *et al*. 2012). DNA metabarcoding made major contributions to the collection of species interaction (Roslin *et al*. 2019). Large-scale application of this method along with methodological optimizations would enlarge the sampling of network data to numerous species at a high resolution. Until then, genetic tools hold the promise to reconstruct ecological networks through entire biomes.

Species interaction along environmental gradients

Beyond a turnover of species and functional properties, environmental gradients are associated to shifts in species interactions within ecological networks (Welti & Joern 2015; Tylianakis & Morris 2017; Pellissier *et al*. 2018). Environmental gradients can shape species networks by influencing species co-occurrence, interaction strengths and specific functional traits that mediate interactions (Tylianakis & Morris 2017). Species turnover along abiotic gradients can impacts ecological networks by affecting whether species co-occur (Poisot *et al*. 2012; Gravel *et al*. 2019). However, co-occurrence is only a prerequisite for interaction; the realization and strength of the interaction may depend on additional factors such as the abundance of the interacting partners (Vázquez *et al*. 2007) and on their ability to form stable links (Dormann *et al*. 2017). In addition, the environmental filtering, which can select for particular traits in assemblage, might modify the prevalence of traits that participate to determine species interactions and cause a wiring of interactions along environmental clines (Dehling *et al*. 2014; Coux *et al*. 2016).

Studying the structural variation of ecological network provides a deeper insight on how species communities come to assemble in ecosystems compared to an analysis of species composition (Proulx *et al*. 2005). Structural differences between networks can be quantified by various metrics related to basic network properties (e.g. species richness, number of links, Poisot *et al*. 2012) or certain aspects of community dynamics (e.g. modularity, nestedness, specialization or centrality measures; Dunne *et al*. 2002; Bascompte *et al*. 2003; Blüthgen *et al*. 2006; Newman 2006). While empirical network analyses along elevation gradients remain scarce, a relatively common pattern as emerged to indicate that networks become more randomly assembled at higher elevations in alpine environments (Ramos-Jiliberto *et al*. 2010;

Miller-Struttmann & Galen 2014; Hoiss *et al*. 2015; see also Pellissier *et al*. 2012, Rasmann *et al*. 2014). For instance, nestedness on plant–pollinator networks has been found to increase toward alpine ecosystems (Flores *et al*. 2018). This pattern can be viewed as an attenuation of the rule limiting interactions of specialists to a subset of generalist species. In parallel, the degree of specialization was found to decrease with elevation in plant–pollinators systems (Miller-Struttmann & Galen 2014; Hoiss *et al*. 2015), between alpine butterflies and their host plants (Pellissier *et al*. 2012) or in the diet of horned lizards (Refsnider *et al*. 2019). These independent studies invoked the severe environmental conditions of the alpine zone and its associated changes in abiotic and biotic parameters to explain shifts in network structuration.

In the context of plant–herbivore interactions, the environmental conditions found at higher elevation leading to more opportunistic interactions have been related to alterations of synchronicity between the interacting partners (Miller-Struttmann & Galen 2014), lower resource availability (Tylianakis & Morris 2017), relaxed plant chemical defence for herbivores (Moreira *et al*. 2018) or reduced search and digestive efficiency in ectothermic animals (Hodkinson 2005). Increasing levels of network generalization are often thought to enhance robustness to species extinction (Lafferty & Kuris 2009; Welti *et al*. 2017; but see Hoiss *et al*. 2015). Thereby, structural indices can be highly informative to measure the response and the resilience of ecological networks to abiotic variation. Contrastingly, opposite patterns of specialization were found in host-parasitoids food web (Maunsell *et al*. 2015; Morris *et al*. 2015). These divergent results suggest that investigations of the multifaceted mechanisms of network assembly are still needed to evaluate how elevation is associated to shifts in the structure of ecological networks. Although progress in our understanding of network structure occurred over the last two decades (Bascompte 2010; Dormann *et al*. 2017; Delmas *et al*. 2019), the comparison of networks along environmental gradients has been hindered by inherent difficulties of network comparison, which still requires methodological development.

The challenging study of ecological networks along environmental gradients

Comparing network structural indices along environmental gradients is methodologically challenging for several aspects. The major difficulties relate to the sensitivity of network structural metrics to sampling size (Blüthgen *et al*. 2008; Pellissier *et al*. 2018). As

network size decreases with species richness under more severe abiotic conditions, and the calculations of network metrics partly depends on network size, reaching conclusion about the network structure independently of the species number is challenging (Blüthgen *et al*. 2006; Galeano *et al*. 2009). To address this issue, several standardization procedures have been developed (Gotelli & Graves 1996; Dalsgaard *et al*. 2013; Albrecht *et al*. 2014; Pellissier *et al*. 2018). For instance, the rarefaction technique normalizes metric calculation by an iterative and random removal of interacting species to equalize network size before metric measurement (Simberloff 1978; Albrecht *et al*. 2014). With similar purpose, null models have been successfully applied to compare ecological networks across contrasting environmental conditions (Gotelli & Graves 1996; Ollerton *et al*. 2007). The method consists in generating random networks of interactions with defined constraint (e.g. network size, connectance) from which is calculated the metric of interest. The distribution of the metric values of random networks is further compared to those obtained from empirical networks. Building on these recent methodological advances in network ecology, how environmental gradients alter the arrangement of species interaction is beginning to be understood (Pellissier *et al*. 2018). Beyond the important insights on life organization that arise from the study of network structure, more predictable understanding of how network ecological rules vary along environmental gradient still requires methodological development.

The spatial variation of network ecological rules

Although, the mechanisms determining the non-random structure of ecological network received increased attention (Dormann *et al*. 2017), most of this research does not account for the spatio-temporal variation of the rules determining species interaction (Baiser *et al*. 2019; Gravel *et al*. 2019). Elevation gradients offer multiple possibilities to explore these questions at a landscape scale. Elevation could be associated to shifts in the ecological rules underlying species interactions in two major ways. First, it can induce a lowering of resource abundance and accessibility which can significantly influence the determinism of species interaction (Miller-Struttmann & Galen 2014; Tylianakis & Morris 2017). Second, if the functional traits involved in an interaction respond to climatic conditions in a decoupled way (Körner *et al*. 1989; Hodkinson 2005; Moreira *et al*. 2018), the trait matching rules between the species could in turn vary along the gradient (Dehling *et al*. 2014). Together with functional shift, the signature of phylogenetic relationships on ecological networks is also expected to be altered by environmental conditions (Tylianakis & Morris 2017). If the conditions under which co-

evolution could arise are not invariably met along the study gradient, the realization of species interaction would be affected resulting in a variation of network structure along the gradient. Abiotic constraint can also directly influence species interaction by affecting behavioural and metabolic traits in cold environments toward a reduced search efficiency and resource uptake (Hodkinson 2005; Wong *et al*. 2019). In these cases, the role of abundance-based processes would be enhanced relatively to trait-based mechanisms. Yet, the versatile nature of the ecological rule governing species interaction in regard to environmental variation is almost unexplored (Pellissier *et al*. 2018; Baiser *et al*. 2019). A quantitative investigation of how network structuration mechanisms are entangled with environmental drivers would help developing network ecology toward the understanding of network structuration at large spatial scales (Tylianakis & Morris 2017; Baiser *et al*. 2019).

Plant–herbivores interaction along elevation

Plant–herbivores interactions are associated to a variety of ecosystem processes by controlling nutrient cycles and serving as food source for higher trophic levels (Olff & Ritchie 1998; Tilman *et al*. 2012). Nevertheless, how environmental gradients influence plant–herbivores interactions has not yet been thoroughly investigated from a network perspective (Pellissier *et al*. 2012; Salgado *et al*. 2016). Interactions between plant and herbivores primary rely on the reciprocal evolution of functional traits related to plant defence and herbivores feeding adaptations, involving both physical and chemical responses (Fox 1981; Schultz 1988). Along elevation, plant and herbivores are influenced by abiotic and biotic parameters that shape functional responses and thereby, may results in a shift of herbivory patterns (Rasmann *et al*. 2014; Galmán *et al*. 2018). For instance, at high elevation plant are generally thought to have lowered chemical defence under declining herbivory pressure (Callis-Duehl *et al*. 2017; Moreira *et al*. 2018). In contrast, under severe conditions of high elevation environments plant physical resistance was shown to increase which can in turn alter plant–herbivores interactions (Körner *et al*. 1989; Descombes *et al*. 2017). Depending on the functional responses of plant and herbivores along elevation, interactions are expected to be wired under the influence of abiotic and biotic parameters (Galmán *et al*. 2018). Therefore, the study of plant–herbivores trophic networks represent a good study system to unravel the mechanisms structuring trophic networks along environmental gradients, which could provide further insight for ecosystem functioning.

Temperate grasslands

Temperate grasslands are among the most altered biomes on Earth, with the vast majority of them being lost to intensive agricultural practices, suffering from habitat fragmentation, air-borne nitrogen pollutants, global warming or alien species invasion (Dengler *et al*. 2014; Fuhlendorf *et al*. 2018). In Europe, direct and indirect human depredation impose significant threats on grasslands which are a home to a range of species assemblages including rare species of plant and arthropods (Pärtel *et al*. 2005; Habel *et al*. 2013; Zong *et al*. 2018). Distributed along the entire elevation gradient, dry meadows and alpine grasslands are essential to maintain a large fraction of the biodiversity of the Alpine regions (Blumer & Diemer 1996; Kampmann *et al*. 2008). By hosting key players of ecosystem processes such as plant and insect interactions, grasslands systems along elevation are particularly suitable to improve our comprehension of species assemblages in link with abiotic shifts.

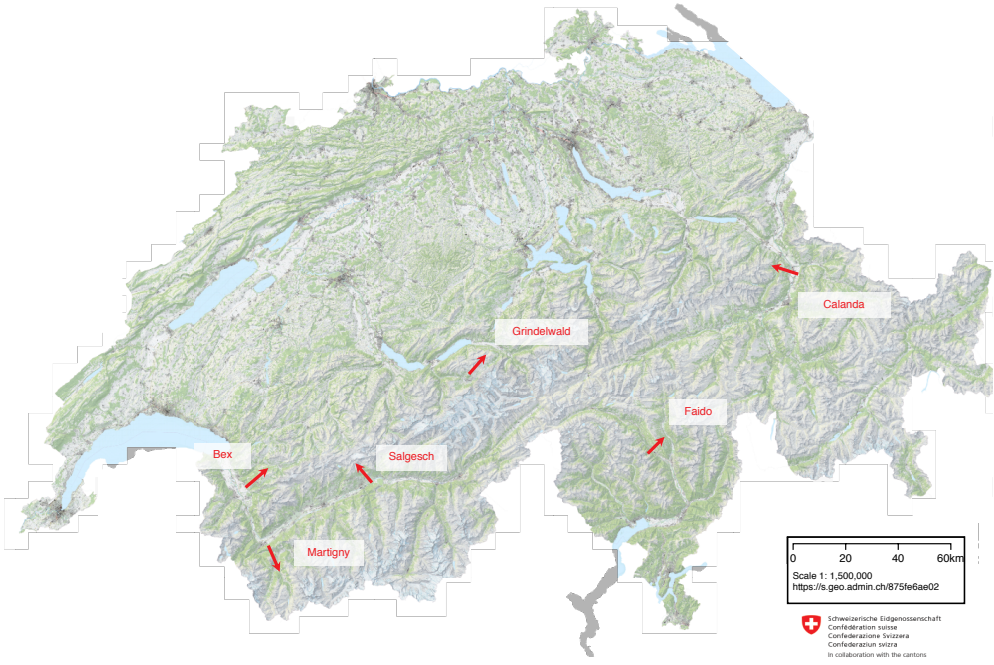

Figure 3 Map of the surveyed areas across Switzerland with the arrows showing the direction from lowest and highest elevation sites for each elevation transect (Bex, Calanda, Faido, Grindelwald, Martigny and Salgesch).

This thesis relies on field data collected in grasslands along elevation gradients of the Swiss Alps (Fig. 3). We focus on natural or semi-natural grasslands in order to specifically study the biological processes as they occur naturally under low or no anthropogenic disturbances. The study sites of low to mid-elevation were selected in dry meadows and pastures while high elevation sites are located in alpine meadows with weak pasture pressure and the absence of mowing activities.

Figure 4 Photographs of a low elevation site along the transect of the southeastern Alps in the region of Salgesch (left panel) and one of the highest elevation sites of the easternmost study area at the Calanda mountain (right panel).

Outline of the thesis

This thesis aims at improving our understanding of the association between elevation gradients and plant–herbivore communities together with the structuration of the trophic networks uniting them, and the rules governing their interactions.

In chapter 1, we aim to perform a direct comparison between above- and belowground herbivores communities along montane clines. We examine the community properties of orthoptera and soil nematodes and their food plants to ask whether these taxonomic groups provide contrasting community response to surface abiotic conditions which shift along elevational gradients. We hypothesize that a decoupling of above- and belowground herbivores communities along the elevation will occur because severe climatic conditions of the Alpine environments are buffered in soil communities. This study is original as it directly compared soil and surface fauna along climatic gradients to raise awareness in considering different ecosystems compartments to study the influence of climate on natural communities.

In chapter 2, we provide a review of the different methodological approaches used to compare ecological networks along environmental gradients. It includes a detailed description of the standardization procedures currently used to allow the comparison of network metrics of different size along gradients and the methods controlling for co-variation with other network variables. A case study, involving plant–hummingbird data, is presented to test the ability of a null models (random and niche) *vs*. hypothesis-based metaweb approach to successfully compare network structure along a gradient of elevation. This rich overview of network comparison methods should contribute to establish of a solid analytical framework that will benefit future investigations on the responses of ecological networks to climatic shifts.

In chapter 3, we investigate how elevation gradients are associated with a shift in the structural wiring of plant–orthoptera trophic networks. Ecological networks are reconstructed using a DNA metabarcoding approach determining the insect diet composition from hundreds of feces samples (Fig. 5). The establishment of an exhaustive plant DNA barcode reference database allows to identify food plant species at a high taxonomic resolution. Using null-models, we quantify variation in network specialization degree with the temperature decrease along elevation. Change in network specialization along the gradient are further related to the resilience of the networks to plant species extinction and to the identification of keystone species important to sustain orthopteran diet. This chapter strongly emphasizes the impact of abiotic conditions on the structuration of ecological networks, which should enrich our understanding of the linkages that exist between climate and the structure of ecological networks.

Figure 5 After capture, species identification and fecal excretion, orthopteran insects are released and feces samples processed along the DNA metabarcoding workflow to reconstruct the trophic networks.

In chapter 4, we explore the mechanisms that underpin plant–orthoptera ecological networks along elevation gradients. Ecological rules related to phylogeny, abundance and trait-based processes are defined to reconstruct networks that are compared against the empirical network reconstructed chapter 2. This allows the discrimination of the major determinants of plant–orthoptera ecological networks. Furthermore, we assessed whether ecological rules uniformly apply to shape interaction patterns along elevation and across biogeographical regions. This study offers promising advances in network ecology intended to understand the ecological mechanisms underlying species interactions at larger spatial scale and under changing environmental conditions.

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CHAPTER I

Contrasting the responses of above- and belowground herbivore communities along elevation

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CHAPTER I

Contrasting the responses of above- and belowground herbivore communities along elevation

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Abstract

Above- and belowground herbivory are key ecosystem processes that can be substantially altered by environmental changes. However, direct comparisons of the coupled variations of above- and belowground herbivore communities along elevation gradients remain sparse. Here, we studied the variation in assemblages of two dominant groups of herbivores, namely, aboveground orthoptera and belowground nematodes, in grasslands along six elevation gradients in the Swiss Alps. By examining variations of community properties of herbivores and their food plants along montane clines, we sought to determine whether the structure and functional properties of these taxonomic groups change along the elevation gradient. We found that orthoptera decrease in both species richness and abundance with elevation. In contrast with aboveground herbivores, the taxonomic richness of nematode does not covary with elevation and increase in abundance towards highlands. We further found a stronger shift in above than belowground functional properties along elevation, where the mandibular strength of orthoptera matched a shift in leaf toughness. Nematodes also showed a weaker pattern of declined sedentary behavior and increased mobility with elevation. In contrast to the direct exposal of aboveground organisms to the surface climate, conditions may be buffered belowground, which together with edaphic factors influencing the biodiversity of soil biota, may explain the differences between elevational patterns of above- and belowground communities. Our study emphasizes the relevance of considering both the above- and belowground compartments to understand the impact of current and future climatic variation on ecosystems, from a functional perspective of species interactions.

Introduction

The study of community properties along environmental gradients is a fundamental and necessary step toward better predictions of the functioning of natural systems (Garnier *et al*. 2016; Mayor *et al*. 2017). In most of the taxonomic groups and trophic levels studied so far, biodiversity shifts along elevation clines has been generally associated with the dramatic climatic variation that exists between low- and highlands (Rahbek 1995; Hodkinson 2005; Mccain & Grytnes 2010; Sergio & Pedrini 2010; Guo *et al*. 2013). One of the most accurate climatic correlates of elevation is temperature, and the linear decrease of temperature with elevation represents a major constraint for species distribution along elevation gradients (Peters *et al*. 2016). Temperature may stimulate metabolic and diversification rates, fostering more species in warm environments (Clarke & Fraser 2004, Allen et al. 2006, Hatfield and Prueger

2015), while cold extremes may generate a stress effect, filtering the composition of communities that can occupy the alpine belt (Körner 2003, Sierra-Almeida et al. 2009, Hoiss et al. 2012, Buckley and Huey 2016). To date, our understanding of the variation of species assemblage along montane gradients is biased toward aboveground organisms (McKenzie *et al*. 2013), while the ecological signature of elevation on soil communities remains largely unknown (Martiny *et al*. 2006; Pellissier *et al*. 2014a).

Above- and belowground communities both influence important ecological processes, including nutrient cycling and trophic interactions (Bardgett *et al*. 1998; Adams & Wall 2000; Wardle *et al*. 2004). Their common role in mediating some of the most important aspects of ecosystem functioning calls for more comprehensive studies on the coupled variation of both systems along environmental gradients. Climatic variation along elevation clines may have different effects on below- compared to aboveground organisms (Adams & Wall 2000). Climatic conditions acting on the surface can be buffered in the soil compartment (Beyens *et al*. 2009), which may result in different species turnover rates between both systems along environmental gradients. For instance, a general decline in species richness along elevation or latitude has been documented for multiple aboveground taxonomic groups of plants and animals (Hodkinson 2005; Sharma *et al*. 2009; Mccain & Grytnes 2010; Guo *et al*. 2013; Descombes *et al*. 2017b, a). In contrast, the few studies conducted on soil systems thus far have not demonstrated a similar association between elevation and taxonomic richness and/or abundance (Margesin *et al*. 2009; Fierer *et al*. 2011; Jarvis *et al*. 2015; Kergunteuil *et al*. 2016; van den Hoogen *et al*. 2019) or even found an increase of these community indices with elevation. For instance, Pellissier et al. (2014a) reported even higher fungal richness and phylogenetic diversity at lower temperatures and higher moisture conditions. Similarly, Kergunteuil et al. (2016) found that the abundance and metabolic footprint of soil nematodes increase when moving from forested low-elevation sites up to Alpine grasslands. Therefore, it appears that community responses to variation in climatic conditions are dependent on where the study system is located, either above- and belowground. If this pattern generalizes to highly important components of trophic networks, such as major herbivore groups, it would lead to a shift and decoupling of ecosystem processes, from above- to belowground, depending on where the system is along the climatic gradient (Adams & Wall 2000; Hooper *et al*. 2000).

The species interactions that drive ecosystem processes are mediated by functional traits. Reciprocally, functional characteristics modulate ecological interactions, and can be used together with community structure indices to understand community responses to environmental gradients (Diaz *et al*. 1998). The choice of relevant functional traits that mediate species–environment interaction through abiotic and biotic mechanisms presupposes a good knowledge of the taxa under study and their ecology (Mlambo 2014; Nock *et al*. 2016). Studies on the elevation variation of functional traits are common for plant traits such as height (Moles *et al*. 2009), specific leaf area (Reich *et al*. 1998), or leaf resource content (Shipley & Vu 2002). For insects, a decrease in body size, in wing length, or shifts in coloration associated with heat absorption have also been related to the elevation gradient (Hodkinson 2005). In contrast, the characterization of functional strategies for soil communities along elevation remain overly marginal (Kergunteuil et al. 2016), although some recent studies have attested to the functional response of soil organisms to the elevation gradient (Bond-Lamberty *et al*. 2016; Looby *et al*. 2016). Yet, as underground systems are less exposed to climatic conditions (Adams & Wall 2000), we expect that functional trait composition and species turnover along elevation is dictated by different structural forces from those acting on the surface.

Climatic variation shapes functional trait composition at the community level (Diaz *et al*. 1998), in turn, potentially modifying the mode and strength of species interactions (Hillyer & Silman 2010), and ultimately, ecosystem processes (Tylianakis *et al*. 2008). For instance, plant-herbivore interactions are modulated by the coupling of plant resistance and feeding-related traits (Moles *et al*. 2013). In this regard, shifts in the composition and functional identity of herbivores along elevation gradients (Hodkinson 2005) has been shown to reduce the intensity of herbivory (Rasmann *et al*. 2014), and should therefore change the investment of plant defences along elevation (Pellissier *et al*. 2012). As a consequence, variation in plant and herbivore functional traits along environmental clines should modify species interactions within ecosystems (Bolnick *et al*. 2011). Consequently, studying both above- and belowground community composition and functional traits could provide valuable information on the plant-herbivores relationships within the two sub-systems along elevation gradients and enable a stratified characterization of ecosystems functioning.

In this study, we explore structural properties and functional constituents of above- and belowground herbivore communities along six elevational transects in the Swiss Alps. We

compare the assemblage of two groups of herbivores: orthoptera that feed on leaves, and herbivore nematodes that feed on roots. Orthoptera are among the most influential aboveground insect herbivores in open habitats, removing high percentages of plant biomass in natural grasslands (up to 30%, Blumer and Diemer 1996). Herbivory pressure exerted by orthoptera has been reported to decrease at high elevation as species richness and abundance are reduced at that level (Scheidel & Bruelheide 2001; Hodkinson 2005; Descombes *et al*. 2017a). Nematodes are belowground organisms characterized by high functional, trophic, and taxonomic diversity. Herbivore nematodes are capable of substantial uptakes of plant root biomass (Ingham & Detling 1990; Hodda *et al*. 2009), while microbivore nematodes graze on soil fungi and bacteria, and omnivores and carnivores prey mainly on other nematodes (Yeates *et al*. 1993). Nematodes have been reported to either decrease (Dong *et al*. 2017) or increase (Kergunteuil et al. 2016) in taxonomic richness and abundance at higher elevation. General patterns of herbivore nematode response to altitude have seldom been described, although high abundances of some herbivore taxa such as *Paratylenchus* and *Pratylenchus* have been found at high elevations in previous studies (Kergunteuil et al. 2016). To date, concomitant investigations of both orthopteran and nematode communities, their functional responses and those of local plant communities along elevation, are still lacking. In this study, we address this question with the following expectations:

1. Species richness and total abundance should decline more above- than belowground along elevation because strong aboveground temperature shifts are buffered compared to belowground. Differential rates of change in communities along elevation may lead to decoupled above- and belowground community composition.
2. The functional properties of aboveground herbivore communities should vary more along elevation than belowground because of attenuated abiotic variations. In particular, we expect that feeding strategies should be
3. Variations in herbivore traits should match those of plant traits along elevation. In particular, we expect the mandibular strength of orthoptera to concurrently vary with changes in leaf toughness of plants. Moreover, we expect a weaker association between elevation and all nematode functional groups, although responses may vary based on feeding type, and depending on how different nematodes' feeding behavior may persist in more unstable environmental conditions.

Materials and methods

Study sites

To study above-belowground plant-herbivore interaction along elevation gradients, we selected six elevation transects spanning the major macro-climatic and environmental conditions (i.e., climate and bedrock type) of the Central Alps (see Fig. S1). We selected eight study sites per elevation transect in open, non-woody areas with elevations ranging from 578 m to 2,417 m, and an average elevation difference between sites of 240 m. Study sites were located in semi-natural grasslands characterized by low impact from agricultural practices in land-use and pasture. Most of the low to medium elevation sites correspond to dry meadows and pastures in the Swiss inventory for national protected areas (Federal Act on the Protection of Nature and Cultural Heritage (LPN), status as of 1 January 2017; Article 18. Protection of animal and plant species, https://www.admin.ch), while high elevation sites were situated in alpine meadows with no mowing and low grazing pressure. Herbivores and plant surveys took place during the summers of 2016 and 2017 within a square area of 10 m x 10 m. Study plots were positioned within the study zone to represent the dominant vegetation type of the surrounding natural environment, and were set a minimum three meters away from forest edges when present. Plant and herbivore inventories were compiled when communities reached maximal species richness and abundance, gradually surveying low to high elevation sites, between early June and the end of August.

Herbivores surveys

Orthoptera surveys were performed under optimal weather conditions for insect activity, between 10 a.m. and 5 p.m. on days with maximal sunshine levels. We focus on Caelifera and Ensifera suborders and species determination was done through visual and auditory identification using the reference work for Swiss Orthoptera (Baur *et al*. 2006). Among orthoptera, the Caelifera suborder includes only strictly vegetarian species, while Ensifera are omnivorous, but largely feed on plant material (Ingrisch & Köhler 1998; Baur *et al*. 2006). We therefore included both suborders in the subsequent analyses. We estimated the abundance of each species following a "Z" sampling pattern across the 100 m^2 study area by counting all adult orthopteran specimens that were visually detected without distinguishing sex. Nematode sampling consisted in a random sampling of 15-20 soil cores (2 cm diameter; 10-25 cm depth) within a 2 m x 2 m area in order to obtain 1 kg of soil after the removal of all rock pieces greater than 2 cm in diameter. The bulk soil was then mixed homogenously, and a

sample of 300 g was collected and stored at 4 °C before nematode extraction. Nematodes were extracted from each sample using a modification of the sieving and Baermann funnel method (Barker, 1985). Once extracted, all nematodes were counted under a binocular microscope. After counting, at least 150 nematodes were identified to the genus or family level according to Bongers (1989) under an inverted microscope.

Plant surveys

The vegetation inventories were first conducted in a circular subplot of 9 m^2 positioned in a floristically homogeneous area within the 100 m^2 plot, across which we further searched for additional rare species. Plant species determination was done following Swiss Floras (Lauber *et al*. 2012; Eggenberg & Möhl 2013). We visually estimated the relative cover of each plant species according to a 9-level scale (<0.25, 0.25-0.5, 0.5-1, 1–5, 5–15, 15–25, 25–50, 50–75 and >75%). The median values of these classes were used in all subsequent statistical analyses.

Herbivore functional traits measurement

We measured orthopteran incisive strength for 90% of the study species using three specimens of each species and sex. When a species was observed in more than one study site, collection points were selected within and between the study areas to cover the full extent of elevation and geographical range of each species. Mandibular trait measurements were performed following the approach described by Ibanez et al. (2013). After the extraction of the left mandible, we took photographs of each mouthpiece in duplicate using a high-resolution measuring digital microscope (Leica DVM6, Leica Microsystems, GmbH, Wetzler, Germany), which, together with the high-resolution photo stacking option available in Leica Application Suite X (LAS X) and Leica Map Premium software (Leica Microsystems), maximizes photographic resolution to increase measurement accuracy. The mandibular incisive strength (F_I) was obtained using the $F_I \sim F_A\, L_A \,/\, L_I\, 1 \,/\, R_I$ formula, where F_A is a proxy for the mandibular section area, L_A is the adductor muscle lever, L_i is the incisive lever and R_I is the incisive region length (Ibanez *et al*. 2013). Values were measured on mandible photographs using the ImageJ image processing program (Rueden *et al*. 2017). For nematodes, we used the Nematode INdicator Joint Analysis system (NINJA, Sieriebriennikov et al. 2014) to assign feeding groups to each nematode taxon, and extracted only the genus corresponding to plant-parasitic nematodes. The functional classification within herbivore nematodes includes a)

epidermal/root hair feeders, which generally induce low phytopathological damage, b) ectoparasites, which feed on superficial root tissues, c) semi-endoparasites, which partially introduce their body into the root tissue, d) migratory endoparasites, which migrate through the root tissue, and d) sedentary endoparasites, which complete their life cycle inside roots, thus establishing a permanent feeding site and inducing the formation of root galls or cysts.

Plant functional traits measurements

Leaf functional traits were collected for 79% of the plant species occurring in the plant surveys. We measured four leaf functional traits that were initially considered as relating to climatic disturbance, but could also confer resistance to herbivory since they reflect leaf toughness and resource acquisition: SLA, LDMC, punch strength, and carbon-to-nitrogen ratio (C/N). We sampled a minimum of three individuals per species, however, when the species occurred at more than one study site along the elevation gradient, we account for potential intra–species variation by increasing the number of replicates across different elevations to a maximum of twelve by multiples of three for each additional site. We selected well-developed and healthy leaves that were moisturized directly after collection and stored at 4°C with additional moisture for a maximum of 24 hours before trait measurement. Measurements of SLA (calculated as the area of a fresh leaf divided by the dry weight and express in $mm^2\ mg^{-1}$) and LDMC (the ratio of the leaf dry mass to the water-saturated weight in $mg\ g^{-1}$) were performed following standard procedures (Cornelissen *et al*. 2003; Vaieretti *et al*. 2007). Dry weight was measured after oven-drying the fresh leaves at 55°C for a minimum of 72 hours. The leaf force to punch, which is considered to be the trait that best captures leaves' mechanical properties that are relevant for herbivory (Sanson *et al*. 2001; Ibanez *et al*. 2013), was measured using a digital force gauge that records the force required to pierce the leaf lamina (IMADA CO., LTD. Toyohashi, Japan). Measurements were taken on fresh leaves with the measurement point selected to avoid leaf veins. Values are expressed in $MN\ m^{-2}$ and were corrected for leaves with widths of less than the diameter of the gauge pin (2 mm). Leaf width was measured using a digital caliper gauge (0.01 mm precision). Total organic carbon (C) and nitrogen (N) amounts were determined for 89% of all species by dry combustion of ground leaf material using a CN elemental analyzer (NC-2500 from CE Instruments, Wigan, Lancashire, United Kingdom).

Taxonomic richness and abundance along elevation

Community-specific richness of plants and orthopteran insects, and the genera richness of herbivore nematodes were calculated as the sum of the number of taxa identified at each study site. Abundance was estimated by using the total specimen count at each site for the orthoptera and as no. of individuals per 100 g of dry soil for nematodes assemblages. The abundance of nematodes for one particular site appears to exceed the median value of the total number of nematodes by a factor of ten. This likely indicates a bias in the sampling process such as the accidental inclusion of soil containing a non-representative high level of organic matter (e.g., dead insect). This site was removed from subsequent analyses. Relationships between elevation and response variables were tested using regression models that included the transect identity as a random factor using functions within the *lme4* and *lmerTest* R packages (Bates 2008, Kuznetsova et al. 2017). All analyses were conducted using R (R Core Team 2019). We related community indices to elevation using generalized linear mixed-effects models for Poisson distribution of count data (glmer) and we use a second-degree polynomial regression when the shape of the relationship was not linear. We also tested the variation of species richness and abundance along the elevation for both orthopteran suborders independently using the same model types. The nAGQ parameter was set to 0 for orthopteran and nematode models to ensure model convergence.

Coinertia analysis

We conducted a coinertia analysis to test the coupling of above- and belowground herbivore communities. For each transect, we first performed a PCA on abundance matrices of orthoptera and nematodes independently using the function dudi.pca in the *ade4* package (Thioulouse *et al*. 2018). We then used the first two factorial axes of the PCAs to apply the coinertia criterion procedure and quantify the co-variance between the two tables (coinertia function) using the RV coefficient. Values close to 1 indicate maximal co-variance between matrices.

Functional traits along elevation

The functional classification of herbivore nematodes was used to reflect functional aspects of belowground communities. We summed the abundance of each herbivore group to quantify their abundance at each site and performed a log-transformation of the data to fulfil model assumptions. The mean community values (CM) of functional traits for orthoptera (i.e.,

incisive mandibular strength) and plants (i.e., SLA, LDMC, punch strength, C/N) were obtained by averaging the sum of the trait values by the total number of species. The community weighted means (CWM) accounting for species abundance were computed for the same traits and each site using $\sum_{i=1}^{R} Pi \times ti$ where R is the number of species, Pi is the relative abundance of the species i, and ti is the mean trait value of the species i. While the two metrics are based on means, CM reflects the average of life-strategies occurring within a community weighting all species equally, and CWM accentuates the ecological role of dominant species (Garnier *et al*. 2004). We used generalized linear mixed-effects models for Poisson data distributions to test whether abundances of the nematodes functional groups were associated with the elevation gradient. For the variation of orthopteran functional trait metrics along elevation (i.e., CM and CWM), we fitted linear mixed-effects models (lmer) for Gaussian data distribution. The weighted and unweighted CM of plant functional traits along the elevation were analyzed using the same models. We excluded the three lowest elevation sites of the Salgesch transect since they were more similar to steppic environments, with plant traits strongly biased toward functional responses that are typical of extremely dry environmental conditions (Volaire 2008; Delarze *et al*. 2015).

Results

Taxonomic richness and abundance along elevation

From vegetation surveys, we identified 526 plant species belonging to 251 genera and 69 families. From orthopteran surveys, 48 species including 19 Ensifera and 29 Caelifera taxa were identified. In total, we identified 55 nematodes genera, comprising 14 herbivore genera that correspond to five different plant-parasitic types (i.e., ectoparasites, epidermal/root hair feeders, semi-endoparasites, migratory, and sedentary parasites). The nematode taxa identified, their functional classification, and percentage contribution of herbivore functional groups to the nematode community are indicated in Table S1. We found that the specific richness of orthoptera and plant species significantly decreases with elevation following linear and hump-shaped relationships, respectively (Fig. 1a, Fig. 1c, Table 1). When tested independently for Caelifera and Ensifera, the same declining trends were found, except for the variation of Caelifera species richness along elevation (Fig. S2, Table S2). This result contrasts with the nematode distribution patterns that presents no variation along the transects for the taxonomic richness and an upward increase of abundance (Fig. 1b, 1e, Table 1). The abundance of

orthoptera displays a significant polynomial relationship with elevation, with the highest values found at mid-elevation (Fig. 1d, Table 1).

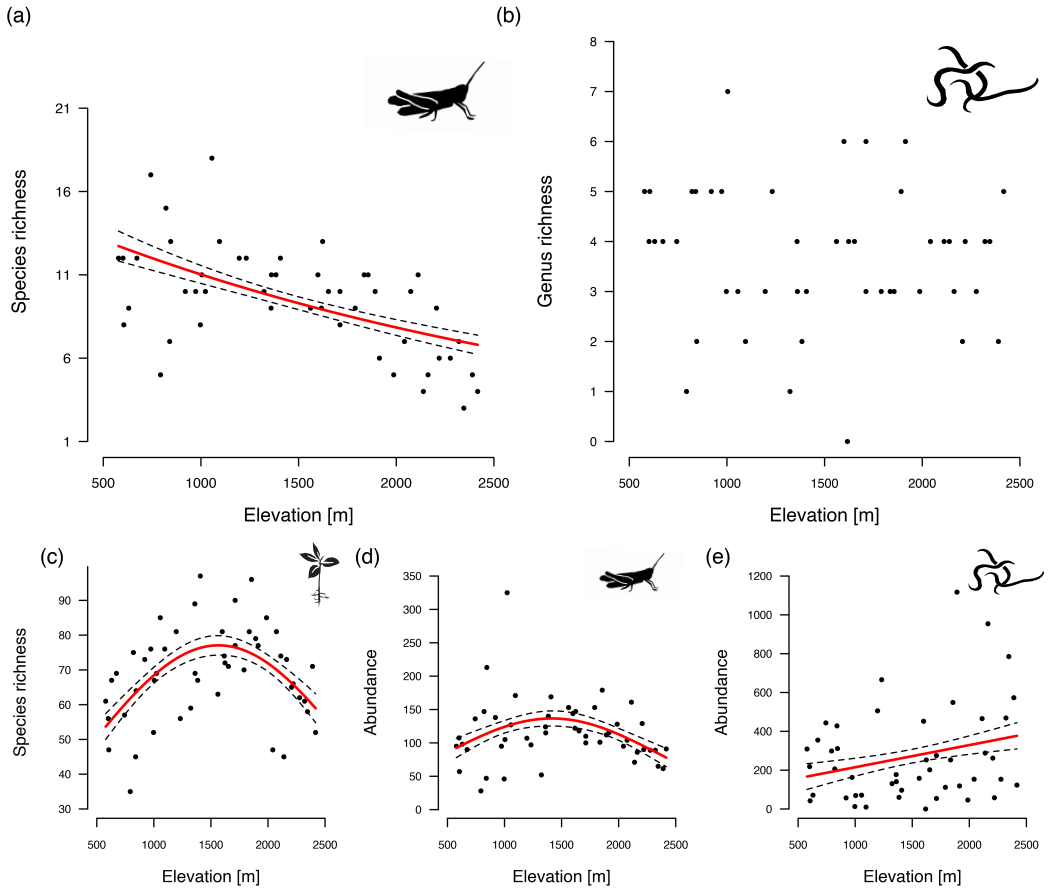

Figure 1 Illustration of the relationships between the elevation and community structure indices using generalized linear and polynomial mixed-effects models with the taxonomic richness of (**a**) orthoptera, (**b**) herbivore nematodes, and (**c**) plants, and the specimen abundance of (**d**) orthopteran communities and (**e**) herbivore nematodes. Regression lines of fitted values and standard error intervals are only displayed for relationships that are statistically significant.

Table 1 Slope coefficients and *p* values for generalized linear mixed-effects models testing the relationships between elevation and community structure indices for species richness of orthoptera, herbivore nematodes, plants, and species abundance of orthoptera and herbivore nematodes.

	Linear model		**2nd degree polynomial model**	
	Slope Estimate	***p* value**	**Slope Estimate**	***p* value**
Species richness				
Orthoptera	-0.0003	<0.001		
Herbivores nematodes	-0.0001	0.584		
Plants	0.246	0.051	-0.698	<0.001
Abundance				
Orthoptera	-0.273	0.006	-1.053	<0.001
Herbivores nematodes	0.0004	<0.001		

Orthopteran and nematodes traits along elevation

Among the five herbivore nematode functional groups, we only found that sedentary parasites decrease significantly in abundance with elevation (Fig. 2d, Table 2). While no clear trend in abundance variation is visible for ectoparasites, epidermal/root hair feeders, or semi-endoparasites (Fig. 2a, 2b, 2e, Table 2), the abundance of migratory endoparasites is significantly higher at high elevation (Fig. 2c, Table 2). The CM of the incisive mandibular strength of orthoptera increases with elevation for males, while for females, values are stable along the gradient (Fig. 2f, Table 2) and largely outreach those of males. The CWM of this trait follows the same pattern (Fig. S3, Table S3).

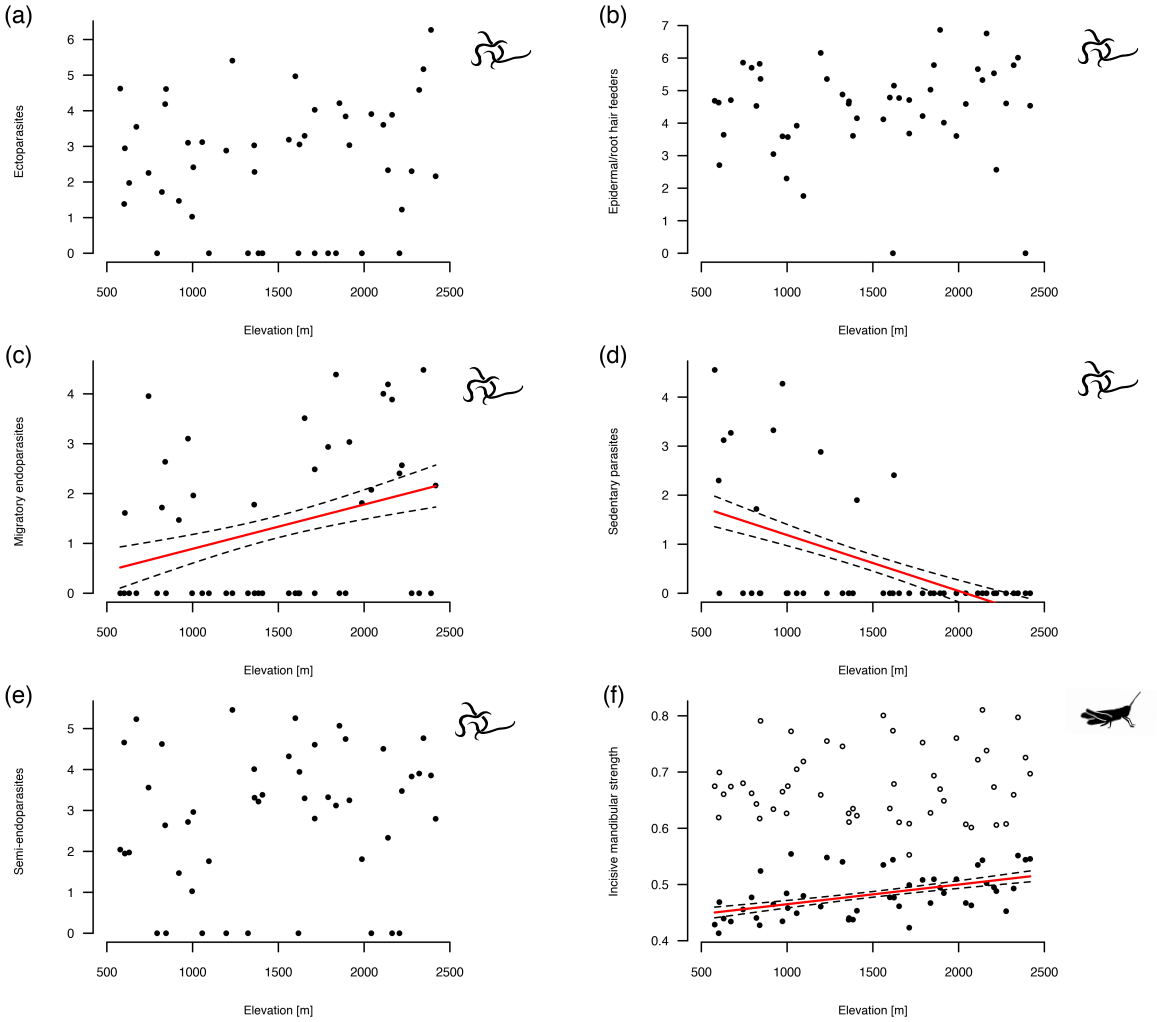

Figure 2 Illustration of the relationships between elevation and the abundance of nematode functional groups of ectoparasites (**a**), epidermal root feeders (**b**), migratory endoparasite (**c**), sedentary parasites (d) and semi-endoparasites (**e**), and the CM of orthopteran incisive mandibular strength (**f**) using generalized linear and linear mixed-effects models. Females are represented by white circles and males by black circles. The regression line of the fitted values and the standard error intervals are displayed only for significant relationships.

Plant traits along elevation

The CM of SLA and LDMC show a significant negative relationship with elevation (Fig.3a, Fig. 3b, Table 2), whereas a positive relationship was found for punch strength and C/N (Fig. 3c, Fig. 3d, Table 2), the latter being concurrently explained by a decrease in nitrogen and an increase in carbon. The results of the regression models applied to CWM generally indicate similar trends to those found for CM (Fig. S4, Table S3)

Table 2 Slope coefficients and *p* values for generalized linear and linear mixed-effects models testing the relationships between the abundance of nematode functional groups, male and female orthopteran incisive mandibular strength, and plant functional traits.

	Slope	
	Estimate	***p* value**
Abundance of nematode functional groups		
Ectoparasites	0.0002	0.340
Epidermal root hair feeders	0.0001	0.555
Migratory endoparasites	0.001	<0.001
Sedentary parasites	-0.003	<0.001
Semi-endoparasites	0.0002	0.328
Orthopteran mandibular strength		
Male	0.00003	<0.001
Female	0.00001	0.55
Plant functional traits		
SLA	-0.001	0.006
LDMC	-0.008	0.004
PUNSH	0.0002	<0.001
C/N	0.001	<0.001

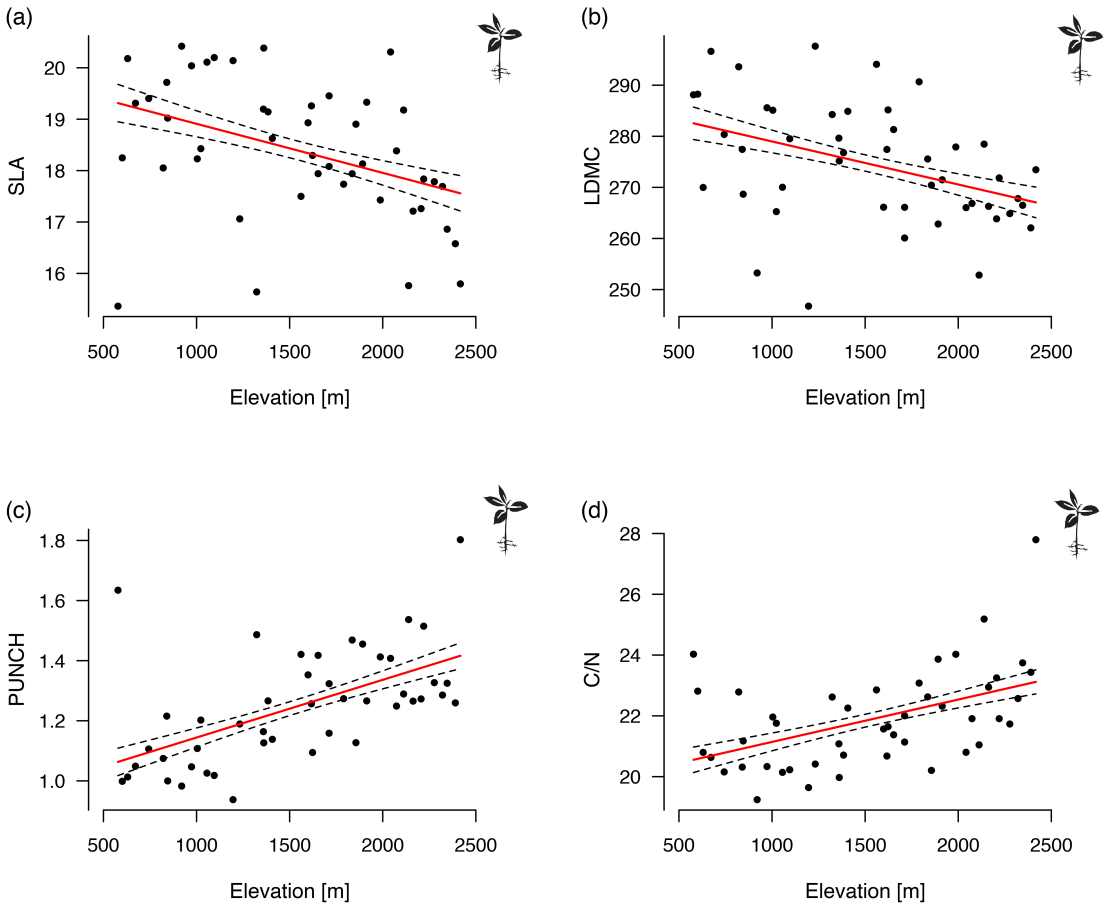

Figure 3 Relationships between plant functional trait community means (CM) and elevation using linear mixed-effects models for (**a**) SLA, (**b**) LDMC, (**c**) punch, and (**d**) C/N. Regression lines of fitted values and standard error intervals are only shown for significant relationships.

Coinertia analysis

For each transect, the PCAs' first two axes together explained more variance in nematode abundance than orthoptera (Table 3), accounting, on average, for 41% and 27% of the variance in orthoptera, and 79% and 15% in nematodes, respectively. The analyses performed on each transect individually suggest a partial decoupling between above- and belowground herbivore communities with RV coefficients ranging from 0.20 to 0.44 (see Table 3).

Table 3 Results of PCAs and the coinertia analyses. For each transect are given the percentage of variance explained by the first two axes of the PCAs applied to orthoptera and nematode abundance matrices and RV coefficients of the coinertia analyses performed on PCA.

	Orthoptera		Nematodes		RV
	Axis 1	Axis 2	Axis 1	Axis 2	coefficient
Transect					
Bex	30.65	29.65	87.4	8.7	0.35
Calanda	55.29	21.74	93.82	5.08	0.25
Salgesch	38.8	32.59	75.29	24.44	0.20
Grindelwald	44.69	26.52	69.43	17.96	0.20
Martigny	37.27	25.63	54.93	29.62	0.44
Faido	38.55	24.54	94.62	4.2	0.25

Discussion

Variations in above- and belowground herbivore communities along the elevation gradient have rarely been directly compared to date. Here, we have focused on two dominant groups of herbivores in grasslands, aboveground orthoptera and belowground nematodes, and studied variation in assemblages along six elevation gradients in the Swiss Alps. We examined variations in community taxonomic properties and the functional traits of above- and belowground herbivores, and their food plants along montane clines. We have shown that variations in community indices along elevation considerably differ between above- and belowground organisms. The evenness of nematode taxonomic richness along the elevation gradient contrasts with the decrease in species richness of orthoptera at the highest elevation and may be related to the tempering effect of stressful abiotic conditions that limits environmental filtering in the soils. The response of above- and belowground communities to elevation is even more contrasted as regard to abundance: we found a negative relationship for orthoptera that is opposite to the upward increase of nematodes along the gradient. We have also found a stronger shift in above- than belowground functional properties along elevation. The mandibular strength of orthoptera matched a shift in leaf toughness along elevation, while nematodes showed a pattern of elevational variation only through a decline in sedentary endoparasites and an increase of migratory endoparasites with elevation. We now discuss our

results in terms of abiotic conditions that act above- and belowground, and examine their implications for shifts in plant-insect herbivore interactions along elevation gradients.

The species richness and abundance of orthoptera decreased with elevation (Fig. 1a, 1d), while for nematodes, no variation or opposite trends were observed (Fig. 1b, 1e). The decrease in aboveground biodiversity is generally associated with a shift in climatic conditions along elevation, although such strong climatic clines may not extend to belowground communities (Bryant *et al*. 2008; Fierer *et al*. 2011). If environmental conditions apply similarly above- and belowground, we should find an equivalent variation in taxonomic richness and abundance for above- and belowground assemblages. Contrary to this expectation, the coinertia analyses indicated generally low congruence between orthopteran and nematode communities (Table 3), suggesting that the response of soil communities to environmental gradients are different from those acting on the surface. Among the environmental conditions that shift strongly with elevation, the temperature gradient may be buffered for soil biota, differentially shaping the structure of above- compared to belowground communities (Beyens *et al*. 2009; Ryalls *et al*. 2013). We found a monotonical decrease in the species richness of orthoptera that directly follows the gradual decrease in temperature with elevation (Barry 2008), while abundance of orthoptera peaks at mid-elevation, where plant specific richness is highest (Fig 1c, Haddad et al. 2001, Descombes et al. 2017b). In contrast, the taxonomic richness of nematodes does not vary with elevation and their abundance increases toward highlands (Fig 1b, 1e). This may indicate the limited influence of temperature decline along the elevation gradient on soil communities. It also suggests that edaphic factors (e.g. soil fertility and humidity) may vary along montane clines to shape soil communities, which, together with buffered surface conditions, may results in patterns that strongly diverge from those observed aboveground (Kergunteuil *et al*. 2016; van den Hoogen *et al*. 2019). In agreement with our study, Kergunteuil et al. (2016), in an analysis of nematode community properties along elevational transects, found that species diversity and abundance are higher in alpine meadows. Hence, indices of community structure that respond to elevation should fundamentally differ depending on whether the study compartment is located above or belowground. Alongside variation in taxonomic richness and abundance, we also expected a weaker variation of functional properties in below- compared to aboveground communities.

Both orthopteran and nematode communities showed some degree of functional changes with elevation. The CM of mandibular strength for male orthoptera increases with increasing elevation, while the community values for females are steady and systematically greater. Morphological differences between sexes have been documented for this insect group, and we show here that sexes respond differently to elevation gradients (Laiolo *et al*. 2013). Because mandibular strength influences plant ingestion, higher values found for females could underscore the greater importance for this sex to bypass plant mechanical barriers at any elevation. This would ensure a nutrient intake that is capital for the production of eggs (Hochkirch & Gröning 2008). For males, although a minimum nutrient intake is necessary to guarantee survival, they may invest less in their feeding ability. Hence, the constant and high mandibular strength of females, and the increase for males with elevation possibly represents a response to plant physical resistance at high elevation where greater mandibular strength would help the ingesting of tougher plants (Ibanez *et al*. 2013). With regards to nematodes, we found a significant increase in abundance for migratory nematodes and a significant decrease for sedentary parasites, with no variation along elevation for nematodes characterized as ectoparasites, semi-endoparasites, or epidermal/root hair feeders. Both sedentary (*Meloidogyne*, *Heterodera*) and migratory (*Pratylenchus*) endoparasitic nematodes are able to feed on large numbers of plant species, being extremely polyphagous (Jones & Fosu-Nyarko 2014; Truong *et al*. 2015). Plant-parasitic nematodes are seldom studied in natural systems, and little information is available on the effects of altitude on parasitic nematodes. The limited information available, however, reports high *Pratylenchus* and low *Meloidogyne* abundances at high elevations in tropical areas (Fogain 2001, Gaidashova et al. 2009, Avelino et al. 2009, Kamira et al. 2013). Our results show that low-specialized belowground herbivory is equally distributed along elevation transects, while the most specialized herbivore conditions, represented by sedentary and migratory endoparasitism (Perrine-Walker 2019), present peak abundances at different elevations. Competition among *Pratylenchus* and *Meloidogyne* has been detected in crops under experimental and natural conditions (Avelino *et al*. 2009; Fontana *et al*. 2015), and similar processes have been described in natural systems between *Pratylenchus* and other endoparasitic nematodes such as *Heterodera* (Brinkman et al. 2005). Besides plant functional traits and other ecological factors, such competition may play a role in structuring herbivore nematode communities across environmental gradients.

If functional traits govern the trophic interplay between plants and herbivores, and reflect community responses to environmental changes, they also reshape plant-insect herbivore interactions along elevation gradients. Influenced or not by abiotic constraints, the abundance of herbivores is also involved in modulating the functional interface between herbivore feeding abilities and plant defence (War *et al*. 2012). We have investigated a set of plant functional traits that represent physical resistance against herbivory. Among the four plant functional traits studied, we found that the CM of SLA and LDMC decreased with increasing elevation, while punch and the C/N ratio showed opposite trends. These increases in C/N and punch traits indicate the greater physical resistance of alpine plant communities. This is in line with the review of Moreira et al. (2018), who concluded that plants adapted to stressful environmental conditions tend to invest more in constitutive defence, which include some chemical but mostly physical defences. Changes in plant leaf traits correspond to the increase in mandibular strength in male orthoptera with increasing elevation. However, we lack data on root properties to properly assess changes in nematode communities. Due to the technical difficulties involved in measuring root functional traits, the imprint of herbivore abundance on root defence is relatively unexplored, with only a few studies focusing on chemical responses to root herbivory (Kaplan *et al*. 2008; Rasmann *et al*. 2011). Without knowledge of root defensive traits against herbivory, it remains difficult to connect nematodes and plant functional responses. However, since root biomass generally increases with elevation (WeiLing *et al*. 2010), plants may support constant herbivory pressures along elevation gradients based on tolerance rather than defensive response. Although this requires further analyses aiming at tracking different defence/tolerance trade-offs in above- and belowground compartments, our study shows that at the surface, herbivore abundance declines with elevation while it increases in the soil. Our study also found that herbivore abundance matrices are partially decoupled and that the variation of functional responses is of greater amplitude for aboveground communities. These findings suggest that the plant-insect herbivore relationships in aboveground systems, in contrast to those belowground, are controlled by a set of abiotic and biotic forces that are unique to the study compartment, and should be studied accordingly. The decline in herbivore abundance along the elevation gradient documented for aboveground insect herbivores (Reynolds & Crossley 1997; Pellissier *et al*. 2014b; Descombes *et al*. 2017a) may not exist belowground. As a result of the increase in nematode abundance along elevation gradients, plant defences in roots may not show the same decline as documented for leaves (Pellissier *et al*. 2012; Callis-Duehl *et al*. 2017). We therefore propose that plant defence and

herbivory relationships of above- *vs*. belowground compartments react differently to environmental change, which calls for a greater effort to document belowground plant-herbivore interactions. However, the factors that operate in the structuring of plant-herbivore interactions remain unexplored, particularly for soil systems. Given the specificities of above- and belowground systems, we believe that a line of research that consider both community types, in a functional and network perspective, is required to identify the drivers of species interaction, and to anticipate how climate change will affect distinct ecosystem compartments and functioning.

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Supplementary materials

1. Supplementary figures

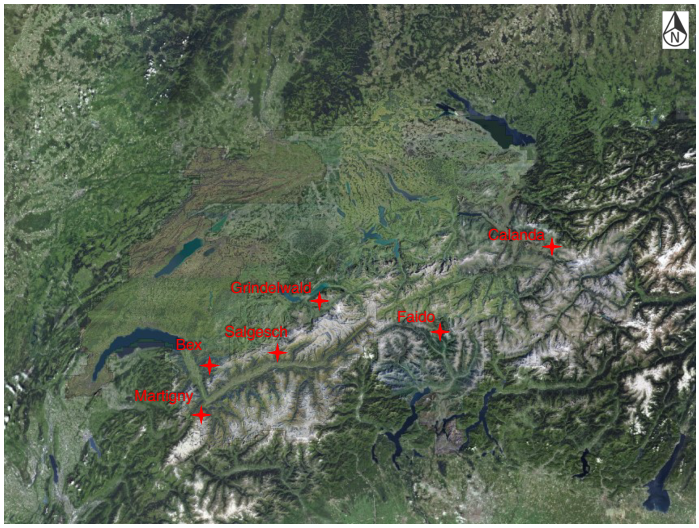

Figure S1 Map illustrating the location of the different study transects across the Swiss Alps

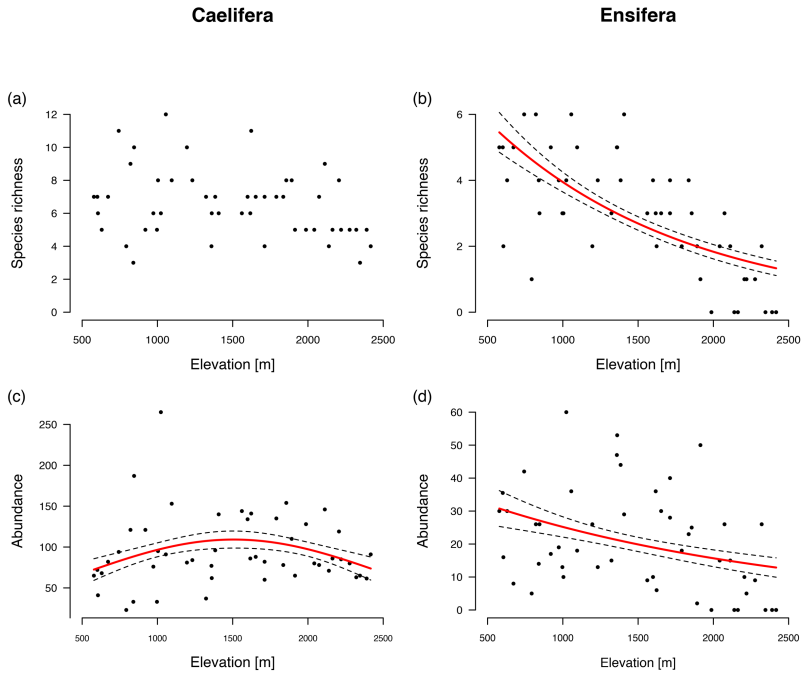

Figure S2 Illustrations of the relationships between elevation and species richness and abundance tested independently for Caelifera and Ensifera suborders using generalized linear mixed-effects model. Regression lines of fitted values and standard error intervals are only shown for significant relationships.

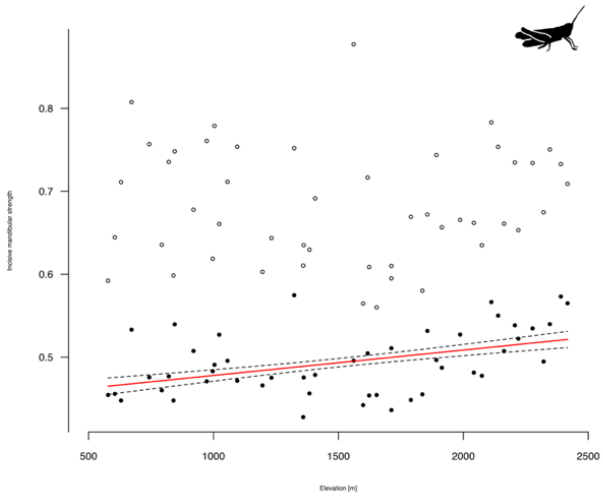

Figure S3 Variation in orthopteran incisive mandibular strength CWM with elevation obtained from a linear mixed-effects model. The significant increase in CWM for males along the elevation gradient, and the steadier relationship found for females are similar to the results obtained through CM calculations. Regression lines of fitted values and standard error intervals are only shown for significant relationships.

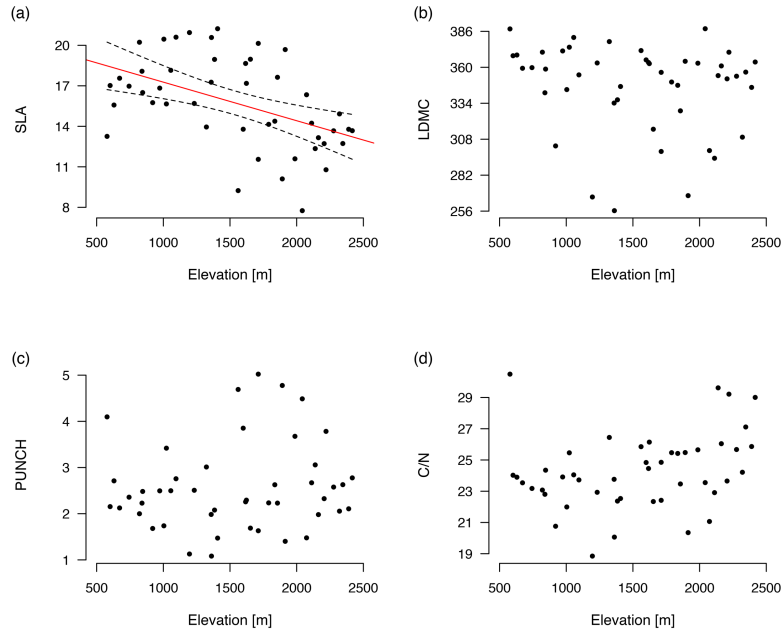

Figure S4 Variation in the plant functional traits (CWM) with elevation obtained from a linear mixed-effects model for (**a**) SLA, (**b**) LDMC, (**c**) punch, and (**d**) C/N. While only the CWM of SLA was found to significantly decrease with elevation, trends for other traits can be compared to the relationships found for CM calculations. Regression lines of fitted values and standard error intervals are only shown for significant relationships.

2. Supplementary tables

Table S1 Mean (± SD) total number of nematodes per transect (including all trophic groups). % of total herbivores and % of different herbivore functional groups are indicated. *Axonchium*, *Gracilacus*, *Longidorella*, *Paratylenchus*, *Trophurus* and *Tylenchorhynchus* were classified as ectoparasites. *Helicotylenchus*, Hoplolaimidae, *Pararotylenchus*, and *Rotylenchus* were classified as semi-endoparasites. *Heterodera* and *Meloidogyne* were considered sedentary parasites. *Pratylenchus* was classified as a migratory endoparasite and Tylenchidae as an epidermal/root hair feeder.

Transect	Bex	Calanda	Faido	Gindelwald	Martigny	Salgesch
Tot. No. Nema.	678.8	1861.4	1221.9	660.2	2056.1	849.7
± SD	±302.3	±2423.833	±853.733	±368.213	±1936.333	±738.3
Herbivores (%)	26.3	20.7	31.4	25.1	31.9	28
Sedentary parasites (% of herbivores)	1	4.7	4.2	4.6	4.3	0
Migratory endoparasites (% of herbivores)	6.6	4.8	4.9	10.1	2.7	4.2
Semi-endoparasites (% of herbivores)	23.2	24.1	16.2	38.5	17.7	8
Ectoparasites (% of herbivores)	5.7	7.3	12.8	9.7	23.5	31
Epidermal/root hair feeders (% of herbivores)	63.4	59.1	61.9	37.1	51.7	56.7

Table S2 Slope coefficients and the *p* values of the generalized linear mixed-effects models testing the relationship of species richness and abundance with elevation for Caelifera and Ensifera suborders.

		Linear model		**2nd degree polynomial model**	
		Slope Estimate	***p*-value**	**Slope Estimate**	***p*-value**
Species richness	Caelifera	-0.0002	0.115		
	Ensifera	-0.001	<0.001		
Abundance	Caelifera	0.104	0.339	-0.891	<0.001
	Ensifera	-0.0004	<0.001		

Table S3 Slope coefficients and the *p* values of the linear mixed-effects models testing the relationship between elevation and CWM of functional traits with respect to orthopteran mandibular strength with values given for males and females and plant SLA, LDMC, punch, and C/N leaf traits.

	Slope Estimate	***p* value**
Orthopteran mandibular strength		
Males	0.00003	0.0014
Females	0.000003	0.882
Plant functional trait		
SLA	-0.003	<0.001
LDMC	-0.010	0.251
PUNCH	0.0002	0.379
C/N	0.001	0.056

CHAPTER I

CHAPTER II

Comparing species interaction networks along environmental gradients

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CHAPTER II

Comparing species interaction networks along environmental gradients

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Abstract

Knowledge of species composition and their interactions, in the form of interaction networks, is required to understand processes shaping their distribution over time and space. As such, comparing ecological networks along environmental gradients represents a promising new research avenue to understand the organization of life. Variation in the position and intensity of links within networks along environmental gradients may be driven by turnover in species composition, by variation in species abundances and by abiotic influences on species interactions. While investigating changes in species composition has a long tradition, so far only a limited number of studies examine changes in species interactions between networks, often with differing approaches. Here, we review studies investigating variation in network structures along environmental gradients, highlighting how methodological decisions about standardization can influence their conclusions. Due to their complexity, variation among ecological networks is frequently studied using properties that summarize the distribution or topology of interactions such as number of links, connectance, or modularity. These properties can either be compared directly or using a procedure of standardization. While measures of network structure can be directly related to changes along environmental gradients, standardization is frequently used to facilitate interpretation of variation in network properties by controlling for some co-variables, or *via* null models. Null models are commonly used to compare the deviation of empirical networks from random expectations and are expected to provide a more mechanistic understanding of the factors shaping ecological networks when they are coupled with functional traits. As an illustration, we compare approaches to quantify the role of trait matching in driving the structure of plant–hummingbird mutualistic networks, i.e. a direct comparison, standardized by null models and hypothesis-based metaweb. Overall, our analysis warns against a comparison of studies that rely on distinct forms of standardization, as they are likely to highlight different signals. Fostering a better understanding of the analytical tools available and the signal they detect will help produce deeper insights into how and why ecological networks vary along environmental gradients.

Introduction

Ecological networks account for both species distributions and their interactions (Reiss *et al*., 2009; Schleuning, Fründ & García, 2015) and provide an integrated representation of communities. They are, however, often considered as fixed entities isolated from one another, and are usually described at a single local site or region. Isolated networks are viewed as the result of deterministic ecological constraints (Clauset, Moore & Newman, 2008), such as forbidden links (Jordano, Bascompte & Olesen, 2003), functional composition (Gravel *et al*., 2016), abundance (Vázquez & Aizen, 2004), morphology (Stang, Klinkhamer & van der Meijden, 2007; Rohr *et al*., 2010) and phylogeny (Cattin *et al*., 2004; Vázquez & Aizen, 2004; Brose, Williams & Martinez, 2006; Petchey *et al*., 2008; Rohr *et al*., 2010; Rohr & Bascompte, 2014). Variation of ecological networks in space or time is a novel and exciting approach to the analysis of community turnover. As shown in recent studies (Tylianakis *et al*., 2008; Kissling *et al*., 2012; Kissling & Schleuning, 2015; Schleuning *et al*., 2015; Tylianakis & Morris, 2017), comparing ecological networks along environmental gradients can generate new insights into the relative importance of environmental filtering and coexistence mechanisms behind community assembly. Beyond analysing general properties that are shared among ecological networks (Bascompte *et al*., 2003), investigations of how networks vary along environmental gradients have the potential to provide insight into how abiotic conditions shape variation in species interactions.

Community ecology has predominantly focused on the structure of species assemblages within a single trophic level, such as plants (Weiher, Clarke & Keddy, 1998; Götzenberger *et al*., 2012) or a guild such as bird communities (Diamond & Cody, 1975; Terborgh *et al*., 1990). The description of assemblages not only by their co-occurrence but also by their interaction has nonetheless a long tradition, as pioneered by the work of Lindeman (1942), Odum (1956) and Margalef (1963). The idea that species are organized into interaction networks was proposed first for terrestrial ecosystems (e.g. plant–herbivore interactions; Elton, 1924) but was later developed mainly in marine ecosystems, e.g. intertidal marine organisms (Paine, 1966), mangroves (Odum & Heald, 1975) and coral reefs (Polovina, 1984). The development of this concept was slower for terrestrial systems and was only recently established as a common approach for studying not just food webs, but also mutualistic (Pimm, 1991; Memmott, 1999; Dunne, Williams & Martinez, 2002; Olesen & Jordano, 2002; Bascompte *et al*., 2003) and host–parasite networks (Lafferty *et al*., 2008). Empirical investigation of ecological networks

requires documenting species presences, along with their interactions and environmental variables. Detection of these can be achieved through direct observation (e.g. records of flower visitors; Fabian *et al*., 2013), use of video-camera systems (Maglianesi *et al*., 2014; Weinstein, 2015), or by indirect methods such as removal experiments (Choler, Michalet & Callaway, 2001), quantification of gut contents (e.g. Barnes *et al*., 2008), isotope analyses (e.g. Vander Zanden *et al*., 1999) or molecular methods (e.g. García-Robledo *et al*., 2013). To provide the most informative ecological signal, quantification of interactions should ideally go beyond the simple observation of the presence or absence of links, and instead estimate the strength of the interactions through time (e.g. interaction frequency between plants for hummingbirds). The documentation of ecological interactions has, however, been very resource-demanding, and only recently have approaches such as molecular barcoding (Jurado-Rivera *et al*., 2009; González-Varo, Arroyo & Jordano, 2014), automated data collection using cameras or other technologies (Weinstein, 2015), as well as data-sharing (Martin González *et al*., 2015; Poisot *et al*., 2016) facilitated the study of ecological networks across sites and along environmental gradients (Wirta *et al*., 2015).

Recent studies comparing the structure of ecological networks along environmental gradients have suggested that ecological and evolutionary constraints may shape networks differently in contrasting environments (Schleuning *et al*., 2012; Hudson *et al*., 2013; Layer, Hildrew & Woodward, 2013; Morris *et al*., 2014; O'Gorman *et al*., 2014; Osorio *et al*., 2015; Martín González *et al*., 2015). These studies highlighted how specific structural properties such as modularity, nestedness, or trophic specialization may vary under the shifting influences of processes such as environmental filtering, competition or facilitation (Layer *et al*., 2010; Schleuning *et al*., 2012; Martín González *et al*., 2015; Cirtwill & Stouffer, 2016). For example, Martin González *et al*. (2015) showed that specialization in plant–hummingbird interaction networks is positively correlated with warmer temperatures and greater historical temperature stability. This can be interpreted as stronger competition for floral resources in warmer and more stable conditions, where specialization favours species co-existence.

Variation of ecological networks along environmental gradients may be driven by multiple factors, since the turnover of species and of interactions may be caused by several abiotic drivers (Poisot *et al*., 2012). Our knowledge of how and why ecological networks vary along environmental gradients is still embryonic, despite increased interest in this field (Warren, 1989; Polis, Anderson & Holt, 1997; Schleuning *et al*., 2011; Dalsgaard *et al*., 2011). Part of

this limitation is caused by the dearth of extensive interaction data sets. In addition, new methods are required to quantify recent networks that contain tens to hundreds of replicated networks (e.g. Krasnov *et al*., 2004) or originate from reconstructed networks based on imposed rules (e.g. Albouy *et al*., 2014). Finally, moving from understanding of 'how networks vary' to 'why networks vary' requires the development of new methodological approaches providing mechanistic insights rather than simple pattern detection (Beaumont, 2010; Gravel *et al*., 2013, 2016).

Species turnover represents the most obvious source of variation of ecological networks along environmental gradients, as interactions between species are primarily conditioned by their co-occurrence (Gravel *et al*., 2016). There are many drivers of species co-occurrence, such as environmental filtering, ecological interactions, dispersal limitations and historical contingencies (Peres-Neto, 2004; Pottier *et al*., 2013). Abiotic conditions may also promote the turnover of interactions for given co-occurrences (Trøjelsgaard *et al*., 2015). Variation in species abundance among sites may influence the frequency and detectability of interactions (Pellissier *et al*., 2013; Bartomeus *et al*., 2016) as more-abundant species are more likely to interact (Petchey, Brose & Rall, 2010; Canard *et al*., 2014). Dominant morphologies or functional traits, for instance body size (Shin & Cury, 2001), both involved in trait-matching constraints (Gravel *et al*., 2013; Albouy *et al*., 2014; Bartomeus *et al*., 2016; Hattab *et al*., 2016), may also vary predictably with the environment (Shipley, Vile & Garnier, 2006). As an example, body size is larger in colder than in warmer conditions (Clarke & Warwick, 1999; O'Gorman *et al*., 2016). Further complicating the picture, co-occurrence is required for an interaction to occur, but the interactions themselves may also affect co-occurrence (Cazelles *et al*., 2016). For example, competitive interactions can potentially exclude a species from locations that would have otherwise favourable abiotic conditions (le Roux *et al*., 2012), or a predator could drive a prey toward an enemy-free location (Wisz *et al*., 2013). When combined, these lines of evidence suggest that strong environmental clines should be associated with significant variation in the structure of ecological networks.

Comparing communities along environmental gradients has traditionally been used to gain a better understanding of how shifting ecological conditions shape the distinct structure of species assemblages, for instance species richness (e.g. Whittaker, Willis & Field, 2001; Macpherson, 2002), functional structure (Cornwell & Ackerly, 2009; Pellissier *et al*., 2010; de

Bello *et al*., 2013), phylogenetic diversity (Graham *et al*., 2009; Pellissier *et al*., 2012) or multiple dimensions simultaneously (Weinstein *et al*., 2014; Dainese, Lepš & de Bello, 2015). Extending the species composition research agenda to ecological networks raises two new questions: what are the network properties to compare, and how to compare them? The first step in such analyses is to extract summary properties from different networks, such as nestedness (Dalsgaard *et al*., 2013) or modularity (e.g. Morris *et al*., 2014), which can be compared directly (Pouilly, Barrera & Rosales, 2006; Fabian *et al*., 2013), or standardized to control for potential covariates (Bascompte *et al*., 2003; Aizen *et al*., 2008; Schleuning *et al*., 2011). Variation in network properties among sites is then interpreted in the light of distinct ecological processes (e.g. matching rules) reflecting different environmental pressures for the stable coexistence of species in communities (Pimm, 1991; Montoya, Pimm, & Sole, 2006). Blüthgen *et al*. (2008) argued that raw metrics, uncontrolled for neutrality or sampling effects, may be substantially flawed resulting in incorrect interpretation of variation across networks. Instead, properties describing network structure should be standardized but the most appropriate method to do so still requires discussion. Here, we review studies which have compared ecological networks along environmental gradients and present the most commonly applied methods with an emphasis on the standardization these methods employ. Using variation in plant–hummingbird mutualistic networks along an elevation gradient as a case study, we compare different methods and discuss their advantages and limitations, along with their ecological interpretation. Our review and case study show that the standardization employed can greatly influence the ecological interpretations of network variation along environmental gradients. We highlight the critical importance of methodological decisions, which should be aligned with the ecological hypotheses that are being tested.

Selecting the network properties to compare

(1) α-properties

Studies of species diversity typically refer to the mean species diversity of a given site at a local scale as alpha diversity (α-diversity; Whittaker, 1972). By analogy, we here refer to α-properties as the characteristics of a local network. Some α-properties are strongly linked to the distribution of interactions such as species specialization or vulnerability (Schleuning *et al*., 2011), while others are related to the topology of the network, including for example connectance (May, 1972; Jordano, 1987; Beckerman, Petchey & Warren, 2006; Santamaria & Rodriguez-Girones, 2007), centrality (Gonzalez *et al*., 2010), nestedness (Bascompte *et al*.,

2003; Santamaria & Rodriguez-Girones, 2007), or modularity (Dalsgaard *et al*., 2013). These properties can be directly extracted from the distribution and structure of nodes and links within each local ecological network. Moreover, the structure of ecological networks can be combined with complementary information, for example with phylogenies (Krasnov *et al*., 2012; Pellissier *et al*., 2013) or with functional traits (Maglianesi *et al*., 2014) to compute more complex indicators within networks. For example, Rezende *et al*. (2007) or Rohr & Bascompte (2014) combined phylogenies with empirical networks and showed a pervasive phylogenetic signal in the structure of species interactions. One may also use traits to compute more-specific metrics, such as ecological matching, when traits of one species should correspond to a trait syndrome of another to allow the interaction (Maglianesi *et al*., 2014; Weinstein & Graham, 2017). One major caveat of the computation of multiple network metrics is that they may show a strong degree of collinearity. Hence, the variation of one metric cannot be interpreted without either considering the variation of its correlate (Poisot & Gravel, 2014), building composite variables using multivariate approaches, or applying a form of standardization.

(2) β-properties

As a complement to the α-properties of ecological networks, β-properties quantify differences between pairs of networks or among multiple networks if a multiple-site dissimilarity measure is required to capture better the heterogeneity of sampled habitats and networks (Diserud & Odegaard, 2007; Melián *et al*., 2015). Poisot *et al*. (2012) proposed quantification of the interactions in common between any pair of localities expressed over the total number of interactions. The total network dissimilarity is then divided into two components, one attributable to the turnover in species composition and the other to the turnover in interactions (Poisot *et al*., 2012). The dissimilarity among ecological networks depends on both the change in the occurrence and the intensity of the interactions (Canard *et al*., 2014). Using this approach, Trøjelsgaard *et al*. (2015) found that distant networks are more dissimilar to one another than closer ones, essentially because of spatial turnover in composition and abundances. As with α-properties, ecological networks can be coupled with species characteristics to compute functional β-properties, for example to quantify whether changes in ecological networks are associated with specific functional or phylogenetic modules. β-properties can be related to environmental differences among sites using a statistical model (e.g. Mantel test). While intuitive and intimately related to the long tradition of β-diversity analysis (Legendre, Borcard & Peres-Neto, 2005), this approach is only

appropriate to compare ecological networks that share many species, whereas it might prove of limited use along environmental clines with significant species turnover. Moreover, the problem of co-varying factors is also relevant when relating β-properties to environmental differences among sites. Depending on the question, applying standardization to avoid biased interpretations can be necessary.

(3) Motif profiles

Ecological networks can be decomposed into smaller modules of interactions, such as omnivory, apparent competition, exploitative competition, and intra-guild predation (Leibold, 1995; Chase, 2003). Whenever these modules are overrepresented in a network, they are generally referred to as 'motifs' (Milo *et al*., 2002). Motifs are hypothesized to be the building blocks of larger network structures (Bascompte & Melián, 2005; Stouffer *et al*., 2007). Ecological networks can thus be described by the combination of all possible motifs of a given number of nodes found in a network (e.g. there are 13 distinct possible motifs of three nodes). The frequency distribution of the different motifs will then reflect the signature of the network topology. This approach can point out conserved regions of the network, which can be key to their functioning under distinct environmental conditions (Baker *et al*., 2015). Motif profiles have been related to certain aspects of community dynamics, such as coexistence and stability (Stouffer & Bascompte, 2011), and have been used to compare networks over space and time. For example, Baker *et al*. (2015) used this approach to investigate the spatial and temporal turnover of host–parasitoid interaction networks in southern Finland. They found that even though there is considerable turnover in species composition, the motif profiles are strongly conserved over spatial and temporal scales, suggesting a consistent network structure. While promising, the rationale of decomposing ecological networks in modules requires further evaluation with empirical data.

Comparing ecological networks along environmental gradients

(1) Comparing raw network properties

Ecological networks can be summarized by structural α- and β-properties, which include nestedness (Bascompte *et al*., 2003), modularity (Olesen *et al*., 2007), and turnover of interactions (Poisot *et al*., 2012). These can be directly related to abiotic variables using various statistical approaches. For instance, Morris *et al*. (2014) evaluated whether connectance, modularity and other properties of antagonistic networks showed a latitudinal trend. After

controlling for sampling effects (species diversity and size of the interaction matrix), they found no consistent latitudinal patterns in 216 quantitative networks of insect parasitoids. Because many network properties are intertwined with each other (Winemiller, 1989; Layer *et al*., 2010), it is essential to control for a possible effect of co-variation, such as with species richness or relative abundance within a standardization procedure. Blüthgen *et al*. (2008) warned that the comparison of raw metrics may be substantially flawed, because of collinearity between network properties or due to underlying variation in species abundance or species richness (see Morris *et al*., 2014). The same limitation applies to high-dimensional properties of network structures involving complementary sources of information such as traits and phylogenies (Rohr & Bascompte, 2014). For example, a direct comparison of the phylogenetic signal (e.g. through a correlation between phylogenetic distances and interactions) among networks only evaluates whether interactions are associated with the phylogenetic distance among species (Aizen *et al*., 2016). Nevertheless, this direct comparison does not evaluate whether the same lineages interact with each other, nor identify the underlying ecological mechanism. A direct comparison of metrics is therefore expected to provide primarily a description of how different aspects of network structures vary along environmental gradients, but is less likely able to answer why they do so. Moreover, due to collinearity among metrics describing ecological networks, a direct comparison generally fails to disentangle the independent variation of a given property.

(2) Residual variation of network properties

The simplest approach to control for the co-variation of network properties is to use a linear regression to remove it and focus on the residuals thereof (e.g. Devoto, Medan & Montaldo, 2005; Tylianakis, Tscharntke & Lewis, 2007; Trøjelsgaard *et al*., 2013; Dalsgaard *et al*., 2013; Morris et al., 2014). For example, connectance is a common metric for describing network complexity, but it is strongly correlated with species richness (Winemiller, 1990; Martinez, 1992; Havens, 1992), which constrains the potential arrangements of links (Poisot & Gravel, 2014). Quantifying the residual variation in connectance among sites that is independent of species richness provides a better measure of the degree of species association in an ecological network (Dunne *et al*., 2002; Olesen & Jordano, 2002). In the situation of multiple collinear variables, structural equation models or path analyses are useful tools for disentangling the relative correlations of collinear variables along environmental gradients (Thébault & Fontaine, 2010). The study of residual variation provides the means to measure

the variation of the property of ecological networks along environmental gradients independently of other co-variables. Although it still does not necessarily identify the underlying mechanisms, it allows us to quantify more precisely the variation of interest among ecological networks.

(3) Rarefaction analysis

Rarefaction techniques provide a way to compare ecological networks that differ in either sampling effort or community complexity across sites (Olesen *et al*., 2011; Albrecht *et al*., 2014; Morris *et al*., 2014). In community ecology, rarefaction curves allow comparison between the observed or expected species richness in a relatively poorly sampled community with the expected species richness of a more extensively sampled community for an equivalent sampling effort, thereby removing confounding sampling effort effects (Simberloff, 1978; Gotelli & Colwell, 2001). In the context of ecological networks, rarefaction analyses allow the comparison of networks that differ in sampling effort, complexity, or species richness. Species and their associated interactions can be randomly removed from the most species-rich network to match the richness level of the species-poor network to which it is being compared. This operation can be repeated multiple times to obtain a statistical distribution of rarefied network properties (Albrecht *et al*., 2014). The value of the property for the species-poor network can be compared to the distribution of the rarefied one. In Fig. 1, we illustrate this approach using 10 parasitic food webs in agricultural landscapes (Fabian *et al*., 2013). Fig. 1 indicates that there is a positive correlation between difference in connectance and difference in the configuration of the agricultural landscape among sites, which is, however, confounded by underlying variation in species richness. When accounting for differences in species richness using a rarefaction approach, only three pairs of sites at similar richness level showed significant differences in connectance. The overall gradient in connectance needs to be robust to differences in species richness before conclusions can be drawn about apparent underlying differences in connectance per site.

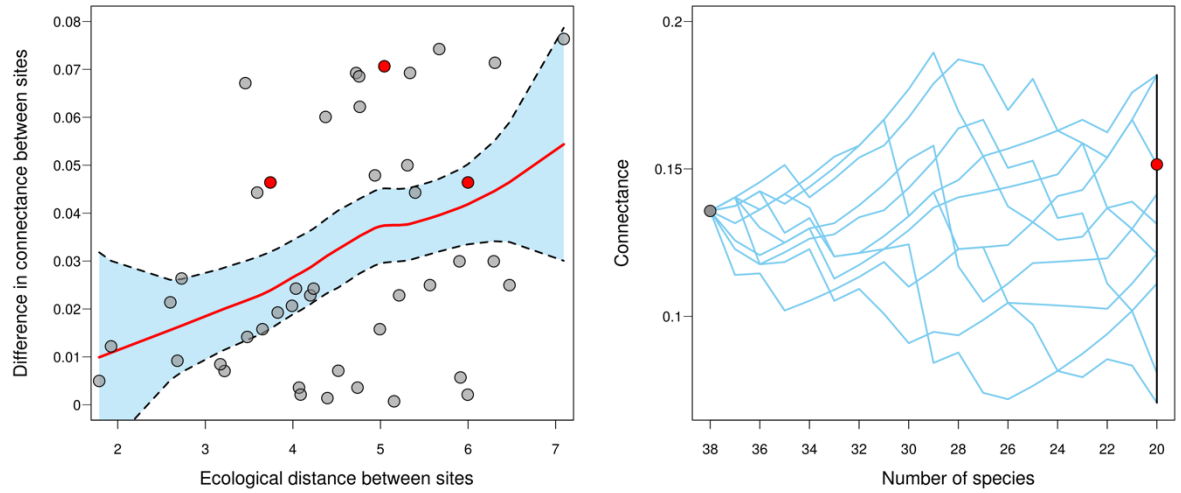

Fig. 1. Comparison of connectance of 10 hymenopteran food webs from Fabian *et al*. (2013) using the rarefaction method to remove species and links randomly. (A) Relation between ecological distance and difference in connectance between sites. The ecological distance between sites was expressed as the Euclidean distance between the percentage cover by six landscape elements on the different sites: (*i*) agricultural fields; (*ii*) extensive meadows, gardens, orchards and hedges; (*iii*) forest; (*iv*) wildflower strips; (*v*) water bodies and (*vi*) urban areas (roads and houses). Red dots on the graph identify the only three pairs of networks that showed a significant difference in connectance when network size was compared after rarefaction. The red line is a local polynomial regression fitted with a confidence interval of 95% (shaded blue). (B) The observed connectance of the smallest network (red dot; 20 species) compared with the distribution of rarefied connectance with 10 iterations from a richer species network (38 species). In this example, the two measures of connectance are not different.

(4) Null models

Null models are useful for evaluating whether a specific structural property may be the result of chance alone in the absence of any particular ecological constraint (Gotelli & Graves, 1996; Gotelli, 2001). This approach has been used widely in spatial community ecology to evaluate whether community structure, such as the distribution of abundance or functional dispersion, differs from random sampling of the regional species pool (Götzenberger *et al*., 2012). Null models are also applied to the analysis of ecological networks (Bascompte *et al*., 2003; Ollerton *et al*., 2007) and along environmental gradients (see Table 1). Here, the value of the network property of interest is contrasted to expected values from the null models, where the links within each network are randomized. The randomization might be constrained, e.g. by fixing the species' relative abundances. Blüthgen *et al*. (2008) showed that the deviation of

network properties from null expectations varies according to the relative abundance of the species. If partners associate randomly, species are more likely to interact with common than with rare partners. Since species abundances and frequencies are known to co-vary with environmental gradients (Lomolino, 2001), a null model accounting for the abundance or frequency of species provides a more relevant baseline to highlight changes in species interactions along the gradient (Schleuning *et al*., 2012; Sebastián-González *et al*., 2015).

Null models have also been used to evaluate the role of functional traits. Trait matching between mutualistic or antagonistic partners is compared to the values expected when the association of species with their traits is randomized. Null models have been used for the evaluation, for example, of whether the functional matching of interactions is stricter than expected under random associations (Fig. 2B). The standard effect size (SES) – the difference of the observation relative to the null distribution – is related to environmental gradients using a statistical model (Schleuning *et al*., 2012). As emphasized by de Bello *et al*. (2013), null models are not "magic wands", and a linear dependence between the SES and the original raw metric is frequently observed. Similarly, it is not known whether standardized measures generated by null models can be properly compared across networks with different dimensions. The architecture of a null model requires careful evaluation (e.g. using simulated data) to understand clearly whether the confounding effects are attenuated as anticipated.

Table 1 Publications where mutualistic or antagonistic ecological networks were compared along environmental gradients, together with the summary network property considered, the environmental gradient, and the standardization approach used. Comparing ecological networks along environmental gradients is an emerging field; most of the 25 studies listed here use either a residual analysis or null models to standardize the comparison.

Network type	Property	Ecological gradient	Method	Reference	Question	Link
Antagonistic	Species richness, trophic composition	Elevation	Comparing raw properties	Pouilly *et al*. (2006)	Changes of taxonomic and trophic structure of fish assemblages along an environmental gradient in the Upper Beni watershed (Bolivia)	http://onlinelibrary.wiley.com/doi/10.1111/j.0022-1112.2006.00883.x/full
Mutualistic	Specialization	Latitude, past and contemporary climate, plant diversity	Null model	Schleuning *et al*. (2012)	Specialization of mutualistic interaction networks towards tropical latitudes	http://www.sciencedirect.com/science/article/pii/S0960982212009438
Antagonistic	Rates of parasitism, linkage density, generality, vulnerability, evenness, connectance, compartment diversity	Habitat modification	Adding a statistical cofactor	Tylianakis *et al*. (2007)	Habitat modification alters the structure of tropical host–parasitoid food webs	http://www.nature.com/nature/journal/v445/n7124/abs/nature05429.html
Mutualistic	Specialization, connectance, number of interactions, species richness	Precipitation, elevation	Adding a statistical cofactor	Devoto *et al*. (2005)	Patterns of interaction between plants and pollinators along an environmental gradient	http://onlinelibrary.wiley.com/doi/10.1111/j.0030-1299.2005.13712.x/full
Mutualistic	Connectance, nestedness, degree of distribution, modularity	Elevation	Null model	Ramos-Jiliberto *et al*. (2010)	Topological change of Andean plant–pollinator networks along an altitudinal gradient	http://www.sciencedirect.com/science/article/pii/S1476945X09000622
Mutualistic	Modularity and nestedness	Historical and contemporary climate change	Comparing raw properties	Dalsgaard *et al*. (2013)	Historical climate change influences modularity and nestedness of pollination networks	http://onlinelibrary.wiley.com/doi/10.1111/j.1600-0587.2013.00201.x/full
Mutualistic	Modularity and nestedness	Latitude, elevation, temperature, precipitation	Null model	Trøjelsgaard *et al*. (2013)	Macroecology of pollination networks	http://onlinelibrary.wiley.com/doi/10.1111/j.1466-8238.2012.00777.x/full

Table 1 continued

Network type	Property	Ecological gradient	Method	Reference	Question	Link
Mutualistic	Specialization	Elevation, historical climate change (velocity), contemporary climate change (precipitation, temperature), species richness and seasonality	Null model	Dalsgaard *et al.* (2011)	Specialization in plant-hummingbird networks is associated with species richness, contemporary precipitation and quaternary climate-change velocity	http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0025891
Mutualistic	Modularity and nestedness	Latitude, climate, topography, human impact	Null model	Sebastián-Gonzáles *et al.* (2015)	Macroecological trends in nestedness and modularity of seed-dispersal networks: human impact matters	http://onlinelibrary.wiley.com/doi/10.1111/geb.12270/full
Antagonistic	Trophic level and contribution of benthic carbon to diet	Inshore–offshore	Comparing raw properties	Kopp *et al.* (2015)	Reorganization of a marine trophic network along an inshore–offshore gradient due to stronger pelagic–benthic coupling in coastal areas	http://www.sciencedirect.com/science/article/pii/S007966111400175X
Antagonistic & mutualistic	Modularity and nestedness	Temperature, precipitation	Adding a statistical cofactor	Welti & Joern (2015)	Structure of trophic and mutualistic networks across broad environmental gradients	http://onlinelibrary.wiley.com/doi/10.1002/ece3.1371/full
Antagonistic	Trophic levels, connectance, generality, vulnerability	Estuarine–costal	Niche model	Vinagre & Costa (2014)	Estuarine–coastal gradient in food-web network structure and properties	http://www.int-res.com/articles/meps2014/503/m503p011.pdf
Antagonistic	Linkage density, connectance, generality, vulnerability, modularity, specialization	Latitude	Comparing raw properties	Morris *et al.* (2014)	Antagonistic interaction networks are structured independently of latitude and host guild	http://onlinelibrary.wiley.com/doi/10.1111/ele.12235/full
Antagonistic	Generality, vulnerability, connectance, interaction evenness	Elevation	Adding a statistical cofactor	Maunsell *et al.* (2015)	Changes in host–parasitoid food web structure with elevation	http://onlinelibrary.wiley.com/doi/10.1111/1365-2656.12285/full
Mutualistic	Number of compartments, modularity, number of modules, nestedness, connectance, pollinator:plant ratio, robustness	Invasion status	Rarefaction analysis	Albrecht *et al.* (2014)	Consequences of plant invasions on compartmentalization and species' roles in plant–pollinator networks	http://rspb.royalsocietypublishing.org/content/281/1788/20140773.short

Table 1 continued

Network type	Property	Ecological gradient	Method	Reference	Question	Link
Antagonistic	Species composition and species interaction	Temperature, isothermality, precipitation, diurnal range	Beta-diversity	Poisot *et al.* (2016)	Hosts, parasites, and their interactions respond to different climatic variables	http://www.biorxiv.org/content/early/2016/11/07/079780.abstract
Antagonistic	Herbivore and predator biomass, and herbivore composition	Productivity	Comparing raw properties	Chase (2003)	Strong and weak trophic cascades along a productivity gradient	http://www.jstor.org/stable/3548357
antagonistic	Phenotypic and ecological specialization	Elevation	Comparing raw properties	Maglianesi *et al.* (2014)	The role of morphological traits (i.e. phenotypic specialization) for ecological specialization in plant–hummingbird networks in three types of Neotropical forests at different elevations	https://www.unioviedo.es/danielgarcia/Papers_ECO2016/Maglianesietal_2014_Ecology.pdf
Antagonistic	Mean species richness, total community abundance, functional group abundance, extinction frequency, and temporal variability in abundance	Latitude	Comparing raw properties	Tuck & Romanuk (2012)	Robustness to thermal variability differs along a latitudinal gradient in zooplankton communities	http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2486.2012.02652.x/abstract
Antagonistic	Species richness, link, chain, omnivory properties	Altitude (river gradient)	Comparing raw properties	Romanuk *et al.* (2006)	The structure of food webs along river networks	https://doi.org/10.1111/j.2005.0906-7590.04181.x

Table 1 Continued

Network type	Property	Ecological gradient	Method	Reference	Question	Link
Antagonistic	Trophic groups, linkage density, connectance, generality, vulnerability, fraction of top and intermediate predators, fraction of basal and herbivore species, mean and maximum trophic level, fraction of omnivorism and cannibalism, mean short-weighted chain length, trophic path length	Human impact	Comparing raw properties	Coll *et al*. (2011)	Food-web structure of seagrass communities across different spatial scales and human impacts	http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0022591
Antagonistic	Specialization	Latitude	Null model	Dalsgaard *et al*. (2017)	Opposed latitudinal patterns of network-derived and dietary specialization in avian plant–frugivore interaction systems	http://onlinelibrary.wiley.com/doi/10.1111/ecog.02604/full
Mutualistic	Specialization	Latitude	Null model	Pauw & Stanway (2015)	Unrivalled specialization in a pollination network from South Africa reveals that specialization increases with latitude only in the Southern Hemisphere	http://onlinelibrary.wiley.com/doi/10.1111/jbi.12453/abstract
Antagonistic	Mass ratios between trophic levels	Latitude	Comparing raw properties	Romero *et al*. (2016)	Food-web structure shaped by habitat size and climate across a latitudinal gradient	http://onlinelibrary.wiley.com/doi/10.1002/ecy.1496/full

Table 1 Continued

Network type	Property	Ecological gradient	Method	Reference	Question	Link
Antagonistic	Vulnerability, generality, link density, interaction diversity, compartment diversity	Site isolation from each other	Generalized linear models relating food-web metrics to descriptors of community species richness, vegetation, landscape and spatial arrangement	Fabian *et al*. (2013)	Importance of landscape and spatial structure for hymenopteran-based food webs in an agro-ecosystem	https://www.ncbi.nlm.nih.gov/pubmed/23863136

(5) Comparison to a hypothesis-based metaweb

The metaweb represents potential interactions among all species from the regional pool (Dunne, 2006) and provides an alternative approach to compare the structure of ecological networks. Instead of assembling each local ecological network by randomly drawing from the overall interaction pool, as is generally done with null models (Schleuning *et al*., 2012; Sebastián-González *et al*., 2015), one can generate a network of expected interactions between all the species in the regional pool under specific constraints (Havens, 1992). The architecture of a metaweb can be based on pure random interactions, which would correspond to a regional random null model, or can further account for the species frequency distribution in the species pool, trait matching (Morales-Castilla *et al*., 2015; Bartomeus *et al*., 2016), or phylogenetic relatedness (Pellissier *et al*., 2013). The deviation of local networks from the metaweb can both inform whether the latter provides a sufficient approximation of realized networks or whether some local structure deviates more than others in particular parts of the environmental gradient. We illustrate in Fig. 3 different metawebs of trophic interactions among Mediterranean fish species built from species co-occurrence, trait or phylogenetic matching. We show that a Mediterranean metaweb built using body size provides a better fit to the local network in the Gulf of Gabes, a southern Mediterranean ecosystem along the Tunisian coast. In this example, only one local network is compared to the metaweb, but this analysis can be extended to an entire gradient (e.g. of bathymetry) and used to determine if there are locations where the body size relationship is not sufficient to explain the network complexity. Deviation of local ecological networks from the metaweb can be quantified using, for example, the True Skill Statistic (TSS; Allouche, Tsoar & Kadmon, 2006) for binary interactions (Fig. 3), or a correlation for quantitative links (Fig. 2C) and thus related to environmental gradients. For instance, Gravel *et al*. (2011) investigated 50 trophic networks in Canadian lakes and found that the structure of many local networks was different from that expected under a random metaweb, with much greater connectance and generality on average than the null expectation. This approach is adjustable to the hypotheses serving to create the metaweb, so that environment-specific deviations from expected rules (e.g. random, abundance-based, and trait-matching) can be quantified. This approach necessitates that the anticipated metaweb is based on ecologically sound assumptions, and will thus require some prior knowledge of the system.

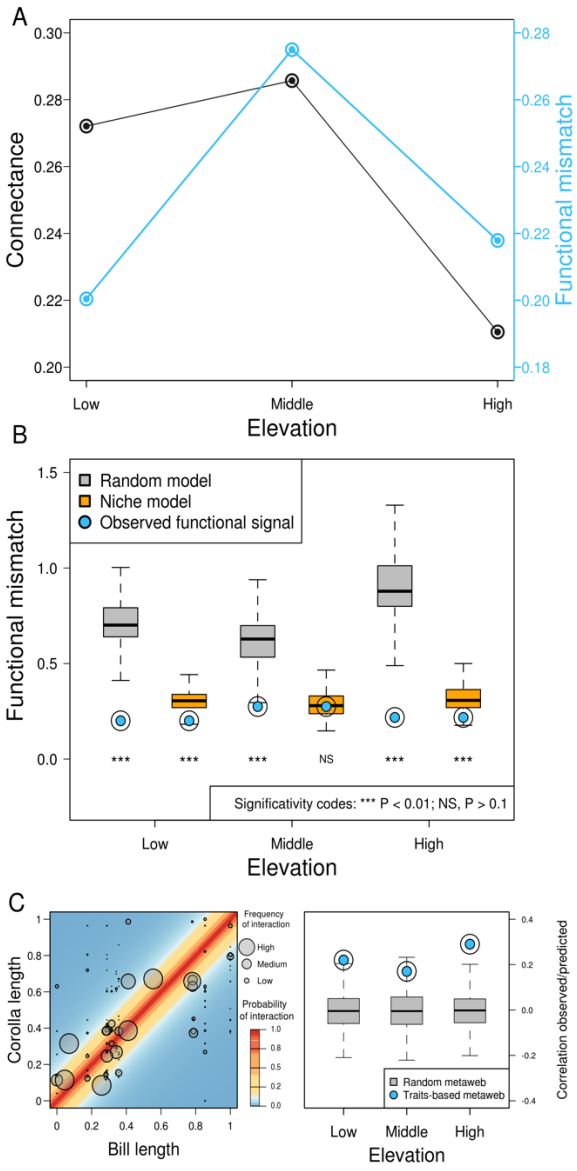

Fig. 2. Methods to compare ecological networks illustrated for the case study of plant–hummingbird mutualistic networks along an elevation gradient in Costa Rica: wet forest (50 m; 10°26′ N, 84°01′ W), pre-montane forest (1000 m; 10°16′ N, 84°05′ W), and lower montane wet forest (2000 m; 10°11′ N, 84°07′ W). For further details about the study site, see the Maglianesi *et al*. (2014). (A) Connectance and functional mismatch (measured as the mean absolute difference between bill and flower corolla length) *versus* elevation. (B) Observed functional matching compared to two null models: randomized 999 times within each local network (grey) and the niche model of species interaction (orange; Williams & Martinez, 2000). The black line represents the median, the top and the down of the box the 1st and 3rd quartile respectively and the whisker represent 1.5 times the distance between the 1st and 3rd quartile. (C) Correlation between the observed interaction frequencies and those expected from a metaweb assuming the highest frequency of interaction for species with matching bill and corolla standardized length.

(6) Network alignment

The alignments of the motifs within networks have been argued to provide a flexible approach to detect whether networks have a common core structure along environmental gradients (Morales-Castilla *et al*., 2015). Alignment may be used to match motifs composed of several nodes among different networks. Conceptually, the method has some similarities with the alignment of sequences of nucleotides performed to compute phylogenies, as it needs to maximize the motif match among networks using a cost function. The cost function could be simple (e.g. by looking at the fraction of matched interactions for each pair of nodes) or use a finer description of the topology. For instance, Stouffer *et al*. (2012) computed the motif profile for each node, i.e. the frequency at which a node belongs to a set of motifs – also called species role – and computed the average correlation between the profiles of pairs of nodes. This approach can be extended to evaluate the recurrence of common motifs across networks in distinct environments and can identify which conserved regions of the network are key to its functioning (Baker *et al*., 2015). This approach enables us to quantify the similarity of the topology between very different pairs of ecological networks, even those with no species in common, such as between marine and terrestrial systems. However, it still requires further development to become a standard tool for network comparison along environmental gradients.

(7) Coupling co-occurrence with interactions

The dissimilarity among ecological networks along environmental gradients can be decomposed using a set of statistical models for species distributions and their interactions (Gravel *et al*., 2016). Models of co-occurrence or co-variation in abundance, so called joint species distribution models, have been developed over the last decade (Pollock *et al*., 2014; Warton *et al*., 2015; Ovaskainen *et al*., 2017). These joint species distribution models predict species distributions based on environmental and spatial variables and allow sharing of information on species distribution and thereby improve the estimation of parameters. Statistical models might not only integrate co-occurrence, but also the interactions that link species to each other to account better for the way abiotic and biotic factors interact with each other to shape species assemblages along environmental gradients (Cazelles *et al*., 2016). For instance, Gravel *et al*. (2016) combined a co-occurrence model with a trait-matching model, both interacting with climatic variations, to understand more mechanistically the drivers of interaction turnover in plant–herbivore networks. The main limitation of this approach, however, is that it requires a large amount of replicated records of interactions along

environmental gradients for calibration and to perform a suitable evaluation of the model parameters, including the interaction between abiotic and biotic effects.

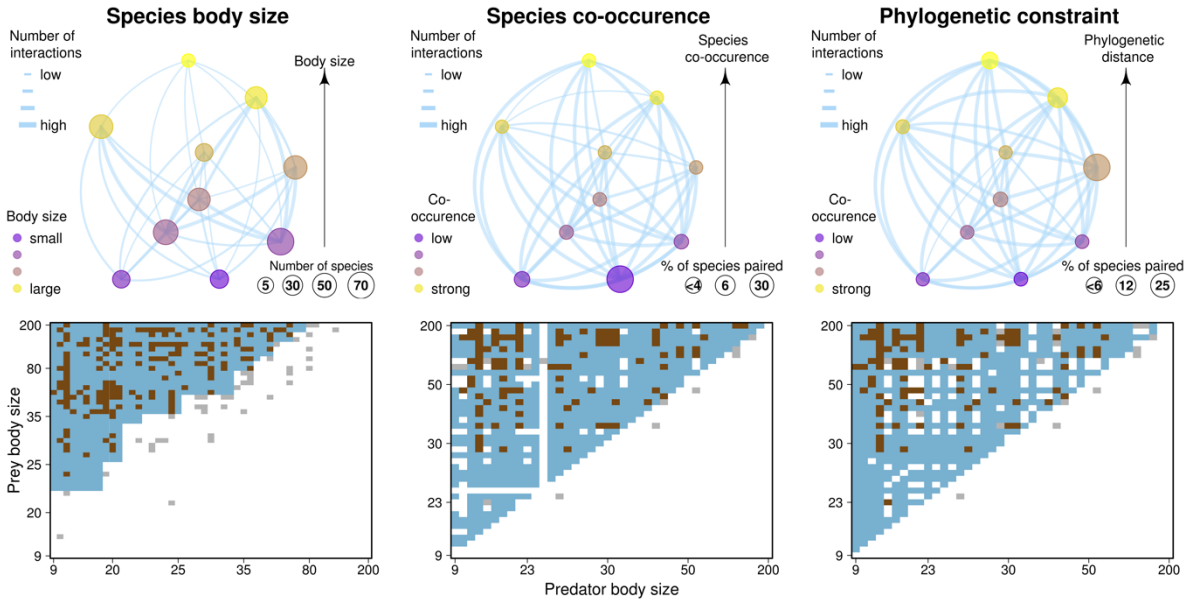

Fig. 3. Hypothesis-based metaweb of fish from the Mediterranean Sea. Upper images show three contrasting hypothesis-based metawebs, based on (A) body size data from Barnes *et al*. (2008), (B) habitat preferences (e.g. benthic, pelagic; Albouy *et al*., 2015) and (C) phylogenetic distance between groups of co-occurred species based on the phylogeny of Mediterranean fish in Meynard *et al*. (2012). Lower images show metaweb expectations compared to the observed food web for the Gulf of Gabes on the Tunisian coast (Hattab *et al*., 2015). The grey colour on the graph represents the observed values, blue represents the expected values according to the hypothesis, and brown is the match between the expected and observed values. The body size hypothesis showed the strongest association to the observed Gabes food web with the highest True Skill Statistic (TSS) values (TSS_{size}= 0.55, $TSS_{habitat}$= 0.5, TSS_{phylo}= 0.44). This comparison can be applied to any food web across the Mediterranean Sea.

What is the best approach for comparing ecological networks?

Studies comparing ecological networks along environmental gradients are relatively scarce in contrast to more traditional community analyses looking at species richness or functional traits within a single trophic level. We reviewed 25 studies (Table 1) that compared ecological networks along a variety of gradients, including elevation (Devoto *et al*., 2005; Ramos-Jiliberto *et al*., 2010; Maunsell *et al*., 2015) and latitude (Sebastián-González *et al*., 2015). This limited number of studies contrasts with the hundreds of publications in community ecology (Götzenberger *et al*., 2012). The use of residual correlations and null models were the most common approaches to standardize and compare ecological networks along environmental gradients. Only one study used a metaweb (Gravel *et al*., 2011) or a full species co-occurrence–interaction coupled model (Gravel *et al*., 2016) to evaluate the role of the abiotic environment in shaping ecological networks. Moreover, most studies compared summary properties based on the distribution of links and network topologies along environmental gradients, and generally did not include functional traits. Researchers investigating the structure of ecological networks along gradients should agree on the most appropriate approach(es) given a data set, and ponder the nature of the variation – and its ecological interpretation – that is quantified.

(1) The plant–hummingbird case study

Here, we compare direct and standardized quantification of the structure of ecological networks using a data set of plant–hummingbird mutualistic interactions along an elevation gradient in Costa Rica. Maglianesi *et al*. (2014) recorded plant visitation by hummingbirds over a year at three different elevations in Costa Rica and constructed quantitative networks of interaction frequencies. Observations of interactions between plant and hummingbird species in the understorey were carried out using videotaping of flowers. Tracked individual plants were randomly selected for each species at each study site. To record visits of hummingbirds to individual plants, unattended cameras were fixed 10 m from open flowers for periods of 120 min between 06:00 and 14:00 h. Morphological traits for hummingbirds and plants were measured, including bill length and corolla length, which are expected to drive interactions in this type of network (Maglianesi *et al*., 2014).

(2) Comparison of plant–hummingbird network properties

We compared the connectance along elevation to exemplify the direct use of a summary metric. We found that connectance decreased with elevation (Fig. 2A), while species richness was constant (low elevation network 28 species; medium elevation network 26 species; high elevation network 28 species). Connectance is a topological measure, representing the ratio of realized links over potential links. Even though they present the same species richness, the configuration of the three networks is different (e.g. 7 bird species and 21 plant species at low elevation; 9 bird species and 19 plant species at high elevation). The shape of the interaction matrices (lines × columns) constrains the number of potential links and the connectance within each network. The variation in connectance may be due to environmental filtering acting on species co-occurrence or a change in how species interact, but a direct comparison of connectance provides limited information on those processes. We therefore combined ecological networks with species functional traits and evaluated the role of trait matching in constraining these interactions. We quantified the absolute mean difference between species bill and corolla length for each observed interaction. This unstandardized measure of functional mismatch was lowest for the low elevation sites, peaked at the middle elevation site and was low again in the highest elevation site (Fig. 2A). Using a direct approach, it remains unclear whether the trait-matching constraint changes over the gradient, or is driven by underlying changes in species functional traits in the species pool.

(3) Comparison of trait matching with two null models

We next compared observed trait matching to two different null expectations, a model where the frequencies of interactions were randomized within each network and the niche model of food-web structure (Williams & Martinez, 2000). Compared to the random null model, all the observed trait matches were significantly lower than random, suggesting that the observed matching cannot be generated by a random distribution of the interactions within each network (Fig. 2B). The use of the niche model as a null hypothesis, as in Dunne, Williams & Martinez (2004), provides more conservative results, with the middle-elevation site not different from the null model. These results suggest that the partitioning of interactions between hummingbirds and plants along a directional niche axis (defined with a centroid and a range) is sufficient to explain the structure of the middle-elevation site, while the other methods suggest a more complex structure. In these cases, the centroid and range of the empirical networks are not random, and show more pronounced niche partitioning due to traits. Hence,

the selection of the appropriate null model, either straight randomization (Schleuning *et al*., 2012), or the niche model (Dunne *et al*., 2004), should be explicitly justified and its hypothesis clearly established.

(4) The use of hypothesis-based metaweb

We built hypothesis-based metawebs to which local ecological networks can be compared. We constructed a metaweb assuming perfect matching between bill and flower length (Maglianesi *et al*., 2014). With this hypothesis, interactions are expected to be more frequent near the 1:1 line of a matrix, in which hummingbird bill and plant corolla are ordered by size. The middle-elevation site is slightly lower, but all sites conform moderately well to the metaweb-based hypothesis of functional matching, with the highest elevation showing the best match (Fig. 2C). For comparison, we generated a set of 999 random metawebs and extracted from each three local webs. We tested whether similar levels of correlation between the observed and modelled interaction arose from random regional metawebs. As found with the randomization performed within each network using the null-model approach, the correlation from a subset of the functional metaweb was higher than from a subset of a random regional metaweb. This indicates that all three networks are more consistent with functional matching than random assembly.

(5) Conclusions from the plant–hummingbird networks

Together, the direct (Fig. 2A, B) and the standardized approaches (Fig. 2C) provide different insights into how and why the structure of plant–hummingbird ecological networks varies along this elevation gradient. Scoring of sites in terms of intensity of matching differed in a direct comparison of the matching values (mean difference between species bill and corolla length in mm: low = 0.2, middle = 0.27, high = 0.22; Fig. 2A), the random null model (SES: low = –4.37, middle = –3.9, high = –3.5; Fig. 2B), the niche model (SES: low = –2.58, middle = –1.28, high = –2.9; Fig. 2B) and after a standardization with a metaweb (correlation to the functional metaweb: low = 0.22, middle = 0.17, high = 0.29; Fig. 2C). While the SES of the null model decreased with increasing elevation, the ranking of SES for the niche model showed a different order, with the greatest value in the high-elevation site. Finally, the highest elevation site also provided a better match for the hypothesis of trait matching as evidenced by the metaweb comparison. Although the plant–hummingbird case provides a first caution regarding the importance of methodological choice in a comparison of ecological networks, evaluating a

greater variety of networks (e.g. antagonistic) across different environmental gradients and with different methods is needed. Our illustration calls for a careful selection of appropriate methods according to prior hypotheses, since the selection of the method will essentially determine the variation being analysed.

Conclusions

(1) There is a limited number of investigations of ecological network variation along environmental gradients because of the difficulty of quantifying interactions among species. Nevertheless, we expect that the rise of molecular techniques will allow better and faster quantification of ecological networks (Pompanon *et al*., 2012; Vacher *et al*., 2016; Roslin & Majaneva, 2016), allowing more spatial replication along environmental gradients. Moreover, the use of automated recording systems (Weinstein, 2015; Bohan *et al*., 2017) is also expected to expedite the collection of interaction data compared with manual techniques.

(2) Species information such as functional traits should be collected together with interactions in order to reach a good ecological understanding of why ecological networks vary along gradients. Alternatively, trait data might be gathered from available databases in isolation from the interaction, but the resulting analyses would not be able to highlight intraspecific co-variation between phenotypic traits and network structure along environmental gradients. When trait data are unavailable, a comparison of ecological networks along environmental gradients is limited to approaches that do not rely on functional traits (e.g. Dalsgaard *et al*., 2013; Sebastián-González *et al*., 2015), but that might provide more limited ecological inferences.

(3) Several approaches have been used to compare ecological networks either by analysing raw properties or using forms of standardization. Our review and case study suggest that different approaches are not directly comparable, and that this precludes, for the present, any meta-analysis of network variation along multiple gradients. Beyond analytical results, we call for further efforts to facilitate the exchange of raw data of species interaction networks along environmental gradients [e.g. MANGAL (Poisot *et al*., 2016), 'Interactionweb' or 'Web of Life']. Finally, studies comparing different approaches using empirical (e.g. bipartite antagonistic or mutualistic networks, food webs) or simulated data sets and discussing methodological bias are critical to provide guidance to select an appropriate methodology when

comparing ecological networks.

(4) We stress the need to agree on the most appropriate methodology to compare ecological networks along environmental gradients – on the one hand, when only data on network structure are available, and on the other when functional traits are also available. It is unlikely that one methodology can be used to answer all possible questions and future research should focus on understanding links between the different methodologies and the questions that they may answer.

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CHAPTER III

The structure of plant–herbivore ecological networks varies along elevation gradients

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The structure of plant–herbivore ecological networks varies along elevation gradients

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Abstract

Ecological gradients are expected to be associated with structural rewiring of species interaction networks. The study of network structures along geographic and ecological gradients, however, remains marginal because documenting species interactions at multiple sites is a methodological challenge. Using a new standardized DNA metabarcoding method applied to feces, we examined how structural properties of plant–orthoptera networks reflecting specialization and resilience vary with elevation. We found an increase in levels of generality and decrease in overall network specialization with decreasing temperature, and the correlation was stronger than in null models. This relationship corresponded to greater robustness and reduced importance of keystone species in alpine habitats. In cold environments, plant–herbivore networks are wired in a way that may reinforce the resilience of the system to species extinction. Our work helps establish a better understanding of the influence of climate and its associated variables on the structure of ecological networks along ecological gradients.

Introduction

Species represent the main building blocks of ecosystems and are connected in webs of positive and negative interactions, which shape ecosystem processes and functioning (Thompson *et al*. 2012). Given the central role of interactions among species for energy and matter flow between ecosystem compartments (Barnes *et al*. 2018), studying the structure of ecological networks helps us understand how ecosystem functioning might be disrupted by global changes (Petchey *et al*. 1999; Tylianakis *et al*. 2008). The wiring among interacting species is hardly random but rather governed by ecological rules (Bascompte 2010; Laigle *et al*. 2018). The strength of interactions between species may depend on the degree of matching between functional traits, which are shaped through co-evolutionary processes (Rausher 2001; Laigle *et al*. 2018). In turn, rules of functional matching might be influenced by variation in environmental conditions, such as temperature (Sentis *et al*. 2014; Gounand *et al*. 2016), or by climatic stability (Dalsgaard *et al*. 2011). By inducing changes in species composition, ecological gradients can be associated with shifts in species co-occurrence and their ability to form stable links (Welti & Joern 2015; Pellissier *et al*. 2018). Moreover, shifting environmental conditions might influence interactions among species even when they are steadily co-occurring (Tylianakis & Morris 2017). As a result, interactions shifts along climatic clines can lead to changes in the structure of networks (Welti & Joern 2015). Nevertheless, the geographic

variation in networks is poorly studied, owing to the difficulty of documenting multiple ecological networks along climatic gradients.

There are major challenges to the large-scale study of ecological networks that relate to the documentation of interactions and the methods used to perform network comparisons at the landscape scale (Pellissier *et al*. 2018). The study of ecological networks along environmental gradients has so far been limited by the difficulty of observing comparable interactions simultaneously at multiple locations. Novel DNA metabarcoding methods, which are increasingly cheaper, faster and more comprehensive, have opened such opportunities (Kaartinen *et al*. 2010; Roslin *et al*. 2019). Deagle *et al*. (2007) were among the first to develop a DNA metabarcoding protocol to reconstruct the trophic regime of the macaroni penguins on Heard Island in the Indian Ocean. Since then, the study of entire ecological networks has been facilitated through the adaptation of DNA metabarcoding techniques to different sample sources, which enables the collection of many samples over a short period of time (Roslin *et al*. 2019). For instance, Pornon *et al*. (2016) developed a protocol to quantify plant–pollinator interactions from pollen samples in the French Central Pyrenees, while Ibanez *et al*. (2013) applied this approach to insect feces for studying the diet of insect herbivores. However, most protocols for network reconstruction were not designed for studies with large spatial scales, and not all were aimed at species-level resolution. In addition, a complete description of the wet lab and bioinformatic procedures is not always accessible, limiting the adaptability and reproducibility of the techniques used to document species interactions. Scaling up the utilization of DNA metabarcoding to entire landscapes, while also sharing methodological workflows as detailed and user-friendly protocols, can spur advances in the study of species interactions along environmental gradients.

From the wide range of natural gradients impacting species distribution and interaction patterns, montane clines represent optimal natural laboratories to understand how species and their interactions vary over environmental gradients (Körner 2003). Changes in climate – most notably temperature – along elevation gradients cause strong environmental filtering in communities (Rahbek 1995; Hodkinson 2005) and can therefore also be expected to influence the structure of ecological networks (O'Connor *et al*. 2009; Welti & Joern 2015). The structure of ecological networks along environmental gradients can change as a result of two main processes: (i) a turnover of the species in the network, or (ii) a turnover of the links in the

network, in which co-occurring species rewire their interactions along the gradient (Gravel *et al*. 2019). In particular, the steady decrease in temperature with increasing elevation has been associated with changes in species richness and abundance (Rahbek 1995; Hodkinson 2005; Descombes *et al*. 2017), likely influencing the networks of species interactions (Adedoja *et al*. 2018; Pellissier *et al*. 2018). Therefore, studying changes in the architecture of species networks along elevation gradients contributes to evaluations of the effect of temperature on community structure and stability.

Changes in species' interactions within networks can be summarized by a set of indicators relating to the degree of structuration and complexity of the network (Delmas *et al*. 2019), including connectance (Martinez 1992), generality (Bersier *et al*. 2002), specialization (Blüthgen *et al*. 2006) and robustness (Dunne *et al*. 2002). Metrics of network structure can also quantify the resilience of the networks to environmental disturbances (Thebault & Fontaine 2010). Complex and specialized networks have been found to be associated with lower robustness against species extinction (Lafferty & Kuris 2009; Tylianakis & Morris 2017; but see May 1973; McCann 2000). This is the result of the existence of keystone species (Paine 1969), which are nodes of interactions associated with specific functional traits (Power *et al*. 1996). The importance and identity of keystone species, but also general structural properties involved in network resilience, may reshuffle along elevation clines. Three main non-exclusive hypotheses have been proposed to support this pattern: (i) at higher elevations the environment is expected to be less predictable (Barry 2008), and survival under these conditions necessitates the evolution of a broader diet breadth (Macarthur & Levins 1967); (ii) more intense competition at low elevations is predicted to select for more specialized diets to decrease niche overlap (Macarthur & Levins 1967; Hodkinson 2005); and, more closely linked to plant–herbivore interactions, (iii) a decline in the capacity of plants to produce efficient defences at higher elevations is expected to facilitate a larger diet breadth of herbivores (Pellissier *et al*. 2012a; Rasmann *et al*. 2014; Moreira *et al*. 2018). In contrast to the expectation of increased herbivore generality at higher elevations, where plant communities are less diverse, it has been proposed that higher plant species richness could benefit insect generalists simply by increasing the availability of species to feed on (Unsicker *et al*. 2008, Welti *et al*. 2017).

The comparison of ecological networks along environmental gradients has the inherent methodological difficulties of network comparison (Pellissier *et al*. 2018). Effective analyses

of network structure isolate the influence of real interaction patterns on network structural indices from the effects of network size or sampling design (Banašek-Richter *et al*. 2004). Studying ecological networks along large-scale environmental clines is challenging in this regard, as the decline in species richness at the extremes of the gradient might result in significant variation in species richness, in turn affecting measures of structural indices sensitive to matrix size (Trøjelsgaard & Olesen 2016; Pellissier *et al*. 2018). For instance, the number of links per species inevitably declines from large to small networks, as larger networks include more possible links. Several strategies have been developed to alleviate the confounding effects implicit in network comparisons (see Pellissier *et al*. 2018 for a review of these approaches). The most commonly used approach is the null model, where networks of randomly distributed interactions are generated and compared with the empirical patterns. This method has been established to isolate the role of observed interaction patterns on the network structure from the effect of matrix size variation when comparing networks along environmental gradients (Vázquez & Aizen 2003).

In this study, we investigated the variation in the structure of plant–orthoptera ecological networks along elevation gradients. Orthoptera are among the most abundant herbivorous arthropods in semi-natural grasslands of the European Alpine system, and they strongly impact the functioning of these ecosystems (Blumer & Diemer 1996). We optimized a protocol for plant DNA metabarcoding applied to orthopteran feces in order to reconstruct plant–orthoptera bipartite networks across 48 study sites situated along six elevation transects in the Swiss Alps. We then applied null models to explore the structural variation in plant–orthoptera bipartite networks and determine if lower levels of network organization and increased robustness are associated with the low temperatures of the alpine environment. Specifically, we proposed the following three hypotheses:

1. Levels of generality in insects should decrease at lower elevations (higher temperatures), while overall network specialization should follow the opposite pattern. Specialization in trophic networks may decrease in alpine environments, according to the following lines of argument: generalist feeders are better equipped to compensate for higher environmental uncertainty; lower interspecific competition attenuates positive selection for specialization; and the reduced plant chemical defences typically found at higher elevations offer more dietary opportunities for insects.

2. The robustness of the network after simulated plant primary extinctions should be higher in colder environments (at high elevations). The increase in insect generalist feeding behavior and the decrease in overall network specialization predicted in the first hypothesis should allow networks of alpine communities to better compensate for possible plant species loss.
3. If more generalist insect herbivores are present and the network is more robust in the alpine setting, the removal of plant keystone species should induce fewer extinctions within orthopteran communities than at low elevation. In addition, coverage of the functional space by keystone species of different botanical groups should vary along the elevation gradient because the functional space of plants shifts under the influence of changing abiotic and biotic variables at different elevations.

Materials and methods

Field data collection

To study variation in the plant–insect trophic network with elevation, we established six elevation transects that covered the diversity of environmental conditions of the Swiss Alps, differing in local climate and bedrock – i.e. in the areas of Bex, Calanda, Faido, Grindelwald, Martigny and Salgesch (Supplementary Materials S1, Fig. S1). Each transect was divided into eight sites, spanning elevations from 578 to 2417 m a.s.l., located on average 240 m of elevation apart from each other. Sites were chosen to be open grasslands with a limited impact from anthropogenic activities. At each site, we defined a 10 m x 10 m survey plot representative of the homogeneous composition of the surrounding vegetation. Orthopteran surveys were conducted under sunny weather conditions during the summer at insect peak activity times. We focused on Caelifera and Ensifera suborders that are known to feed on living plant material (Baur *et al*. 2006). We identified orthoptera by visual inspection, caught on average 10 individuals per species, kept them in falcon tubes for c. 2 h, for collecting fecal excretions, before releasing them again all at once. We performed the vegetation surveys in a 9 m^2 circular plot located in the most homogeneous zone of the 100 m^2 plot and searched for additional rare species within the 100 m^2 plot. We used temperature as the main environmental variable that changed along the elevation gradient (Supplementary Materials S1, Fig. S2). Soil temperature data were collected for half of the sites per transect using temperature loggers (DS1921G-F5 HomeChip, Newton Longville, England) that were parameterized at a 0.5°C resolution with a sampling rate of 240 minutes, wrapped in parafilm, protected by a silicone capsule and buried

4 cm deep in the ground at each site (from October 2017 to October 2018). Summer soil temperature was extrapolated for unmonitored sites by linear regression (Supplementary Materials S1, Fig. S3). To study the keystone plant species, we measured plant functional traits that related to physical resistance or nutrient content: specific leaf area (SLA), leaf dry matter content (LDMC), force required to pierce the leaf lamina (punch), and carbon-to-nitrogen ratio (C/N). We sampled well-developed, healthy leaves to measure the traits of all species with a minimum of three replicates across their elevation range (76% of the total number of surveyed species). SLA and LDMC were measured using standard procedures (Pérez-Harguindeguy *et al*. 2013). Punch was calculated using a digital force gauge (IMADA CO., LTD. Toyohashi, Japan), following Sanson *et al*. (2001). C/N was determined by dry combustion of ground leaf material (4mg +/- 0.2mg) of intraspecific replicates pooled to equal weight using an elemental analyzer (NC-2500 from CE Instruments, Wigan, Lancashire, United Kingdom). The collection of trait data was completed with published datasets (Kattge *et al*. 2011; Körner *et al*. 2016; Descombes *et al*. 2017).

Plant–orthoptera network reconstruction

The reconstruction of plant–insect trophic networks from fecal samples relies on a DNA metabarcoding procedure that uses a two-step DNA amplification PCR-based approach in which samples are individually tagged by dual-indexing. A full protocol of the wet-lab procedure, from DNA extraction to sequencing, is provided in Supplementary Materials S2, section Methods 1. In short, after DNA extraction from the insect feces, the ITS2 nuclear plant marker (360bp) was amplified in the amplicon PCR. We selected this marker based on its ease of amplification, high taxonomic resolution, good coverage of the reference database and successful application to degraded DNA samples (Li *et al*. 2011; García-Robledo *et al*. 2013). In parallel to DNA metabarcoding library preparation and sequencing, we compiled an ITS2 reference database by recovering sequences from Genbank (Clark *et al*. 2016). The database was filtered using in-silico PCRs that allow only one mismatch between the primers and the priming sites. We expanded the reference database with custom sequences generated for 54% of the plant species (see Supplementary Materials S2, section Methods 2). After processing of the raw sequencing data, OTU calling and taxonomic assignment against the DNA reference database, the OTU table was streamlined to reconstruct individual networks using R (R Core Team 2019; see complete descriptions of the bioinformatic and OTU table cleaning procedures in Supplementary Materials S2, section Methods 3). We discarded OTUs that were non-

monophyletic and/or identified above the family level, summed the OTUs belonging to the same taxon, used vegetation surveys to filter out the OTUs with low read numbers, and redistributed the count of OTUs assigned above the species level. In a final step, we computed the relative read abundance (RRA) for each sample as an estimate of the interaction intensity (Deagle *et al*. 2019, Roslin *et al*. 2019).

Statistical analyses of variation in network metrics

We computed network metrics using the *bipartite* R package (Dormann *et al*. 2008) and extracted: i) The number of links per species, calculated as the total number of links divided by the total number of species. ii) The generality index, which estimates the mean number of plant species per orthopteran species weighted by the marginal counts (Bersier *et al*. 2002) and is calculated from presences and absences of interactions instead of their intensities. iii) The overall network specialization (H2'), which represents the degree of specialization of the entire bipartite network. This metric is derived from the Shannon diversity index and was specifically developed and tested to control for the effects of network size (Blüthgen *et al*. 2006). It uses non-integer values to represent interaction intensity. iv) The robustness of the networks (Dunne *et al*. 2002), which involves calculating the cumulative proportion of secondary extinctions caused by the sequential removal of plant species until all insect species are extinct. As implemented in *bipartite*, the function uses a quantitative estimation of the robustness introduced by Burgos et al. (2007). It measures the area under the attack tolerance curve (ATC), which describes the relationship between the proportion of species removed and the proportion of surviving insect species, until all species are extinct. The sequential species removal was done randomly for 100 replicates, excluding plant taxa that were not ingested. Relationships between mean summer temperature and the observed network metrics were tested using linear mixed-effects models including transect identity as a random factor (packages *lme4* Bates *et al*. 2008 and *lmerTest* Kuznetsova et al. 2017). We used a null model approach to discriminate the effect of the non-random interactions on the metric from the influence of inherent bias of network metric calculation (e.g. network size). We generated 999 random metawebs, where interactions were fully randomized and impossible links excluded. Individual random networks were then reconstructed for each study site according to their species composition. We further measured network properties for each network and metric variation along the gradient, following the same procedure as applied for the observed networks. Statistical significance of the metric variation was confirmed if the observed slope of the relationship between the

temperature and the network metric fell outside the 2.5–97.5% quantile interval of the slopes obtained for the randomized network of interactions. We also calculated the standardized effect size (SES) to quantify the difference between the observed relationships and the null models. The approach we used here does not suppress the metrics' sensitivity to sampling effects, but slope values outside the 2.5–97.5% quantile interval of the slopes obtained from random networks (and large values of SES) indicate that the interactions of empirical networks contribute more to the metric variation along the gradient than expected by chance.

Identification of keystone species

Keystone species are defined here as plant taxa that play a major role in providing a food source for the orthopteran herbivores (Mills *et al*. 1993; Power *et al*. 1996). We identified keystone species using custom R scripts, submitting each network, preliminarily transformed into an igraph object (Csardi & Nepusz 2006), to a sequential and random removal of plant species. Insect species were considered to be extinct upon loss of all the plant species they feed on. Plant species were removed until all insect species became extinct. This was repeated for n*(n–1) simulations, with n equaling the total number of plant taxa in the network. The mean number of secondary extinctions caused by plant removal, to which we refer hereafter as the keystone score, was then calculated for each plant species. To examine the distribution and the keystone score of species within the plant functional space at the low (<1050 m a.s.l.) and high elevation (>2000 m a.s.l.), we performed a principal components analysis on plant traits with the function dudi.pca from the ade4 package (Thioulouse *et al*. 2018). We compared the distribution of species with different keystone scores in the functional space of plant traits of species at the lowest and highest elevation sites. For both elevation classes, we extracted plant species based on their presence within the corresponding elevation range. We further determined the 10 species with the highest keystone scores for each network, averaged their weights for each elevation class and placed them in the functional space of plant trait.

Results

Field surveys and network reconstruction

We identified 45 orthopteran species, including 29 and 16 species of the Caelifera and Ensifera suborders, respectively, and we collected 403 feces samples. Vegetation surveys led to the identification of 496 plant species, belonging to 265 genera and 63 families. The DNA barcoding reference database compiled 5969 reference sequences covering the taxonomic

diversity of the vegetation surveys for 95.2% of the families, 92.2% of the genera and 88.5% of the species, with 50% of the missing species having their genus represented in the database. The MiSeq v3 2x300 PE sequencing run provided 31 M reads, which decreased to 15.5 M after

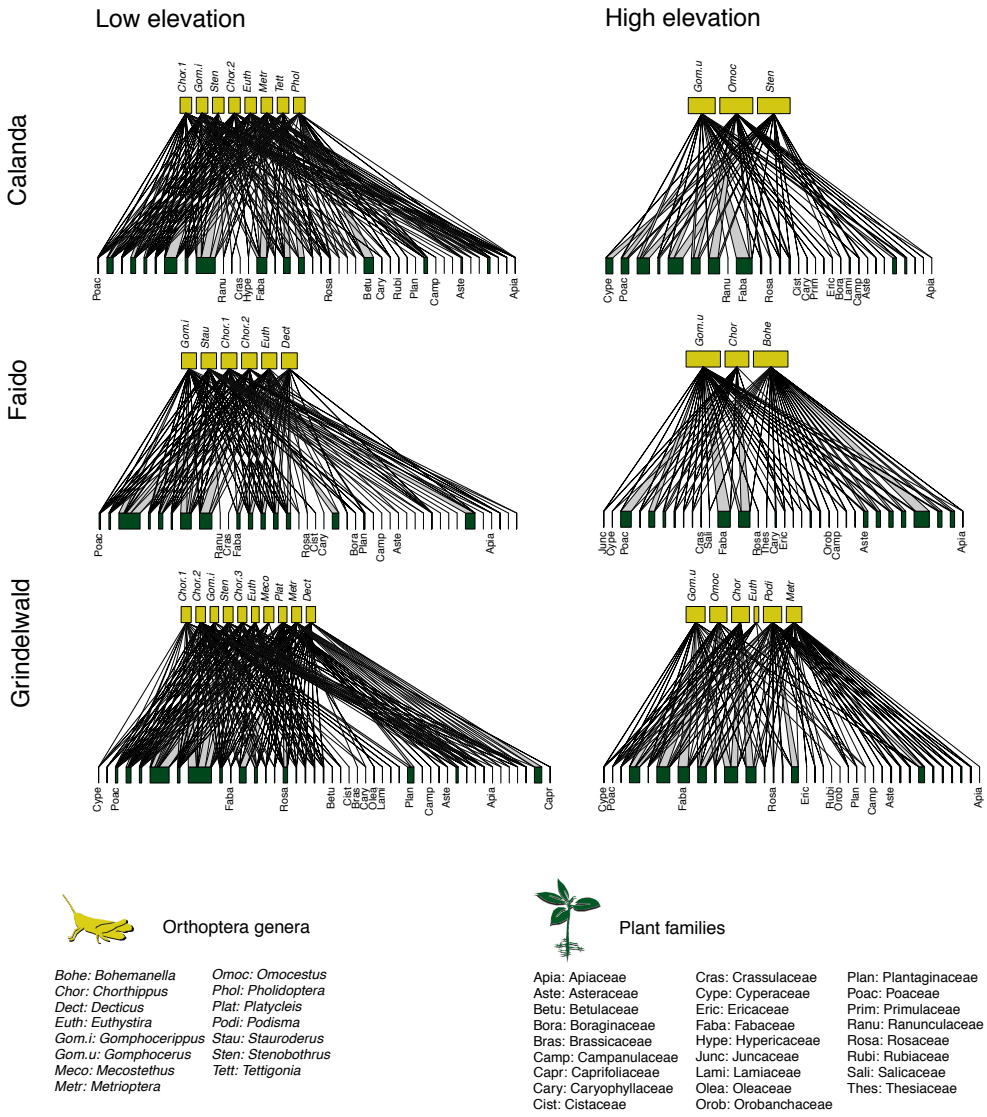

Figure 1 Plant–orthoptera ecological networks for low- and high-elevation sites situated along three elevation transects. For each network, yellow bars represent species of orthoptera and green bars species of plant. To ease graph interpretation, species labels are grouped into genera for orthoptera and into families for plants, sorted according to the phylogeny. For species belonging to non-monophyletic genera (e.g. *Chorthippus*), taxa are indicated with a number following the genus name. The reconstruction of plant and insect phylogenies is detailed in Supplementary Materials S1, section method. Bars with no label correspond to the taxa of the preceding labelled bar, reading from left to right. The width of the links represents interaction intensity.

filtering, paired-end merging and trimming steps (https://github.com/lh3/seqtk; Magoc & Salzberg 2011; Martin 2011), with an average sequencing depth of >36,500 reads, and to 1774 OTUs after OTU calling (Edgar 2016). Following taxonomic identification that used a stringent threshold of assignment of 0.95, we discarded 105 OTUs that were not monophyletic and 176 OTUs with a taxonomic assignment higher than the family level. After the merging of OTUs belonging to the same taxon, the equal redistribution of read counts to lower taxonomic ranks (Methods and Results sections in Supplementary Materials S2) and the addition of species that had not been consumed, the OTU table comprised 601 taxa, including 496 species, 99 genera and 6 families corresponding to taxa that were not identified to a lower taxonomic level in the field. The total number of interactions recorded was 10,615 out of 28,127 possible links. The reconstruction of individual networks exemplified for low- and high-elevation sites of three transects are illustrated in Fig. 1.

Variation in network metrics

In agreement with our first hypothesis, we found that the generality of the observed networks decreased with increasing temperature (for generality: observed slope = -0.4973, p value = 0.035; for specialization: observed slope = 0.0075, p value = 0.046; Fig. 2, Table 1). The variation in these metrics differed significantly from null models, as we found their observed slopes to be outside the 2.5–97.5% quantile interval of the slopes obtained from random networks and SES values were high (generality: 2.5–97.5% quantile interval = (-0.4066, -0.1868), SES = -3.436; overall network specialization: 2.5–97.5% quantile interval = (-0.0073, 0.0013), SES = 4.861). We found a positive relationship between robustness and temperature (slope = 0.0033, p value = 0.023), but the observed decrease in robustness in cold environments was lower than expected from nulls models (2.5–97.5% quantile interval = (0.0057, 0.0082), SES = -5.766), indicating a role of the wiring of interactions in attenuating the decrease in robustness toward colder conditions (Fig. 2). We further found a negative relationship between weighted nestedness and temperature for empirical webs that was different from null models (Fig. S4, Table S1). Variation in the number of links per species (Fig. 2, Table 1), the connectance and the trophic niche overlap of orthoptera (Supplementary Materials S1, Fig. S4, Table S1) were not different between the observed and the randomized networks. We found a positive relationship between temperature and the number of links per species in empirical networks (Fig. S4, Table S1) but not for the connectance or the niche overlap (Supplementary Materials S1, Fig. S4, Table S1).

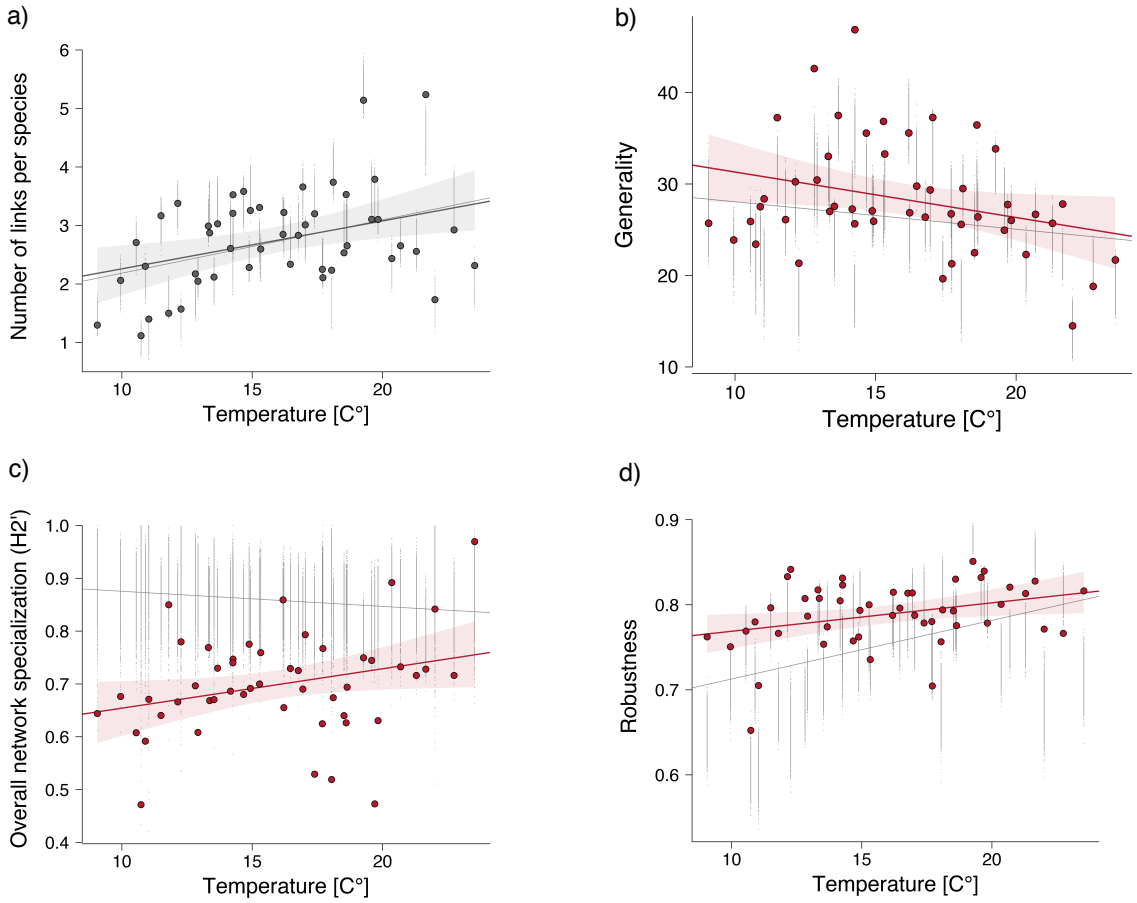

Figure 2 Relationship between the mean summer temperature at each site along the elevation gradients and various plant–orthoptera network metrics: (a) number of links per species, (b) network generality, (c) overall network specialization, and (d) network robustness. Expected metrics based on null models are represented in light gray. Regression lines result from linear mixed-effects models, where solid lines indicate a significant relationship between the observed or random network metrics and the temperature. For empirical networks, the confidence interval and regression line of the temperature *vs*. metric relationship is shown. When the slope of the empirical relationship is outside the 2.5–97.5% quantile interval of the slopes obtained from random networks, the regression line and metric values are red, while they are dark grey when the observed slope is not outside the slope interval expected from null models.

Table 1 Coefficients obtained for the observed metric variation along the temperature gradient and the null models. For each metric, the slope of the relationship between the observed metric and the temperature, the intercept estimate, the *p* value, the degrees of freedom (df), the t-value, the standardized effect size (SES) measured between the observed metric slopes and those obtained from random networks of interactions, and the 2.5% and 97.5% quantile interval limits of the slopes obtained for random networks are given. For generality, overall network specialization and robustness, observed slopes were found outside the 2.5% and 97.5% quantile interval limits obtained with null models.

	Slope Estimate	**Intercept Estimate**	***p* value**	**df**	**t-value**	**SES**	**2.5% quantile**	**97.5% quantile**
Links per species	0.0819	1.44	0.003	42.56	3.20	-1.820	0.0816	0.1032
Generality	-0.4973	36.28	0.035	43.66	-2.17	-3.436	-0.4066	-0.1868
Overall specialization	0.0075	0.58	0.046	44.08	2.35	4.861	-0.0073	0.0013
Robustness	0.0033	0.74	0.023	43.62	2.06	-5.766	0.0057	0.0082

Keystones species in the functional space of plant traits

We found higher keystone plant species scores in warmer environments (0.41 on average; Table 2) than in colder environments (0.26 on average; Table 2). The keystone scores were generally low, with the removal of a single plant species resulting, on average, in less than one insect secondary extinction (Supplementary Materials S1, Table S2). The top 10 keystones species retrieved from alpine and lowland trophic networks had same dietary preferences for botanical groups, as illustrated by their similar distribution patterns within the functional space of the plant traits (Fig. 3). In both warm and cold environments, the top 10 keystone species occupied the functional space characterized by high punch values (to the maximal extent of this axis; Fig. 3). The top three keystone species of warm and cold environments all belonged to the Poaceae family (Table 2). Forb keystone species were located in the functional space along the opposing axes of C/N, LDMC *vs*. SLA in both low- and high-elevation networks; while legume keystone species were mostly distributed along the SLA trait axis, at high but not at low elevation (Fig. 3).

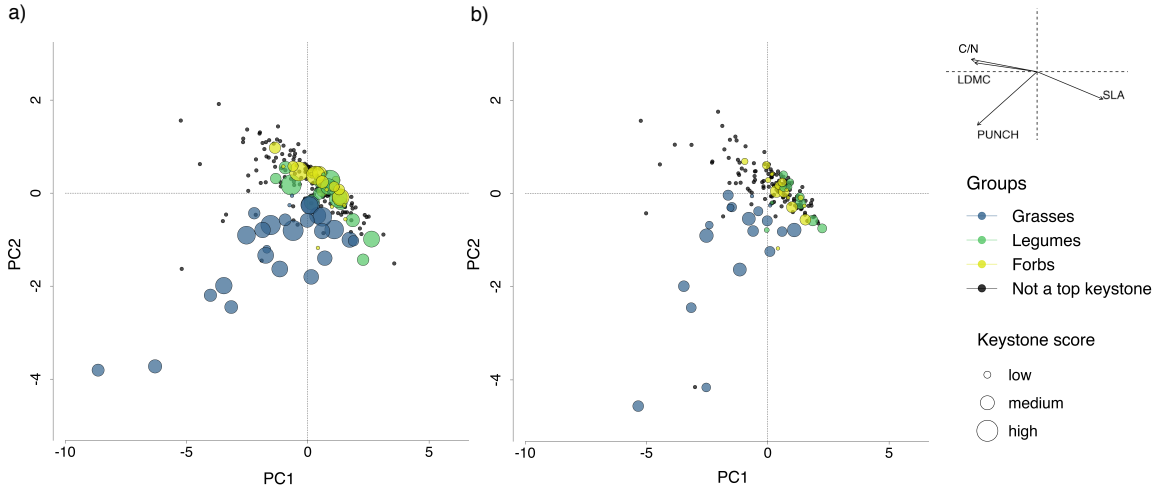

Figure 3 Distribution of keystone species in the functional space of the plant traits between low- (**a**, <1050 m a.s.l.) and high-elevation (**b**, >2000 m a.s.l.) plant communities. Plant species are projected onto the first two axes of the principal components analysis (PCA) performed on plant functional traits, which explain 58.8% (PC1) and 15.6% (PC2) of the variance. The top 10 keystone species are colored by plant taxonomic group, while all other species are shown in black. The keystone score corresponds to the mean number of secondary extinctions caused by the removal of the plant species. It varies between 0.12 and 0.75 secondary extinctions across all networks but is summarized in the legend as three circle sizes (low, medium and high). Correlations between plant functional traits (C/N, LDMC, punch and SLA) and the two first axes of the PCA are given in the plot in the top right corner.

Table 2 Keystone species score and identity for low- and high-elevation plant communities. The keystone score corresponds to the number of insect secondary extinctions resulting from the removal of the focal plant species from the network averaged over 1000 simulations. The mean, minimum and maximum scores, averaged for low- (<1050 m a.s.l.) and high-elevation (>2000 m a.s.l.) sites, are shown for the top three keystones species (see Supplementary Materials S1, Table S2 for the complete list of top 10 keystone species).

	Keystone score			Keystone species		
	Mean	Minimum	Maximum	top 1	top 2	top 3
Low elevation	0.41	0.21	0.75	*Festuca* sp.	*Bromus erectus*	*Arrhenatherum elatius*
High elevation	0.26	0.12	0.43	*Helictotrichon pubescens*	*Festuca ovina*	*Nardus stricta*

Discussion

The development of novel molecular methods has paved the way for a better understanding of how environmental factors shape the structure of species interaction networks (Nielsen *et al*. 2018; Roslin *et al*. 2019). Using an improved and non-invasive metabarcoding procedure based on insect feces, we reconstructed the structure of plant–orthoptera networks across multiple sites along elevation gradients, thus helping advance the field of 'landscape network ecology'. We showed that networks exhibited structural variation along the ecological gradients, as a result of both the rewiring of species interactions and shifts in network size. Networks of high-elevation cold environments displayed reduced levels of specialization, which resulted in greater robustness than expected from null models. We also found an increase in the network weighted nestedness with temperature, which is theoretically expected to be a central component of stability (Supplementary Materials S1, Fig. S4, Table S1). We argue that lower specialization and increased generality confer higher network resilience, presumably through a more homogeneous distribution of the herbivore interaction over the available plant species functional space. Theoretical work on the structure–stability relationship of ecological networks suggests a positive association between network resilience to species extinction and structural indices, including connectance and nestedness (Dunne *et al*. 2002; Memmott *et al*. 2004; Lafferty & Kuris 2009). Our empirical analyses along several elevation transects support theoretical expectations, where networks in cold environments are less specialized, a quality presumably associated with increased robustness. Novel molecular methods enabling the monitoring of network variation in space, as done in our study, but also in time, should provide new perspectives for understanding the trophic architecture of species assemblages.

The observed lower specialization for alpine plant–orthoptera networks agrees with three underlying arguments supporting biotic and abiotic shifts along elevation gradients: (i) lower environmental predictability, (ii) less species competition for resources, and (iii) declining plant chemical defences (Macarthur & Levins 1967; Hodkinson 2005; Rasmann *et al*. 2014). First, greater environmental stresses and variation in the alpine belt (Körner 2003; Barry 2008) may impose constraints for insects to complete developmental and reproduction cycles (Hodkinson 2005). In particular, environmental fluctuation at high elevations may increase resource stochasticity, which translates into greater spatio-temporal variation of the host plants than at low elevations (Billings & Mooney 1968). Cooler and more variable temperatures might also reduce search and digestive efficiency in ectothermic animals

(Hodkinson 2005). In turn, such environmental unpredictably could be offset through the reinforcement of generalist feeding behavior (Macarthur & Levins 1967). The Orthoptera order is mainly composed of generalist feeders (the median number of host plants in our study was 26). Hence, while food plant fluctuation should largely impact the evolutionary specialization of more specialized clades, such as the butterflies (median of 8 host plants calculated from published dataset, Pellissier *et al*. 2012b), orthoptera should more easily compensate for the demographic fluctuations of food plant species by maintaining a large diet breadth (Cates 1981). Second, higher species richness of orthoptera at low elevations (Supplementary Materials S1, Fig. S5) might pressure species to escape competition by focusing on distinct and more specialized diets (Macarthur & Levins 1967; Hodkinson 2005). However, we found no relationship between the species richness of orthoptera and network specialization (Supplementary Materials S1, Fig. S6) and species niche overlap among orthoptera did not vary along the temperature gradient, indicating that interspecific competition for plant resources is weakened in orthoptera (Supplementary Materials S1, Fig. S4, Table S1). Third, it was previously shown that alpine plant communities are less resistant to herbivores than low-elevation plant communities (Rasmann *et al*. 2014; Callis-Duehl *et al*. 2017). These plant defence patterns could promote a stronger generalist feeding behavior in colder environments, through easier digestibility of various plant materials (Moreira *et al*. 2018). Our results indicating lower selectiveness of orthoptera for alpine plants are in agreement with a generalized reduction in defence levels in plants growing at high elevations (Rasmann *et al*. 2014). We documented that orthopteran communities from cold environments feed on a broader range of plant families and target more intensively some of these, as for instance the Apiaceae, Boraginaceae, Caryophyllaceae and Fabaceae, compared with the feeding habits of lower-elevation orthoptera (Supplementary Materials S1, Fig. S7). Because our results did not support the hypothesis of higher generality of orthoptera in more species-rich plant communities, plant species chemical composition, rather than the number of host plant species *per se*, may have a greater impact on the level of specialization of orthoptera along elevation gradients, but this conjecture needs further in-depth assessment.

The increase in generality and decrease in specialization of networks with higher elevation (lower temperatures) were associated with an increase in network robustness in cold environments (Fig. 2, Table 1). These results support previously documented co-variation between generality and network robustness (Welti *et al*. 2017), and a negative association

between temperature and network robustness (Welti *et al*. 2019). We found that specialization and robustness metrics were associated with the underlying temperature gradient, which suggests that ecological or evolutionary factors have led to more robust networks in more stressful environments. In general, orthoptera feed on multiple plant species, so the loss of one plant species is never sufficient to cause the loss of one species of orthoptera (averaged keystone score <1), but they still show some degree of preference as regard to the functional traits of the plant they are feeding on. In our study, orthoptera showed a preference for plants with tougher leaves (Fig. 3, Table 2), which typically correspond to monocotyledons (Supplementary Materials 1, Fig. S8), some of which were particularly dominant in the studied grasslands (e.g. *Bromus, Festuca* and *Nardus*), as these herbivores are equipped with enough mandibular strength to cut through such leaves (Ibanez *et al*. 2013a). We found lower and more even keystone scores for alpine plant species, meaning that the removal of plant species at higher elevations was associated with lower secondary extinctions (Table 3). The decrease in the keystone score of grasses at higher sites might also be associated with the decline in the cover of grass vegetation with increasing elevation (Supplementary Materials S1, Fig. S9). At high-elevation sites, keystone species also had other functional attributes, including higher SLA but lower C/N values (Fig. 3) compared with low-elevation plants, corresponding to more palatable and resource-rich host plants (Pérez-Harguindeguy *et al*. 2013), providing herbivores with higher nutritive content during the short growing season of the alpine environment. These results suggest that the identity of the keystone species in plant–orthoptera bipartite systems is determined by a combination of factors involving plant species abundances and co-evolutionary mechanisms between insect feeding ability and plant defence, presumably resulting from mechanical and chemical defence tradeoffs.

Compared with traditional methods based on visual analyses of feces or gut content or literature-based documentation of interactions (Nielsen *et al*. 2018), the DNA metabarcoding procedure represents an effective and easily adaptable method for documenting interactions involving plants and insect species. As a compromise between the spatial coverage of our study and the available sampling resources, potential impacts of sampling replication and seasonality and year-to-year change on diet composition were not assessed here (Mata *et al*. 2019). Overall, our approach may open fields of investigation on the possible spatio-temporal variation in plant–insect interactions by expanding the means for collecting species interaction data.

Taken together, our results show a decline in plant–herbivore network specialization with increasing elevation, which drives variation in network robustness along the gradient and ultimately gives lower weights to keystone species in alpine than in lowland environments. Shifts in abiotic components can alter the structuring of species interactions directly or indirectly (Welti & Joern 2015; Tylianakis & Morris 2017), by influencing the different aspects of the species interface through both abiotic and biotic pressures. We suggest that the observed patterns of network structural variation regarding elevation represent entangled responses of networks to environmental predictability and plant chemical defence, although further investigation would be required to confirm this possibility. Generally, orthoptera are not very sensitive to extinction, in that the loss of multiple plant species is necessary to cause secondary extinctions. Nevertheless, land use practices in lower-elevation mountain grasslands, for instance the use of fertilizers, can regularly cause the loss of multiple plant species, which could then lead to extinctions in orthoptera (Chisté *et al*. 2016). Our study helps pave the way to a better understanding of the eco-evolutionary factors underlying network structure along large-scale ecological gradients, but also highlights how resilient species assemblages are to the accelerated rate of species extinction given these structural constraints.

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Supplementary materials S1

1. Supplementary methods

Plant and orthopteran phylogenies

We used the Daphne published phylogeny for European flora (Durka & Michalski 2012), pruned to the species list of our study. The orthopteran phylogeny relies on sequences of COI, COII, CytB and ITS2 retrieved from Genbank and completed with unpublished data from colleagues and custom sequencing data. DNA was extracted from insect leg muscle tissue using a Sbeadex livestock kit following the manufacturer instructions (LGC Genomics, Berlin, Germany). A portion of the COI genetic barcode with a length of 1500 bp was amplified for 7 species using the forward primer UEA1 (gaataattcccataaatagatttaca) and the reverse primer UEA10 (tccaatgcactaatctgccatatta, Lunt *et al*. 1996). The PCR reaction master mix contained the following component concentrations: 1x PCR Gold Buffer without $MgCl_2$ (provided with the Taq), 2 mM $MgCl_2$ (provided with the Taq), 0.2 mM dNTPs, 0.04 U/µl AmpliTaq Gold DNA Polymerase (ThermoFisher, Waltham, MA, USA), and 0.2 µM of each primer (Sigma-Aldrich, St. Louis, MO, USA). Molecular grade water was added to reach 20 µl and 5 µl of DNA extraction product diluted to 2 ng/µl. The PCRs were run under the following conditions: 10 minutes at 95°C; 40 cycles of amplification for 30 seconds at 95°C; 55 seconds at 56°C; 1.5 minutes at 72°C; and 10 minutes at 72°C. PCR products were purified using AMPure (ratio 0.5x, Beckman Coulter, Brea, CA, USA) and sent to Microsynth AG for Sanger sequencing (Balgach, Switzerland). Raw sequences were trimmed and paired-end merged in Geneious (Kearse *et al*. 2012). Sequences were aligned through multiple alignment using a Geneious algorithm (Kearse *et al*. 2012) with a cost matrix of 93% similarity threshold. Alignments of each marker were concatenated and the phylogeny was generated using the RaxML program (Stamatakis & Ott 2008) on the CIPRESS portal (Miller *et al*. 2010). Since *Oedipoda germanica* could not be amplified successfully with PCR, the species was manually added to the tree as a sister species of *Oedipoda caerulescens*. This placement is supported by the high morphological congruence of these two species, which are the only two in the phylogeny belonging to the genus *Oedipoda*.

2. Supplementary figures

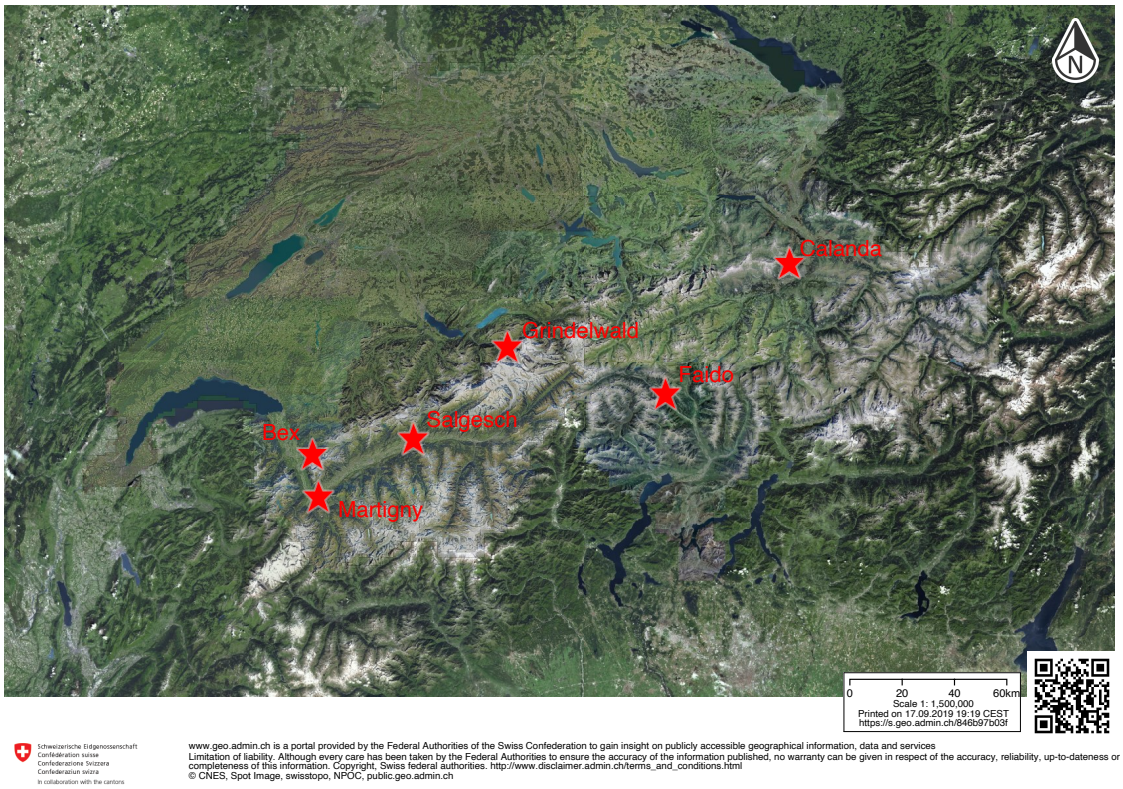

Figure S1 Map illustrating the locations of the different study transects across the Swiss Alps.

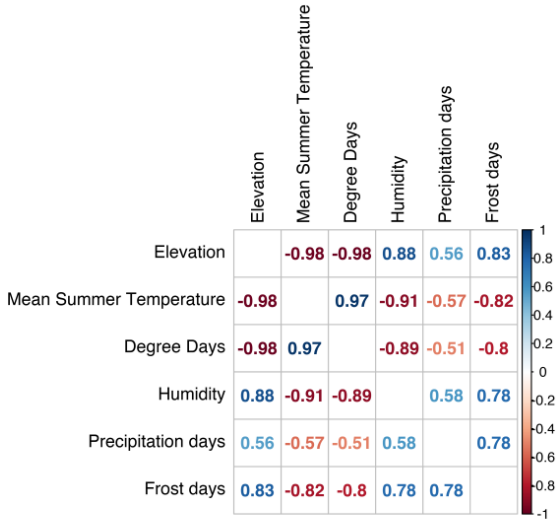

Figure S2 Correlation plot between abiotic variables extracted at each study site: elevation, mean summer temperature, degree days, humidity, precipitation days and frost days. Elevation was measured in the field; temperatures were extrapolated for each site using data from temperature loggers (Fig. S3); values for degree days, humidity, precipitation days and frost days were calculated from meteorological stations using a Digital Elevation Model (DEM) at 100 m resolution and interpolated following Zimmermann & Kienast (1999).

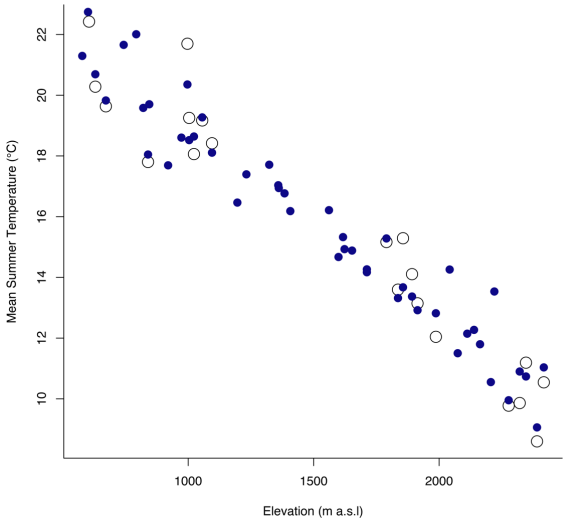

Figure S3 Elevation plotted against mean summer temperature (°C). White circles correspond to temperature data obtained from data loggers and collected from 1 May to 29 September 2018, while blue circles correspond to temperatures of unmonitored study sites extrapolated from linear regression models applied to each transect individually.

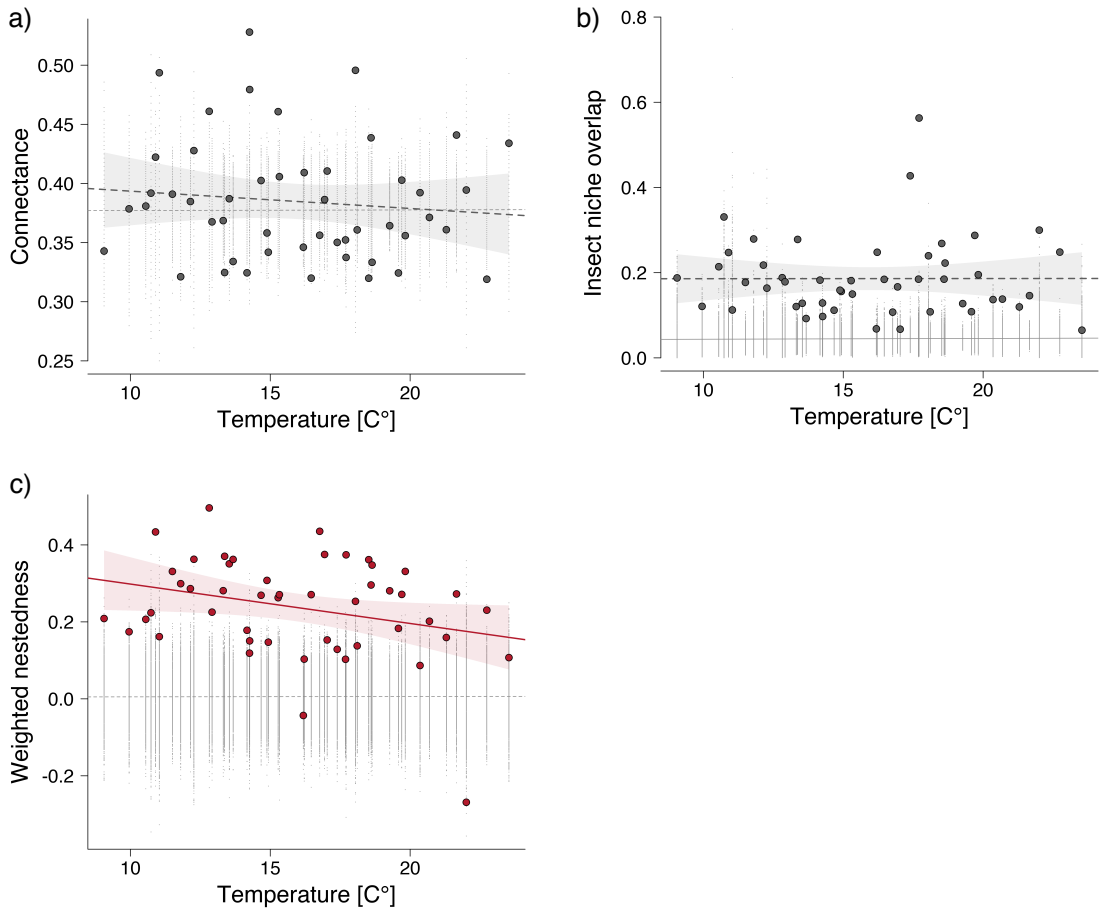

Figure S4 Variation in plant–orthoptera network connectance (a), insect niche overlap (b) and weighted nestedness (c) with the mean summer temperature at each site along the elevation gradients. We measured network connectance as the number of realized links divided by the total number of all possible links, including plant species for which no interaction was observed. Insect niche overlap was calculated as Horn's index using interaction intensities (Horn 1966). Nestedness, in a bipartite network where both levels are organized from specialist to generalist species, reflects the extent to which generalists to specialists of one level interact with generalists and specialists of the other level. We used weighted nestedness, which considers interaction intensity and was quantified following Galeano *et al.* (2009). The metrics calculated from a network of random interactions are displayed in light gray points. When the slope of the empirical relationship is outside the 2.5–97.5% quantile interval of the slopes obtained from random networks, the relationship and metric values are red, while they are dark grey when the observed slope is within the slope interval expected from null models. Dashed lines indicate a non-significant relationship between the observed or random network metrics and the temperature.

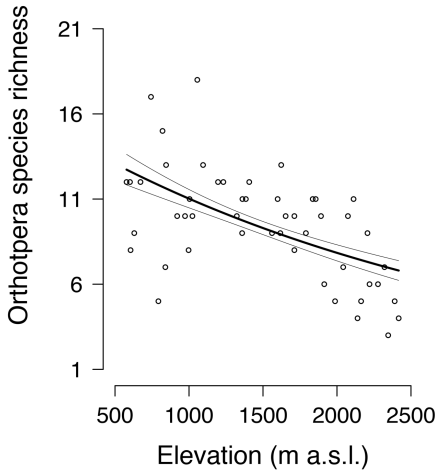

Figure S5 Variation in species richness for orthoptera with elevation (quantified as the total number of species identified at each site) was measured using generalized linear mixed-effects models with a Poisson distribution for count data, including transect as a random factor, using the *lme4* and *lmerTest* R packages (Bates 2008, Kuznetsova et al. 2017). The decline in species richness with elevation was significant (p value ≤ 0.001, slope estimate = -0.0003, t-value = -4.091).

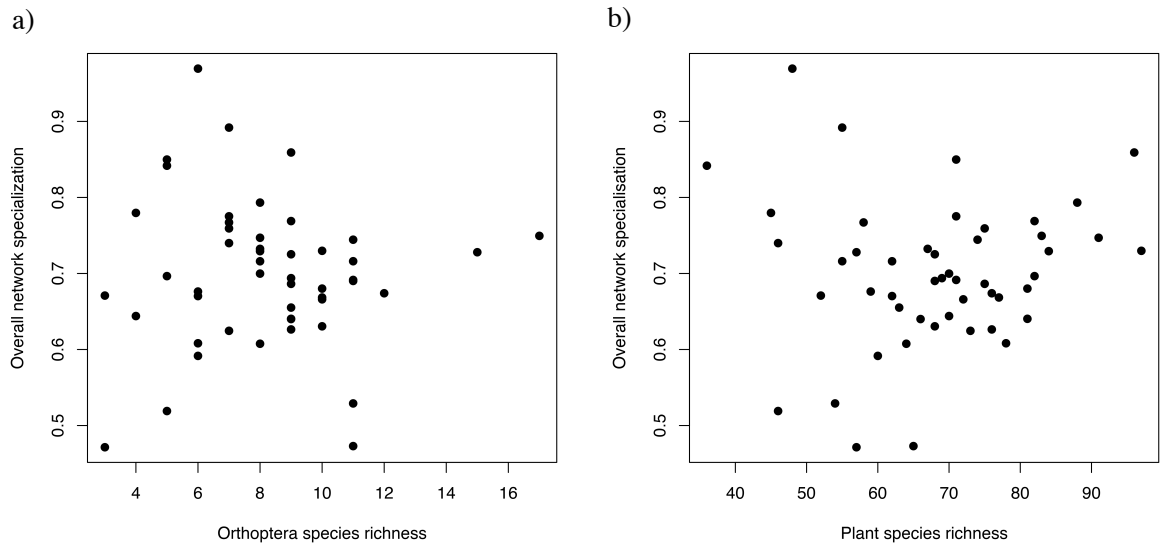

Figure S6 Species richness of orthoptera (a) and plants (b) at each study site against the overall network specialization, a metric that is robust to the variance in network size (Blüthgen *et al*. 2006).

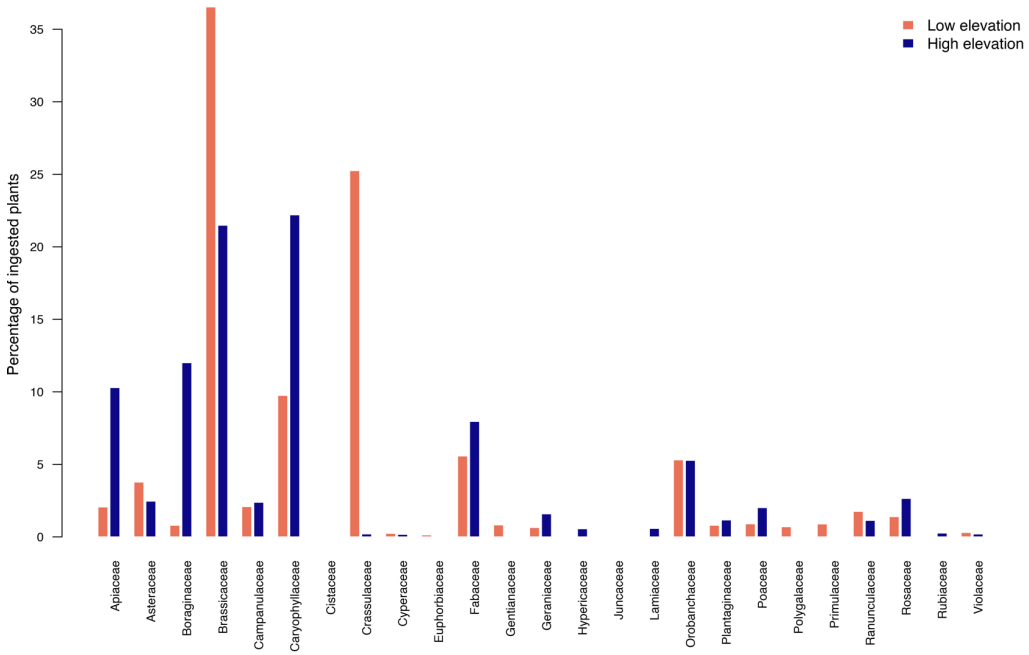

Figure S7 Percentage of plant families ingested. Bars represent the percentage of plant families ingested at low-elevation (<1050 m a.s.l.) and high-elevation (>2000 m a.s.l.) sites, corrected for the mean abundance of the plant families estimated at each site. Only plant families found at both extremes of the elevation gradient are shown. Bars that do not appear in the graph correspond to extremely low percentages of ingested plant families.

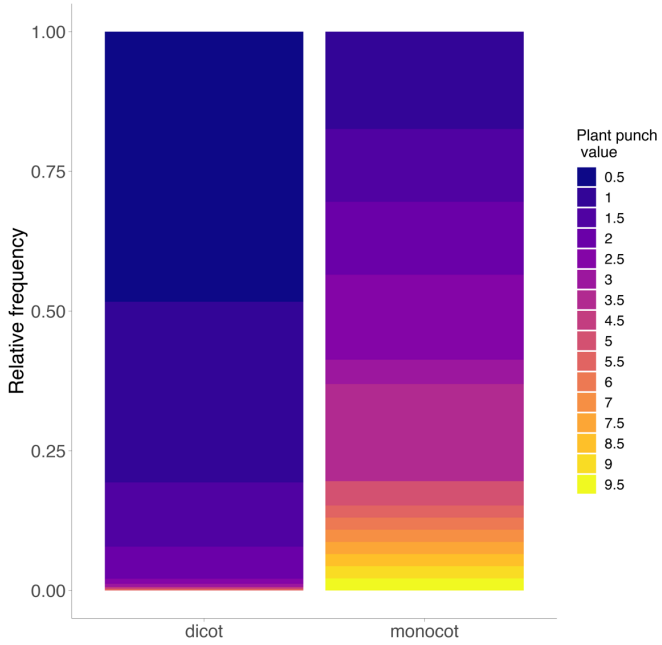

Figure S8 Relative frequency of punch values (by 0.5 increments) in dicotyledon and monocotyledon plant species.

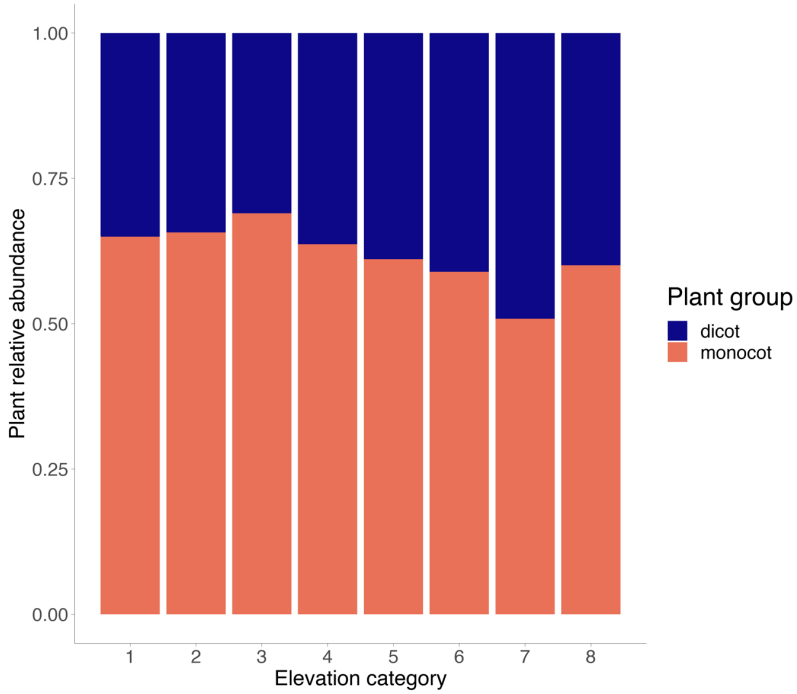

Figure S9 Histogram of plant abundance. The scaled sums of the monocotyledon and dicotyledon abundances are given for each elevation category, ranging from low (category 1) to high elevation (category 8). The categories 1 to 8 have the average elevations: 654, 849, 1062, 1384, 1606, 1879, 2123 and 2329 m a.s.l. A gradual decrease in the cover of monocotyledons is observed along the elevation gradient, from approx. 60% to 50%, as reported in previous studies (Descombes *et al*. 2017).

3. Supplementary tables

Table S1 Coefficients of the statistical models used to quantify the relationship between temperature and the indices connectance, insect niche overlap and weighted nestedness: slope of the relationship between the observed metric and the temperature, intercept estimate, *p* value, degrees of freedom, t-value, standardized effect size (SES) measured between the observed metric slope and those obtained from random networks of interactions, and the 2.5% and 97.5% quantile interval limits of the slopes obtained for random networks.

	Slope Estimate	**Intercept Estimate**	***p* value**	**df**	**t-value**	**SES**	**2.5% quantile**	**97.5% quantile**
Connectance	-0.0015	0.41	0.467	43.63	-0.73	-1.547	-0.0018	0.0019
Insect niche overlap	0.00004	0.19	0.992	44.28	0.01	-0.094	-0.0029	0.0027
Weighted nestedness	-0.0102	0.40	0.042	43.91	-2.1	-3.51	-0.0054	0.0060

Table S2 Table of species keystone scores. Values are provided for the top 10 keystone species of each network.

Species	Keystone score	Site ID	Elevation (m)	Transect
Bromus erectus	0.713	B1	601	Bex
Briza media	0.690	B1	601	Bex
Hippocrepis comosa	0.687	B1	601	Bex
Lotus corniculatus	0.664	B1	601	Bex
Peucedanum oreoselinum	0.650	B1	601	Bex
Brachypodium pinnatum	0.596	B1	601	Bex
Trifolium montanum	0.583	B1	601	Bex
Securigera varia	0.550	B1	601	Bex
Trifolium rubens	0.549	B1	601	Bex
Medicago sativa	0.484	B1	601	Bex
Festuca	0.747	B2	743	Bex
Arrhenatherum elatius	0.718	B2	743	Bex
Holcus lanatus	0.705	B2	743	Bex
Trisetum flavescens	0.675	B2	743	Bex
Dactylis glomerata	0.675	B2	743	Bex
Brachypodium pinnatum	0.674	B2	743	Bex
Helictotrichon pubescens	0.654	B2	743	Bex
Leontodon hispidus	0.617	B2	743	Bex
Briza media	0.601	B2	743	Bex
Bromus erectus	0.590	B2	743	Bex
Festuca pratensis	0.752	B3	1056	Bex
Dactylis glomerata	0.708	B3	1056	Bex
Bromus erectus	0.644	B3	1056	Bex
Anthoxanthum odoratum	0.624	B3	1056	Bex
Holcus lanatus	0.605	B3	1056	Bex
Brachypodium pinnatum	0.602	B3	1056	Bex
Koeleria pyramidata	0.582	B3	1056	Bex
Briza media	0.558	B3	1056	Bex
Helictotrichon pubescens	0.547	B3	1056	Bex
Molinia caerulea	0.531	B3	1056	Bex
Festuca	0.452	B4	1384	Bex
Cynosurus cristatus	0.413	B4	1384	Bex

Festuca pratensis	0.393	B4	1384	Bex
Deschampsia cespitosa	0.358	B4	1384	Bex
Nardus stricta	0.352	B4	1384	Bex
Dactylis glomerata	0.347	B4	1384	Bex
Holcus lanatus	0.335	B4	1384	Bex
Briza media	0.330	B4	1384	Bex
Agrostis capillaris	0.327	B4	1384	Bex
Bromus erectus	0.313	B4	1384	Bex
Cynosurus cristatus	0.496	B5.1	1361	Bex
Festuca rubra	0.480	B5.1	1361	Bex
Trisetum flavescens	0.464	B5.1	1361	Bex
Anthoxanthum odoratum	0.458	B5.1	1361	Bex
Dactylis glomerata	0.449	B5.1	1361	Bex
Sesleria caerulea	0.448	B5.1	1361	Bex
Phleum	0.444	B5.1	1361	Bex
Brachypodium pinnatum	0.431	B5.1	1361	Bex
Agrostis capillaris	0.421	B5.1	1361	Bex
Festuca pratensis	0.394	B5.1	1361	Bex
Dactylis glomerata	0.209	B5.2	1712	Bex
Bromus erectus	0.200	B5.2	1712	Bex
Pimpinella major	0.193	B5.2	1712	Bex
Carex sempervirens	0.190	B5.2	1712	Bex
Poa pratensis	0.188	B5.2	1712	Bex
Anthoxanthum odoratum	0.183	B5.2	1712	Bex
Festuca	0.181	B5.2	1712	Bex
Bupleurum falcatum	0.178	B5.2	1712	Bex
Centaurea scabiosa	0.177	B5.2	1712	Bex
Festuca ovina	0.177	B5.2	1712	Bex
Danthonia decumbens	0.361	B6	1836	Bex
Nardus stricta	0.347	B6	1836	Bex
Festuca rubra	0.345	B6	1836	Bex
Sesleria caerulea	0.336	B6	1836	Bex
Festuca ovina	0.329	B6	1836	Bex
Agrostis capillaris	0.324	B6	1836	Bex
Koeleria pyramidata	0.322	B6	1836	Bex
Helictotrichon pubescens	0.321	B6	1836	Bex

Phleum hirsutum	0.316	B6	1836	Bex
Dactylis glomerata	0.311	B6	1836	Bex
Phleum hirsutum	0.397	B7	2074	Bex
Anthoxanthum odoratum	0.364	B7	2074	Bex
Helictotrichon pubescens	0.363	B7	2074	Bex
Dactylis glomerata	0.358	B7	2074	Bex
Sesleria caerulea	0.343	B7	2074	Bex
Festuca rubra	0.338	B7	2074	Bex
Lotus corniculatus	0.338	B7	2074	Bex
Poa alpina	0.324	B7	2074	Bex
Nardus stricta	0.315	B7	2074	Bex
Trifolium badium	0.249	B7	2074	Bex
Agrostis alpina	0.344	B8	2277	Bex
Festuca rubra	0.307	B8	2277	Bex
Sesleria caerulea	0.293	B8	2277	Bex
Poa alpina	0.292	B8	2277	Bex
Anthoxanthum odoratum	0.292	B8	2277	Bex
Helictotrichon pubescens	0.291	B8	2277	Bex
Ligusticum mutellina	0.288	B8	2277	Bex
Trifolium pratense	0.280	B8	2277	Bex
Festuca ovina	0.273	B8	2277	Bex
Helictotrichon versicolor	0.250	B8	2277	Bex
Festuca ovina	0.384	C1	630	Calanda
Dactylis glomerata	0.378	C1	630	Calanda
Bromus erectus	0.373	C1	630	Calanda
Brachypodium pinnatum	0.369	C1	630	Calanda
Trisetum flavescens	0.344	C1	630	Calanda
Arrhenatherum elatius	0.343	C1	630	Calanda
Festuca rubra	0.336	C1	630	Calanda
Festuca pratensis	0.329	C1	630	Calanda
Holcus lanatus	0.312	C1	630	Calanda
Lotus corniculatus	0.260	C1	630	Calanda
Centaurea jacea	0.504	C2	821	Calanda
Bromus erectus	0.501	C2	821	Calanda
Festuca rubra	0.468	C2	821	Calanda
Briza media	0.459	C2	821	Calanda

Agrostis capillaris	0.451	C2	821	Calanda
Cynosurus cristatus	0.430	C2	821	Calanda
Lotus corniculatus	0.428	C2	821	Calanda
Trifolium pratense	0.403	C2	821	Calanda
Lolium perenne	0.400	C2	821	Calanda
Brachypodium pinnatum	0.400	C2	821	Calanda
Koeleria pyramidata	0.480	C3	1004	Calanda
Briza media	0.467	C3	1004	Calanda
Phleum pratense	0.466	C3	1004	Calanda
Brachypodium pinnatum	0.458	C3	1004	Calanda
Bromus erectus	0.452	C3	1004	Calanda
Agrostis capillaris	0.449	C3	1004	Calanda
Danthonia decumbens	0.426	C3	1004	Calanda
Dactylis glomerata	0.426	C3	1004	Calanda
Lotus corniculatus	0.366	C3	1004	Calanda
Trifolium pratense	0.360	C3	1004	Calanda
Festuca	0.335	C4	1407	Calanda
Festuca pratensis	0.288	C4	1407	Calanda
Bromus erectus	0.284	C4	1407	Calanda
Danthonia decumbens	0.271	C4	1407	Calanda
Poa pratensis	0.266	C4	1407	Calanda
Anthoxanthum odoratum	0.259	C4	1407	Calanda
Dactylis glomerata	0.247	C4	1407	Calanda
Brachypodium pinnatum	0.244	C4	1407	Calanda
Agrostis capillaris	0.240	C4	1407	Calanda
Briza media	0.237	C4	1407	Calanda
Festuca rubra	0.495	C5	1623	Calanda
Phleum alpinum	0.469	C5	1623	Calanda
Sesleria caerulea	0.456	C5	1623	Calanda
Briza media	0.448	C5	1623	Calanda
Dactylis glomerata	0.419	C5	1623	Calanda
Agrostis capillaris	0.412	C5	1623	Calanda
Cirsium acaule	0.389	C5	1623	Calanda
Brachypodium pinnatum	0.374	C5	1623	Calanda
Achillea millefolium	0.370	C5	1623	Calanda
Trifolium pratense	0.365	C5	1623	Calanda

Agrostis	0.177	C6	1987	Calanda
Poa alpina	0.163	C6	1987	Calanda
Helictotrichon versicolor	0.159	C6	1987	Calanda
Sesleria caerulea	0.158	C6	1987	Calanda
Agrostis rupestris	0.158	C6	1987	Calanda
Phleum alpinum	0.156	C6	1987	Calanda
Agrostis capillaris	0.151	C6	1987	Calanda
Phleum	0.150	C6	1987	Calanda
Anthoxanthum odoratum	0.148	C6	1987	Calanda
Lotus corniculatus	0.148	C6	1987	Calanda
Trifolium	0.306	C7	2163	Calanda
Silene vulgaris	0.296	C7	2163	Calanda
Trifolium pratense	0.296	C7	2163	Calanda
Alchemilla xanthochlora	0.231	C7	2163	Calanda
Alchemilla conjuncta	0.227	C7	2163	Calanda
Anthoxanthum odoratum	0.167	C7	2163	Calanda
Festuca rubra	0.163	C7	2163	Calanda
Carex sempervirens	0.161	C7	2163	Calanda
Phleum alpinum	0.159	C7	2163	Calanda
Leontodon hispidus	0.158	C7	2163	Calanda
Agrostis	0.158	C8	2346	Calanda
Anthyllis vulneraria	0.156	C8	2346	Calanda
Festuca rubra	0.154	C8	2346	Calanda
Sesleria caerulea	0.145	C8	2346	Calanda
Alchemilla hybrida	0.144	C8	2346	Calanda
Helictotrichon versicolor	0.139	C8	2346	Calanda
Poa alpina	0.135	C8	2346	Calanda
Helictotrichon pubescens	0.133	C8	2346	Calanda
Alchemilla conjuncta	0.128	C8	2346	Calanda
Anthoxanthum odoratum	0.117	C8	2346	Calanda
Trifolium montanum	0.283	F1	840	Faido
Potentilla erecta	0.278	F1	840	Faido
Trifolium pratense	0.274	F1	840	Faido
Dactylis glomerata	0.267	F1	840	Faido
Agrostis capillaris	0.264	F1	840	Faido
Bromus erectus	0.233	F1	840	Faido

Festuca rubra	0.230	F1	840	Faido
Brachypodium pinnatum	0.228	F1	840	Faido
Holcus lanatus	0.219	F1	840	Faido
Molinia arundinacea	0.218	F1	840	Faido
Festuca rubra	0.301	F2	920	Faido
Trifolium pratense	0.291	F2	920	Faido
Digitaria sanguinalis	0.290	F2	920	Faido
Trifolium repens	0.290	F2	920	Faido
Brachypodium	0.288	F2	920	Faido
Trifolium campestre	0.287	F2	920	Faido
Phleum	0.286	F2	920	Faido
Dactylis glomerata	0.283	F2	920	Faido
Bromus erectus	0.283	F2	920	Faido
Trifolium arvense	0.276	F2	920	Faido
Trifolium repens	0.362	F3	1196	Faido
Trifolium pratense	0.344	F3	1196	Faido
Dactylis glomerata	0.340	F3	1196	Faido
Trifolium arvense	0.328	F3	1196	Faido
Holcus lanatus	0.315	F3	1196	Faido
Trifolium campestre	0.313	F3	1196	Faido
Agrostis capillaris	0.302	F3	1196	Faido
Festuca rubra	0.295	F3	1196	Faido
Anthoxanthum odoratum	0.286	F3	1196	Faido
Pimpinella saxifraga	0.286	F3	1196	Faido
Dactylis glomerata	0.386	F4	1599	Faido
Festuca ovina	0.381	F4	1599	Faido
Anthoxanthum odoratum	0.376	F4	1599	Faido
Festuca rubra	0.371	F4	1599	Faido
Lolium perenne	0.356	F4	1599	Faido
Nardus stricta	0.350	F4	1599	Faido
Briza media	0.346	F4	1599	Faido
Festuca	0.342	F4	1599	Faido
Danthonia decumbens	0.326	F4	1599	Faido
Agrostis capillaris	0.326	F4	1599	Faido
Briza media	0.477	F5	1712	Faido
Dactylis glomerata	0.445	F5	1712	Faido

Anthoxanthum odoratum	0.443	F5	1712	Faido
Nardus stricta	0.425	F5	1712	Faido
Phleum alpinum	0.418	F5	1712	Faido
Agrostis capillaris	0.416	F5	1712	Faido
Festuca rubra	0.378	F5	1712	Faido
Chaerophyllum villarsii	0.362	F5	1712	Faido
Trifolium pratense	0.349	F5	1712	Faido
Trifolium montanum	0.338	F5	1712	Faido
Briza media	0.470	F6	1892	Faido
Nardus stricta	0.460	F6	1892	Faido
Trifolium pratense	0.451	F6	1892	Faido
Festuca ovina	0.442	F6	1892	Faido
Dactylis glomerata	0.436	F6	1892	Faido
Agrostis capillaris	0.428	F6	1892	Faido
Anthoxanthum odoratum	0.419	F6	1892	Faido
Agrostis rupestris	0.416	F6	1892	Faido
Trifolium montanum	0.407	F6	1892	Faido
Poa alpina	0.397	F6	1892	Faido
Festuca rubra	0.261	F7	2139	Faido
Festuca varia	0.256	F7	2139	Faido
Agrostis rupestris	0.254	F7	2139	Faido
Anthoxanthum	0.251	F7	2139	Faido
Lotus corniculatus	0.241	F7	2139	Faido
Helictotrichon versicolor	0.215	F7	2139	Faido
Potentilla grandiflora	0.214	F7	2139	Faido
Potentilla aurea	0.203	F7	2139	Faido
Cardamine resedifolia	0.164	F7	2139	Faido
Asteraceae	0.148	F7	2139	Faido
Anthoxanthum	0.150	F8	2417	Faido
Nardus stricta	0.149	F8	2417	Faido
Poa alpina	0.143	F8	2417	Faido
Helictotrichon versicolor	0.140	F8	2417	Faido
Agrostis rupestris	0.139	F8	2417	Faido
Trifolium alpinum	0.137	F8	2417	Faido
Lotus corniculatus	0.137	F8	2417	Faido
Loiseleuria procumbens	0.137	F8	2417	Faido

Festuca ovina	0.129	F8	2417	Faido
Leontodon hispidus	0.129	F8	2417	Faido
Festuca	0.488	G1	672	Grindelwald
Briza media	0.488	G1	672	Grindelwald
Brachypodium pinnatum	0.482	G1	672	Grindelwald
Bromus erectus	0.482	G1	672	Grindelwald
Holcus lanatus	0.476	G1	672	Grindelwald
Festuca rubra	0.474	G1	672	Grindelwald
Agrostis capillaris	0.454	G1	672	Grindelwald
Arrhenatherum	0.451	G1	672	Grindelwald
Dactylis glomerata	0.446	G1	672	Grindelwald
Alchemilla xanthochlora	0.338	G1	672	Grindelwald
Briza media	0.344	G2	973	Grindelwald
Anthoxanthum odoratum	0.330	G2	973	Grindelwald
Trifolium pratense	0.327	G2	973	Grindelwald
Brachypodium pinnatum	0.321	G2	973	Grindelwald
Poaceae	0.315	G2	973	Grindelwald
Bromus erectus	0.314	G2	973	Grindelwald
Cynosurus cristatus	0.313	G2	973	Grindelwald
Arrhenatherum	0.296	G2	973	Grindelwald
Festuca rubra	0.295	G2	973	Grindelwald
Holcus lanatus	0.294	G2	973	Grindelwald
Bromus erectus	0.528	G3	1095	Grindelwald
Brachypodium pinnatum	0.524	G3	1095	Grindelwald
Arrhenatherum elatius	0.519	G3	1095	Grindelwald
Trisetum flavescens	0.514	G3	1095	Grindelwald
Briza media	0.509	G3	1095	Grindelwald
Festuca ovina	0.492	G3	1095	Grindelwald
Dactylis glomerata	0.476	G3	1095	Grindelwald
Cynosurus cristatus	0.473	G3	1095	Grindelwald
Centaurea scabiosa	0.430	G3	1095	Grindelwald
Phleum pratense	0.426	G3	1095	Grindelwald
Trifolium	0.265	G4	1359	Grindelwald
Dactylis glomerata	0.254	G4	1359	Grindelwald
Festuca rubra	0.252	G4	1359	Grindelwald
Phleum	0.249	G4	1359	Grindelwald

Bromus erectus	0.246	G4	1359	Grindelwald
Molinia	0.240	G4	1359	Grindelwald
Trifolium pratense	0.236	G4	1359	Grindelwald
Anthoxanthum odoratum	0.234	G4	1359	Grindelwald
Trifolium repens	0.234	G4	1359	Grindelwald
Briza media	0.234	G4	1359	Grindelwald
Agrostis capillaris	0.432	G5	1561	Grindelwald
Festuca rubra	0.370	G5	1561	Grindelwald
Nardus stricta	0.358	G5	1561	Grindelwald
Festuca ovina	0.355	G5	1561	Grindelwald
Bromus erectus	0.354	G5	1561	Grindelwald
Poaceae	0.351	G5	1561	Grindelwald
Dactylis glomerata	0.349	G5	1561	Grindelwald
Sesleria	0.332	G5	1561	Grindelwald
Trifolium repens	0.315	G5	1561	Grindelwald
Briza media	0.311	G5	1561	Grindelwald
Sesleria caerulea	0.347	G6	1790	Grindelwald
Trifolium repens	0.340	G6	1790	Grindelwald
Festuca rubra	0.336	G6	1790	Grindelwald
Trifolium montanum	0.335	G6	1790	Grindelwald
Bromus erectus	0.329	G6	1790	Grindelwald
Trifolium pratense	0.328	G6	1790	Grindelwald
Carum carvi	0.235	G6	1790	Grindelwald
Alchemilla xanthochlora	0.230	G6	1790	Grindelwald
Lotus corniculatus	0.213	G6	1790	Grindelwald
Leontodon hispidus	0.203	G6	1790	Grindelwald
Phleum	0.366	G7	2042	Grindelwald
Festuca violacea	0.352	G7	2042	Grindelwald
Poaceae	0.334	G7	2042	Grindelwald
Poa	0.318	G7	2042	Grindelwald
Trifolium pratense	0.310	G7	2042	Grindelwald
Anthoxanthum odoratum	0.304	G7	2042	Grindelwald
Trifolium repens	0.290	G7	2042	Grindelwald
Festuca rubra	0.285	G7	2042	Grindelwald
Agrostis capillaris	0.279	G7	2042	Grindelwald
Trifolium badium	0.264	G7	2042	Grindelwald

Agrostis rupestris	0.334	G8	2220	Grindelwald
Poa	0.308	G8	2220	Grindelwald
Phleum	0.294	G8	2220	Grindelwald
Nardus stricta	0.289	G8	2220	Grindelwald
Festuca violacea	0.283	G8	2220	Grindelwald
Agrostis capillaris	0.281	G8	2220	Grindelwald
Anthoxanthum	0.279	G8	2220	Grindelwald
Festuca rubra	0.276	G8	2220	Grindelwald
Helictotrichon	0.276	G8	2220	Grindelwald
Poaceae	0.272	G8	2220	Grindelwald
Trifolium dubium	0.438	M1	578	Martigny
Trifolium arvense	0.437	M1	578	Martigny
Peucedanum oreoselinum	0.396	M1	578	Martigny
Festuca valesiaca	0.366	M1	578	Martigny
Dactylis glomerata	0.345	M1	578	Martigny
Poa bulbosa	0.336	M1	578	Martigny
Hippocrepis comosa	0.326	M1	578	Martigny
Bromus squarrosus	0.324	M1	578	Martigny
Vicia sepium	0.318	M1	578	Martigny
Bromus erectus	0.301	M1	578	Martigny
Trifolium pratense	0.485	M2	845	Martigny
Trifolium montanum	0.473	M2	845	Martigny
Festuca	0.469	M2	845	Martigny
Brachypodium pinnatum	0.464	M2	845	Martigny
Dactylis glomerata	0.460	M2	845	Martigny
Briza media	0.454	M2	845	Martigny
Anthoxanthum odoratum	0.454	M2	845	Martigny
Trifolium repens	0.446	M2	845	Martigny
Koeleria pyramidata	0.439	M2	845	Martigny
Festuca rubra	0.435	M2	845	Martigny
Bromus erectus	0.500	M3	1023	Martigny
Festuca ovina	0.480	M3	1023	Martigny
Arrhenatherum elatius	0.472	M3	1023	Martigny
Briza media	0.466	M3	1023	Martigny
Poa	0.465	M3	1023	Martigny
Koeleria pyramidata	0.455	M3	1023	Martigny

Poa bulbosa	0.454	M3	1023	Martigny
Helictotrichon pubescens	0.450	M3	1023	Martigny
Dactylis glomerata	0.431	M3	1023	Martigny
Trifolium montanum	0.319	M3	1023	Martigny
Festuca	0.681	M4	1232	Martigny
Poa	0.676	M4	1232	Martigny
Koeleria pyramidata	0.624	M4	1232	Martigny
Melica ciliata	0.622	M4	1232	Martigny
Bromus erectus	0.610	M4	1232	Martigny
Trisetum flavescens	0.600	M4	1232	Martigny
Bromus tectorum	0.580	M4	1232	Martigny
Helictotrichon pubescens	0.571	M4	1232	Martigny
Achillea millefolium	0.536	M4	1232	Martigny
Bromus squarrosus	0.507	M4	1232	Martigny
Festuca arundinacea	0.325	M5	1653	Martigny
Anthoxanthum odoratum	0.308	M5	1653	Martigny
Briza media	0.308	M5	1653	Martigny
Festuca ovina	0.307	M5	1653	Martigny
Trifolium pratense	0.306	M5	1653	Martigny
Poa angustifolia	0.300	M5	1653	Martigny
Dactylis glomerata	0.294	M5	1653	Martigny
Festuca rubra	0.292	M5	1653	Martigny
Helictotrichon pubescens	0.292	M5	1653	Martigny
Bromus erectus	0.288	M5	1653	Martigny
Koeleria pyramidata	0.398	M6	1856	Martigny
Festuca rubra	0.392	M6	1856	Martigny
Anthoxanthum odoratum	0.391	M6	1856	Martigny
Phleum phleoides	0.379	M6	1856	Martigny
Festuca ovina	0.378	M6	1856	Martigny
Bromus erectus	0.371	M6	1856	Martigny
Agrostis capillaris	0.370	M6	1856	Martigny
Briza media	0.356	M6	1856	Martigny
Brachypodium pinnatum	0.355	M6	1856	Martigny
Dactylis glomerata	0.340	M6	1856	Martigny
Nardus stricta	0.431	M7	2112	Martigny
Festuca ovina	0.423	M7	2112	Martigny

Helictotrichon pubescens	0.404	M7	2112	Martigny
Phleum alpinum	0.400	M7	2112	Martigny
Anthoxanthum odoratum	0.383	M7	2112	Martigny
Poa alpina	0.378	M7	2112	Martigny
Trisetum flavescens	0.374	M7	2112	Martigny
Koeleria pyramidata	0.371	M7	2112	Martigny
Festuca rubra	0.364	M7	2112	Martigny
Lotus corniculatus	0.326	M7	2112	Martigny
Festuca varia	0.301	M8	2321	Martigny
Sesleria caerulea	0.286	M8	2321	Martigny
Festuca ovina	0.285	M8	2321	Martigny
Poa alpina	0.271	M8	2321	Martigny
Festuca rubra	0.270	M8	2321	Martigny
Helictotrichon versicolor	0.268	M8	2321	Martigny
Carex sempervirens	0.266	M8	2321	Martigny
Anthoxanthum odoratum	0.246	M8	2321	Martigny
Trifolium badium	0.230	M8	2321	Martigny
Trifolium pratense	0.221	M8	2321	Martigny
Koeleria vallesiana	0.341	S1	605	Salgesch
Poa bulbosa	0.339	S1	605	Salgesch
Bromus erectus	0.308	S1	605	Salgesch
Bothriochloa ischaemum	0.305	S1	605	Salgesch
Stipa pennata	0.300	S1	605	Salgesch
Artemisia campestris	0.280	S1	605	Salgesch
Artemisia absinthium	0.275	S1	605	Salgesch
Melica ciliata	0.273	S1	605	Salgesch
Ononis pusilla	0.270	S1	605	Salgesch
Elymus	0.269	S1	605	Salgesch
Potentilla	0.423	S2	793	Salgesch
Melica ciliata	0.362	S2	793	Salgesch
Bothriochloa ischaemum	0.357	S2	793	Salgesch
Stipa pennata	0.352	S2	793	Salgesch
Koeleria vallesiana	0.343	S2	793	Salgesch
Centaurea scabiosa	0.329	S2	793	Salgesch
Hippocrepis emerus	0.320	S2	793	Salgesch
Erigeron annuus	0.292	S2	793	Salgesch

Sedum album	0.225	S2	793	Salgesch
Minuartia rubra	0.213	S2	793	Salgesch
Coronilla minima	0.346	S3	997	Salgesch
Stipa pennata	0.345	S3	997	Salgesch
Koeleria vallesiana	0.338	S3	997	Salgesch
Bothriochloa ischaemum	0.320	S3	997	Salgesch
Ononis pusilla	0.314	S3	997	Salgesch
Bromus erectus	0.312	S3	997	Salgesch
Prunus mahaleb	0.312	S3	997	Salgesch
Leontodon hispidus	0.303	S3	997	Salgesch
Hippocrepis comosa	0.296	S3	997	Salgesch
Lotus corniculatus	0.273	S3	997	Salgesch
Sesleria caerulea	0.440	S4	1323	Salgesch
Festuca ovina	0.431	S4	1323	Salgesch
Lotus corniculatus	0.406	S4	1323	Salgesch
Koeleria pyramidata	0.393	S4	1323	Salgesch
Trifolium montanum	0.387	S4	1323	Salgesch
Dactylis glomerata	0.384	S4	1323	Salgesch
Brachypodium pinnatum	0.379	S4	1323	Salgesch
Koeleria vallesiana	0.345	S4	1323	Salgesch
Bromus erectus	0.345	S4	1323	Salgesch
Briza media	0.332	S4	1323	Salgesch
Bromus erectus	0.289	S5	1617	Salgesch
Festuca pratensis	0.279	S5	1617	Salgesch
Festuca rubra	0.270	S5	1617	Salgesch
Trifolium pratense	0.238	S5	1617	Salgesch
Lolium perenne	0.231	S5	1617	Salgesch
Koeleria pyramidata	0.223	S5	1617	Salgesch
Briza media	0.207	S5	1617	Salgesch
Carex montana	0.207	S5	1617	Salgesch
Agrostis	0.196	S5	1617	Salgesch
Trifolium montanum	0.189	S5	1617	Salgesch
Deschampsia cespitosa	0.236	S6	1914	Salgesch
Carex sempervirens	0.234	S6	1914	Salgesch
Festuca rubra	0.231	S6	1914	Salgesch
Sesleria caerulea	0.231	S6	1914	Salgesch

Melica nutans	0.228	S6	1914	Salgesch
Helictotrichon pubescens	0.227	S6	1914	Salgesch
Festuca ovina	0.224	S6	1914	Salgesch
Trifolium badium	0.222	S6	1914	Salgesch
Festuca pratensis	0.221	S6	1914	Salgesch
Briza media	0.220	S6	1914	Salgesch
Festuca rubra	0.392	S7	2206	Salgesch
Festuca ovina	0.375	S7	2206	Salgesch
Trifolium pratense	0.362	S7	2206	Salgesch
Senecio doronicum	0.336	S7	2206	Salgesch
Agrostis alpina	0.321	S7	2206	Salgesch
Poa alpina	0.320	S7	2206	Salgesch
Phleum hirsutum	0.314	S7	2206	Salgesch
Sesleria caerulea	0.307	S7	2206	Salgesch
Anthyllis vulneraria	0.305	S7	2206	Salgesch
Carduus defloratus	0.300	S7	2206	Salgesch
Anthyllis vulneraria	0.201	S8	2390	Salgesch
Oxytropis jacquinii	0.198	S8	2390	Salgesch
Hedysarum hedysaroides	0.193	S8	2390	Salgesch
Helictotrichon versicolor	0.190	S8	2390	Salgesch
Festuca rubra	0.188	S8	2390	Salgesch
Sesleria caerulea	0.187	S8	2390	Salgesch
Agrostis alpina	0.180	S8	2390	Salgesch
Hippocrepis comosa	0.176	S8	2390	Salgesch
Lotus corniculatus	0.171	S8	2390	Salgesch
Poa alpina	0.168	S8	2390	Salgesch

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Supplementary materials S2

Contents of the file

This document provides a complete and detailed methodological workflow for reconstructing plant–herbivore trophic networks from insect feces samples using DNA metabarcoding. It includes explanations on the wet-lab protocol design (section Methods, 1A), a user-friendly wet-lab protocol (section Methods, 1B), a description of the bioinformatic pipeline (section Methods, 1C) and results of the amplification success and database coverage (section Results).

Supplementary methods

1. ITS2 DNA metabarcoding library preparation

The key aspects of this protocol are the adaptation of the DNA metabarcoding method to insect feces samples, the use of a dual-indexing approach that limits the costs associated with oligos and the generation and sharing of a high-quality plant-specific ITS2 reference database. It relies on a modified version of Chen et al. (2010) for the set-up of the amplicon PCR reaction and on an adaptation of Illumina reference's protocol for the preparation of the indexing libraries using Nextera XT Index adapters (Illumina, Humabrich).

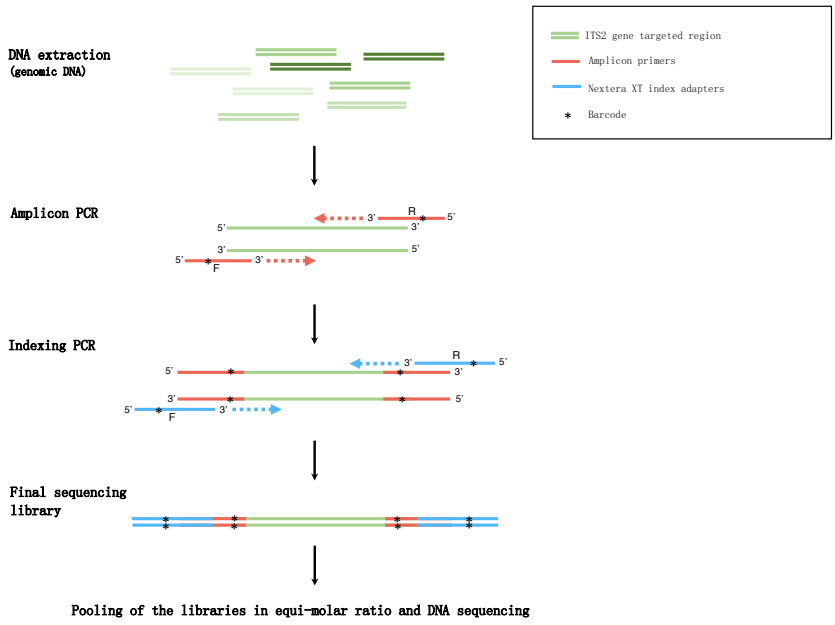

Figure S1 Schematic representation of the DNA metabarcoding library preparation workflow

A. Design of the wet lab protocol

A.1 ITS2 marker choice

ITS2 was chosen because of its high ability to amplify deeply diverged plant taxa within Spermatophyta, an ease of PCR amplification and a large discriminatory power between taxa (Yao *et al*. 2010; Hollingsworth *et al*. 2011; Li *et al*. 2011; Staats *et al*. 2016; Moorhouse-Gann *et al*. 2018). We used the primer pair ITS2-S2F (Chen *et al*. 2010) / ITS4_rev (White *et al*. 1990) that generates a 360bp amplicon which is particularly convenient for retrieving intact sequences from degraded DNA from feces, gut or soil samples (García-Robledo *et al*. 2013; Fahner *et al*. 2016; Moorhouse-Gann *et al*. 2018). The universality of this primer pair was underlined in several studies (Chen *et al*. 2010; García-Robledo *et al*. 2013; Fahner *et al*. 2016). However, we recommend consulting the recent publication of Moorhouse-Gann et al. (2018) who proposed a new ITS2 primer pair (UniPlantF/ UniPlantR) that amplifies a smaller DNA fragment that completely overlaps with the region targeted by the ITS2-S2F/ITS4_rev primer pair and includes additional wooble bases improving the universality of the priming sites.

A.2 Dual-indexing approach

The preparation of the sequencing DNA metabarcoding libraries follows a dual-indexing procedure that allows to individually tag each sample. This procedure permits to increase the number of tagged samples while reducing the number of oligos to be ordered and the associated handling and financial costs. The first PCR (i.e. amplicon PCR) amplifies the targeted genetic marker using the locus-specific primers extended by an overhang used as a priming site in the next round of amplification, the indexing PCR (see Fig. S1). The indexing PCR incorporates in the DNA construct sequences that are compatible with the Illumina sequencing primers for achieving competent-sequencing library (Table S1). This approach is often referred to as a two-step based PCR, and contrasts to the ligation approach that bind technical sequences to blunt-end DNA fragments. Barcodes are inserted in both the amplicon primers and the Nextera XT Index adapters and are used in combination. In the present protocol, we propose a dual-indexing design that allows to pool up to 576 samples. The amplicon primers are composed of the ITS2 locus-specific primer, linkers (to balance the fluorescent signals in the sequencing process), 8-nucleotide barcode, and the complementary sequences to Nextera XT Index adapters (Fig. S1). We designed two sets of forward and reverse primer pairs to tag in parallel two separated batches of samples during the amplicon PCR. Using custom scripts, we generated four barcodes, different from each other by at least 4

substitutions that are further incorporate in each set of forward and reverse primer pairs (Table S1). Each of the four amplicon primer are ordered in two variants differing by the linker length (two or three nucleotide). Explanation on how to mix the primers is provided in section B2. The individual tagging of the samples is done at the indexing PCR by applying the Nextera XT Index adapters set (A, B, D) in parallel to each sample batch of amplicon PCRs.

Table S1 Oligos sequences of ITS2 amplicon primers.

Primer name	Primer direction	Mix	Primer sequence
ITS2_S2F_B1.1	forward	1	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNACCTGCTTATGCGATACTTGGTGTGAAT
ITS2_S2F_B1.2	forward	1	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNACCTGCTTATGCGATACTTGGTGTGAAT
ITS2_S2F_B2.1	forward	2	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNGAAGTTGCATGCGATACTTGGTGTGAAT
ITS2_S2F_B2.2	forward	2	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNGAAGTTGCATGCGATACTTGGTGTGAAT
ITS_4_rev_B1.1	reverse	1	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNAACGACGTTCCTCCGCTTATTGATATGC
ITS_4_rev_B1.2	reverse	1	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNAACGACGTTCCTCCGCTTATTGATATGC
ITS_4_rev_B2.1	reverse	2	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNTGGAGGCCTCCTCCGCTTATTGATATGC
ITS_4_rev_B2.2	reverse	2	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNTGGAGGCCTCCTCCGCTTATTGATATGC

Compatible sequence with Nextera XT Index adapters
Linker (two or three bp)
Unique barcode
Locus-specific primer (ITS2)

NB. Each set of Nextera XT Index adapters consists in 8 forward (S) and 12 reverse (N) adaptors that are used in combination to tag 96 samples. Since the S adapters of the set A and B and the N adapters of the sets B and D are the same, we could create the set D (and C) by ordering only the sets A and D (See Reference Guide for Nextera XT DNA Library Prep Kit, Document # 15031942 v05). Doing so, one should ensure that the quantity of each Nextera XT Index adapter is sufficient to tag the desired number of samples, i.e., 2µl * 96 * n, with n the number of sample batches defined at the amplicon PCR.

B. Step-by-step wet lab protocol

B.1 DNA extractions

DNA extractions are performed using the column-based FastDNA™ SPIN Kit for Soil (MP Biomedicals, Santa Ana, USA) according to manufacturer's instructions, in series of 24 samples at a time, including negative controls to verify for cross-contaminations. DNA extraction are eluted in 110µl of 10mM Tris-HCl pH 8.5. Samples are thoroughly disrupted with a TissueLyser (Schieritz & Hauenstein AG, Laufen, Switzerland) at the maximal speed (30 Hz) for a minimum of two rounds of 30 seconds using tungsten beads. If the plant fibers are not properly fragmented, the grinding time should be increased. For the extraction and the first PCR steps, the equipment is meticulously decontaminated using DNA-Exitus Plus ™ IF (PanReac AppliChem, Chicago, USA), the manipulations are conducted under a pre-PCR Hepa Hood (UVP, Upland, USA) and all the hand material is disinfected with UV light for 30 minutes.

B.2 Amplicon PCR

1. Preparation of the primer mixes

   Resuspend each primer to 100µM (Table S1). Mix all primers B1 and B2 into two separate mixes in order to have a final concentration of 2.5uM of each primer in each mix. Use these primer mixes to tag two separate batches of samples in the amplicon PCR reaction.

2. Prepare the following Master Mix for the required number of reactions.

Reagent	**Volume (µl) for one reaction**
H20	13.8
PCR Gold Buffer without MgCl2 (10x)	2.5
MgCl2 (25mM)	2
dNTPs (10mM)	0.5
AmpliTaq Gold DNA Polymerase (5U/µl)	0.2
Primer mix (2.5µM)	1
	20

NB. For each Master Mix, use 1.2x of each reagent volume to compensate for pipetting bias.

3. Add 5µl of DNA extraction product (diluted to 1:10) to 20µl of Master Mix.
4. Run the PCR under the following cycling conditions:

• 95°C for 10 min	
— 95°C for 30 sec	35x
— 56°C for 30 sec	
— 72°C for 45 sec	
• 72°C for 10 min	

5. Verify the amplification success on a 1.5% agarose gel.
6. Purify 19µl of PCR product with purification beads (AMPure XP, Beckman coulter, Switzerland) at a ratio of 0.8x (15.2µl) and elute in 22µl of 10mM Tris-HCl pH 8.5.

B.3 Indexing PCR

1. Prepare the following Master Mix for the required number of reactions.

Reagent	**Volume (µl) for one reaction**
KAPA HiFi HotStart ReadyMix	10
H20	4

2. Distribute 14 µl of Master Mix
3. Add 2µl of **N** and 2µl of **S** Nextera XT Index adapter in each reaction. Along each rows/column is distributed a unique N (1 of 8) / S (1 of 12) adapter combination.
4. Add 2µl of purified amplicon PCR product to 18µl of Master Mix.
5. Run the PCR under the following cycling conditions:

• 95°C for 3 min	
— 95°C for 30 sec	8x
— 55°C for 30 sec	
— 72°C for 30 sec	
• 72°C for 5 min	

6. Verify the amplification success on a 1.5% agarose gel.
7. Purify 15µl of each library with purification beads at a ratio of 0.8x (12µl) and elute in 22µl of 10 mM Tris-HCl pH 8.5.

Table 2 List of reagents used for the PCRs

Reagent	**Company**
AmpliTaq Gold DNA Polymerase (5U/µl)	ThermoFisher, Waltham, USA
PCR Gold Buffer without $MgCl_2$ (10x)	Provided with the Taq
$MgCl_2$ (25mM)	Provided with the Taq
KAPA HiFi HotStart ReadyMix	Roche, Basel, Switzerland
Amplicon primers	Sigma-Aldrich, St. Louis USA
Nextera XT Index kit v2	Illumina, San Diego, USA

B.4 Pooling of the amplicon libraries in equimolar ratio

1. Measure the DNA concentration of each library, e.g. with PicoGreen fluorescent dyes (Quant-iT™PicoGreen™dsDNA Assay Kit, ThermoFisher) on Spark 10M Multimode Microplate Reader (Tecan, Männedorf, Switzerland).
2. Pool the indexed libraries in equimolar ratio so that each sample contributed equally (in ng of DNA) to the final pool. Be aware that there is a minimum concentration of pooled libraries for sequencing and that losses will occur during the last purification (step 3.)
3. Purify the library pool using purification beads at a ratio of 0.8x.
4. Verify that the profile of the purified library pool using a bioanalyzer matches the representation in Fig. S2 (2200 TapeStation, Agilent, Santa Clara, USA). If primers or primer dimers are still visible, repeat step 3.

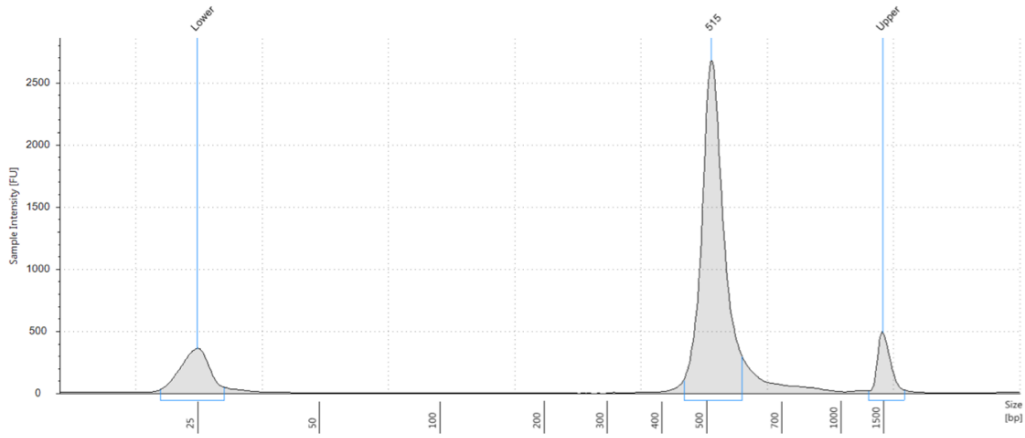

Figure S2 Expected profile of the final pooled ITS2 metabarcoding libraries. The length of the marker (360 bp) added to that of the technical sequences must reach approximately 517 bp.

B.5 DNA sequencing

Libraries are sequenced following the Illumina MiSeq v3 2x300 PE protocol using 10% of PhiX.

2. Plant–specific ITS2 reference database

We created a plant-specific ITS2 reference based on NCBI records (Benson *et al*. 2014) and supplement it using Sanger sequencing. In a first step, we obtained plant-specific ITS2 sequences from NCBI and removed species not expected at the study side. In a second step, we searched for full-length sequences containing both primer sites. In a next step, we merged overlapping records of the same species and used the consensus sequences to find full-length ITS2 sequences. We also kept almost full-length records (minimum length 300 nt) if we could find at least one primer site. The reference database was completed with Sanger sequences for 54% of the plant species identified on sites. Plant specimens were collected during the field season of 2017, air dried and stored in sealed plastic bags with silica gel (ROTH AG, Obergerlafingen, Switzerland). DNA extractions were automated using a high-throughput DNA-extraction KingFisher 96 instrument (Thermo Fisher Scientific, Waltham, USA), following a modified version of the Sbeadex mini plant kit protocol (LGC Genomics, Berlin, Germany). The main modification includes an increase by four times the amount of lysis buffer and β-mercaptoethanol. We amplified the ITS2 marker of each plant sample under the same PCR conditions used for the amplicon PCR, but used five extra cycles of amplification. Sanger sequencing of purified PCR products was processed by Microsynth AG (Balgach, Switzerland). After trimming of the sequences ends on Geneious (Kearse et al. 2012) using an

error probability cutoff value of 0.05, the sequences were merged using the highest sensitivity method and the consensus sequences were appended to the ITS2 reference database.

3. Sequencing processing workflow for DNA metabarcoding libraries

The raw reads were end-trimmed prior to merging (https://github.com/lh3/seqtk) to improve merging rate (Magoc & Salzberg 2011). We used an in-silico PCR approach to identify and remove primer site (Martin 2011). The merged and primer trimmed reads were subsequently quality filtered (Schmieder & Edwards 2011). We used UNOISE2 to obtain ZOTUs (Edgar 2016b) with additional clustering at 97% identity. Taxonomic level were predicted using for each ZOTU using SINTAX (Edgar 2016a) and the ITS2 reference describe above. A confidence threshold of 0.95 was applied. For values below this threshold, we considered the next lower taxonomic level. An OTUs phylogeny was generated using Usearch and Muscle (Edgar 2004, 2010) to identify and we remove OTUs that were not monophyletic. We further discarded OTUs of a taxonomic assignment above the family level and sum the OTU counts belonging the same taxonomic affiliation. Only OTUs of plant taxa that were identified on the study site were kept in the final OTU table. For OTUs with a taxonomical assignment not reaching the species level, we distributed the read counts equally across all known genus or species of this taxa. To control for differences in sequencing depth between samples, we transformed the OTUs absolute abundance (read counts) into relative read abundance (RRA) by sample. This step provides a semi-quantitative estimation of the diet components that was further used as interaction intensity in the downstream network analyses (Deagle *et al*. 2006). From the OTU table, we extracted one trophic network per study site, considering plant species that were not retrieved in the sequencing data as non-interacting species.

Supplementary results

A. PCR on insect feces samples

After optimizing the protocol, all feces samples were successfully amplified during the amplicon PCR. At the first round of PCR only 75% of all feces samples did amplify. To reduce the effect of potential PCR inhibitors, we increase the dilution of DNA extracts (from 10x to 100x) and added five additional PCR cycles. This adjustment enhanced the PCR success rate to 95%. As long as the amount of extracted DNA is sufficient, dilution improved the PCR efficiency. Common in herbivores feces samples (Schrader *et al*. 2012), PCRs inhibitors can be the cause of difficult amplification. The remaining 5% of samples were re-extracted and successfully amplified. Increasing the grinding time to 2 minutes allowed the PCR reaction to be more effective. For a highly effective first round of PCRs (using orthoptera feces samples and the FastDNA™ SPIN Soil Kit), we thus recommend a dilution of 10x minimum and a grinding time of 2 minutes. Additionally, the FastDNA™ SPIN Soil Kit provides good quality results but can be time-consuming. Therefore, we would recommend using 96 well kits, typically the DNeasy PowerSoil HTP 96 kit (Qiagen, Hilden, Germany) developed for soil samples and highly optimized against the effects of PCR inhibitors.

B. Generation of Sanger sequences to complete the plant–specific ITS2 reference database

The combined (NCBI records and Sanger sequences) ITS2 reference database covered about 95% of all the families found on the study sites, 92% of the genera, and 88.5% of the plant species with 5% of the missing species not having their genus represented. The first round of PCR amplification of the plant material sampled to complete the reference database succeeded for 80% of the samples. In cases of unsuccessful PCR, dilution up to 100x coupled with 5 additional PCR cycles allowed reaching 99% of amplification success rate. Due to insufficient data quality and/or fungal contamination 22% of the samples had to be excluded after Sanger sequencing. Unsuccessful amplifications from plant material could be caused by plant secondary compounds that can strongly inhibits the PCR reaction (Schrader *et al*. 2012) or results from a poor primer match for certain species.

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CHAPTER IV

The ecological rules of interaction networks are not systematically conserved along environmental gradients

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The ecological rules of interaction networks are not systematically conserved along environmental gradients

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Abstract

Network ecology aims to unravel the structural organization of species interactions and their underlying ecological rules. While non-random structure of single networks and their associated ecological determinants have been documented, it remains unclear whether network ecological rules are conserved along environmental gradients and across biogeographic regions. Here, we sampled 48 plant-herbivore interaction networks along six elevation gradients in the Central Alps using DNA metabarcoding on orthopteran feces. We developed a set of a priori hypotheses in the form of ecological rules expected to structure interaction networks, based on the plant phylogeny, plant abundance, leaf toughness, nitrogen content and metabolomics richness. We show that the phylogenetic position and the species abundance rules have the largest explanatory power across all networks. The explanatory power of ecological rules was not constant along the elevation gradient and across transects, where the fit of plant abundance and nitrogen content decreased in alpine environments. Our hypothesis-based approach shows that rules underlying species interaction can shift across along environmental gradients and provide a general framework to study the mechanisms that structure how species interact with one another.

Introduction

Species are far from being isolated from each other, and form complex ecological networks through various types of interactions that span the entire spectrum from antagonist to mutualistic relationships (Reiss *et al*. 2009; Bascompte 2010). The structure of ecological networks are not random and present architectures, such as the presence of modules or the ordination of their interactions (e.g. nestedness structuration), which can be driven by species traits related to feeding capacity (Gravel *et al*. 2013; Laigle *et al*. 2018) or to their defences (Gravel *et al*. 2016; Poelman & Kessler 2016). Studying the structure of ecological networks is challenging as their construction are the outcomes of multiple entangled causes (Dormann *et al*. 2017; Laigle *et al*. 2018). Progress in our understanding of the structure of ecological network could be achieved by formulating distinct hypotheses on the causes of species interaction tested against empirical data (Pellissier *et al*. 2018). Furthermore, the determining factors of species interactions could be context-dependent, where interaction rules may shift along environmental gradients (Welti & Joern 2015; Pellissier *et al*. 2018), possibly as a result of modification in the function and the evolutionary history present along the gradients (Tylianakis & Morris 2017). Nevertheless, the field of network ecology only recently

considered the spatio-temporal variations of species interaction and their underlying drivers (Gravel *et al*. 2019) probably due to the large effort needed to acquire network data sets. The development of DNA metabarcoding techniques and their application in trophic ecology opens new perspectives for addressing the biogeography of ecological networks (Roslin & Majaneva 2016).

The structure of ecological networks is expected to be driven by species attributes that can modulate interaction links between species (González-Varo & Traveset 2016; Dormann *et al*. 2017), including functional traits (Laigle *et al*. 2018), phylogeny (Rohr & Bascompte 2014) or demographic properties (Vázquez *et al*. 2007). Species traits can allow or forbid species interactions such as feeding-related or defence traits in trophic interactions (Monteiro & Faria 2018). For example, in plant–pollinators networks, morphological trait-matching rule between floral traits and pollinator feeding appendages explains the distribution of interactions in networks (Garibaldi *et al*. 2015). Supplementing species trait, phylogenetic positions of species reflect the shared evolutionary history of close relatives, and may carry the co-evolutionary signal between interacting species explaining their degree of interactions (Rohr & Bascompte 2014; Brousseau *et al*. 2018). Moreover, some demographic parameters can be associated with species interaction, where, for example, interactions are more probable between abundant species than between rare ones (Vázquez *et al*. 2007; Krishna *et al*. 2008; Canard *et al*. 2014; Fagundes *et al*. 2016). Trait values, and their matching, phylogenetic similarities and species abundances could all shape the structure of ecological networks, but with different influences and thus with variable explanatory power (Tylianakis & Morris 2017). Formulating hypotheses based on the different attributes of species can help comparing empirical ecological network to quantify their agreement with data (Pellissier *et al*. 2018; Gravel *et al*. 2019).

Interaction patterns and network structure may vary along environmental gradients as result of turnover in species taxonomic composition (Pellissier *et al*. 2018), turnover in species traits (Lamanna *et al*. 2014; Descombes *et al*. 2017) and turnover in interaction rules (Tylianakis & Morris 2017). An hypothesis-based approach could help determine whether ecological rules equally determine species interactions along environmental gradients (Pellissier *et al*. 2018). Steep environmental clines such as along elevation gradients exert a strong pressure on organisms that is primarily reflected by a turnover in species composition, abundance and traits (Rahbek 1995; Hodkinson 2005; Kergunteuil *et al*. 2018). Shifting abiotic

conditions are expected to alter both species interaction and the structuring mechanisms that control the realization of the interactions and their intensities (Ramos-Jiliberto *et al*. 2010; Morris *et al*. 2015). Moreover, the strength of the mechanisms determining species interactions may vary along climatic gradients, due to a shift in traits that respond to climatic variations (Tylianakis & Morris 2017), but which also determine the interaction between species (Pellissier et al. 2018). Together, changes in species abundance, functional or lineages composition along environmental gradients could be associated to shifts in the ecological rules underlying the structure of ecological networks (Mccain & Grytnes 2010; Hoiss *et al*. 2012)

Here, we investigate the role of ecological rules in structuring ecological networks and in particular, whether their explanatory power vary along environmental gradients. As a case study, we investigate the role of species traits, phylogenetic position, and plant cover in determining the interactions in plant-orthoptera bipartite networks along elevation. We reconstructed 48 plant–herbivores networks of natural grasslands located along six elevation gradients of the Central Alps (Fig. S1) using DNA metabarcoding method applied on insect feces (Pitteloud *et al*. in prep. - chapter 3). We designed hypotheses-based networks that are driven by (*i*) plant abundance, where herbivore feed on the plant species with highest cover (Bernays & Chapman 1970; Cates 1980); (*ii*) phylogenetic position of plant taxa, where orthoptera are expected to mainly feed on Poaceae (Joern 1979; Baur *et al*. 2006); (*iii*) mechanical trait matching, where we considered the positive association between mandibular strength and leaf toughness (Ibanez *et al*. 2013); (*iv*) plant traits that reflect nutritional qualities or chemical defences, which should influence herbivore preference (Bernays *et al*. 1994; Joern & Behmer 1998), and (*v*) chemical trait matching, where a diverse microbiome should help digest plants with high metabolomic richness (Hammer & Bowers 2015). We apply a hypothesis-based framework to quantify the relative contribution of these rules in explaining species interactions and to test their variation along the temperature gradient associated with elevation.

Results

We built hypotheses in the form of quantitative metawebs (i.e. all possible feeding links between co-occurring species determined by specific ecological rules, Fig. 1). We evaluated the explanatory power of ecological rules through correlations with local empirical interactions using binary and quantitative metrics. The explanatory power of most ecological rules

significantly differed from random expectations, but further showed contrasted levels of explanatory power among ecological rules (Fig. 2a, 2c; Table 1).

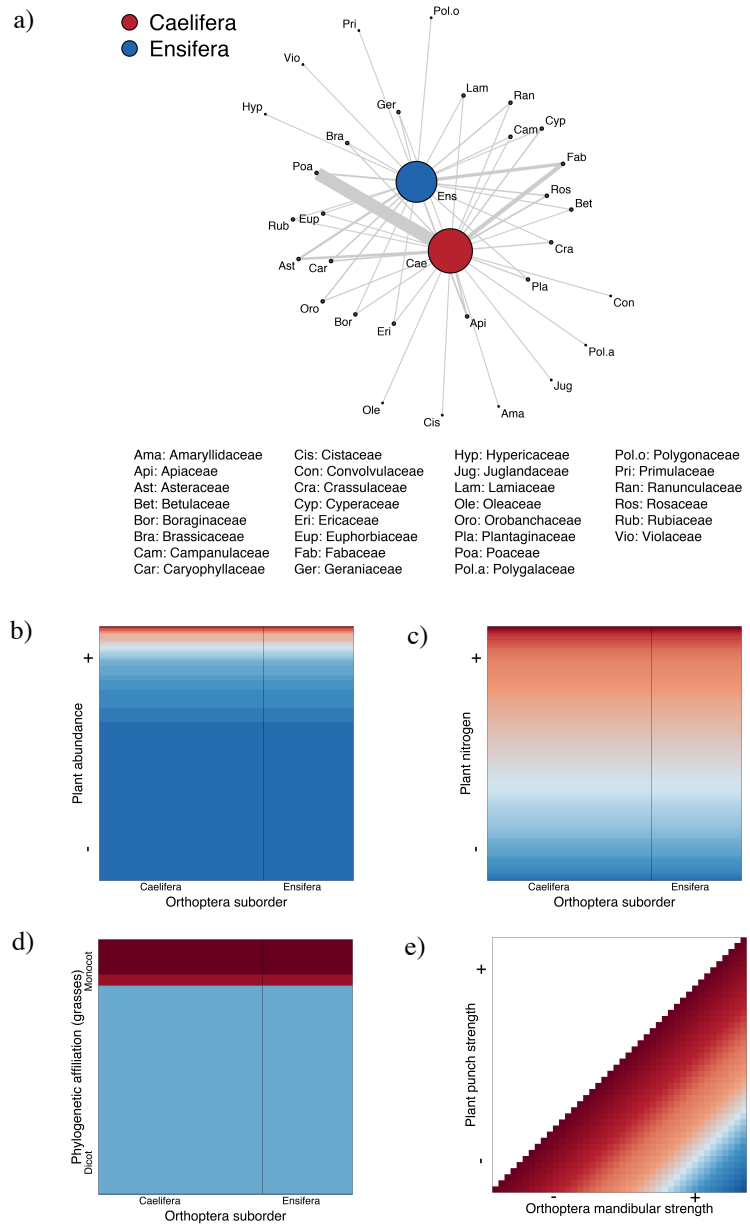

Figure 1 Representation of the metaweb for empirical plant–orthoptera interactions (a) and reconstructed metawebs from interaction rules (b-c). Projection of the hypothesized ecological rules on the metawebs for the plant abundance (b), the nitrogen content (c), the plant phylogenetic distances to define a trophic regime firstly relying on grasses (d), the mechanical trait matching between the insect mandibular strength and plant punch strength (e). The gradient from blue to red indicates the intensity of the interaction, where red indicates the strongest and blue weakest interactions.

We found the highest correlation between local empirical interactions and the phylogenetic rule along the temperature gradient (quantitative value with mean tau: slope=0.343, t-value=21.73, $P \leq 0.001$, Fig. 2a; presence-absence with mean TSS: slope=0.210, t-value=14.21, $P \leq 0.001$, Fig. 2c; Table 1). The empirical networks show high level of modularity (median modularity Q across all networks of 0.58, calculated following Newman 2006) associated with species phylogenetic positions, where Caelifera are more strongly interacting with Poaceae (Fig. 1a). Plant abundance rule showed the second highest correlation, with dominant plant species being more likely to be eaten (mean tau=0.189, $P \leq 0.001$, t-value=11.85, Fig. 2a; mean TSS=0.150, $P \leq 0.001$, t-value=10.16, Fig. 2c; Table 1), which further support that species abundance is a major factor structuring interaction networks (Vázquez *et al*. 2007). Rules based on plant traits further indicate that interactions are determined by SLA (mean tau=0.079, $P \leq 0.001$, t-value=5.02, Fig. 2a; mean TSS=0.115, $P \leq 0.001$, t-value =7.82, Fig. 2c; Table 1) and nitrogen content (mean tau=0.068, $P \leq 0.001$, t-value=4.33, Fig. 2a; mean TSS=0.093, $P \leq 0.001$, t-value=6.31, Fig. 2c; Table 1). Because nitrogen is a major limiting resource for insects, plants with higher nitrogen concentration are usually preferred (Joern & Behmer 1997). While we found a signal of the mechanical trait matching (mean tau=0.077, $P \leq 0.001$, t-value=5.16, Fig. 2a; mean TSS=0.095, $P \leq 0.001$; t-value=6.43, Fig. 2c; Table 1), in agreement with Deraison *et al*. (2015a), leaf toughens in relation to mandibular strength was comparatively weaker than when tested under controlled experimental conditions (Ibanez et al. 2013). The chemical matching rule had no explanatory power (Fig. 2a, 2c, Table 1, see also Fig. S2), which was confirmed by analyzing the co-structuration between the composition of the microbiome and the composition of chemical compounds screened by metabolomics (Mantel test p value > 0.05, see Supplementary methods). This absence of signal can be explained by the generalist feeding behavior of orthoptera potentially diluting plant toxins (Singer *et al*. 2002) or by a poor representation of effective secondary metabolites in the complete metabolome richness measure. Our results are generally consistent with the work of Bernays & Chapman (1970, see also Joern 1979) suggesting that orthoptera are more sensitive to plant nutritive qualities, or leaf physical properties, but less to secondary metabolites.

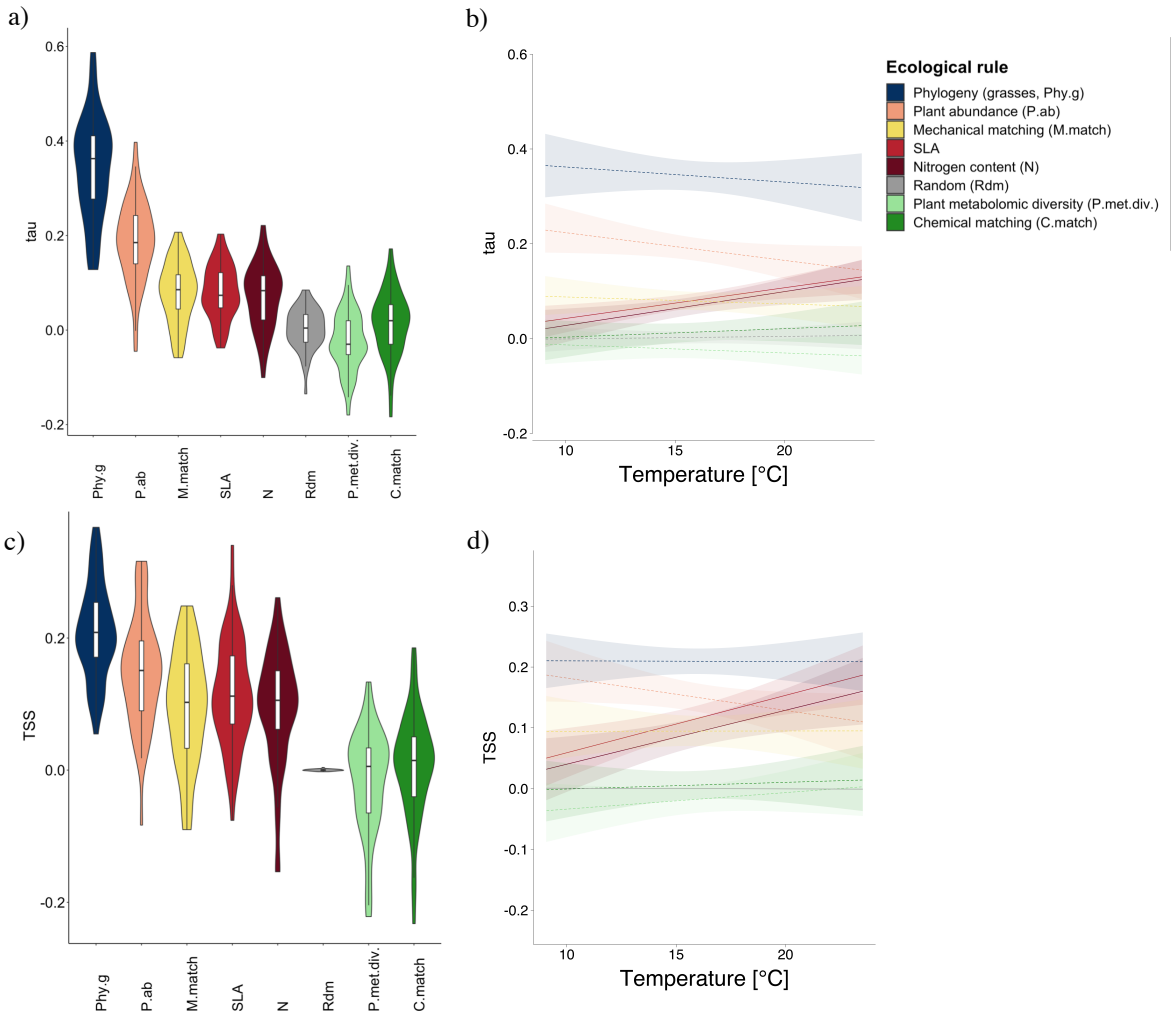

Figure 2 Violin plots and regressions obtained from linear mixed-effect models representing the relationship between the temperature and tau and TSS coefficients calculated for each interaction rules. The mean of the tau (a) and TSS (c) coefficients significantly differ from an interaction rule based on a random distribution of interaction for all variables except for the chemical trait matching and the metabolomic plant richness (Table 1). Interaction rules hypothesized to underpin plant–orthoptera were generally conserved along the temperature as shown by constancy of tau (b) and TSS (d) coefficients along the gradient expect for SLA and nitrogen content that contributed significantly less to species interaction in cold environments (Table 2).

Table 1 Linear mixed-effect models table for evaluating the ability of each hypothesis-based rule to underpinned plant–orthoptera interactions. The model tests the difference between the tau and TSS coefficient mean values for each hypothesis against a random assembly of interaction. Greater values of tau and TSS denote a higher explanatory power of the interaction rule compared to the reference. For each rule tested is given the mean value of the tau and TSS coefficients, the *p* value (*P*) indicating the significance of the difference to the random rule, the degrees of freedom (df), the t-value and the standard error.

Interaction rule	Test coefficient	Mean	*P*	df	t-value	Std. error
Phylogeny	tau	0.343	≤ 0.001	426.9	21.73	0.0154
(grasses)	TSS	0.210	≤ 0.001	432	14.21	0.0149
Plant abundance	tau	0.189	≤ 0.001	426.9	11.85	0.0154
	TSS	0.150	≤ 0.001	432	10.16	0.0149
Mechanical matching	tau	0.077	≤ 0.001	426.9	4.90	0.0154
	TSS	0.095	≤ 0.001	432	6.43	0.0149
SLA	tau	0.079	≤ 0.001	426.9	5.02	0.0154
	TSS	0.115	≤ 0.001	432	7.82	0.0149
Nitrogen content	tau	0.068	≤ 0.001	426.9	4.33	0.0154
	TSS	0.093	≤ 0.001	432	6.31	0.0149
Plant metabolomic	tau	-0.025	0.10	426.9	-1.63	0.0154
richness	TSS	-0.017	0.24	432	-1.17	0.0149
Chemical matching	tau	0.011	0.47	426.9	0.73	0.0154
	TSS	0.006	0.69	432	0.40	0.0149

We evaluated the constancy of the explanatory power of each hypothesis along elevation and showed a change in signal for several hypotheses (Fig. 2b, 2d, Table 2). We related the explanatory power of the ecological rules applied to each network to temperature and tested the significance of the relationship using linear mixed-effect regression models. The hypothesis based on SLA (tau: slope=0.006, $P \leq 0.01$, t-value=3.10, Fig. 2b; TSS: slope=0.009; $P \leq 0.0$, t-value=3.27, Fig. 2d; Table 2) and nitrogen content (tau: slope=0.007, $P \leq 0.01$, t-value=2.92, Fig. 2b; TSS: slope = 0.009; $P \leq 0.01$, t-value=2.74, Fig. 2d; Table 2) decreased with temperature, indicating that plant-herbivore interactions are weakly determined by SLA and nitrogen content in cold environments. In parallel, we found a decrease in the community mean for SLA and nitrogen values at higher elevation (Pitteloud *et al*. minor revision - chapter 1; Read *et al*. 2014). Plant with higher nutrient content are expected to be preferentially consumed at high elevation, where the season to complete the life cycle is shorter (Hodkinson 2005). However, a decline in the average and variance of nitrogen content (Pitteloud *et al*.

minor revision - chapter 1; Read *et al*. 2014) might decrease the benefit of diet selectivity, favoring diet mixing (Joern & Behmer 1997; Unsicker *et al*. 2008; Franzke *et al*. 2010) and promote higher levels of generalism at high elevation (Pitteloud *et al*. in prep. - chapter 3). Together, our results suggest that plant-herbivore interaction networks might be less determined by species differences in leaf quality in cooler environments which aligns with former studies (Lemoine *et al*. 2013).

Table 2 Linear mixed-effects models table for quantifying the variation of the explanatory power of each hypothesis along the temperature gradient. The relationship between the tau and TSS coefficients and the temperature is described by the intercept and the slope estimate, the *p* value (*P*), the degrees of freedom (df) and the t-value.

Interaction rule	Test coefficient	Slope estimate	Intercept estimate	*P*	df	t-value
Phylogeny	tau	-0.003	0.394	0.46	47	-0.75
(grasses)	TSS	-0.0001	0.211	0.97	47	-0.04
Plant abundance	tau	-0.006	0.282	0.06	42.9	-1.90
	TSS	-0.005	0.235	0.07	42.7	-1.86
Mechanical matching	tau	-0.001	0.103	0.57	43.5	-0.58
	TSS	0.0001	0.094	0.99	43	0.02
SLA	tau	0.006	-0.022	≤ 0.01	47	3.10
	TSS	0.009	-0.035	≤ 0.01	47	3.27
Nitrogen content	tau	0.007	-0.044	≤ 0.01	43.4	2.92
	TSS	0.009	-0.049	≤ 0.01	47	2.74
Random	tau	0.001	-0.006	0.76	47	0.31
	TSS	0	0.002	≤ 0.05	43.8	-2.10
Plant metabolomic	tau	-0.002	0.004	0.47	43	-0.72
richness	TSS	0.003	-0.061	0.35	42.9	0.95
Chemical matching	tau	0.002	-0.015	0.46	42.6	0.74
	TSS	0.001	-0.012	0.71	42.8	0.37

We contrasted the explanatory power of hypotheses across the six transects across the Swiss Alps, to evaluate the differences among regions associated to macroclimate or bedrock types, which influence the community composition (e.g. Michalet *et al*. 2002) and possibly interaction networks (Poisot *et al*. 2020). We found that the ecological rules of plant abundance (tau: F=2.71, $P \leq 0.05$; TSS=3.61; $P \leq 0.05$; Table 3), plant metabolomic richness (tau: F=2.47, $P \leq 0.01$; TSS = 2.77, $P \leq 0.05$; Table 3) and chemical matching (tau: F=3.86, $P \leq 0.05$; TSS=2.91; $P \leq 0.05$; Table 3) had a significantly different explanatory power in the different transects. In particular, the set of networks in the southeastern Alps, located on a siliceous rock type (Fig.

S1), showed a higher influence of plant abundance, but a weaker influence of plant metabolomic richness and chemical matching (Fig. S3). The bedrock may influence the magnitude to which interaction rules are exerted on ecological networks via the leaf properties of the plants (Michalet *et al*. 2002). Moreover, we found that the mechanical trait matching had a lower explanatory power on the transect located in the most arid climate (Fig. S3). Dry condition are typically associated to plant species with high leaf toughness and leaf-dry matter content, which might condition the association between plants and herbivores (Bernays & Chapman 1994). By using empirical data rather than reconstructed webs (Hattab *et al*. 2016; Albouy *et al*. 2019) together with multiples ecological rules, we highlight the biogeographic dimension of the ecological rules underlying species interaction networks.

Table 3 Analysis of variance table computed on the linear models testing the relationships between the transect identity and the tau and TSS coefficients. For each interaction rule tested individually is given the F-ratio (F), the *p* value (*P*), the mean squares (MS) and the degrees of freedom (df). The hypotheses of plant abundance, plant metabolomic richness and chemical matching explain the plant–herbivores interaction with a significant variation among transect.

Interaction rule	Test coefficient	F	*P*	MS	df
Phylogeny	tau	0.73	0.61	0.01	5
(grasses)	TSS	0.22	0.95	0.001	5
Plant abundance	tau	2.71	≤ 0.05	0.02	5
	TSS	3.61	≤ 0.05	0.02	5
Mechanical matching	tau	1.68	0.16	0.01	5
	TSS	2.36	0.06	0.01	5
SLA	tau	0.67	0.65	0.00	5
	TSS	0.34	0.88	0.002	5
Nitrogen content	tau	1.37	0.25	0.01	5
	TSS	0.28	0.92	0.002	5
Random	tau	0.96	0.45	0.00	5
	TSS	1.44	0.23	0.00	5
Plant metabolomic	tau	2.47	≤ 0.01	0.01	5
richness	TSS	2.77	≤ 0.05	0.01	5
Chemical matching	tau	3.86	≤ 0.05	0.01	5
	TSS	2.91	≤ 0.05	0.02	5

Discussion

We here propose a general framework to identify the ecological rules underlying interaction networks and to compare these rules along environmental gradients and across multiple geographical regions. We illustrate this framework using a data set of plant–herbivore interaction networks obtained with DNA metabarcoding. We found that the phylogenetic relationship of plants and orthoptera, and plant abundance showed the highest explanatory power in the structuring of the plant–herbivores networks, while trait-based rules related to mechanical constraints and nutritive requirements showed weaker explanatory power (Fig. 2a, 2c, Table 1). Our findings suggest that the phylogeny, abundance, functional traits all partly contribute to explain species interaction in networks (Vázquez *et al*. 2007; Bersier & Kehrli 2008; Dormann *et al*. 2017; Laigle *et al*. 2018). The strong phylogenetic signal found in our plant-herbivore interactions system relates with the findings of Brousseau *et al*. (2018), in which they showed that the ability of a trait-based model to predict interactions in a prey predator-system increases when the phylogenetic information is included. Our study agrees with a generally strong phylogenetic signal in antagonistic systems (Rohr & Bascompte 2014). Plant abundance was the second best predictor for describing plant–herbivore interactions across all networks (Fig. 2a, 2c, Table 1). Interactions intensity should increase with the probability of encounter, which should relate to plant species cover or herbivore abundances (Tylianakis & Morris 2017). Species abundance was evidenced by other studies to be major determinant of interaction in networks (Vázquez *et al*. 2007; Canard *et al*. 2014; Sam *et al*. 2017) making the signal of other facets of ecological interactions better detectable after removing its effect (Dormann *et al*. 2017), notably of the functional traits (Laigle *et al*. 2018). Functional traits are expected to predict both antagonistic (Gravel *et al*. 2013; Ibanez *et al*. 2013) and mutualistic relationships (Dehling *et al*. 2014), but these were less important than phylogeny and abundance than previously assessed for plant–orthoptera networks (Ibanez *et al*. 2013). This might be due to the fact that functional trait values in the community are the reflection of phylogenetic inertia (Rohr & Bascompte 2014), as well as to the abundance of individuals (Carnicer *et al*. 2009). Therefore, both phylogeny and species abundance values might overshow a direct link between plant and herbivore functional traits during network analyses. Our study illustrates the challenge of disentangling the role of multiple mechanisms to explain the interaction networks in nature but also provide a methodological way forward.

We showed that the rules underlying plant–herbivores interaction networks are not systematically conserved along elevation and across biogeographical regions. Studies have investigated how structural properties of ecological networks change along environmental gradients (Ramos-Jiliberto *et al*. 2010; Miller-Struttmann & Galen 2014; Maunsell *et al*. 2015; Morris *et al*. 2015), but generally did not consider the potential variation of ecological rules with abiotic conditions. Measure of network variation along environmental for instance show that the specialization of plant-pollinator networks decreases with latitudes (Schleuning *et al*. 2012) or elevation (Pellissier *et al*. 2012; Miller-Struttmann & Galen 2014; Refsnider *et al*. 2019), a pattern that was directly linked to animal feeding traits or floral morphology. In parallel, studies have investigated the mechanisms ruling species interaction, including for instance phylogeny, abundance and functional traits (Dormann *et al*. 2017), but did not in regard to the influence of abiotic parameters on network structuration. Our study indicates that ecological rules determining interaction networks can vary along environmental gradients and across biogeographic regions. A recent study using trait-matching constraints indicates that ecological networks might display geographic patterns alike at a global scale (Albouy *et al*. 2019), however, in this case, ecological networks were inferred rather than homogenously measured in the field. That said, our and previous work, indicate that ecological rules determining species interactions are not constant across the landscape, and should be studied in the context of the surrounding environmental conditions.

The identification of the mechanisms that determine species interactions serves two important purposes. First, it allows quantifying structural properties of ecological networks, which in turn can inform on their changes and resilience when faced with environmental disturbances, such as species extinction (Dunne *et al*. 2002). Second, knowledge of ecological rules enables formulating predictions on the properties of future ecological networks, between species that have not been so far observed as interacting (Hattab *et al*. 2016; Albouy *et al*. 2019). So far, most ecological network studies have not considered the possibility that ecological rules may not be constant along environmental gradients or across biogeographic regions (Baiser *et al*. 2019). Ignoring this spatial and temporal variation in predictive modelling could lead to biases in network inferences under environmental changes. Our results demonstrate the standing variability of the explanatory power of network ecological rules through space. Our effort opens the way to the development of an emerging research field of

'spatial network ecology' aiming to understand the nature of the processes shaping the structure of biological communities through in space and time.

Materials and methods

Study design, vegetation surveys and temperature data collection

We selected six elevation gradients that are representative of the diversity of macro-climatic conditions and bedrocks in the Central Alps (Fig. S1). Each transect consists in eight study sites ranging from low to high elevation (minimum 578 m; maximum 2,417 m) separated by an average elevation distance of 240m. Low to medium elevation sites correspond to the dry meadow (mostly Mesobromion or Stipopoion grasslands, Delarze *et al*. 2015) with low impact from agricultural practices in land-use and pasture while high elevation sites are typical alpine meadows with no mowing and occasional grazing. We conducted insect and vegetation inventories during summer 2016 and 2017, gradually surveying from low to high elevation following the peak in species richness of each community throughout the season, within a plot of 10m x 10m that covers the plant–insect interactions found at each site. Plant species identification are based on Swiss Floras (Lauber *et al*. 2012; Eggenberg & Möhl 2013) and vegetation cover estimated using a 9 levels scale (<0.25, 0.25-0.5, 0.5-1, 1–5, 5–15, 15–25, 25–50, 50–75 and >75%). The vegetation surveys were first performed in a circular subplot of 9 m^2 located within the 100m^2 plot where the vegetation was floristically the most homogenous. We further extend the surveys to the 100m^2 plot where we searched for rare species and refined abundance estimations. We used the median values of the vegetation cover categories in downstream statistical analyses. Vegetation surveys resulted in the identification of 492 plant species, representing 264 genera and 62 families. To represent abiotic variation along elevation, we collected the temperature for four sites per transect using temperature loggers (DS1921G-F5 HomeChip, DS1921G-F5 HomeChip, Newton Longville, England, Fig. S4) The data loggers were protected by silicone capsules, wrapped in parafilm and buried at 4cm deep in the ground at the center of the study plot. Loggers recorded data every 240 minutes at a resolution of 0.5°C for one year. Temperatures for unsampled sites were inferred from a linear regression model applied on each transect individually, using the relationship between the elevation and the summer mean temperature from May, 1 2018 to September 29, 2018.

Reconstruction of trophic networks using DNA metabarcoding

The reconstruction of 48 plant–orthoptera bipartite networks was performed through DNA metabarcoding applied on 403 insect feces samples. We conducted field surveys within the 100m² plot during weather conditions maximizing insect activity. Species were identifying through visual identification (Baur *et al*. 2006) and feces were sampled for 10 specimens per species on average, ranging from 1 to 40 individuals depending on the insect abundance. Insect were released after 2h fecal excretion and the collected feces were kept at 4°C for a maximal of eight hours before storage at -20°C. After this phase of sampling, we performed the DNA extraction by using FastDNA™ SPIN Kit for Soil (MP Biomedicals, Santa Ana, USA). The DNA metabarcoding procedure consists in two consecutive PCR steps along which samples are individually tagged by double-indexing, i.e., the amplicon PCR and the indexing PCR. We choose the ITS2 plant genetic barcode relying on recent works concluding that this marker is the most competent to identify Streptophyta taxa to the species level (Moorhouse-Gann *et al*. 2018). Following library preparation, samples were sequenced on the MiSeq v3 2X300 Illumina platform. The DNA extraction, library preparation procedures and processing of sequenced libraries are further detailed in Supplementary Methods. We generated an OTUs table of plant sequences by completing the following key steps: read trimming (https://github.com/lh3/seqtk), paired-ends merging, (Magoc & Salzberg 2011), primer removal (Martin 2011), quality filtering (Schmieder & Edwards 2011), size selection and ZOTU calling (Edgar 2016b). The taxonomical assignment of plant sequences was done with the SINTAX classifier (Edgar 2016a) against a DNA barcode reference database that we established by fetching sequences from Genbank (Clark *et al*. 2016) and producing custom sequences of ITS2. The reference DNA barcoding reference database use spans the plant species richness observed on the field for 95.2% of the families, 92.2% of the genera and 88.5% of the species with 50% of the missing species having their genus represented in the database. Based on assignment probabilities provided for each taxonomic level, we retained affiliations above a threshold of 0.95. From the OTU table (1774 plant entries), we removed the taxa identified above the family level (176) and those that were not monophyletic (105), sum the count of the taxa sharing their affiliation, filtered the taxa count by site-specific species composition as low count may refer to both sequencing artefacts or low amount of plant intake. The streaming of the OTUs table resulted in 459 unique plant taxa to what was added 138 taxa that were never consumed by the insects. The list of plant taxa included 495 species and 99 genera and 6 families that could not be further identified on the field. To account for differences

in sequencing depth, we computed the sample relative read abundance (RRA) used in downstream analyses as quantitative interaction to reflect relative dietary preferences (Deagle *et al*. 2019; Roslin *et al*. 2019). The methodological procedures used for the reconstruction of the DNA barcode reference database, taxonomical assignment and OTU table filtering are detailed in the Supplementary Methods. We recorded in total, 10'615 realized interactions out of 28'064 possible links between 45 species of orthoptera (29 Caelifera, 16 Ensifera) and 597 plant taxa.

Collection of plant and insect functional traits

Orthopteran incisive strength was measured following the procedure described in Ibanez et al. (2013), for 93% of the orthoptera species identified on the field, using six specimens per species that were collected across their respective elevation and geographical ranges. For each specimen, mandibular strength was measured from pictures of mouthpiece taken in duplicate with a high-resolution digital microscope (Leica DVM6, Leica Microsystems, GmbH, Wetzler, Germany) and averaged per species. For microbiome analyses, we collected 368 specimens along one of the transect (i.e. Bex), sampling 2-3 specimens per species and sex at each site to cover 53% of the orthoptera diversity across all transects. Insect were killed by a cold treatment at -20°C, their entire gut was extracted by aseptic dissection and further stored at -20°C. We extracted the microbial DNA using the DNeasy® PowerSoil® HTP 96 Kit (Qiagen, Hilden, Germany). The preparation of the 16s DNA metabarcoding libraries and the sequencing procedure were conducted by AIMethods (Munich, Germany). The pre-processing of the raw sequencing reads of microbiome samples was performed following commonly recommended best practices and resulted in an average sequencing depth of 18,350 reads. Further details from the DNA extraction to the building of the OTUs table are provided in Supplementary Methods. The ZOTUs calling program identified 3128 potential microbial units. While the selected 16s primer pair was shown to be highly specific to bacterial DNA (Klindworth *et al*. 2013), we discarded a 121 OTUs identified as chloroplastic DNA. The read counts were then normalized using RRA (McMurdie & Holmes 2014; Weiss *et al*. 2017). We computed the microbial phylogenetic diversity for each sample as the total phylogenetic branch length with the *picante* R package (Kembel *et al*. 2010) and retrieved the median values per species. We collected plant leaves functional traits that respond to abiotic variations but also relates to nutritional qualities and resource acquisition – i.e. the nitrogen content and the specific leave area (SLA, Pérez-Harguindeguy *et al*. 2013); and to chemical resistance to

herbivory – i.e. plant metabolomic richness. To represent leave mechanical resistance, we measured the force required to pierce lamina leave that translate the leaves' mechanical properties relevant for herbivory (Sanson *et al*. 2001). For each species, we collected on average six replicates that were selected across the species elevational range. Measurements were done on well-developed and healthy leaves. For SLA and punch traits, we sampled individuals in triplicate per collecting sites. To measure the SLA we calculated as the area of a fresh leaf divided by the dry weight following standard procedures (Pérez-Harguindeguy *et al*. 2013). For the punch strength, we used a digital force gauge for the punch (IMADA CO., LTD. Toyohashi, Japan) equipped with a measuring tip that pierce the leave lamina on a circular surface of 2mm diameter and that we positioned outside major leave nerves. From the measured values, we calculated the punch strength in MN/m^2. For a few grasses species that had a leave width smaller that the tip diameter, we measured the leave width using a digital caliper gauge (0.01 mm precision) to estimate the tip contact area and calculated the punch strength over this area. We further used the averaged value of the traits for each species.

To measure plant nitrogen content and metabolomic richness, five healthy and fully expanded leaves from all plant species were desiccated at 40°C for one week. We quantified the nitrogen content by dry combustion (CN elemental analyzer, NC-2500 from CE Instruments, Wigan, Lancashire, United Kingdom). For untargeted metabolomics analyses, we pooled 20 mg of each species across replicates, which were extracted with 0.5 ml of extraction solvent (MeOH: MilliQ water: formic acid; 80:19.5:0.5). After centrifugation, the supernatant was placed in a HPLC vial, and a volume of 2.5 µl of the pure extract was injected into an Acquity UPLC™ C18 column (50 mm × 2.1 mm, 1.7 µm) and analyzed via ultra-high-pressure liquid chromatography—quadrupole time-of-flight mass spectrometry (UHPLC-QTOFMS) using an Acquity UPLC™ coupled to a Synapt G2 MS (Waters, Milford, MA, USA). We used a binary solvent system consisting of H_2O and acetonitrile, both supplemented with 0.05% formic acid. The chromatographic separation was carried out at a flow rate of 0.6 ml/min under a temperature of 40°C using a linear gradient of 2%–100% acetonitrile in 6.0 min. MS detection was done in positive electrospray ionization over a mass range of 85–1,200 Da. The MS source was cleaned before each of the five batches running over five days. Data was acquired in the data-independent acquisition (DIA) mode, in which all precursors ions from the full mass range are fragmented to yield MS/MS spectra. For peak detection and assignment of the parent mass to each of the fragmented spectra of the DIA data, we used MS-DIAL

(Tsugawa *et al*. 2015) The output of MS-DIAL was implemented in the Global Natural Products Social (GNPS) to cluster the MS/MS spectra into compound families based on their cosine similarity and molecular networking (Wang *et al*. 2016). Finally, to estimate the metabolomic richness, we summed the number of chemical families present each plant species or communities. Because MS/MS spectra were clustered by their fragmentation profile, the total number of chemical families reflects the maximal potential functional chemical richness. The data collection of the SLA, N and C traits was completed with published datasets (Kattge *et al*. 2011; Körner *et al*. 2016; Descombes *et al*. 2017). Plant trait measurements of SLA, nitrogen content, punch strength and plant metabolomic richness encompass respectively 79%, 83%, 76%, 77% of the plant species found in the field.

Definition of hypotheses

We defined five ecological rules that are expected to structure ecological networks: (i) plant relative abundance: orthoptera should preferentially feed on species of plant with higher surface cover; (ii) the phylogenetic position of plant taxa: orthoptera are expected to feed preferentially on grasses; (iii) a mechanical trait matching: we expected a stronger interaction intensity for corresponding values of mandibular strength and leaf toughness (iv) leaf nutritional qualities or chemical defences: orthoptera should preferentially feed on species with more nitrogen and lower chemical richness, and (v) chemical trait matching: we expected a stronger interaction intensity for corresponding values of orthopteran microbiome and plant metabolomic richness. For each hypothesis, we constructed a metaweb of expected interactions between all species pairs. Ecological rules are directly based on the ranking of plant cover (Fig. 1b), SLA and nitrogen content (Fig. 1c) and metabolomic richness. For the phylogenetic rule, we hypothesized that insect have lower interaction intensity with plants that are phylogenetically distant from grasses (Fig. 1d). We used a well-resolved phylogeny for European flora (Durka & Michalski 2012) that was pruned to the species found in sites and made ultrametric (function force.ultrametric, R package *ape*, Paradis & Schliep 2019). We collapsed the species tips corresponding to grasses and measured the phylogenetic distance of each plant to the collapsed tip (function cophenetic, package *stats*, R Core Team 2019). For the mechanical matching hypothesis (Fig. 1e), expected interaction intensity was highest when mandibular strength corresponds to punch strength, impossible when above and decreasing when below this correspondence. The matching rule was calculated by scaling the mandibular strength and plant punch values between zero and one, subtracting the mandibular strength

from the plant punch and adding one to all values. Mandibular strength smaller than the punch force were finally set to zero (i.e., the upper half of the matrix, Fig. 1e). We applied a similar approach for the trait matching between the metabolomic richness and the microbiome phylogenetic diversity. We also added a random rule of interaction that corresponds to a randomization of the empirical interaction values applied on each network individually. On overall, ecological rules were weakly correlated with each other indicating a marginal interdependency of the hypotheses defined in our methodological framework (Fig. S5).

Comparison of hypotheses to empirical data

We used both quantitative and presence-absence data to assess the explanatory power of each ecological rule, and to quantify their variation along elevation and across biogeographic regions. For quantitative data, we computed the Kendall rank correlation coefficient tau (function cor.test, package *stats*, R Core Team 2019) between the observed interaction intensity for each network and each ecological rule. For presence-absence data, we computed the True Skills Statistic coefficient (TSS) from the error matrices obtained by comparing empirical and hypothetical networks (function confusionMatrix, R package *caret*, Kuhn *et al*. 2018). To transform quantitative rules into binary, we assigned presences of interaction to the highest value of the rule but limited to the same number of presences as found in empirical networks. Since several ecological rules are categorical rather than quantitative, several correspondences are possible between the rule value and the assignment to presence or absence. In these cases, we applied a randomization of 1000 times. We then reconstructed the trophic networks and computed the TSS coefficients. Next, we compared the explanatory power of the ecological rules by testing the difference between tau and TSS coefficients against the randomly-generate rule values using linear-mixed regression models by including transect identity as a random factor using the *lme4* and *lmerTest* R packages (Bates 2008, Kuznetsova et al. 2017). We quantified the relationship between temperature and the explanatory power of each ecological rule using linear-mixed regression models with the transect identity as a random factor (Bates 2008, Kuznetsova et al. 2017). We finally performed an analysis variance (package *stats*, R Core Team 2019) to ask whether the explanatory power of ecological rule vary across biographic regions.

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Supplementary materials

1. Supplementary methods

1.1 Reconstruction of plant–orthoptera trophic networks using DNA metabarcoding

DNA metabarcoding library preparation

The documentation of plant–insect trophic interaction relies on a DNA metabarcoding procedure that uses the ITS2 plant genetic barcode (Yao *et al*. 2010; Li *et al*. 2011; Pompanon *et al*. 2012; Staats *et al*. 2016; Moorhouse-Gann *et al*. 2018). We use the universal primers pair ITS2-S2F (Chen *et al*. 2010) / ITS4_rev (White *et al*. 1990) that generates a short DNA product (360pb) that can be retrieve in degraded DNA such as feces (García-Robledo *et al*. 2013; Fahner *et al*. 2016). All steps of the wet-lab procedure from the DNA extraction to the amplicon PCR were performed following strict DNA decontamination procedures (incl. the use of a pre-PCR Hood, bleech and UV cleaning of all hand material). DNA extractions were performed using FastDNA™ SPIN Kit for Soil (MP Biomedicals, Santa Ana, USA) without modification of the manufacturer's instructions. Feces samples were grinded with tungsten beads using a TissueLyser (Schieritz & Hauenstein AG, Laufen, Switzerland) at the maximal speed (30 Herz) for a minimum of 2x30 seconds, which was increased until all plant fibers were fully disrupted. DNA was extracted for series of 24 samples, including blank to control for cross-contaminations. One amplicon PCR reaction consists in 13.8µl of molecular-grade water, 2.5µl of PCR Gold Buffer without $MgCl_2$ (10x), 2µl of $MgCL_2$ (25mM), 0.5µl of dNTPs (10mM), 0.2 µl of AmpliTaq Gold DNA Polymerase (5U/µl, ThermoFisher, Waltham, USA), 0.5µl of each ITS2 primer (2.5µM), 5µl of extracted DNA. We designed four pairs of individual tagged amplicon ITS2 primers, with variable linker length, to barcode two batches of samples separately during the amplicon PCR (Table S1) and limit the cost associated with the Illumina Nextera XT Index (Illumina, San Diego, USA) used in the indexing PCR. The PCR was run under the following conditions: initial denaturation step at 95°C for 10min; 35 cycles of denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds, elongation at 72°C for 45 seconds; final elongation step at 72°C for 10min. After verifying the PCR success by electrophoresis, PCR products were cleaned with purification beads (AMPure XP, Beckman coulter, Switzerland) with a ratio of 0.8x and eluted in 22µl of 10mM Tris-HCl pH 8.5. Difficult samples were successfully amplified through dilution of the DNA extraction products

(up to 1:100) and 40 cycles. Indexing PCR were conducted using Illumina Nextera XT Index kit v2; the reaction mix included 10µl of KAPA Hifi HotStart ReadyMix (Roche, Basel, Switzerland), 4µl of H20, 2µl of each Nextera XT Index adapter and 2µl of purified amplicon PCR product to reach 20µl. After cleaning of the indexing products using purification beads (ratio 0.8x), libraries were pooled in equimolar ratio and sequenced in one run using the MiSeq v3 2X300 PE protocol.

Reference database

The reference database was compiled by retrieving sequences from Genbank (Clark *et al*. 2016). The database was complemented using custom sequences for 54% of the plant species. Plant were collected during the field season of 2017, DNA was extracted using Sbeadex mini plant kit protocol (LGC Genomics, Berlin, Germany) with the DNA-extraction KingFisher 96 instrument (Thermo Fisher Scientific, Waltham, USA). PCR were generated using the same conditions than for the DNA metabarcoding amplicon PCR but for 40 cycles, further sequenced with Sanger, trimmed and paired-end merged using Geneious (Kearse *et al*. 2012).

Processing of sequencing data of the DNA metabarcoding libraries

Raw sequencing data of the DNA metabarcoding libraries were processed through the following steps: quality control of the sequencing run (Andrews S 2010), trimming of the raw reads (https://github.com/lh3/seqtk), paired-end merging (Magoc & Salzberg 2011), removal of the primers (Martin 2011), quality filtering (Schmieder & Edwards 2011), size selection and deduplication of the reads with custom scripts, ZOTU calling using UNOISE (Edgar 2016b) with a 97% identity threshold, taxonomical assignment with the SINTAX classifier (Edgar 2016a) using the reference database.

Taxonomical assignment

For OTUs that were assigned below a threshold of a taxonomic assignment probability of 0.95, we consider the next highest taxonomic level. After merging OTUs count belonging to the same taxonomic affiliation, only OTUs count of plant taxa that were observed on the study site were retained. While OTUs assigned above the family level were discarded, OTUs count of family and genus were equally distributed to the lower taxonomic level identified on the site (i.e. genus or species). We finally computed samples relative read abundance (RRA)

that were used in subsequent analyses as interaction intensities (Deagle *et al*. 2019; Roslin *et al*. 2019).

1.2 Microbiome data generation and processing

After bacterial DNA extraction of orthoptera gut samples using DNeasy® PowerSoil® HTP 96 Kit (Qiagen, Hilden, Germany), the genetic marker was amplified using the 16s primer pair 341f / 785r that generate a fragment of 444 bp (Klindworth *et al*. 2013). After a purification step with ethanol DNA precipitation, the PCR products were used in a ligation PCR using Nextera Illumina i5/i7 indices. Libraries were pooled in equimolar ratio and sequenced on the MiSeq Illumina platform. Using custom R scripts, we conducted the quality control of the sequencing run (Andrews S 2010), the trimming (https://github.com/lh3/seqtk), paired-end merging of the raw sequencing reads (Magoc & Salzberg 2011), the removal of the primers (Martin 2011), the quality filtering (Schmieder & Edwards 2011), the size selection and deduplication of the reads. The ZOTU calling was performed using UNOISE (Edgar 2016b) with an identity threshold of 100% and the taxonomical assignment using the SINTAX classifier (Edgar 2016a) and the Silva database (Quast *et al*. 2013). We excluded from the analyses 12 samples that were under-sampled with a sequencing depth lower than 1000 to avoid an under-representation of microbial richness (Clooney *et al*. 2016). Screening of the OTU table and normalization of the sample count using RRA were achieved in *phyloseq* R package (McMurdie & Holmes 2013).

1.3 Microbiome and metabolome co-structuration

In complement to the richness analyses of the insect microbiome and plant metabolomics, we performed analysis evaluating the correlation between compositional differences related to these traits. We calculated for each orthoptera species, the median of the OTUs counts found across samples to obtain the microbiome matrix. Then, we weighted the matrix of the chemical families obtained through metabolomics by the intensities of pairwise interaction for each orthoptera. The sum within each chemical family was computed to obtain a unique vector per orthoptera species. Vectors were collated to generate the metabolomic composition matrix. We further calculated the Bray-Curtis dissimilarity matrix for both microbiome and metabolome matrices. The similarity between distance matrices was not significant (Mantel test p value > 0.05).

2. Supplementary figures

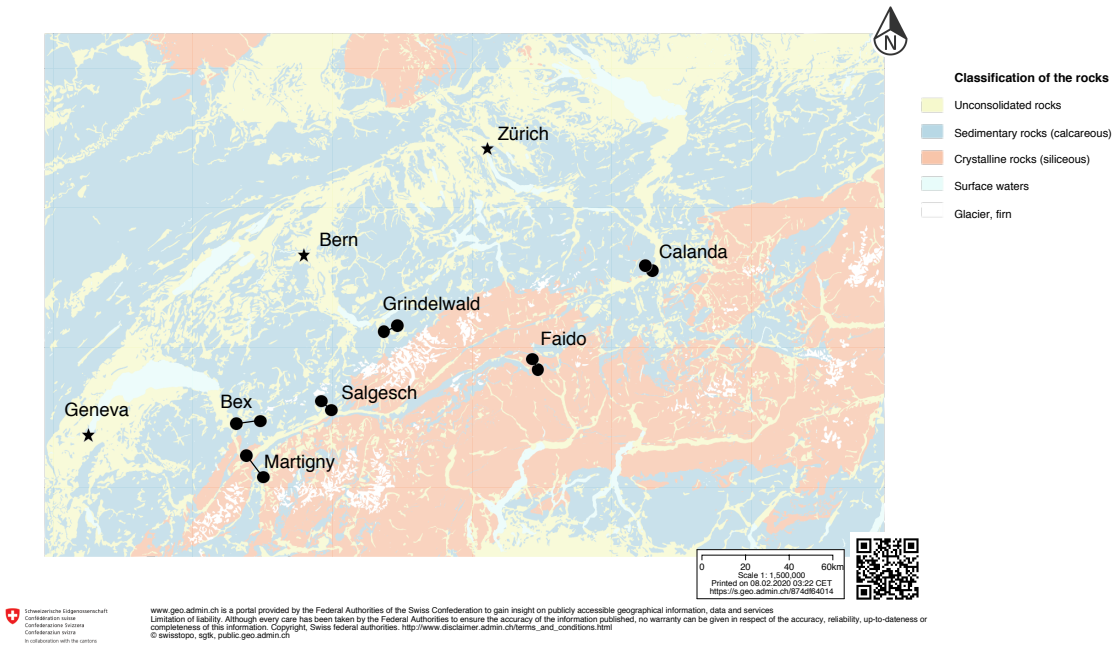


Figure S1 Location of the elevational transects across the Swiss Alps. Position of the lowest and highest elevation site of each transect are displayed. The color legend refers to type of rock substrates.

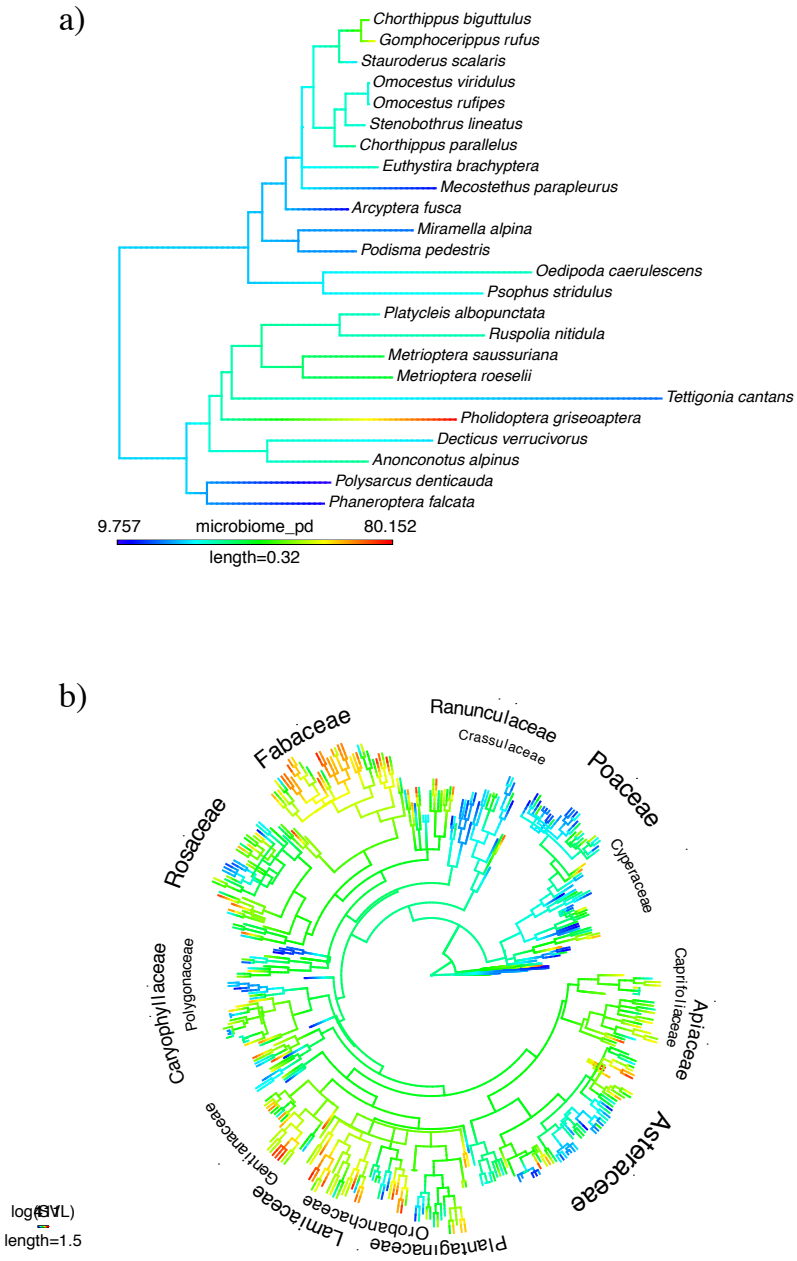
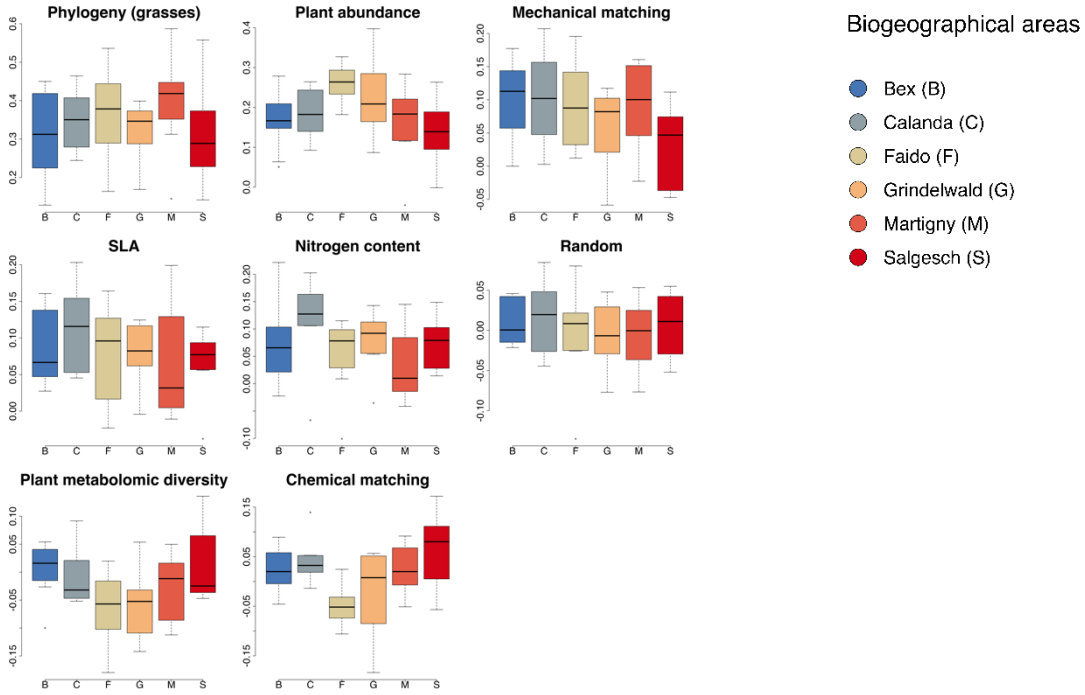


Figure S2 Distribution of the microbiome and metabolomics richness through orthopteran (a) and plant (b) phylogenies. The gradient from blue to red indicates the microbiome and metabolomics richness, where blue corresponds to low richness and red to high richness.

a)



b)

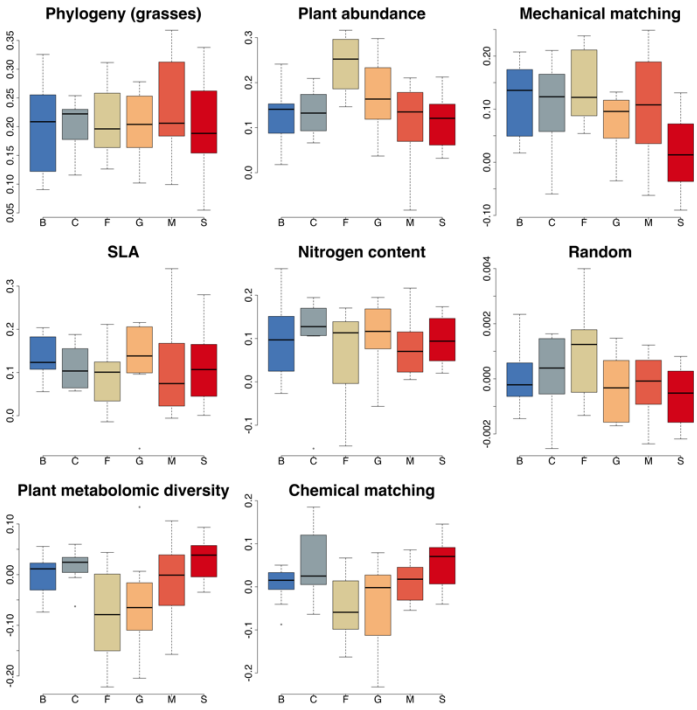


Figure S3 Boxplots of the tau (a) and TSS (b) coefficients plotted individually for each bioregions and ecological rule.

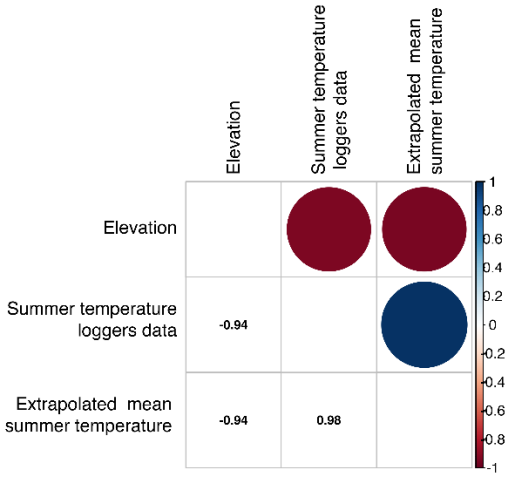


Figure S4 Plot of the correlation matrix between elevation and temperature data. Spearman correlation is calculated between the elevation, summer temperature data loggers collected on the field and the data extrapolated for the summer period from linear regression models applied on each transect individually.

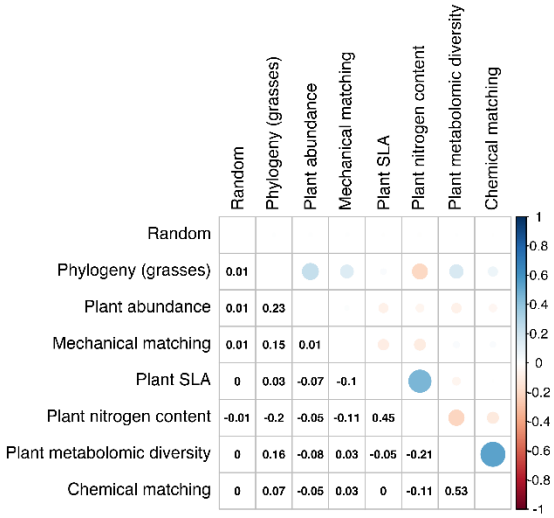


Figure S5 Plot of the correlation matrix between the ecological rules. Spearman correlations between hypotheses are generally weak suggesting a limited interdependency of the ecological rule as defined in our methodological framework

3. Supplementary tables

Table S1 Amplicon primers used in the DNA metabarcoding library preparation

Primer name	Primer direction	Primer sequence
ITS2_S2F	forward	**ATGCGATACTTGGTGTGAAT**
ITS4_rev	reverse	**TCCTCCGCTTATTGATATGC**
ITS2_S2F_B1.1	forward	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**NNACCTGCTTATGCGATACTTGGTGTGAAT**
ITS2_S2F_B1.2	forward	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**NNNACCTGCTTATGCGATACTTGGTGTGAAT**
ITS_4_rev_B1.1	reverse	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**NNAACGACGTTCCTCCGCTTATTGATATGC**
ITS_4_rev_B1.2	reverse	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**NNNAACGACGTTCCTCCGCTTATTGATATGC**
ITS2_S2F_B2.1	forward	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**NNGAAGTTGCATGCGATACTTGGTGTGAAT**
ITS2_S2F_B2.2	forward	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**NNNGAAGTTGCATGCGATACTTGGTGTGAAT**
ITS_4_rev_B2.1	reverse	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**NNTGGAGGCCTCCTCCGCTTATTGATATGC**
ITS_4_rev_B2.2	reverse	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**NNNTGGAGGCCTCCTCCGCTTATTGATATGC**

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CONCLUSIONS & PERSPECTIVES

The primary objective of this dissertation was to investigate how plant and herbivore species assemblages change along elevation in grassland systems of the Swiss Alps. Through the study of plant and herbivores communities and their interactions, we show that: (i) different ecological rules contribute to explain the presence and strength of interaction links between plant and herbivores; (ii) the structure of plant–orthoptera networks varies along the elevation gradients; (iii) the explanatory power of the ecological rules supporting species interactions vary along elevation and across biogeographic regions; (iv) the responses of herbivore species assemblages to elevation differ between above and belowground organisms.

The ecological rules of plant–orthoptera networks

By means of the DNA metabarcoding method applied to insect feces, we show that plant–orthoptera networks are non-random and structured by multiple ecological rules of interaction (chapter 3, chapter 4). In particular, the reconstructed networks had a modular structure suggesting that clusters of species interact more often with each other possibly under the constraint of ecological rules (Rohr & Bascompte 2014; Dormann *et al*. 2017). The non-random structure of networks has been associated to several factors including phylogenetic association between species, functional traits and the abundance of species (Vázquez *et al*. 2007; Eklöf & Stouffer 2016; Laigle *et al*. 2018). Individually, each of these factors has been recognized to shape the organization of networks and those were especially studied in mutualistic plant–bird networks (Carnicer *et al*. 2009; Maglianesi *et al*. 2014; Maruyama *et al*. 2014; Rohr & Bascompte 2014), but also in other taxa (Gravel *et al*. 2013; González-Castro *et al*. 2015; Spaniol *et al*. 2019). Because the structure of ecological networks is expected to be associated with multiple factors, we proposed to investigate them together by collecting a large amount of functional trait data in addition to the species interaction networks.

We developed an analytical framework to test a set of *a priori* ecological rules that are based upon plant phylogeny, abundance and functional traits, integrated or not into a matching constraint (chapter 4). Generally, our analyses reveal that plant–orthoptera interactions are jointly ruled by phylogeny, abundance, biomechanical and nutrient constraints, while we found no evidence of the role of chemical matching or plant metabolomic richness. We found that the ecological rules that rely on plant phylogeny and abundance showed the highest explanatory power of plant–herbivore interactions. Since Caelifera are generally more frequent within networks compared to Ensifera, this signal is essentially supported by the strong

relationship observed between Caelifera and grasses (Joern 1979). The general preference of Caelifera for grasses may be explained by the digestibility of food plant with a lower number of secondary metabolites compared to dicotyledons according to former studies (Bernays & Chapman 1994). Accordingly, we found that keystone species in chapter 3 were frequently among the Poaceae family. However, we further did not find conclusive evidence on the role of plant chemistry in structuring plant–orthoptera networks (chapter 4). The second factor best explaining interactions between plants and herbivores was the plant abundance, which corresponds to the mechanisms generally proposed to explain high level of trophic generalism (Joern 1979; Cates 1980). Since grasses show a higher cover than forbs in the surveyed plant communities, phylogenetic affiliation might be further associated with plant abundance to explain the modular structure of plant–orthoptera networks. In agreement, modularity in ecological networks was found to be driven, at least partially, by phylogeny in other systems (Olesen *et al*. 2007; Donatti *et al*. 2011; Végvári 2019). Our hypothesis-based framework testing for the signal of phylogeny relies on a coarse grouping of plant, which could be refined in the future by implementing specific phylogenetic hypotheses and methods (Balbuena *et al*. 2013). Nevertheless, our approach allowed demonstrating that a phylogenetic rule only based on grasses consumption is explaining a significant fraction of orthopteran interactions with plants.

Functional trait constraints were shown to be associated to species interactions in both antagonistic and mutualist networks (Dormann *et al*. 2017; Laigle *et al*. 2018) and our analyses supported the role of both insect and plant traits in determining species interactions (chapter 4). In the analyses of the chapter 4, hypotheses based on functional traits indicate that the interactions between plants and herbivores are associated to mechanical trait matching and plant traits related to nutritional value. While the importance of biomechanical constraints and plant nutritive quality were proposed as drivers of plant–orthoptera interactions through experimental testing (Joern & Behmer 1997; Ibanez *et al*. 2013), their relative contribution to structuring networks have not been investigated so far, and particularly not for species assemblages in natural conditions. We found a signal of trait matching, but weaker than in a cafeteria experiments (Ibanez *et al*. 2013), which suggests that the species interactions in natural systems are associated to a more complex set of factors that might attenuate the individual signals. The plant chemical richness and the chemical trait matching were weakly associated to species interactions. This absence of signal can be caused by the ability of

orthoptera to dilute specific plant toxins found in any of the plant lineages ingested through a broader diet (Bernays & Chapman 1994; Singer *et al*. 2002). Moreover, the metabolomics diversity measure used in the hypothesis could be a poor proxy of the secondary metabolites in each plant lineage, because the metabolomes contains molecules associated to other functions not related to plant defenses (Schauer & Fernie 2006). Specifically, extracting the secondary metabolites from the metabolomic profiles could allow to test more directly the relationship between chemical defense and herbivory but the analytical tools to identify the function of individual plant chemical molecules need further development to be readily applicable (Maag *et al*. 2015). Similarly, some gut bacteria are most likely involved in the digestive processes of plant secondary metabolites (Hammer & Bowers 2015; Smith *et al*. 2017), which add noises to the use of microbiome diversity as a measure of digestion capacity. Overall, our work reveals that plant–orthoptera interactions are determined by multiple factors, but are mostly ruled by phylogeny, abundance and traits related to nutritional values and mechanical matching.

Investigating the diversity of assembly rules associated to ecological networks allows ecologists to appreciate the complex mechanisms underlying species interactions (Bascompte 2010; Dormann *et al*. 2017). Although these topics are central to network research, they do not account for the abiotic factors that might alter network structuration through space. These questions can typically be addressed using steep environmental gradients (Tylianakis *et al*. 2007; Welti & Joern 2015; Pellissier *et al*. 2018) and the chapter 3 and chapter 4 of this thesis were intended to answer them.

The structure of plant–orthoptera networks along elevation

Ecological networks are expected to show variability in their structure along environmental gradients (Welti & Joern 2015; Tylianakis & Morris 2017; Pellissier *et al*. 2018) and we found such variations in the plant–herbivore networks along elevation (chapter 3). Changes in species composition, abundance, functional traits and coevolutionary history are all expected to contribute to the wiring of species interactions (Tylianakis & Morris 2017). The marked species turnover in assemblages along environmental gradients evidenced by former studies (Gaston 2000) and also revealed in chapter 1 could provide the substrate for differences in network structure along elevation (Tylianakis & Morris 2017; Pellissier *et al*. 2018).

Moreover, shifts in species functional characteristics along environmental gradients have been documented for both animal and plant with modifications of chemical and mechanical species properties (Lenfant 1973; Callis-Duehl *et al*. 2017; Descombes *et al*. 2017; Wong *et al*. 2019). In chapter 1, we found that the functional traits of plant and orthoptera involved in herbivory relationships vary with elevation, which might in turn shape different interaction rules in high elevation compared to low elevation grasslands. Consequently, we predicted biotic and abiotic variation to translate into shifts of network structure along elevation.

As a result of a shift in climate regime, species taxonomic and functional composition, plant–orthoptera trophic networks are expected to display significant structural changes along the elevation gradient. In chapter 3, we showed that increased elevation is associated with more generalist networks, which were more resilient to plant species extinction. We proposed several hypotheses to explain higher generalism at high elevation. First, the reduced environmental predictability and harsh abiotic conditions of high elevation might result in a decrease in network specialization (Macarthur & Levins 1967; Rasmann *et al*. 2014a). At higher elevation, climate is more variable with unpredictable freezing events, as well as the duration of the growing season that can strongly vary from year to year (Körner 2003). These factors might affect the behavioural and physiological traits of insect such as search efficiency and metabolomic rates (Hodkinson 2005; Wong *et al*. 2019). In support of this hypothesis, Lemoine *et al*. (2013) proposed that temperature can influence the diet breadth in generalist insect herbivores by modulating the insect ability to handle plant chemical content. Second, functional traits involved in species interactions might change along elevation and thus rewire ecological networks into differently organized structure (Tylianakis & Morris 2017; Pellissier *et al*. 2018). In particular, if insects did not coevolve with plants by adapting their mandibular strength to the increase of leave toughness, a weaker mechanical match could be compensated by higher generalism for instance by feeding on forbs (Wende *et al*. 2017). However, in chapter 1, we found a change in the mandibular trait with elevation, which suggest an adaptation to tougher leaves in alpine grasslands. Moreover, the results of chapter 4 indicate that the trait matching rule explains similarly low and high elevation networks. Our third hypothesis involved a decrease in plant chemical defense in alpine floras (Callis-Duehl *et al*. 2017; Moreira *et al*. 2018), which might promote a generalist feeding habits (Pellissier *et al*. 2012; Rasmann *et al*. 2014a). However, in chapter 4 we did not find evidence that plant chemistry is involved in plant–orthoptera interactions when using the complete metabolome diversity as an

ecological rule. Shift in the average and variance of plant nutritive content (Pitteloud *et al*. minor revision - chapter 1; Read *et al*. 2014) might also modulate insect dietary choices along elevation which might translate into less organized plant–orthoptera networks. Together, we propose that a combination of climate together with change in species morphological and physiological traits is altering the way herbivore select their food plants toward greater foraging opportunism.

Elevation is also expected to alter the resilience of ecological networks to species extinctions by influencing the organization of interactions and how links are oriented toward a few core species (Dunne *et al*. 2002; Memmott *et al*. 2004; Tylianakis & Morris 2017). In chapter 3, we found a change in network organization along elevation, which was associated with a decrease in robustness compared to expectations from null models. In addition, we showed that high elevation networks include less plant species with higher keystone scores. These findings suggest that in colder environments, plant species that are eaten by herbivores are more widely distributed across the plant communities, which may result in a decrease in network specialization (Lafferty & Kuris 2009; Tylianakis & Morris 2017; Welti *et al*. 2017). Since May's foundational paper (1973), there is an ongoing debate over whether organization level in ecological networks is a central component of stability (Thebault & Fontaine 2010; Jacquet *et al*. 2016). While we found empirical evidence that less specialized networks of high elevation are also more resilient to species extinction, experimental testing is required to conclusively inform on whether a relationship between network complexity and stability exists. Our conclusions on the structural variation of plant-orthoptera networks along elevation are consistent with former studies that evidenced network structural variation along environmental gradients (Welti & Joern 2015; Tylianakis & Morris 2017). By evidencing that the structure of plant–herbivores network responds to abiotic variations, our work offers fundamental knowledge to increase our understanding of the network functioning, in particular for grasslands systems, under abiotic changes.

The spatial variation of the ecological rules governing species interaction

The study of how environmental conditions influence the structure of ecological networks through space should be coupled to the identification of the ecological rules supporting species interactions (Baiser *et al*. 2019). Changes in abiotic conditions are associated to shifts in species distribution, abundance and functional traits (Pitteloud *et al*.

minor revision - chapter 1, Gaston 2000; McGill *et al*. 2006), which can in turn alter the potential for interaction, the structure of networks (chapter 3, Tylianakis & Morris 2017; Pellissier *et al*. 2018) and the underlying ecological rules (Baiser *et al*. 2019). We measured the variation in the explanatory power of the hypothetical plant–orthoptera ecological rules along elevation and across biogeographic regions (chapter 4). Our results indicate that the ecological rules of species interaction can vary along elevation gradients and across biogeographic regions. In particular, we found that the rule based on plant nutritional value is associated to species interactions in warm environments but less so at high elevation. Plant nutritive qualities, and in particular the nitrogen found in amino acids (Chen 1966), play a major role in defining the selection of host plant by insect herbivores as nutrient intake is a most limiting factor for growth (Bernays & Chapman 1994). Therefore, if climate influences plant nitrogen and its use by insects, herbivore interactions with plant species might vary under shifting abiotic conditions (Lemoine *et al*. 2013). Consequently, the strength of the ecological rules relying on this trait to structure interaction networks might not be constant along environmental clines. In addition, behavioral adaptations (e.g. diet mixing, Franzke *et al*. 2010) were also shown to help coping with variation in nutritive content among plant lineages (Yang & Joern 1994) and with the negative effect of temperature on search time and digestion efficiency (Logan *et al*. 2002), typically lower at high elevation (Hodkinson 2005). Change in ecological rules might also be associated with the level of generalism documented at high elevation found in chapter 3. We found a marginal signal that plant abundance rule is more associated network interactions at higher elevation, which supports this hypothesis. Overall, these analyses suggest that elevation is associated with a change in the ecological rules underlying the structure of plant–orthoptera networks by influencing the abiotic and biotic determinants of species interaction.

Together with a gradual abiotic shift along environmental gradients, geography can be associated to varying strength of ecological rules through changes of abiotic and biotic conditions across biogeographic regions (Baiser *et al*. 2019). Testing this hypothesis on plant–orthoptera trophic networks, we found that the explanatory power of ecological rules changes according to the biogeographical area. In particular, the explanatory power of the ecological rules differ between transects located on siliceous rocks or in extremely dry environments. Collectively, the results of chapter 4 suggest that bedrock and regional climate, together with the elevation, can condition the performance of interaction rules to determine plant–orthoptera

interactions. The few studies that rely on ecological rules to study networks at larger spatial scale mostly infer network structure rather than use direct observations of interactions (Hattab *et al*. 2016; Albouy *et al*. 2019). Understanding how species are interacting with each other is crucial to anticipate the consequences of climate change on ecosystem resilience (Harvey *et al*. 2017). Yet, a methodological framework combining empirical data and multiple assembly rules is required to understand the structure of ecological networks under shifting abiotic conditions and refine prediction of future interactions accordingly. With the hypothesis-based framework develop here to test the variation of ecological rules across the landscape, we propose a path toward the study of biogeography of species interaction networks.

How elevation may influence plant–nematodes trophic networks

The study of trophic networks along environmental gradients can uncover how abiotic variables influence ecosystem processes operating in different compartments such as above and belowground herbivory. Initially, the research plan aimed to investigate more than one trophic level, and in particular the comparison of both orthoptera– and nematodes– plant networks. However, the achievement of this goal was impeded by major methodological challenges. First, the micron-size of nematodes complicate the use of DNA metabarcoding for building trophic networks. Second, a standard framework for the characterization of functional traits within herbivore nematodes feeders is not yet well established (Bongers & Bongers 1998). Finally, the scale of our study design did not allow measuring root traits for all species and open-access database (Iversen *et al*. 2017) only covers 19% of the surveyed plant diversity, which would have limited the study of underground ecological rules. As a proxy, we used community descriptors to inform on the different response of above and belowground herbivores communities to elevation (chapter 1). In contrast to surface biotas, we found that nematodes richness does not vary along elevation, while their abundance increases. Our result align with the limited number of studies conducted so far on soil nematodes (Kergunteuil *et al*. 2016; van den Hoogen *et al*. 2019). Our results also indicate that soils can buffer climatic conditions suggesting that surface abiotic filtering does not preclude the existence of rich soil biotas at high elevation (Bryant *et al*. 2008; Kergunteuil *et al*. 2016). The edaphic factors affecting belowground communities are mostly soil texture, pH, soil organic carbon (van den Hoogen *et al*. 2019) and nitrogen content (Fierer *et al*. 2009). In regard to ecological interactions, plant root responses to both abiotic (e.g. soil moisture and chemical composition, Russell 1977; Monti & Zatta 2009) and biotic factors (e.g. root exudates involve in defense,

Rovira 1969), were found to influence belowground communities (Rasmann & Agrawal 2008; Liu *et al*. 2010). At high elevation, the presence of a highly diverse and abundant nematodes communities may be facilitated by denser root systems providing more micro-habitats (Kergunteuil *et al*. 2016). In addition, while a decrease of chemical defense in leaves is generally associated to the decline of herbivores abundance (Rasmann *et al*. 2014b; Moreira *et al*. 2018), plant root chemistry may not decrease under increased nematodes feeding pressure at high elevation (Rovira 1969), although this remains to be investigated. Because root traits are involved in species interaction, we also expect elevation to wire plant–nematodes interactions but through edaphic determinants instead of climatic. Therefore, a comparative analysis of above and belowground ecological networks would provide a unified understanding of the changes in multitrophic interactions along environmental gradients.

Perspectives

Evaluating the association between large-scale environmental variations and the structure of natural communities has increasingly gained attention (Tylianakis & Morris 2017), particularly for providing insights in the response of assemblages to global change. This thesis enables a better understanding of the ecological factors associated to species interactions along elevation gradients in mountains. This work allows the identification of the ecological rules structuring plant–orthoptera networks and provides hints on the action of surface abiotic conditions in structuring above and belowground herbivores communities.

Collectively, our findings indicate that the tools required to explore the functioning of natural systems at higher spatial scales and organizational levels are now available. The DNA metabarcoding method presented here is adequate for documenting plant–animal interaction networks at a high resolution. Important effort is now required to collate complete, multitrophic ecological networks and capture the complexity of life across large spatial scales (Dormann *et al*. 2017; Poisot *et al*. 2020). In this vein, O'Connor *et al*. (2019) compiled an literature-based metaweb to assess the variation of trophic diversity through space (see also Braga *et al*. 2019). However, a recent review of available network metadata reveal that the information on species interaction is fragmented over space and bias toward certain types of ecological interactions and taxonomic groups (Poisot *et al*. 2020). Here, we show that the rapid collection of thousands of species interactions using genetic tools can greatly contribute to fill the gap of the currently scattered database of species interactions (Clare 2014; Evans *et al*. 2016; Vacher *et al*. 2016).

In parallel, gaining knowledge on the impact of climatic parameters on ecological networks is necessary to assess the effects of global change on community structure and stability (Tylianakis *et al*. 2008; Gao *et al*. 2016). In particular, the hypothesis-based framework enables investigations of the assembly mechanism that support ecological networks and can be applied to multiple bipartite networks to increase our understanding of networks structuring mechanisms across broad spatial scales.

Before this thesis, the analysis of ecological networks was hampered by the limited availability of joint dataset of ecological networks and species traits (Poisot *et al*. 2020). Progress in the collection of species interaction at large-scale coupled to mechanistic understanding of network structure are expected to fuel a wave a new research in network ecology (Joly *et al*. 2014; Dormann *et al*. 2017). By contributing to understanding network assembly rules and structure, it becomes possible to measure the response of ecosystems to perturbation affecting their stability through space and time (Schleuning *et al*. 2016; Hui & Richardson 2019). The network approach used in this thesis can further provides new perspectives for conservation practices through the identification of keystones species and measures of propagation of extinctions and invasions through ecosystems (Mills *et al*. 1993; Hui & Richardson 2019). Focusing on these aspects of network ecology could help developing appropriate conservation strategies and evaluate ecosystem resilience under broad environmental changes (Harvey *et al*. 2017). In addition, our work has also significant implications for the forecasts of species interactions as not only taxonomic and functional composition can exhibit spatial heterogeneity but also the rules structuring ecological networks. By pursuing these research lines, future research should aim to develop better understanding and predictions of biodiversity patterns under global change to preserve the ecosystem functions (Dubois *et al*. 2019). Through explorations of networks assembly across landscapes, the results of this thesis accentuate the importance of integrating network ecology into the study of diversity patterns to reach a multifaceted comprehension of natural systems under present and future environmental constraints.

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july 2015	Congress of the European Society of Evolutionary Biology, Lausanne, Switzerland (poster)
may 2014	Conference for the Entomological Society of Geneva, Geneva, Switzerland
nov. 2014	Lepidopterologen-Tagung, Berne

PUBLICATIONS

In preparation

Camille Pitteloud, Patrice Descombes, Sara Sànchez-Moreno, Alan Kergunteuil, Sébastien Ibanez, Sergio Rasmann, Loïc Pellissier. *Contrasting the responses of above- and belowground herbivore communities along elevation*. Accepted with minor revision in Oecologia.

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* These authors are considered as co-first authors.

SKILLS

Ecological network analyses – next-generation sequencing methods (DNA metabarcoding, museum genomics) – field inventories (botany, entomology, soil) – functional traits measurement – R programming – statistical analyses – phylogenetic inferences

LANGUAGE

French	native
English	fluent
German	good knowledge

HOBBYS

Reading – kayaking – travelling – daydreaming