Functional Traits 2.0:

The power of the metabolome for ecology

A manuscript in consideration as an expanded editorial review for the special feature "*Functional Traits 2.0: the power of the metabolome for ecology*" in *Journal of Ecology*

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Abstract

1. A major aim of ecology is to upscale attributes of individuals to understand processes at population, community and ecosystem scales. Such attributes are typically described using functional traits, *i.e.* standardised characteristics that impact fitness *via* effects on survival, growth and/or reproduction. However, commonly-used functional traits (e.g. wood density, specific leaf area) are becoming increasingly criticised for not being truly mechanistic and for being questionable predictors of ecological processes.

2. This Special Feature reviews and studies how the metabolome (*i.e.* the thousands of unique metabolites that underpin physiology) can enhance trait-based ecology and our understanding of plant and ecosystem functioning.

3. In our Editorial Review, we explore how the metabolome relates to plant functional traits, with reference to life history trade-offs governing fitness between generations and plasticity shaping fitness within generations. We then identify solutions to challenges of acquiring, interpreting and contextualising metabolome data, and propose a roadmap for integrating the metabolome into ecology.

4. We next summarise the seven studies composing the Special Feature, which use the metabolome to examine mechanisms behind plant community assembly, plant-organismal interactions and effects of plants and soil microorganisms on ecosystem processes.

Synthesis: We demonstrate the potential of the metabolome to improve mechanistic and predictive power in ecology by providing a high-resolution coupling between physiology and fitness. However, applying metabolomics to ecological questions is currently limited by a lack of conceptual, technical and data frameworks, which needs to be overcome to realise the full potential of the metabolome for ecology.

1. Introduction

A central tenet of functional ecology is the understanding that organisms display characteristics that go beyond taxonomy in explaining how they affect, and respond to, their environment (Calow 1987). This understanding has led to the widespread adoption of trait-based approaches to link organismal functioning to ecological processes operating at population, community and ecosystem scales. Trait-based approaches are most famously embodied by the concept of functional traits - a suite of morphological, physiological, phenological or behavioural characteristics of an organism that impact fitness *via* their effects on survival, growth and/or reproduction (Violle *et al.* 2007). Functional traits are notable because they not only contain information about how organisms are adapted to and interact with their environment (Lavorel & Garnier 2002; Violle *et al.* 2007), but also integrate processes across different levels of biological organisation, from organs, to species, to ecosystems (McGill *et al.* 2006; Gross *et al.* 2017). Functional traits have been extensively used to characterise organisms across the tree of life (Wong, Guénard & Lewis 2019; Echeverri *et al.* 2020; Lundgren *et al.* 2021). However, their origin, and most common usage, is with reference to the plant kingdom (Calow 1987). For instance, plant functional traits have been used to explain variation in plant life history trade-offs (Wright *et al.* 2004; Adler *et al.* 2014; Díaz *et al.* 2016), community composition (Bruelheide *et al.* 2018), species interactions (Kunstler *et al.* 2016) and ecosystem processes (De Deyn, Cornelissen & Bardgett 2008; Cornwell *et al.* 2008). Nevertheless, the plant functional traits most commonly used by ecologists (e.g. plant height, seed mass, leaf area, specific leaf area and leaf carbon, nitrogen and phosphorus contents; Díaz *et al.* 2016) often leave a large fraction of variation in ecological processes unexplained (Adler *et al.* 2014; Yang, Cao & Swenson 2018), limiting the possibility to provide mechanistic insights into plant effects on ecosystem processes (Walker *et al.* 2019; Firn *et al.* 2019; Laughlin *et al.* 2020; van der Plas *et al.* 2020).

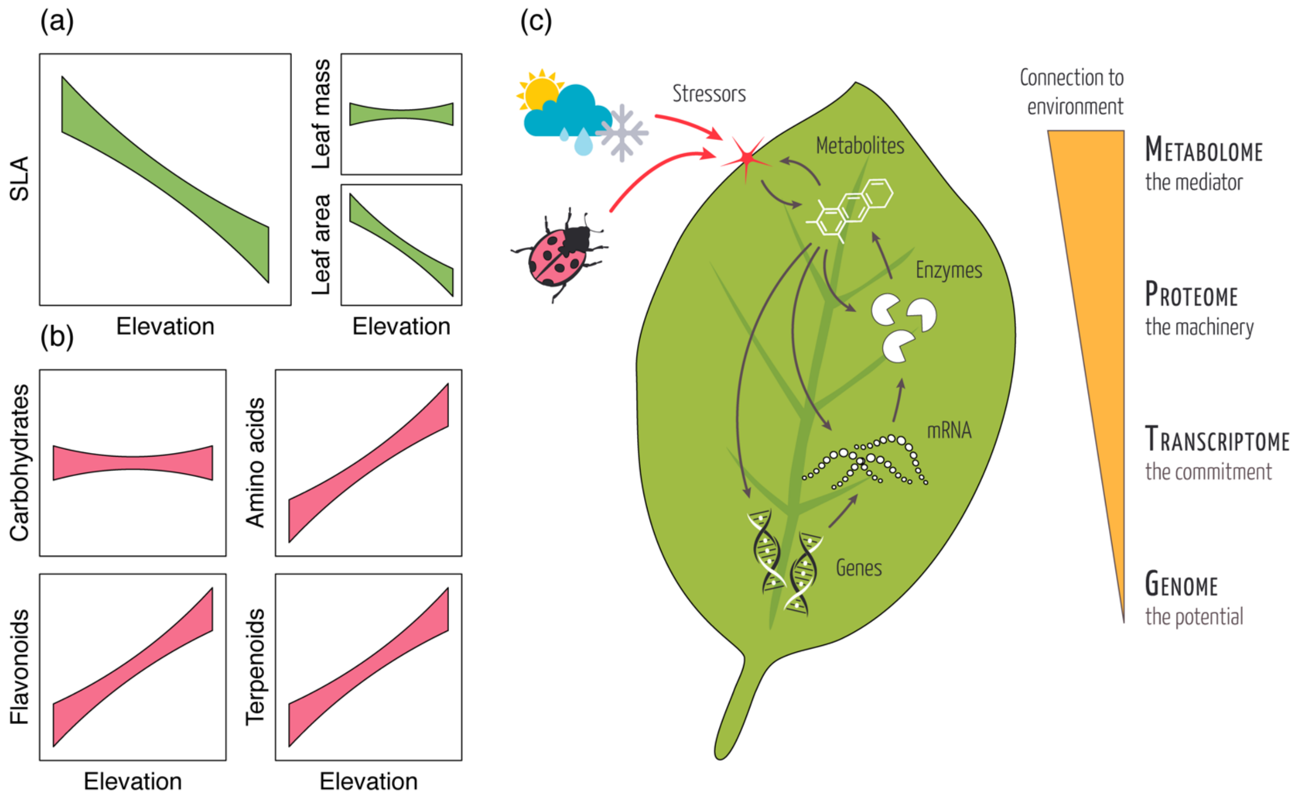

Figure 1 | Contextualising the plant metabolome. (a) A hypothetical relationship between elevation above sea-level and specific leaf area (SLA), including its constituent traits (leaf mass and area), whereby one interpretation would be that leaves become less productive at higher elevation. (b) The same relationship captured through changes within the plant metabolome, namely concentrations of carbohydrates, amino acids, flavonoids and terpenoids, through which it is clear that reductions in SLA are influenced by increased investment in tissue defence (*i.e.* flavonoids, terpenoids) at higher elevation. (c) A schematic showing how the plant metabolome is the interface between genes and the environment.

One reason for the low predictive power of functional traits is that they are aggregates of multiple physiological processes that can each be differently constrained by evolutionary history and environmental factors, and thus represent the combined product of homology, convergent evolution and plasticity. For instance, specific leaf area, which describes a trade-off between investment in photosynthesis (*i.e.* leaf surface area) and protection against stressors (*i.e.* leaf mass), is controlled by evolutionary history and local environmental factors and to this day remains unpredictable in its response to global change (Leishman *et al.* 2007; Dwyer, Hobbs & Mayfield 2014; Firn *et al.* 2019). Such complexity in interpreting the mechanistic basis of functional traits can mask the true nature of links between physiology and an ecological process of interest (Funk *et al.* 2017). As a consequence, while functional traits are central to characterising how plants function, they are imperfect proxies of physiology and so often cannot provide a mechanistic basis for plant functioning and its influence over populations, communities and ecosystems (Fig. 1a,b).

The metabolome, *i.e.* the collective name for the tens of thousands of metabolites that are the substrates and products of enzymatic reactions, offers a mechanistic bridge between plant physiology and ecological processes (Fig. 1c). Plants collectively produce an astonishing diversity of metabolites – somewhere between 200,000 and 1,000,000 throughout the plant kingdom and tens of thousands within an individual (Wang *et al.* 2019). For example, plants create rigid structures from metabolites called lignans, capture energy from sunlight and carbon dioxide in the metabolite glucose and respond to drought, temperature and herbivore stress with specialised metabolites, such as alkaloids and flavonoids (Fernandez *et al.* 2016; Peters *et al.* 2019). While some families of metabolites are restricted to particular taxa, such as glucosinolates in Brassicales (Fahey, Zalcmann & Talalay 2001), many are produced throughout the plant kingdom (Wink 2003). Thus, the metabolome varies among organs, individuals, populations and species due to genetic and environmental factors that operate over multiple timescales (Wink 2003; Hilker 2014; Schuman & Baldwin 2016). In this sense, the metabolome is regulated by the same suite of evolutionary and ecological processes that limit the interpretation of classical functional traits. However, the metabolome may provide better mechanistic understanding in ecological and evolutionary contexts. The metabolome, as some functional traits, lies at the interface between genes and the environment (Fig. 1c), but is unique in encompassing the physiological processes that act to maximise fitness both over an individual's lifetime and between generations. Hence, by comprising the composition, abundances and interplay of many thousands of metabolites, the metabolome provides a direct and multidimensional measure of the molecular mechanisms through which evolutionary and ecological processes shape plant functioning – but does not aggregate such mechanisms into a small number of variables (Box 1; Weckwerth 2003; van Dam & van der Meijden 2018). From this, it follows that the metabolome itself is a vast repository of functional traits. Nevertheless, the metabolome remains on the periphery of ecology, and few attempts have been made to integrate the metabolome into trait-based approaches.

Over recent decades, advances in analytical chemistry and mass spectrometry have led to the advent of metabolomics, which allows for the characterisation of the metabolome in a cost-effective and fast manner (Weckwerth 2003; Wishart 2005). Plant metabolomics was developed by molecular biologists to understand biochemical mechanisms behind phenotypes, and has found traction throughout the life sciences, including biotechnology (Yang *et al.* 2019), pharmacology (Newman & Cragg 2016) and medicine (Clish 2015). However, with notable exceptions (Inderjit *et al.* 2011), the use of metabolomics in ecology is more recent (van Dam & van der Meijden 2018). Early studies in chemical ecology used analytical standards to determine the presence or abundance of a limited set of pre-determined metabolites in a sample (Everette *et al.* 2010). The field has now shifted towards untargeted metabolomics, which instead attempts to detect and identify all of the thousands of metabolites in a sample (Schrimpe-Rutledge *et al.* 2016). Untargeted metabolomics is a powerful holistic tool that requires only small amounts of material and uses simple laboratory extractions relative to other “omics” approaches (Ryan & Robards 2006). However, it employs a complex analytical pipeline and yields many thousands of often-unknown metabolites, both of which create major challenges for interpretation (Allard, Genta-Jouve & Wolfender 2017). Accordingly, most ecological metabolomics studies are restricted to plants, in particular model species (Nagler *et al.* 2018) or within-species treatment comparisons (Wiggins *et al.* 2016), with few studies attempting multi-species comparisons (Rivas-Ubach *et al.* 2012; Sedio *et al.* 2017; Endara *et al.* 2018; Sedio *et al.* 2018b; Forrister *et al.* 2019; Ristok *et al.* 2019; Gargallo-Garriga *et al.* 2020). Recent pushes towards "big data" ecology (e.g. Maldonado *et al.* 2015; Díaz *et al.* 2016; Kattge *et al.* 2020; Katz *et al.* 2021), coupled with decreasing analytical costs and improved data analysis tools (Wang *et al.* 2016; Allard *et al.* 2017), make it increasingly possible to undertake the multi-species studies necessary to explore the power of the metabolome to enhance trait-based assessments of plant and ecosystem functioning. Nevertheless, no coherent framework yet exists for harmonising data acquisition, standardisation and analysis across such studies.

This Special Feature represents a coordinated effort to bring focus to the potential of the metabolome to provide deeper mechanistic insight and predictive power to a variety of ecological questions. In this editorial review, we focus on the link between the metabolome and plant functional traits, which are established and commonly-used metrics of plant functioning. We consider the capacity of the metabolome to shed light on the mechanisms behind existing plant functional traits and to unlock a new generation of molecular functional traits that underpin links between physiology and ecological processes (Sedio *et al.* 2017; Sardans *et al.* 2020), placing particular emphasis on life history trade-offs that govern fitness over multiple generations and plasticity that shapes fitness within an individual's lifetime. We then examine and propose solutions to major challenges of acquiring, interpreting and contextualising metabolomics data in ecological studies. Following this, we showcase the seven papers that comprise the Special Feature, which reveal the potential for metabolomics to help resolve mechanisms behind plant community assembly, plant-organismal interactions and the effects of plants and soil microorganisms on ecosystem processes, as well to improve predictions of them. Finally, we synthesise these insights to build a roadmap for integrating metabolomics into ecology, including key questions for future research.

2. The metabolome in plant ecology

2.1. The metabolome and plant life history

A key contribution of trait-based ecology has been the description of broad trade-offs in plant functioning that occur throughout the plant kingdom (Díaz *et al.* 2016). A notable example is the leaf economics spectrum (Wright *et al.* 2004), which describes correlations among leaf traits along an axis from small, long-lived, unproductive leaves (e.g. low specific leaf area, low photosynthetic rates) to large, short-lived, productive leaves (e.g. high specific leaf area, high photosynthetic rates). These traits also correlate with the relative importance of survival, growth and reproduction for plant population performance (Adler *et al.* 2014; Pistón *et al.* 2019), demonstrating their relevance to life history. Other schemes have sought to classify plants into life-history strategies using functional traits, for example by distinguishing competitive species from ruderal and stress-tolerating species (Grime 1977). Regardless of the system, trait-based life history trade-offs reflect relatively conserved characteristics of species generated by selection and phylogenetic constraints that operate over millennia – although it should be noted that local adaptation over several generations can also drive life history variation at the population level (Halbritter *et al.* 2018).

Comparative ecological metabolomics studies are relatively rare, and those that do exist are mostly limited to single growth forms or ecosystems (Rivas-Ubach *et al.* 2012; Sedio *et al.* 2017; Endara *et al.* 2018; Sedio *et al.* 2018b; Forrister *et al.* 2019; Ristok *et al.* 2019; Gargallo-Garriga *et al.* 2020). However, there is evidence that the metabolome plays a central role in plant life history. This is particularly true when considering the role of the metabolome in specialisation towards herbivory and climate, which are important factors governing plant fitness (Bruelheide *et al.* 2018). Plants generate a plethora of specialised metabolites to defend themselves against biotic stressors, such as herbivory, pathogens and competitors, as well as abiotic stressors, such as extreme temperatures or drought (Sardans *et al.* 2020). Such metabolites can be produced throughout a plant's growth and development (*i.e.* constitutively) or in response to a specific event (*i.e.* facultatively; see Section 2.2, below; Feeny 1976). Recent studies examining the metabolomes of temperate and tropical plant species suggest that herbivory generally selects for genotypes possessing more diverse metabolomes (Kursar *et al.* 2009; Becerra 2015; Salazar *et al.* 2018; Sedio *et al.* 2018b). Moreover, the selection pressure exerted by herbivores appears to drive metabolic variation that reflects a trade-off between investment in specialised metabolism (e.g. flavonoids, anthocyanins) *vs.* central metabolism (e.g. amino sugars, nucleotide sugars; Gargallo-Garriga *et al.* (2020)). Studies examining the impact of climate on the plant metabolome also suggest a coupling between plant metabolic variation and life history. In a comparative study of temperate plant species (Defossez *et al.* 2021), it was found that alpine species possess metabolites that are not present in low elevation species, suggesting that cold temperatures may select for unique metabolites not required under a warmer climate. Moreover, a recent study on the sedge *Eriophorum vaginatum* L. demonstrated that populations genetically adapted to alpine climates invest more in cold-stress metabolites than populations adapted to low elevation climates, and that this is associated with slower growth rates overall (Walker *et al.* 2019).

In general, species with functional trait values at the conservative end of the leaf economics spectrum (Wright *et al.* 2004) or competitor-stress-ruderal axes (Grime *et al.* 1997) are more resistant to herbivores and better adapted to cold climates. We thus propose that the metabolome not only underpins plant life history trade-offs, but also has the potential to provide further mechanistic insight into such trade-offs by describing finer scale variation nested within them (Box 1; Schuman *et al.* 2016; Forrister *et al.* 2019). While further work is necessary to explore the full interplay between the metabolome and plant life history, the examples discussed here provide clues about what to expect. For instance, we might expect metabolites that associate with life history trade-offs to display strong phylogenetic signals that reflect known constraints on ecological strategies (e.g. gymnosperms *vs.* angiosperms; Díaz *et al.* 2016). We might also suppose that such metabolites provide life-long fitness benefits and so will be consistent throughout an individual's lifetime or among cohorts of leaves, in spite of diurnal or seasonal fluctuations in other parts of the metabolome. Finally, given that all trait-based life history trade-offs make a distinction between acquisitive (*i.e.* fast-growing) and conservative (*i.e.* slow-growing) species (Grime *et al.* 1997; Wright *et al.* 2004; Díaz *et al.* 2016), it is likely that such a distinction also exists in the plant metabolome. This could, for example, manifest as a varying investment in central versus specialised metabolism or changes in the abundances of metabolites associated with fast rates of growth. While the idea of a metabolic basis to life history is not new (Feeny 1976; Coley, Bryant & Chapin 1985), it is only now becoming possible to go beyond isolated examples and examine how the metabolome supports, and expands upon, established life-history trade-offs widely across the plant kingdom.

2.2. The metabolome & plant phenotypic plasticity

As well as describing broad trade-offs in plant life history, functional traits are commonly used to characterise plant phenotypic plasticity in response to changing environmental conditions. However, with notable exceptions (Jung *et al.* 2014; Siefert *et al.* 2015), classical functional traits are usually not measured at sufficient frequency to capture changes in plant functioning over short temporal scales. Even when they have been, widely used functional traits, such as leaf carbon and nitrogen concentrations and specific leaf area, show strong, mixed or no responses to short-term environmental change (Koricheva 2002; Leishman *et al.* 2007; Dwyer *et al.* 2014; Hu *et al.* 2018; Walker *et al.* 2019, 2020; Firn *et al.* 2019). As we discuss below, the lack of consistent functional trait plasticity might not be because changes to plant functioning do not occur, but because classical functional traits are too coarse to detect them (Fig. 1a,b) or do not capture aspects of physiology that are responsive to environmental changes (Box 1). Here, we propose that measurements of the metabolome offer potential to expand the mechanistic insight provided by existing functional traits.

Comprehensive reviews already exist on the effects of temperature stress, drought, nitrogen availability and biotic interactions on the plant metabolome (Bundy, Davey & Viant 2009; Sardans *et al.* 2011, 2020; Rivas-Ubach *et al.* 2012; Schuman & Baldwin 2016). In general, temperature or drought stress rapidly stimulates production of simple sugars and amino acids (Sardans *et al.* 2020), as well as phenolic compounds, such as flavonoids (Yang *et al.* 2018). By contrast, mild to moderate warming, which can occur over hours to days in temperate ecosystems and last for days to weeks during heat waves, has been shown to lower concentrations of phenolic compounds in a number of vascular plant species and bryophytes (Jassey *et al.* 2011; Pellissier *et al.* 2014). The onset of herbivory, as well as pathogen attack, is also known to increase production of phenolic compounds, as well as alkaloids, terpenoids and other specialised molecules, in many plant species (Schuman & Baldwin 2016; Peters *et al.* 2019).

The production of specialised metabolites in response to biotic or abiotic stress acts to maximise fitness by prioritising immediate defence over growth or reproduction (Herms and Mattson, 1992). However, although dynamic coupling between the plant metabolome and leaf traits has been found (Rivas-Ubach *et al.* 2012; Gargallo-Garriga *et al.* 2015; Monson *et al.* 2021), it does not follow that such plasticity in the metabolome is always evident in classical functional traits, for two reasons. First, while specialised metabolites are relatively rich in carbon or nitrogen (Züst & Agrawal 2017), the number of carbon and nitrogen atoms allocated to such metabolites is several orders of magnitude lower than that allocated to morphological structures. This means that diverting nitrogen into amino acids to resist drought may occur without affecting leaf nitrogen concentration and converting sugars into phenolic compounds during a herbivore attack may not alter leaf carbon concentration (Campbell & Vallano 2018). Coupled with this, the metabolites involved in a plastic response usually compose only a small fraction of total tissue mass (Heiling *et al.* 2010) and can thus impact fitness without affecting mass-based functional traits (e.g. leaf mass, specific leaf area, aboveground biomass, seed mass). Second, the production of specialised metabolites normally induces a downregulation of growth processes *via* signalling, even if energy or nutrients are not limiting (Monson *et al.* 2021), which can impact fitness without affecting functional traits (van Dam & Baldwin 2001; Strauss *et al.* 2002; Guo *et al.* 2018). Indeed, it is now known that investment in plant growth versus defence is coordinated at the cellular scale by two transcription factors (*jaz*, *fitness*; Monson *et al.* 2021) and is widely detectable in the metabolome (Strauss *et al.* 2002; Schuman & Baldwin 2016), whereas associated changes to functional traits may only occur in subsequent generations (Van Dam & Baldwin 2001), if at all (Strauss *et al.* 2002; Guo *et al.* 2018). More generally, changes to the metabolome occur within seconds to hours of an environmental stimulus (Schuman & Baldwin 2016), whereas changes to functional traits can take weeks or years to become apparent (Jassey & Signarbieux 2019). As such, while effects of ephemeral stressors (e.g. drought) and fluctuating environments on plant fitness are detectable in the metabolome, they may be missed entirely by classical functional traits.

In short, environmental variation over the lifetime of an individual plant causes alterations to the plant metabolome that directly impact plant functioning and fitness. As such, considering the metabolome will likely improve the power of functional traits to explain mechanisms behind plant and ecosystem functioning. For instance, metabolomics studies have convincingly shown how neighbouring plant identity and diversity affect plant performance (Scherling *et al.* 2010; Chiapusio *et al.* 2018), and have provided a mechanistic basis to root competition (Latif, Chiapusio & Weston 2017), plant-soil feedback effects (Ristok *et al.* 2019) and facilitation (Rasmann & Turlings 2016). More broadly, we might expect that metabolites that mediate plasticity in response to environmental factors are facultative (i.e. induced only when needed) and vary in concentration, for example over the lifetime of a plant or among cohorts of leaves. Such metabolites may thus be distinct from constitutive (i.e. always present) metabolites associated with long-lived strategies (see Section 2.1, above). We may also hypothesise that, with the exception of metabolites restricted to specific taxa (e.g. glucosinolates in Brassicales; Fahey *et al.* (2001)), such plastic metabolites display a weaker phylogenetic signal, although the capacity to modify their concentrations in response to stressors may still be under strong selection (Kergunteuil, Röder & Rasmann 2019).

3. Measuring, interpreting and contextualising the plant metabolome

Ecological research is increasingly gravitating towards big data studies that employ large sampling campaigns or draw from vast data repositories to make inferences beyond site-specific factors and context dependency (Dietze & Lynch 2019; Schrodt *et al.* 2019). Moving forwards, ecological metabolomics studies will also need to involve multi-species and multi-biome comparisons to explore the full relevance of the metabolome to functional ecology. To achieve this, researchers will need to overcome known challenges relating to the design of ecological metabolomics studies and the acquisition and analysis of metabolomics data. Researchers will also need to identify the limitations of using metabolomics in ecology, both generally and compared to existing trait-based approaches, which are currently unknown. In this section, we summarise those known challenges and propose solutions to help ecological metabolomics enter the era of "big data ecology".

3.1. Challenge 1: designing ecological metabolomics studies

The recent development of data science approaches specifically adapted to large and complex datasets yields exciting possibilities for ecologists to interrogate links between the plant metabolome and functional trait theory over large spatial, environmental or taxonomic scales. At the same time, movements to make scientific data openly available are providing motivation to share raw metabolomics data on digital platforms, such as GNPS (Wang *et al.* 2016), the Metabolomics Workbench (https://www.metabolomicsworkbench.org/) and MetaboLights (Haug *et al.* 2013). However, inferring links between the plant metabolome and variation in classical functional traits requires that there is sufficient overlap between metabolomics and ecological databases (e.g. TRY (Kattge *et al.* 2020), GBIF (www.gbif.org)). This is currently not the case. Molecular biologists apply metabolomics mostly to small numbers of model plant species, and often to specific organs or tissues. Phytochemists typically screen ecosystems for metabolites of pharmaceutical value or focus on the metabolomes of species from extreme environments or certain taxonomic groups. Chemical ecologists have chiefly employed metabolomics to identify metabolites involved in organismal interactions, and mostly for individual species, families or growth forms. As such, metabolomics data are currently restricted to subsets of targeted species, which is of limited use in large-scale ecological studies.

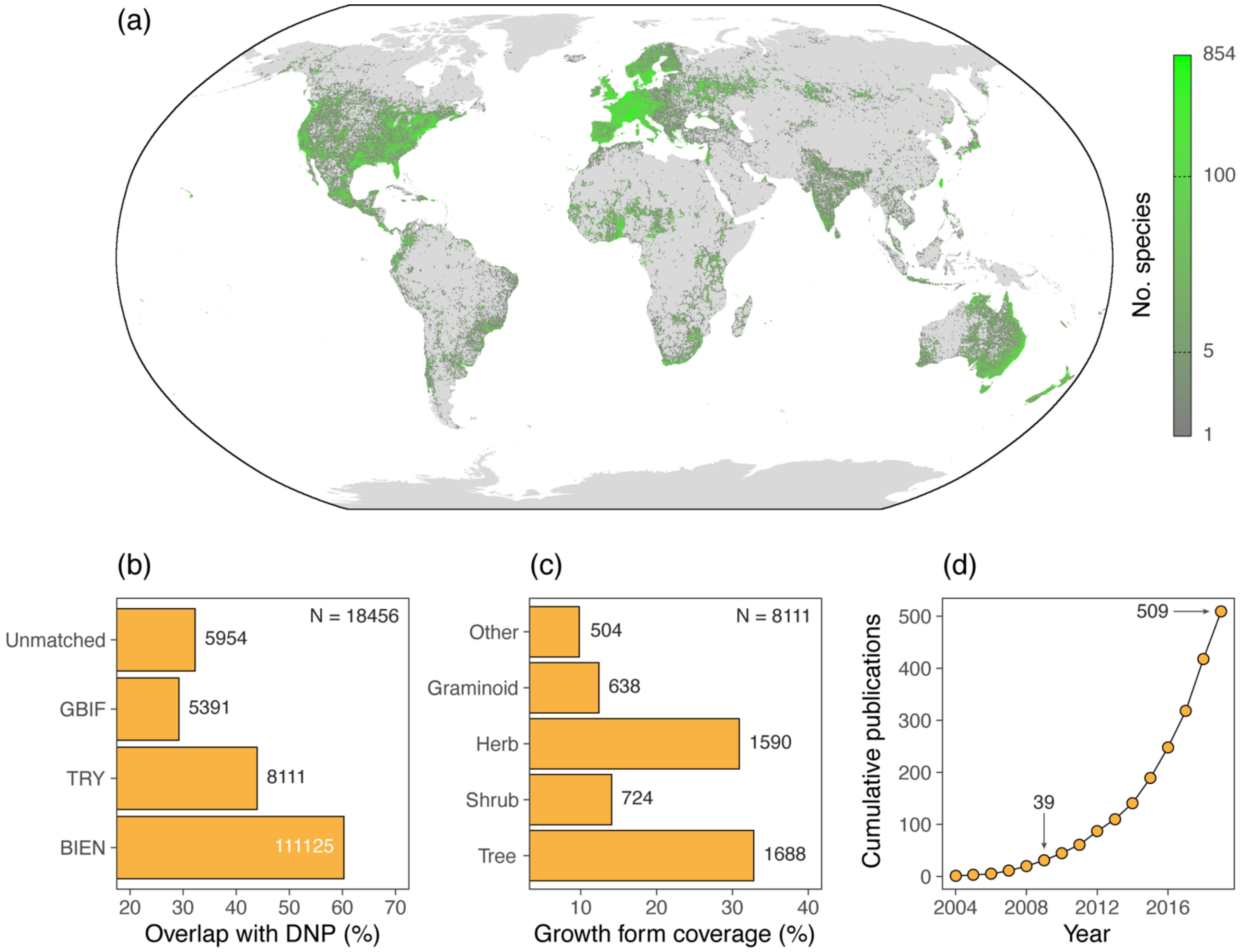

Figure 2 | Overlap between metabolomics and ecology. (a) The estimated geographical distribution of all plant species matched from metabolomics data in the Dictionary of Natural Products (DNP; N = 18,456 species) (Chassagne *et al.* 2019) to cleaned occurrence records in GBIF (www.gbif.org). Each point denotes the presence of at least one species in a 0.25º grid cell, with colour intensity increasing as a function of the number of species present (maximum 854 species, natural log-scale). (b) The percent matches between plant species in the DNP and plant species in the GBIF, TRY (Kattge *et al.* 2020) and BIEN (Maitner *et al.* 2018) databases. (c) The percent coverage of different plant growth forms present in the DNP, considering species for which data are available (N = 8,111; other: bryophytes, ferns, herb/shrub, shrub/tree, herb/shrub/tree). (d) The cumulative number of manuscripts published on ecological metabolomics (from a Web of Science search for the keywords "ecolog* AND metabolom*"). Annotated points highlight the rapid change within the last 10 years.

Despite the limited overlap between existing ecological and metabolomics data, comparative ecological metabolomics studies are now beginning to emerge (Rivas-Ubach *et al.* 2012; Sedio *et al.* 2017; Endara *et al.* 2018; Sedio *et al.* 2018b; Forrister *et al.* 2019; Ristok *et al.* 2019; Gargallo-Garriga *et al.* 2020), demonstrating efforts from ecologists to overcome these issues (Fig. 2d). Moreover, we estimate that there is already sufficient overlap between metabolomics and ecological trait databases to make a robust start towards a global comparative ecological metabolomics study (Fig. 2a-c). Such an effort would strongly depend on compatibility between analyses (see Section 3.2, below) and would require active information exchange between two distinct fields. On the metabolomics side, the inclusion of raw data and metadata will play a crucial role in allowing researchers to reprocess data within the context of their study. On the ecology side, there is a need to engage metabolomics researchers early on in a project cycle, preferably before sampling species or ecosystems of interest (Uthe *et al.* 2021). Despite the challenges ahead, the success of the ecological genomics revolution (Baldwin 2012) is a clear demonstration that combining distinct fields is possible and has the capacity to yield a powerful tool for ecology.

3.2. Challenge 2: metabolomics data acquisition

Even after samples have been selected, the data they yield are sensitive to a number of decisions made during collection and analysis. This used to be true for existing functional trait measurements, but has been effectively eliminated due to huge effort from ecologists to establish and share best practice for acquiring standardised trait data (Pérez-Harguindeguy *et al.* 2013; Dawson *et al.* 2018). The search for best practice in ecological metabolomics has now begun (Sedio, Boya P & Rojas Echeverri 2018a; Uthe *et al.* 2021), although the field is still missing a handbook that sets out standardised approaches for acquiring and analysing metabolomics data in ecological applications. Developing such a handbook is an essential step in bringing metabolomics approaches to ecology.

During fieldwork, researchers must decide whether to cool or freeze samples immediately or leave them to air-dry. Flash-freezing samples in liquid nitrogen provides the most holistic view of the metabolome, in that it prevents less stable metabolites from decaying before analysis (but see Sedio *et al.* 2018a). Freezing (or at least cooling) samples on collection may be important in situations where the metabolome shifts subtly under manipulation (e.g. plasticity; see Section 2.2, above), but is practically challenging - especially in remote locations. For this reason, air-drying samples is common practice in some disciplines (Chassagne *et al.* 2019). In deciding to air-dry samples, researchers sacrifice the ability to detect less stable metabolites in favour of collecting more samples overall. Such an approach may thus be suited to comparative metabolomics studies, such as detecting persistent differences in metabolomes among a large number of species (e.g. life-history variation; see Section 2.1, above).

On return to the laboratory, samples must be extracted and analysed. Numerous extraction methods exist, all of which vary in their capacity to extract different aspects of the metabolome (e.g. polar versus non-polar metabolites, lipids). Settling on one appropriate extraction method for ecological metabolomics studies is an ongoing task for ecologists and metabolomics researchers. Further work is also needed to harmonise the analytical side of metabolomics, which varies greatly between studies due to intrinsic variability in mass spectrometry (and see Richards et al. (2018) for other approaches). Briefly, gas- and liquid-chromatography, when coupled with mass spectroscopy, characterise samples by quantifying a unique set of spectral features. Each feature is described based on its relative signal intensity, retention time (i.e. physicochemical properties) and mass spectrum (i.e. mass-to-charge ratio of the intact – and in tandem mass spectrometry, fragmented – feature). While relative signal intensity is used as a proxy for relative abundance, it is also a function of a feature's ionization response, which can vary due to a feature's chemical structure, a sample's overall composition or an instrument's design or protocol (De Vijlder *et al.* 2018). Moreover, retention time, which is used to align features between samples, is variable between runs on the same instrument, let alone between laboratories (Watrous *et al.*

2017). As such, while synthesising datasets from multiple sources is a common practice in ecology, it is challenging to do so with untargeted metabolomics datasets.

One solution for synthesising metabolomics data is to treat separate datasets as independent replicates, but a better long-term solution is to focus on mass spectra. Recent advances in computational metabolomics make it now possible to do this, and specifically use similarities between mass spectra to build molecular networks of related features and group them into spectral clusters (Wang *et al.* 2016). This approach can deliberately ignore retention time and signal intensity and can thus be applied to multiple datasets simultaneously. Moreover, spectral information relating to each cluster can be cross-referenced against public libraries to annotate it with a putative chemical structure or metabolite name, which is helpful for interpretation. However, while generating spectral clusters is reasonably robust, assigning chemical structures to them is not. Metabolite annotation remains a major bottleneck in metabolomics and is far from being a routine operation (Box 2; Peters *et al.* 2018). Nevertheless, by treating assigned chemical information as putative, it is still possible to gain ecological insight into the metabolomes of multiple species, and the annotation step can be repeated as new approaches are developed.

3.3. Challenge 3: statistics for ecological metabolomics

The amount of information present in untargeted metabolomics data provides a vast resource for examining links between the metabolome and ecological processes. However, the nuances and complexity of these data create three common traps for statisticians.

First, it is not meaningful to compare the abundances of different metabolic features within a sample, because the abundance of a feature is also dependent on its chemical structure (see Section 3.2, above). This precludes the use of common ecological diversity indices (e.g. Shannon, Simpson), which compare abundances both between and within metabolic features. Metabolic diversity can thus currently only confidently be expressed through metabolic feature richness.

Second, metabolomics datasets contain a large number of zeros, owing to many features being unique to individual samples, while also possessing many features that do not co-vary. This is important because it is common to reduce the complexity of metabolomics data by performing ordinations (e.g. Principal Components Analysis, Principal Coordinates Analysis, Non-Metric Multidimensional Scaling), which can be sensitive to both of these factors. Methods like sparse Partial Least Squares analysis, Random Forest, or Support Vector Machines are increasingly being applied to metabolomics data (e.g. Defossez *et al.* 2021) because they are less biased to zero-inflated data than classical ordinations.

Finally, most commonly used distance matrices assume that all metabolic features are independent and do not take into account the varying degrees of chemical similarity or relatedness among them. For this reason, developing statistical approaches that accurately reflect the chemical space of metabolomics data is the subject of active research. For instance, several research groups are developing chemically-informed metrics that take into account spectral relatedness across individual metabolic features (Sedio *et al.* 2017; Salazar *et al.* 2018; Endara *et al.* 2018). Other groups are using chemically-informed tree structures to constrain tree-based distance metrics (e.g. UniFrac; Junker 2018). Until a consensus emerges, ordinations of metabolomics data should be performed carefully and interpreted with caution. Assuming these traps are avoided, metabolomics data are similar to other multivariate data, with the exception that they contain thousands of metabolic features (Box 1) and give users the flexibility to choose how precise or confident their interpretation should be (Box 2).

4. Special Feature Content

Thus far, we have provided evidence that measurements of the metabolome, once properly incorporated into classical functional trait theory, may improve our understanding of the coupling between physiology and fitness in trait-based studies. The papers in this Special Feature build on this foundation to explore how the metabolome can help characterise the mechanisms behind plant community assembly, plant-organismal interactions and the effects of plants and soil microorganisms on ecosystem processes, as well to improve predictions of them.

Beginning with plant community assembly, Endara *et al.* (2021) couple phylogenetic and metabolomics analyses on the neotropical genus of trees *Inga* across five locations in the Amazon to show that individuals with dissimilar metabolomes are more likely to co-occur, but that regional variation in metabolites related to herbivore defence occurs independently of phylogenetic relatedness. These findings suggest that the metacommunity for any regional community in the Amazon is potentially the whole Amazon itself, and that community assembly in this region likely depends on how herbivores interact with plant metabolic diversity. Fernandez-Conradi *et al.* (2021) examine such interactions directly by assessing rates of herbivory on experimental plant communities possessing high *vs.* low community-wide metabolic diversity in the Swiss Alps. While the expectation was that plant communities with high metabolic diversity would be more resistant to herbivores than communities with low metabolic diversity, this effect only occurred at low elevation, whereas communities with high metabolic diversity were less resistant to herbivores at high elevation.

One explanation for the findings of Fernandez-Conradi *et al.* (2021) is that metabolite richness represents only one aspect of plant metabolic diversity that has consequences for plant-herbivore interactions. Indeed, Philbin *et al.* (2021) perform metabolomics analyses on two *Piper* shrub species to show that herbivores interact with two distinct aspects of plant metabolic diversity, namely metabolic composition and metabolite structural complexity. This distinction explains how closely related species support diverse herbivore assemblages, and confirms that plants are able to draw on multiple aspects of metabolic diversity in response to herbivore pressure. Another explanation for the findings of Fernandez-Conradi *et al.* (2021) could be that the metabolome is not static and represents the collective outcome of many interacting biotic and abiotic factors. In this regard, Weinhold *et al.* (2021) exploit a biodiversity ecosystem functioning experiment in China to show that tree diversity is an important determinant of the leaf metabolome in four subtropical tree species. At the same time, Rawlings *et al.* (2021) use controlled experiments on wood-decay fungi in UK beech woodlands to show that generalist pioneer and stress-tolerant species display contrasting metabolic responses to constant versus fluctuating temperature regimes. Finally, the studies by Fernandez-Conradi *et al.* (2021) and Philbin *et al.* (2021) suggest that the metabolome responds not only directly to abiotic shifts (in both studies induced by elevation change), but also indirectly *via* interactions between the metabolome and herbivores, which alter a plant's capacity to respond to abiotic change itself.

The above studies collectively demonstrate that the metabolome provides deeper mechanistic insight into plant community assembly and plant-herbivore interactions. However, they also suggest that the metabolome is an important regulator of ecosystem processes. Indeed, Fernandez-Conradi *et al.* (2021) and Philbin *et al.* (2021) both show that the plant metabolome shapes herbivore community dynamics, and Endara *et al.* (2021) suggest that the metabolome dictates plant community assembly processes. Similarly, Rawlings *et al.* (2021) show the potential for wood-decay fungi with different ecological strategies to display different metabolic responses to fluctuating temperature, with consequences for wood decomposition – an important biogeochemical process in forest ecosystems.

The remaining papers in this Special Feature examine the metabolome as the foundation of plant-soil interactions. Specifically, Williams *et al.* (2021) characterise root traits and the rates and metabolic composition of root exudates in experiments involving 18 European grassland species. They show that the root exudate metabolome is a key component of root functioning, and posit that the root exudate metabolome characterises an aspect of root functional space not currently described by existing functional trait frameworks. Finally, Sytiuk *et al.* (2021) undertake a comparative study across five European peatland ecosystems on a latitudinal gradient to determine how climate, edaphic characteristics and the phylogeny, functional traits and metabolites of peat-forming *Sphagnum* mosses affect the composition and functioning of peatland soil microbial communities. They find that *Sphagnum* metabolites have the strongest bearing over the soil microbial food web, revealing that the *Sphagnum* metabolome is an important regulator of peatland soil processes.

5. Conclusions and future directions

In this review, we have shown that the metabolome comprises the pool of molecular mechanisms that underpins physiology and represents a highly resolved lens through which to examine plant fitness, both between and within generations. By focussing on the metabolome as the biochemical basis of organismal functioning and the language of organismal interactions, the studies that form this Special Feature illustrate how metabolomics can provide deeper mechanistic insight into open ecological questions, and show how using the metabolome within a functional ecology framework holds great promise for improving predictive power in ecology. Yet, applying metabolomics approaches to ecological questions requires careful experimental design, as well as the development of standardised approaches for acquiring and analysing ecological metabolomics data. In our opinion, ecologists and metabolomics researchers must now work together to develop three key frameworks necessary for integrating the plant metabolome into ecology (Fig 4): (i) a **conceptual framework**, placing the metabolome within the context of established trait-based approaches and theory; (ii) a **technical framework**, equipping ecologists with the tools and expertise for measuring the metabolome in a wide range of species; and (iii) a **data framework**, providing the digital infrastructure and data processing pipelines needed for the use of existing and future high throughput metabolomics data in comparative ecological studies. In Box 3, we outline nine key research directions that we believe will help to achieve this vision.

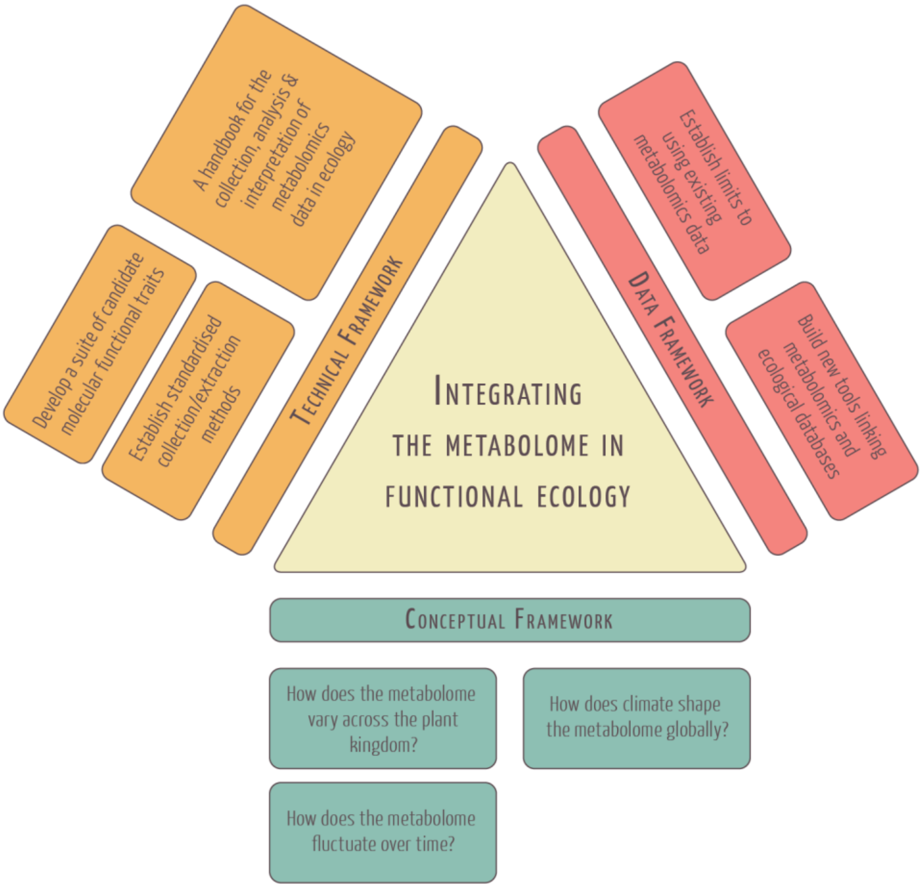

Figure 3 | Research needs for integrating the metabolome into functional ecology. The conceptual (green), technical (yellow) and data (red) frameworks needed to establish measurements of the metabolome into a functional trait framework. Expanded boxes correspond to research directions proposed in Box 3.

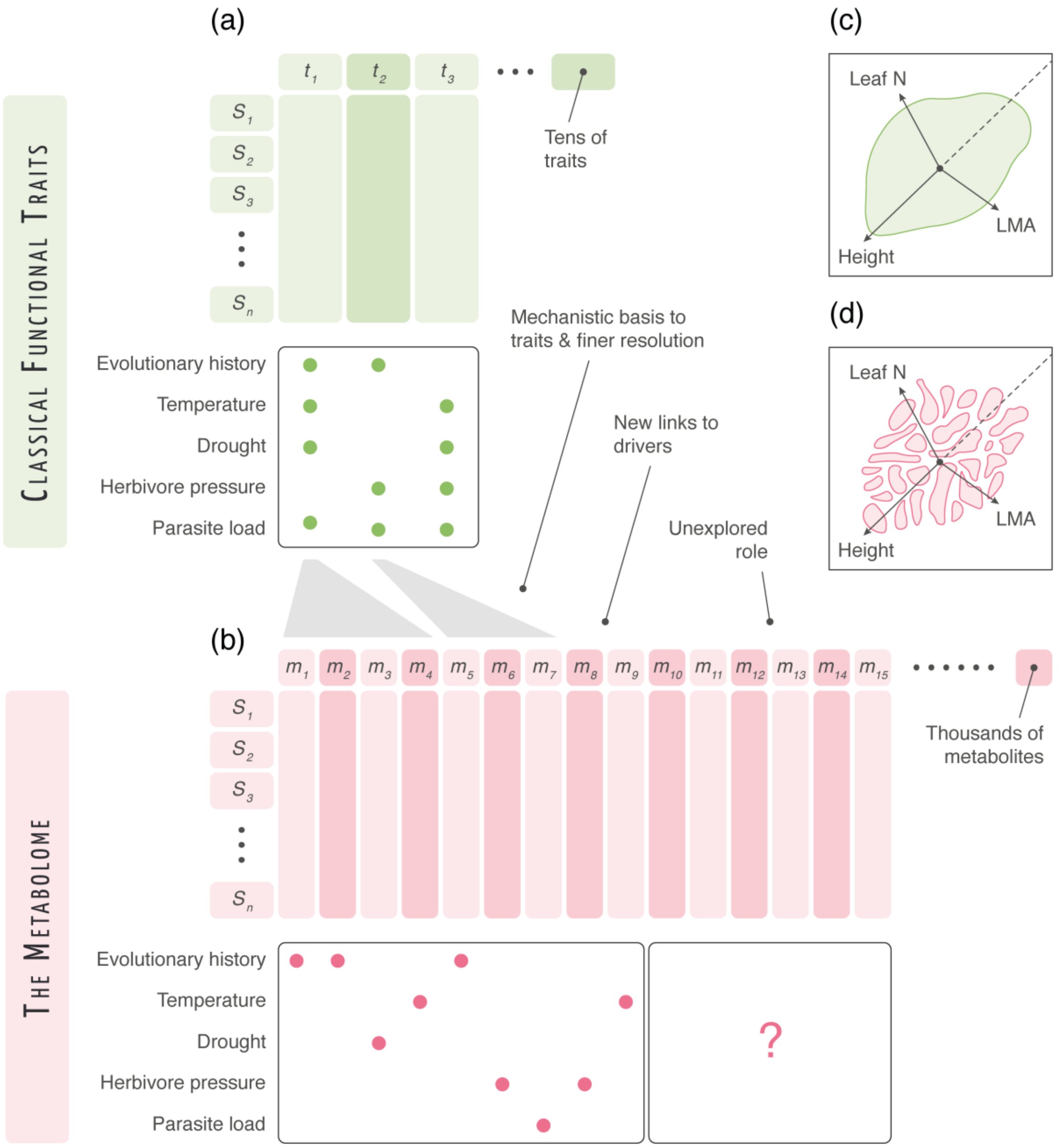

Figure 4 | The dimensionality and resolution of the metabolome versus functional traits. (a,b) Tables illustrating the dimensionality of data describing (a) classical functional traits (green) or (b) the metabolome (red) of n species (rows). Columns represent a small selection of traits (t_x) or metabolites (m_x), and estimates are given for the total number of traits/metabolites attainable from one sampling effort. Hypothetical relationships are also shown between traits/metabolites and some drivers of plant fitness or function (white boxes, coloured dots), as well as plausible coupling between some functional traits and some metabolites (grey shading). (c,d) Hypothetical separations of species from (a,b) into distinct groups using ordinations based on (c) classical functional traits (following Díaz *et al.* 2016) or (d) the metabolome. See Box 1.

Box 1 | The Resolution and Explanatory Power of the Metabolome

At the most basic level, datasets describing classical functional traits and the metabolome share a similar structure (Fig. 4a,b), albeit comprising tens of traits versus thousands of metabolites (commonly expressed as presences/absences or abundances; see Section 3.2). However, a unique facet of metabolomics data is the additional provision of background chemical information about detected metabolites (see Box 2). Such chemical information allows researchers to characterise not just the metabolites associated with a change among samples, but also the types of chemical structures and/or biochemical pathways involved (da Silva, Dorrestein & Quinn 2015; Djoumbou Feunang *et al.* 2016). As such, measurements of the metabolome provide a large amount of molecular context with which to interpret each variable therein, which is not automatically the case for classical functional traits.

To further illustrate the explanatory power of the metabolome, let us imagine that a set of measured functional traits is sufficient to capture all variation in plant fitness or function among samples. In this situation, measuring the metabolomes of the same samples would provide two further interpretative tools. First, by identifying which metabolites are responsible for changes to traits (Fig. 4a,b: grey shading), it would be possible to determine the molecular mechanisms behind changes among samples (e.g. Walker *et al.* 2019). Second, most functional traits are the aggregation of many interacting physiological processes (Funk *et al.* 2017), making it challenging to isolate effects of external factors, such as evolutionary history, climate or organismal interactions, on plant fitness (Fig. 4a, white box; Koricheva 2002; Leishman *et al.* 2007; Dwyer *et al.* 2014; Hu *et al.* 2018; Walker *et al.* 2019, 2020; Firn *et al.* 2019). The metabolome provides a direct measure of such physiological processes (Weckwerth 2003; van Dam & van der Meijden 2018), making it easier to pinpoint which external factors affect which aspects of plant fitness or function (Fig. 4b, white box). In short, by measuring the metabolome alongside classical functional traits, researchers would gain greater mechanistic insight and finer resolution than provided by functional traits alone.

In reality, any set of measured functional traits is unlikely to encompass all variation in plant fitness or function (see Section 3; Adler *et al.* 2014; Díaz et al. 2016). Measurements of the metabolome thus provide a vast repository of molecular information through which to capture variation missed by classical functional traits. For example, by interrogating links between external factors and those metabolites unrelated to existing functional traits, it may be possible to identify novel “molecular functional traits” that characterise variation among apparently similar samples (Fig. 4b). The amount of biological information captured by the metabolome should also allow for much greater separation of species along established life history trade-offs than classical functional traits (Fig. 4c,d), although this remains to be empirically tested. Importantly, the roles of most metabolites in shaping plant fitness and function remain unexplored (Fig. 4b, white box), making the metabolome a frontier for improving explanatory power in trait-based ecology.

Box 2 | Annotating Mass Spectra with Chemical Structures

Mass spectrometry allows users to detect thousands of mass spectra in a single analysis. However, resolving such spectra into distinct metabolites with associated chemical structures is challenging. In contrast to DNA, RNA and proteins, which are mostly polymeric materials (bases or amino acids, respectively), metabolites can possess one of many thousands of possible chemical structures and thus cannot be systematically deconstructed into their component parts (Lipinski & Hopkins 2004). As such, typically only 2% to 5% of features detected during an untargeted mass spectrometry analysis are matched to known metabolites in public libraries (da Silva, Dorrestein & Quinn 2015). Assigning chemical structures to spectra, *i.e.* **metabolite annotation**, is a major research objective and the subject of recent developments in computational metabolomics. Here, we present some of the most promising approaches emerging from such developments.

It is now possible to propagate chemical information from known metabolites in a sample to unknown features using molecular networks (see Section 3.2) (Wang *et al.* 2016). At the same time, the coverage of chemical structures in existing public spectral libraries is being improved using approaches such as *in silico* spectral calculations (Allard *et al.* 2016; da Silva *et al.* 2018), spectral fingerprint matching to structural databases (Dührkop *et al.* 2019) and unsupervised sub-structure discovery (van der Hooft *et al.* 2016). Computational metabolite annotation approaches have been further improved by including taxonomic information (e.g. up to seven-fold improvements; Rutz *et al.* 2019) or combining them (e.g. 60% of features annotated; Ernst *et al.* 2019). Moreover, researchers can increase the number of features annotated by aggregating at broader organisational levels of a chemical taxonomy (e.g. chemical subclass, to class, to superclass; Djoumbou Feunang *et al.* 2016) or propagating chemical classifications to unannotated compounds that are spectrally similar (e.g. among clusters of a molecular network). However, doing so involves a trade-off between precision and confidence. A loss of precision in metabolite annotation is acceptable, in that it allows users to eliminate instrument and protocol biases in raw data and standardise data between analytical platforms (see Section 3.2). However, once spectral clusters have been generated, merging chemical information into broader categories sacrifices precision for confidence in interpretation. For instance, in a dataset describing the metabolomes of 472 tropical plant species (unpublished data), we identified a total of 515 annotated metabolites metabolites from 7649 spectral clusters, but this could be collapsed into 146 metabolite classes or 15 metabolite superclasses. Such information loss is not unique to metabolomics – in metagenomics, which has become a powerful tool in soil microbial ecology, it is common to map less than 1% of genes to a function (Bahram *et al.* 2018). Ultimately, while metabolite annotation is under development, we argue that it is already sufficiently developed to be useful in ecology. Indeed, given both ongoing developments in metabolite annotation and the growing usage of metabolomics in ecology (Fig. 3), we are on the cusp of a revolution in ecological metabolomics akin to that seen in ecological genomics over the past decade (Baldwin 2012).

Box 3 | Nine Key Questions for the Future of Ecological Metabolomics

Conceptual framework

Q1. How does the metabolome vary across the plant kingdom? Which aspects are strongly phylogenetically constrained, and which relate to established life history trade-offs? Does measuring the metabolome capture additional axes of specialisation not apparent from classical functional traits?

Q2. How does global environmental variation (e.g. climate) shape the plant metabolome? Do species with larger geographical ranges possess more diverse, or less specialised, metabolisms? Do species from distinct environmental contexts also possess distinct metabolomes?

Q3. How does the metabolome fluctuate over time? Which aspects remain static (i.e. providing a lifetime fitness benefit; see Section 2.1), and which aspects are dynamic (i.e. providing a short-lived fitness benefit; see Section

2.2)? Can this knowledge be used to disentangle the mechanisms behind the (lack of) classical functional trait variation under short-term environmental change?

Technical framework

Q4. Can insights from the conceptual framework, above, as well as from the growing number of ecological metabolomics studies (Fig. 2d), be combined to derive a suite of candidate molecular functional traits that can be measured in the metabolome?

Q5. What are the limitations of using metabolomics approaches in ecology? For which applications, and in which situations, does the metabolome not provide additional value to existing trait-based approaches?

Q6. What are the most appropriate sample collection and extraction methods for ecological metabolomics? Is there a "one size fits all" solution, or should researchers pick from a number of standardised options, each with their own benefits and limitations?

Q7. Following the lead of functional trait ecology (Pérez-Harguindeguy *et al.* 2013; Dawson *et al.* 2018) and building on early momentum (Sedio *et al.* 2018a; Uthe *et al.* 2021), can we develop a handbook for the standardised collection, acquisition and analysis of ecological metabolomics data?

Data framework

Q8. To what extent can existing metabolomics data be used in comparative ecological studies? Which comparisons are valid (i.e. what are the limits to inferences)? For which species and ecosystems is there already sufficient coverage, and where do we need to focus future effort?

Q9. Can further momentum be generated for sharing and reusing metabolomics data hosted on online repositories? What tools are necessary for integrating and synthesising existing and future metabolomics and ecological data (e.g. knowledge graph exploitation, *R* packages, new statistical methods, cross-talk between database owners, adequate metadata)?

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