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FROM THE COVER

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Alternative developmental and transcriptomic responses to host plant water limitation in a butterfly metapopulation

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Abstract

Predicting how climate change affects biotic interactions poses a challenge. Plant–insect herbivore interactions are particularly sensitive to climate change, as climate-induced changes in plant quality cascade into the performance of insect herbivores. Whereas the immediate survival of herbivore individuals depends on plastic responses to climate change-induced nutritional stress, long-term population persistence via evolutionary adaptation requires genetic variation for these responses. To assess the prospects for population persistence under climate change, it is therefore crucial to characterize response mechanisms to climate change-induced stressors, and quantify their variability in natural populations. Here, we test developmental and transcriptomic responses to water limitation-induced host plant quality change in a Glanville fritillary butterfly (*Melitaea cinxia*) metapopulation. We combine nuclear magnetic resonance spectroscopy on the plant metabolome, larval developmental assays and an RNA sequencing analysis of the larval transcriptome. We observed that responses to feeding on water-limited plants, in which amino acids and aromatic compounds are enriched, showed marked variation within the metapopulation, with individuals of some families performing better on control and others on water-limited plants. The transcriptomic responses were concordant with the developmental responses: families exhibiting opposite developmental responses also produced opposite transcriptomic responses (e.g. in growth-associated transcripts). The divergent responses in both larval development and transcriptome are associated with differences between families in amino acid catabolism and storage protein production. The results reveal intrapopulation variability in plasticity, suggesting that the Finnish *M. cinxia* metapopulation harbours potential for buffering against drought-induced changes in host plant quality.

KEYWORDS
insects, lepidoptera, life-history evolution, phenotypic plasticity, species interactions, transcriptomics

1 | INTRODUCTION

Because changes in the abiotic environment have different effects on different species, human-induced climate change affects species interactions (van Asch & Visser, 2007; Bale et al., 2002; Tylianakis et al., 2008; Voigt et al., 2003). As species interactions are abundant even in the simplest of natural systems (Wirta et al., 2015), much of the effects of climate change are manifested indirectly, rendering any predictions on how climate change affects natural populations difficult (Gilman et al., 2010; Siepielski et al., 2018; Tylianakis et al., 2008; Van der Putten et al., 2010). How much individuals' fitness will be affected by quantitative and/or qualitative changes in interacting species depends on their ability to mount plastic responses to compensate for the abrupt changes. In addition, to ensure long-term persistence of populations via adaptive evolution and increased demographic stability, intrapopulation variation in the plastic responses is required (Forsman & Wennersten, 2016; Gotthard & Nylin, 1995; Miner et al., 2005; Price et al., 2003; Via & Lande, 1985; Wennersten & Forsman, 2012). Therefore, to clarify prospects for population persistence under climate change, it is crucial to identify and characterize the response mechanisms associated with changes in interspecific interactions, and examine how they vary within populations.

Interactions between terrestrial insect herbivores and their host plants are particularly susceptible to climate change (van Asch & Visser, 2007; Bale et al., 2002). Climate change not only alters the spatial and phenological availability of host plants, but also modifies their quality, which then cascades further to the behaviour and performance of insect herbivores (Jamieson et al., 2012, 2017; Pincebourde et al., 2017). In particular, changes in spatiotemporal variability of precipitation and the resulting water stress can greatly impact the concentrations of plant primary (e.g. amino acids, carbohydrates and lipids) and secondary metabolites (e.g. alkaloids, terpenes and complex phenols), the former of which are central to insect growth and development, and latter influence the rate of nutrient intake and absorption (Behmer, 2009; Dobler et al., 2011; Gershenzon, 1984; Isah, 2019; Rizhsky et al., 2004). How insect populations respond to changes in plant quality varies tremendously across species and studies, with some of the variability attributed to different feeding guilds of the insect herbivores and different drought stress severities across studies (Cornelissen et al., 2008; Gely et al., 2020; Gutbrodt et al., 2011; Huberty & Denno, 2004; Larsson, 1989; White, 1974).

Insect herbivores are central for nutrient cycling and functioning of entire ecosystems (Hunter, 2016; Kalinkat et al., 2015; Post, 2013; Rosenblatt & Schmitz, 2016; Weisser & Siemann, 2004), and therefore we need to gain detailed understanding of the abilities of insect populations to persist during global climate change. As intrapopulation variability in genetically determined traits and plastic responses are frequently associated with improved population persistence during environmental change (Forsman & Wennersten, 2016; Hughes et al., 2008; Miner et al., 2005; Reed et al., 2010; Wennersten & Forsman, 2012), it is of great importance to quantify intrapopulation variability in insect responses to host plant water stress and describe its mechanistic basis. However, we are aware of very few studies explicitly focusing on intrapopulation or among-population variability in insect herbivore responses to host plant water stress (Dai et al., 2015; Gibbs et al., 2012).

To characterize insect herbivore response mechanisms to water stress-induced changes in plant quality and examine intrapopulation variability therein, we need to (i) test how water stress changes plant quality, (ii) identify insect developmental responses to plant quality, (iii) characterize the associated transcriptomic/genetic responses, and finally (iv) quantify how the developmental and transcriptomic responses differ between individuals from different genetic backgrounds (Hoffmann & Sgrò, 2011; Rosenblatt & Schmitz, 2016). Despite long-lasting interest in insect responses to plant water stress (reviewed by Cornelissen et al., 2008; Huberty & Denno, 2004; Larsson, 1989), and despite the fact that studies on transcriptomic responses of insect herbivores to host plant compounds are emerging (Nallu et al., 2018; Seppey et al., 2019; Vogel et al., 2014), we are not aware of studies combining the two.

Here, we investigate how water stress in the ribwort plantain (*Plantago lanceolata*) cascades into the performance of the specialist Glanville fritillary butterfly (*Melitaea cinxia*) larvae, and how the responses vary within a Finnish metapopulation of the butterfly. Twenty-five years of survey data have revealed that precipitation across larval stages is positively associated with regional population growth rates of *M. cinxia* (van Bergen et al., 2020; Kahilainen et al., 2018; Tack et al., 2015). The tight connection between precipitation and population growth rate challenges the long-term persistence of the metapopulation, because any change in precipitation can have large impacts on the size and dynamics of the metapopulation. Indeed, prolonged drought events and a reduction in the spatial variability of precipitation in the last decade have resulted in abrupt declines in the number of larval nests, synchronization of regional population growth rates and increased year-to-year size fluctuations of the metapopulation (van Bergen et al., 2020; Hanski & Meyke, 2005; Kahilainen et al., 2018; Tack et al., 2015). Therefore, to evaluate the sensitivity of the metapopulation to the harmful effects of climate change, we need to map the individual-level processes that couple population growth rates with precipitation (Johnston et al., 2019).

To examine the mechanisms via which host plant water stress cascades into larval performance, we combined host plant metabolic profiling with development assays and full-transcriptome sequencing of herbivore larvae. First, we profiled metabolic differences between well-watered and water-limited host plants using proton nuclear magnetic resonance spectroscopy (^{1}H-NMR). Second, we tested how the performance of developing larvae was affected by host plant water limitation. Third, we examined larval gene regulatory responses to water-limited host plants by sequencing full transcriptomes of 77 female larvae (RNA-seq). Finally, to examine intrapopulation variation in the plastic responses, we compared the phenotypic and transcriptomic responses across full-sib families originating from different parts of the metapopulation.

2 | MATERIAL AND METHODS

2.1 | The ribwort plantain

The ribwort plantain (*Plantago lanceolata*, Plantaginaceae) is found across the studied *Melitaea cinxia* metapopulation and it is the more abundant of the two host plant species of the butterfly (see below) (Ojanen et al., 2013). *P. lanceolata* produces antiherbivore and antifungal chemicals (iridoid glycosides and phenolic compounds), amounts of which can vary with plant genotype, age and environmental conditions (Bowers et al., 1992; Bowers & Stamp, 1993). Iridoid glycoside concentrations in *P. lanceolata* have previously been found to correlate positively with *M. cinxia* oviposition preference and larval growth rate (Nieminen et al., 2003; Saastamoinen et al., 2007) and they increase feeding rate in *Junonia coenia*, another Nymphalid butterfly specialized on host plants with these defensive compounds (Bowers & Puttick, 1989).

We collected seeds from several *P. lanceolata* individuals within a single natural population in the Åland Islands (60.196°N, 20.704°E) and after germination planted 360 plants in 0.75 L pots (two saplings each). We reared the plants for 3 months in controlled glasshouse conditions (~40 ml water per pot daily, 15 h:9 h light–dark photoperiod with 26:18°C temperature cycle) before initiating the water limitation treatment. We exposed 240 plants to a water limitation treatment in which daily watering was reduced by 50% compared to controls (20 ml per pot). This watering scenario was developed in a pilot study in which we experimented with minimum watering allowing the plants to stay alive. More plants were allocated to the water limitation treatment than to the control treatment, because plants produced substantially less leaf biomass in the water limitation treatment (Figure S1). To minimize temporal trends in plant quality caused by the plants acclimatizing to altered water availability, we initiated the water limitation treatment well in advance (47 days) to the larval exposure (see below).

2.2 | Leaf metabolomics assays

Each morning prior to watering (9–10 AM), we randomly harvested *P. lanceolata* leaves from control and water-limited plants and cut them into 2.25 cm^2 pieces, discarding the basal and tip parts of the leaves. We used these pieces to feed larvae during the experiment (see below), and selected a random subset of six pieces from both treatments for metabolomics assays. For the assays, we recorded the fresh biomass of each piece of leaf, snap froze the pools in liquid nitrogen and stored them at −80°C. We then freeze dried the samples for 48 h after which we measured their dry weight, estimated relative water content in the sample (i.e. [fresh mass − dry mass]/fresh mass), and prepared the samples for ^{1}H-NMR following the protocol described by Kim et al. (2010). The ^{1}H-NMR spectra of the pool samples were then recorded at the Finnish Biological NMR Center (Institute of Biotechnology, University of Helsinki) and we further processed the obtained spectra for statistical analyses using MNOVA version 10.0.2 software (Mestrelab Research S.L.) (Supporting Information methods).

2.3 | The Glanville fritillary butterfly metapopulation in Finland

The Glanville fritillary butterfly (*M. cinxia*; Lepidoptera: Nymphalidae) is widespread across Eurasia. In Finland, *M. cinxia* is found in the Åland islands archipelago where it exists as a metapopulation inhabiting a network of ~4400 discrete habitat patches (mean patch area = 1,932 m^2; *SD* = 4617 m^2) containing at least one of its two host plants, *P. lanceolata* (see above) or *Veronica spicata* (Plantaginaceae) (Nieminen et al., 2004). Whereas *P. lanceolata* is present in practically all habitats suitable for *M. cinxia*, *V. spicata* is found in less than 15% of habitats. The *M. cinxia* metapopulation and its host plants have been surveyed annually since 1993, and the extinction–(re) colonization dynamics of the system have been documented in detail (Hanski et al., 1994; Hanski et al., 2017; Nieminen et al., 2004; Ojanen et al., 2013; T. Schulz et al., 2019).

In the Åland islands *M. cinxia* is univoltine and the hatching larvae spend their prediapause development in gregarious full-sib groups on a single or a couple of host plant individuals and typically enter diapause in the 5th larval instar (Fountain et al., 2017; Kuussaari et al., 2004; Wahlberg, 2000). The prediapause larvae are therefore highly susceptible to any changes in host plant quality caused by fluctuations in environmental conditions (Kuussaari et al., 2004).

2.4 | Experimental *M. cinxia* families

We created nine experimental full-sib larval family groups by mating individuals originating from different parts of the range of the natural Åland islands metapopulation (Figure S2). We collected diapausing larvae from nine localities in which *P. lanceolata* was the only host plant species present and which differed in their average percentages of *P. lanceolata* desiccation (Figure S2, Table S1). We allowed the larvae to continue diapause in a climate chamber (+5°C, 95% air humidity) for 5 months and after breaking diapause reared them to adults (12 h:12 h light–dark photoperiod, 28:15 °C temperature cycle) with daily *ad libitum* provision of control reared *P. lanceolata* leaves.

We mated virgin females with males derived from the same habitat patch but different overwintering nests. We placed the mated females on host plants immediately after mating, collected daily the egg clutches laid on the plant and—to have two replicates per full-sib family per treatment—picked two larval groups of a minimum of 80 larvae each from each female to enter the experiment. To minimize potential quality differences caused by clutch rank (Rosa & Saastamoinen, 2017), we selected the larval groups from within the first three larval groups.

This design allowed us to maintain the original population genetic structure while minimizing the risk of inbreeding in the larval

groups. Thus, it best captures the potential range of variability within the metapopulation. However, although we have aimed to maximize genetic differences between families, the *M. cinxia* metapopulation is characterized by dispersal and rapid population turnover at each site, and thus the among-family variability best corresponds to intra(meta)population variability (DiLeo et al., 2018; Fountain et al., 2017; Orsini et al., 2008).

2.5 | Treatments and developmental assays

On the day after hatching, we divided the larval groups into four smaller groups of 20 larvae each and placed them on separate Petri dishes (9 cm diameter, 1.5 cm deep) lined with filter paper. We then randomly assigned each of the dishes to one of four different treatments mimicking different temporal exposures to drought-stressed host plants. In addition to a control treatment, in which we fed the larvae with control reared host plants only, the larvae experienced water-limited host plants at different stages during prediapause development: (i) at late prediapause development during 3rd and 4th larval instars (late water limitation), (ii) at early prediapause development during 1st and 2nd larval instars (early water limitation), and (iii) throughout their prediapause development from the 1st to the 5th larval instar (constant water limitation) (Figure S3). In all treatments, we fed the larvae daily with pieces of host plant leaf tissue corresponding to the treatment. We provided *ad libitum* food such that—to avoid feeding on old leaf tissue with potentially altered phytochemistry—the larvae consumed most but not all of the leaf tissue during the next 24 h after provisioning (Supporting Information methods).

During the experiment, we monitored daily the development and mortality in all larval groups, and recorded development time to diapause, body mass at diapause and mortality during development. Once the last larva on a Petri dish had entered diapause, we allowed them to spend another 4 days under normal rearing temperature and photoperiod, after which we measured their body mass and placed them in climate chambers (+5°C, 95% air humidity) for diapause. We allowed the larvae to diapause for 6 months, after which we woke them up and recorded overwintering mortality.

With one exception, unexplained mortality during the rearing (i.e. mortality that could not be explained by accidents during handling) was low in all families and in all treatments (mean = 1.1 larvae per Petri dish, *SD* = 1.4 larvae). Only the control treatment of the first replicate larval group of family F-5 had a mortality of 55% (11 larvae). As the larvae in this group were developing poorly in general, we concluded it to be an outlier case, potentially suffering from a disease or some other unknown agent, and decided to exclude this larval group from any further analyses.

2.6 | Transcriptomics sampling and sequencing

When more than 50% of the larvae on a Petri dish had spent two full days in the 4th larval instar, we sampled either 10 (from the first larval group of each full-sib family) or five (from the second larval group of each full-sib family) larvae for RNA and DNA extraction (see Table S2 for exceptions). Before noon on the day of sampling, we provided the larval groups with leaf tissue matching their treatment and monitored that they fed on the plant before sampling to ensure sampling larvae that are feeding. We weighed and sampled the larvae for RNA and DNA extraction ~1.5–2 h after feeding, by immersing them in liquid nitrogen and stored the sampled larvae at -80 °C until further processing.

To extract RNA and DNA, we placed the larvae individually in dry ice and homogenized the frozen larvae in their entirety and separated RNA and DNA following a TRIzol-chloroform purification protocol in combination with QIAamp DNA Mini Kit protocol (Qiagen) (Supporting Information methods). We then used the extracted DNA for determining the sex of the sampled individuals using sex-specific markers (Supporting Information methods). To ensure an adequate sample size and eliminate expression differences between sexes, we chose to focus on the transcriptomes of females. We thus selected five females per family per treatment for transcriptome sequencing (see Table S2 for exceptions).

Library preparation from whole RNA and sequencing was conducted at the University of Helsinki Institute of Biotechnology (http://www.biocenter.helsinki.fi/bi/). The libraries were prepared using an Illumina TruSeq Stranded mRNA Library Prep Kit and sequenced to a depth of a minimum of 13.3 million reads per sample (mean = 17.3 million, *SD* = 1.2 million) in three separate sequencing runs (all samples included in all runs) using Illumina NextSeq 500, with 85 bp + 65 bp forward and reverse paired-end reads, respectively.

2.7 | Sequence data preprocessing, *de novo* transcriptome assembly and expression quantification

Prior to downstream analyses we removed all Illumina adapter sequences and trimmed low-quality sequences using TRIMMOMATIC (version 0.33; Bolger et al., 2014) and verified family structure of the larvae by determining pairwise genetic distances of the individuals from single nucleotide polymorphisms (SNPs) observed in the sequenced reads (Supporting Information methods).

Despite a reference genome (Ahola et al., 2014), we decided to map the obtained reads against a *de novo* transcriptome. We chose this approach because the gene models in the available genome were predicted using RNA-seq data collected primarily from adult butterflies or postdiapause larvae and may have thus produced unreliable or inadequate predictions of genes expressed only during prediapause larval stages or during nutritional stress.

To build one complete and diverse *de novo* transcriptome, we first built two transcriptomes using the obtained preprocessed reads with both the TRINITY (Grabherr et al., 2011; Haas et al., 2013) and VELVET/OASES (M. H. Schulz et al., 2012) pipelines. We then combined the two to obtain a single transcriptome of 69,182 putative transcripts using EVIDENTIALGENE (Gilbert, 2016, 2019). We then mapped the transcriptome against the mitochondrial genome of *M. cinxia* using GMAP (Wu & Watanabe, 2005) and removed all transcripts that exhibited

any probability to map to the mitochondrial genome (29 transcripts in total). We then checked the transcriptome for potential contaminants using AAI PROFILER (Medlar et al., 2018) and electronically annotated the combined transcriptome for transcript protein product descriptions and biological process Gene Ontology terms (BP GO terms; Ashburner et al., 2000) using the PANNZER2 annotation web server (Törönen et al., 2018). For both protein product descriptions and BP GO terms we accepted only annotations above a 0.7 positive predictive value.

Finally, to obtain expected read counts for each sample we mapped the preprocessed reads against the transcriptome using RSEM (B. Li & Dewey, 2011) with BOWTIE ALIGNER (Langmead et al., 2009). For further details on building the *de novo* transcriptome and mapping the reads, see Supporting Information methods.

2.8 | Statistical analyses

To analyse the proportional water content and the metabolomic response of *P. lanceolata*, we fitted a generalized linear model (GLM) with a Beta-distribution and a constrained correspondence analysis (CCA), respectively. We implemented the GLM following Bayesian inference in the STAN statistical modelling platform (Carpenter et al., 2017) via R (version 3.6.1; R Core Team, 2019) by using the packages BRMS (version 2.7.0; Bürkner, 2017, 2018) and RSTAN (version 2.17.3; Stan Development Team, 2018) (Supporting Information methods). We implemented the CCA using the R package VEGAN (version 2.5–2; Oksanen et al., 2018). We included the water limitation treatment, days since the start of the experiment, and their interaction with the water limitation treatment as explanatory variables in the GLM and as constraints in the CCA. In addition to the simple temporal trends modelled in the CCA, we tested for convergence of metabolomes in the control and water-limited plants. For this, we extracted Euclidean distances between the metabolomes of the two treatments and fitted a Bayesian Gamma distribution GLM in STAN, with days since the beginning of the experiment as an explanatory variable (Supporting Information methods).

We analysed the different phenotypic responses of the larvae by fitting a series of Bayesian generalized linear mixed effects models (GLMMs). We modelled development time using a shifted lognormal distribution, diapause mass using a normal distribution and overwintering mortality using a binomial distribution. We included water limitation treatment as the only explanatory variable in the models and allowed the estimates to vary among families (i.e. a group-level slope). Additionally, to account for average response differences among families and egg clutches, we added group-level intercept terms for both family and egg clutch identity nested within the larval family (Supporting Information methods). As with the above described GLM for water content, we did the GLMMs in STAN via the R packages BRMS and RSTAN.

Because the majority of larvae in three of the families (F-7, F-8 and F-9; Figure S4, Table S1) entered diapause in the 4th larval instar instead of the 5th (in which we typically observe diapause under laboratory conditions), we analysed the phenotypic responses in families exhibiting primarily 5th instar and 4th instar diapause in separate models. We chose to do this because the distributions of the phenotypic responses of the 4th instar diapausing larvae are widely different from those diapausing in the 5th.

Additionally, we observed that the metabolomes of the control and water-limited plants converged as the experiment proceeded (see below), with metabolomes being more distinct during the first two instars of the larvae (~10 days; Figure S5). We thus chose to focus on water limitation during early development and combined the control treatment with late water limitation and early water limitation with constant water limitation (Figure S3). Throughout the text we focus primarily on comparisons done for the combined treatments, and refer to these as control and early development water limitation, respectively. We report results differentiating between all temporal water limitation treatments in the Supporting Information.

To explore patterns across the transcriptomic data set, we analysed the effects of family identity, treatment and their interaction on gene expression patterns using redundancy analysis (RDA) as implemented in the R package VEGAN (version 2.5–2; Oksanen et al., 2018). Prior to model fitting, we normalized the expected count data according to weighted trimmed mean of M-values (TMM; Robinson & Oshlack, 2010) as implemented in the BIOCONDUCTOR R package EDGER (McCarthy et al., 2012; Robinson et al., 2010), and retained only transcripts with >1 normalized count-per-million in a minimum of four samples. As in the analyses regarding developmental performance, we combined the control treatment with late water limitation and early water limitation with constant water limitation (Figure S3). We then constrained the ordination of the log-transformed counts-per-million values of each transcript with family identity, treatment and their interaction while partialling out potential effects of larval body mass (i.e. model conditional on larval mass).

We approached the biological mechanisms associated with the RDA results in two ways. First, we checked for enrichment of BP GO terms within the set of transcripts among the 5% highest absolute loadings along the RDA axes. Second, we examined individually the annotated protein products in a smaller group of strongest loading per cent of the transcripts. For the former approach we checked for enrichment of BP GO using the BIOCONDUCTOR R package TOPGO (Alexa & Rahnenfuhrer, 2019) and retained terms using a statistical significance threshold of <0.01 (Fisher's exact test with "elim" algorithm; Alexa et al., 2006). We then clustered the retained BP GO terms hierarchically according to their semantic similarity (Schlicker et al., 2006) using the BIOCONDUCTOR R package VISEAGO (Brionne et al., 2019). For illustration of average expression in transcripts closely associated with the enriched BP GO terms, we extracted the average *z*-score across all transcripts annotated for the enriched GO BP term or its direct offspring terms (including those not among the 5% with strongest associations). For individual examination of transcripts in the latter approach, we examined transcripts that were annotated for descriptions of protein products and validated the annotations manually with NCBI nucleotide BLAST (Boratyn et al., 2013).

Next, we proceeded to test family-specific transcriptomic responses. For this we fitted transcriptwise negative binomial GLMs with quasi-likelihood F-tests as implemented in the BIOCONDUCTOR R package EDGER (McCarthy et al., 2012). We conducted filtering and

normalization as described above for the RDA and fitted the model such that each family-by-treatment combination was treated as a separate treatment level. Again, to account for the fact that plant metabolites in the different treatments converged during the experiment, we contrasted the coefficients of control and late water limitation with those of early and constant water limitation. We then selected transcripts with a false discovery rate (FDR) below 0.05 and checked for enrichment of BP GO terms using the BIOCONDUCTOR R package TOPGO (Alexa & Rahnenfuhrer, 2019) as described above for the RDA across families and treatments.

3 | RESULTS

3.1 | Host plants shift metabolome but not water content upon water limitation treatment

We observed that the leaf tissue water content did not differ between the control and water-limited plants (Table 1). However, water content may have increased slightly with time in both treatments, but the coefficient differed from zero only once the credible interval was narrowed down to 90% (not shown).

The metabolic profiles differed between the control and water-limited plants, and both treatments exhibited slightly different temporal trends. These are illustrated by the statistically significant treatment (pseudo-$F_{1,32}$ = 9.759, p <0.001), temporal trend (pseudo-$F_{1,32}$ = 15.308, $p < 0.001$) and their interaction constraints (pseudo-$F_{1,32}$ = 3.308, $p = 0.023$) in the CCA model. The metabolic profiles of plants in the two treatments were separated along two statistically significant constrained axes (CCA1: pseudo-$F_{1,32}$ = 23.393, $p < 0.001$; CCA2: pseudo-$F_{1,32}$ = 3.779), that together account for 45.5% of the variation in the sample metabolite contents (Eigenvalues: CCA1 = 0.038, CCA2 = 0.006, total =0.099; Figure 1a). In both treatments the metabolite composition changed in the positive direction along CCA1, whereas the temporal trends were opposite along CCA2 (Figure 1a). Despite the opposite trends along CCA2, the overall metabolic profiles of the control and water-limited plants converged with time, suggesting that the plants in the two treatments became more similar as the experiment proceeded (Figure S5, Table S3).

A closer examination of the metabolites associated with the CCA axes revealed that water-limited plants had higher concentrations of amino acids, aromatic compounds and organic acids, and that in carbohydrates the responses were mixed (Figure 1b,c). Compounds that we could reliably identify and that clearly increased in response to water limitation (i.e. positively associated with CCA1or CCA2) included, for example, the amino acids proline, glutamine and glutamate, the sugars glucose and xylose, the iridoid glycoside catalpol and the phenylethanoid glycoside verbascoside (Figure 1b). The few identifiable compounds that were clearly more abundant in the control treatment (i.e. negatively associated with CCA1 or CCA2) included sucrose and the iridoid glycoside aucubin. The accumulation of proline, glutamine and glutamate is a well-characterized response of water stress and indicates that water limitation treatment was stressful (Hayat et al., 2012; Verbruggen & Hermans, 2008).

3.2 | Divergent developmental responses to host water stress across *Melitaea cinxia* families

Feeding on water-limited plants did not induce phenotypic responses that could be generalized across the studied nine larval families. Instead, we discovered that the responses differed considerably between families (Figure 2; Table S4). We observed three kinds of responses in the studied families: (i) families in which early development water limitation decreased performance in at least one of the development-related traits (i.e. increased development time, decreased diapause body mass or increased probability of overwintering mortality) (F-2, F-7 and F-9), (ii) families in which early development water limitation improved the performance in at least one of the phenotypic traits (F-3, F4, F-5, F-6) and (iii) families in which early development water limitation led to mixed responses across traits (F-1 and F-8) (Figure 2). The two families in the last category exhibited larger diapause body masses and higher overwintering mortality when exposed to early development water limitation (Figure 2a,c–f). Additional analyses maintaining all temporal water limitation levels produced very similar results and supported combining treatment levels according to early water limitation (Figure S6, Table S5).

3.3 | Transcriptomic differences between families and divergent transcriptomic responses to water-limited host plants

Both the overall transcriptomes and the transcriptomic responses to water limitation differed between *M. cinxia* families. In addition, the transcriptomic patterns were parallel to the phenotypic patterns: families that exhibited developmental responses opposite

TABLE 1 The association between plant leaf tissue water content, drought treatment and time.

Covariate	Est. coef.	*SE*	95% Cr.I.	
			Lower	Upper
Intercept	1.033	0.053	0.931	1.137
Water limitation	−0.086	0.073	−0.233	0.058
Time	0.004	0.002	0.000	0.009
Water limitation × Time	0.001	0.003	−0.005	0.008

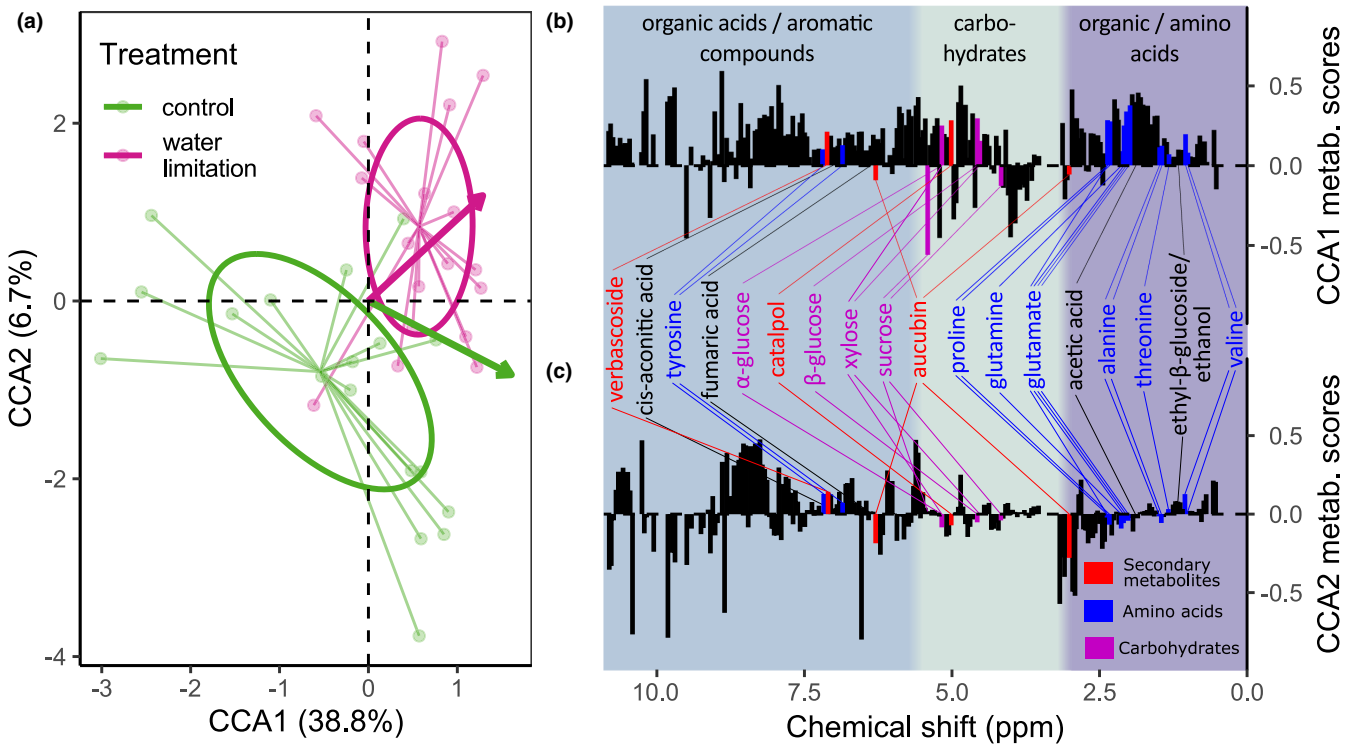

FIGURE 1 The metabolome differences between control and water-limited *Plantago lanceolata*. (a) The sample scores (points), group centroids (the centre of line spiders) and the 95% confidence interval of the centroids (ellipses) along the first two constrained axes (CCA1 and CCA2) of a constrained correspondence analysis (CCA) on the associations between plant metabolite composition, water limitation and time since the initiation of the experiment. Arrows depict the temporal trends along the displayed axes. (b,c) The association between plant chemical compounds with the first two constrained CCA axes. The compounds are arranged according to their known ^{1}H-NMR chemical shifts and the typical ranges of different types of compounds are shaded with different background colours

to each other also produced opposite transcriptomic responses (Figures 2 and 3a). From the RDA model, we identified family (pseudo-$F_{3,68} = 13.044$, $p < 0.001$), treatment (pseudo-$F_{1,68} = 1.703$, $p = 0.033$) and their interaction (pseudo-$F_{3,68} = 1.977$, $p < 0.001$) as statistically significant constraints, with the constraints together capturing 35.9% of the total variance in the data (variance: total =2385.2, constrained =856.8, body mass conditioned =282.6). Of the resulting seven constrained axes, four were statistically significant (RDA1: pseudo-$F_{1,68} = 18.049$, $p < 0.001$; RDA2: pseudo-$F_{1,68} = 12.662$, $p < 0.001$; RDA3: pseudo-$F_{1,68} = 9.010$, $p < 0.001$; RDA4: pseudo-$F_{1,68} = 3.738$, $p < 0.001$) and together they explain 37.9% of the variability in the gene expression data (Eigenvalues: RDA1 = 330.7, RDA2 = 232.0, RDA3 = 165.1, RDA4 = 68.5, total =2102.6).

The first three of the significant constrained axes primarily illustrate the overall transcriptomic differences between families and together explain 34.6% of the variability. RDA1 separates families in which performance was improved in the early development water limitation (families F-5 and F-6) from family F-2, in which performance was decreased in the water limitation treatment (Figure 3a). Family F-1, a family that exhibited mixed developmental responses, separates from the others along RDA2 (Figure S7a).

The fourth constrained axis (RDA4) highlights both the responses to host water limitation and the response differences between the families (i.e. a family-by-treatment interaction) and explains 3.3% of the variability in the transcriptomes (Figure 3a). For most families, the transcriptomes of the early development water limitation experiencing larvae were negatively associated with RDA4 (and vice versa for the control), but for F-2 the pattern was exactly opposite (Figure 3a). Therefore, it seems that RDA4 separates individuals based on their developmental performance, with individuals exhibiting reduced performance grouping on the positive side of RDA4. An additional RDA model maintaining all temporal water limitation levels produced practically identical results (Table S6).

3.4 | Transcriptomic differences between families reflect differences in metabolic and nutrient storage-related processes

A large part of the overall among-family transcriptomic variability relates to differences in, for example, metabolizing amino acids, activity of the intracellular transport system, nutrient storage and development of the sensory system. The first two are revealed by the enrichment of BP GO terms among the transcripts that have a large contribution to the among-family transcriptomic variability (strongest loading 5% of transcripts on RDA1) (Figure 3b; Tables S7 and

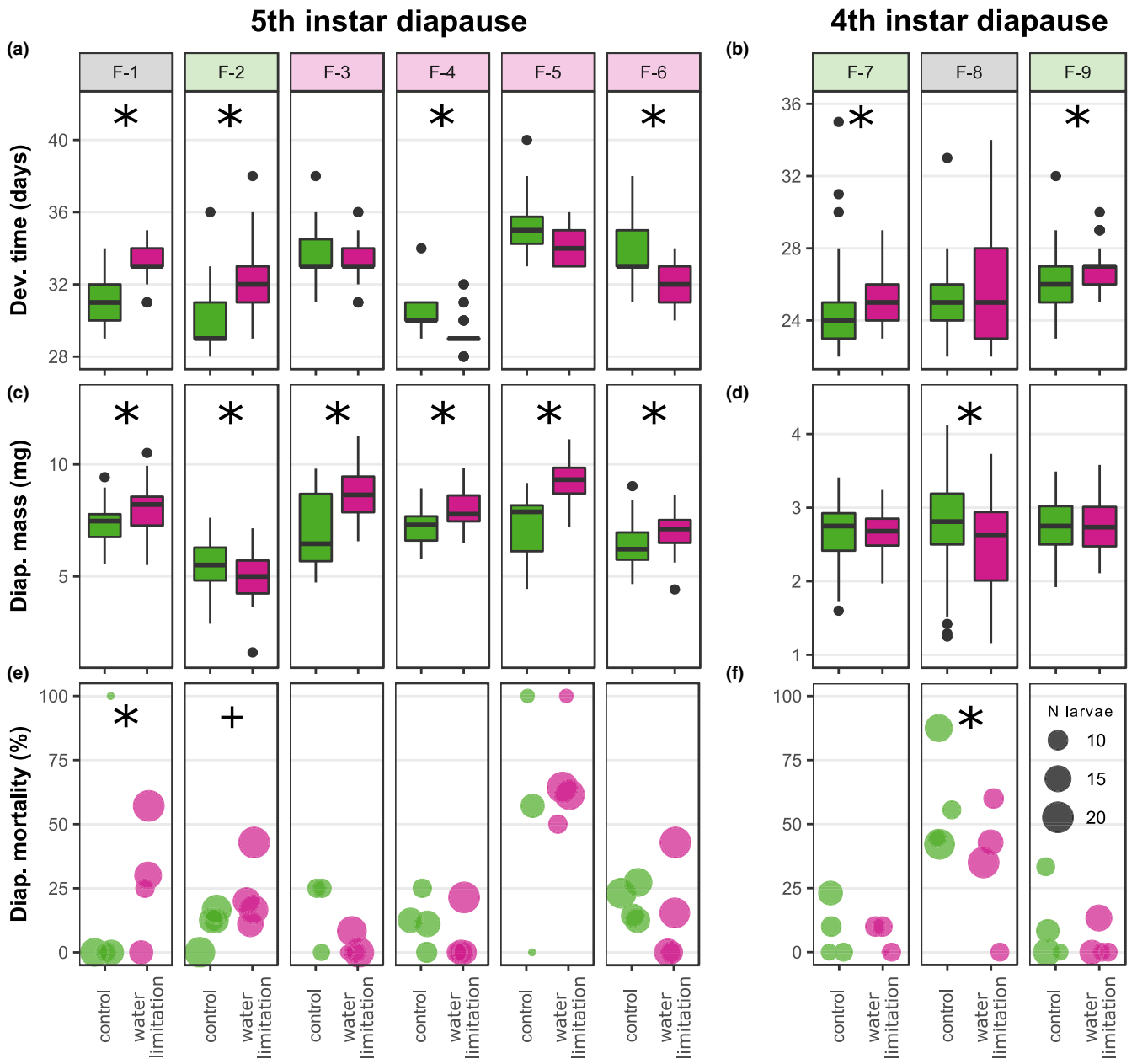

FIGURE 2 Opposite phenotypic responses of *Melitaea cinxia* to feeding on water-limited *Plantago lanceolata* during the first two instars of their prediapause development. Between-treatment comparisons in which the 95% and 90% credible intervals of the coefficients do not overlap are indicated with a star and a plus sign, respectively. The panel colour for each family illustrates the treatment in which performance was better. Grey panel colour indicates ambiguous responses in different traits. Note the different scales for development time and diapause mass between the 5th (a, c) and 4th instar (b, d) diapause families

S8), and the last two stand out when examining individual influential transcripts (strongest loading 1% of transcripts on RDA1) (Table S9).

The BP GO terms enriched among the transcripts associated with RDA1 suggest that families F-5 and F-6 had higher expression of transcripts involved in allantoin metabolism, drug catabolism, intraciliary transport and microtubule-based movement (Figure 3b). The first two are enriched due to a partly overlapping set of transcripts, and thus both actually point towards increased catabolism of amino acids and allantoin, a purine metabolism product (Bursell, 1967) (Tables S7 and S8). Intraciliary transport and microtubule-based movement are central in the intracellular transportation system involved in a wide range of cellular processes such as endocytosis, autophagy, vesicle trafficking and cell cycle control (Berbari et al., 2009; Finetti et al., 2019; Hancock, 2014; Hua & Ferland, 2018).

The individual transcripts that contribute most to differences between families reveal that family F-2 (and to some extent F-1) produce more compounds related to nutrient storage and development of the sensory system. Transcripts related to nutrient storage include those coding for transmembrane protein 135, moderately methionine-rich storage protein a, and arylphorin subunit alpha

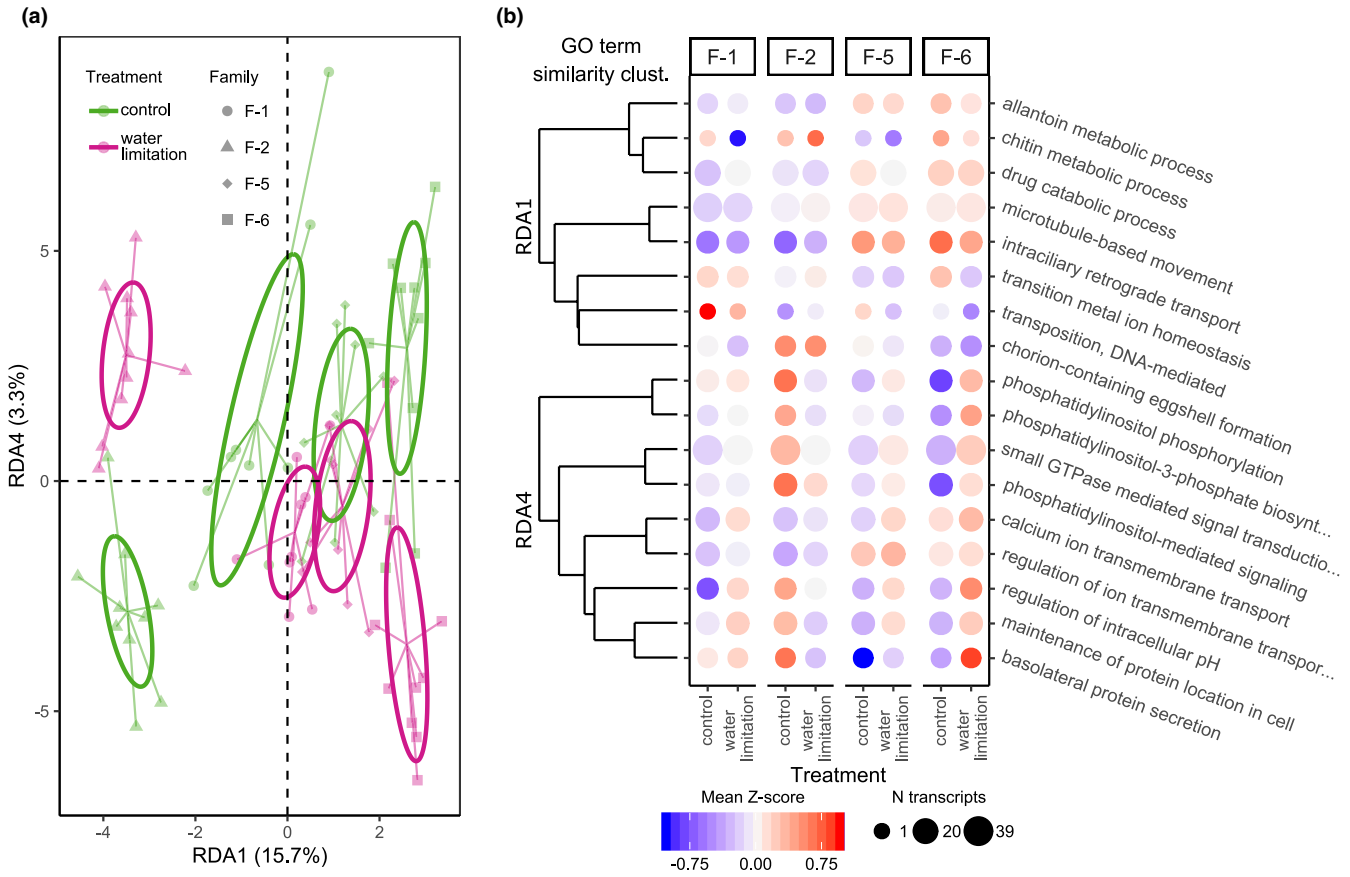

FIGURE 3 Differences between *Melitaea cinxia* families in the overall transcriptomic profiles and transcriptomic responses to plant water limitation. (a) The individual scores (points), group centroids (the centre of line spiders) and the 95% confidence interval of the centroids (ellipses) along two constrained axes (RDA1 and RDA4) of an redundancy analysis (RDA) examining the associations between family, treatment and their interaction with the transcriptome of *M. cinxia* larvae. (b) Biological processes enriched in the transcripts showing strongest associations with RDA1 and RDA4, their clustering according to GO-term semantic similarity, and the average expression levels of transcripts associated with the processes. Z-score standardization of expression levels was conducted across families

(Table S9). In holometabolous insects, these proteins are typically associated with preparing for metamorphosis, diapause or periods of environmental/nutritional stress (Ashfaq et al., 2007; Denlinger, 2000; Exil et al., 2010; Sonoda et al., 2007). Transcripts related to the development of the sensory system include those that translate into peripherin-2-like protein, lachesin and gustatory receptor 22 (Table S9). All of the above transcripts are negatively loaded with RDA1 and thus have greatest expression levels in family F-2 and lowest in F-6.

3.5 | Divergent transcriptomic responses to water limitation reflect differences in growth

As RDA4 essentially separates poorly developing individuals from those performing better (see above), the transcripts and the processes involved with RDA4 highlight a stress response that is similar across families, but induced by different treatments in different families. Here, the evidence seems to point towards processes directly or indirectly related to growth and cell proliferation.

The BP GO terms enriched among the transcripts associated with RDA4 (strongest loading 5% of transcripts on RDA1) reveal that poorly performing individuals had lower expression in transcripts that are connected to endocytosis, endosome formation and endosome trafficking, all of which are associated with nutrient intake, TOR signalling X and ultimately growth (Corvera et al., 1999; Flinn et al., 2010; Hennig et al., 2006;. Li et al., 2013) (Figure 3b; Table S10). For example, the phosphatidylinositol- and small GTPase-mediated signalling are negatively associated with RDA4 and thus expressed less in individuals with reduced developmental performance (Figure 3). Both signalling processes are central for endocytosis (Berridge, 2016; Corvera et al., 1999; X. Li et al., 2013), and are further connected to calcium ion transmembrane transport and regulation of intracellular pH, which are also among the enriched processes (Berridge, 2016; Corvera et al., 1999; X. Li et al., 2013).

Detailed examination of individual transcripts (strongest loading 1% of transcripts on RDA4) further strengthens the interpretation that the responses are mostly related to TOR signalling and larval growth (Table S11). In addition to TOR itself, proteins directly (i.e. headcase protein) and indirectly (i.e. lysine-specific demethylase

LID, transient receptor potential cation channel TRPM and inositol 1,4,5-trisphosphate receptor) associated with TOR are negatively associated with RDA4. Some transcripts also point towards the production or functioning of specific tissues such as the cuticle (i.e. larval cuticle protein F1-like) or the nervous system (i.e. neurexin-1 alpha).

3.6 | Within families differential gene expression analysis suggests family-specific drought responses

A differential gene expression analysis conducted within each of the families revealed clear family dependency in the transcriptomic response to host plant water limitation (Figure 4). Families showed considerable differences in the numbers and identities of differentially expressed transcripts (Figure 4b). Within most families, individuals grouped quite clearly into the different treatments (Figure 4a). This finding is consistent with the results of the RDA in the sense that individuals in most families exhibit an observable transcriptomic response to early development exposure to a drought-stressed host (Figure 3a).

When we compared the groups of transcripts that were differentially expressed within each family, we noticed that family F-2 stands out as responding with many of the same transcripts that are differentially expressed in other families, but the expression is regulated in the exact opposite direction (Figure 4c). In fact, over half of the differentially expressed transcripts in family F-2 are ones that respond in the opposite direction in other families. This can also be seen in individual-level expression patterns (Figure 4a) and it highlights the family-by-treatment interaction observed along RDA4 (Figure 3a). Thus, both within-family differential expression analysis and global RDA identify a transcriptomic stress response that is shared between the families but is induced by different treatments in different families.

The shared (but opposite) transcriptomic response between families F-2 and F-6 is also reflected in enrichment of similar BP GO-terms (Figure 5; Table S12) and is, again, concordant with the RDA. In addition to prominent involvement of endocytosis and endosome trafficking-related signalling (small GTPase- and phosphatidylinositol-mediated signalling), both families also respond with protein (de-)ubiquitination, which is involved in targeting particles during endosome trafficking (Piper & Lehner, 2011) (Figure 5; Table S12).

In families F-1 and F-5, the processes were not as clearly associated with endocytosis. Nevertheless, the BP GO-terms enriched in family F-1 (i.e. regulation of DNA replication, proteasomal protein catabolism, and metabolism of chitin, AMP and glycerol-3-phosphate; Figure 5) suggest differences in cell replication and in nutrient metabolism between the control and water-limited host plant feeding individuals. The few enriched processes in F-5 are associated with immune responses (Figure 5), which could indicate a general stress response or exposure to infections. Based on the developmental responses, and high prediapause mortality in the control treatment of another larval group replicate of the same family, one would expect stress/infection-related responses in individuals feeding on the control treated plants (Figure 2). The expression patterns, however, do not support this as the immunity-related responses seems to be upregulated in the water limitation treatment (Figure 5), in which the developmental performance of this family was improved.

4 | DISCUSSION

We discovered that the Åland islands *Melitaea cinxia* metapopulation harbours divergent plastic responses to water limitation-induced changes in *Plantago lanceolata* nutritional quality. First, we found that water limitation resulted in an increase in amino acids and aromatic compounds, but the responses in carbohydrates and the iridoid glycosides aucubin and catalpol were mixed (Figure 1). Second, we found that whereas the larvae of four *M. cinxia* families grew larger, developed faster and/or had lower overwintering mortality when feeding on water-stressed host plants during early larval developmental stages, the opposite was true for three of the families, and mixed responses were observed in two families (Figure 2). Third, in a subset of four families, we observed transcriptomic patterns parallel to the divergent developmental responses, revealing a stress response in which growth-related signalling processes were downregulated in poorly performing individuals. However, in some families the response was induced by the water-limited plants and in others the control treated plants (Figures 3 and 4). Fourth, we observed between-family transcriptomic differences associated with the divergent phenotypic responses and—consistent with observed water limitation-induced changes in plant quality—discovered that they reflect differences in metabolizing amino acids, nutrient storage and intracellular transport (Figure 3b).

4.1 | Intrapopulation variability in responses to water-stressed host plants

Whereas an abundance of studies have focused on the between-species ecological differences (e.g. feeding guild, specialization and phenology) that influence population-level responses of insect herbivores to host plant water stress (Che-Castaldo et al., 2019; Cornelissen et al., 2008; Gely et al., 2020; Gutbrodt et al., 2011; Huberty & Denno, 2004), our study is among the few that have examined how the responses vary within lower levels of biological organization, namely within species or within populations (Dai et al., 2015; Gibbs et al., 2012). Gibbs et al. (2012) and Dai et al. (2015) reported among-population variability in life-history trait responses to water stress in Belgian *Pararge aegeria* butterflies and northern Chinese *Sitobion avenae* aphids, respectively. Interestingly, the latter study also reported that the contribution of clonal variability within each population was for many traits greater than among-population variability. Our study adds to these studies by highlighting that variability within a single dynamic metapopulation inhabiting a small

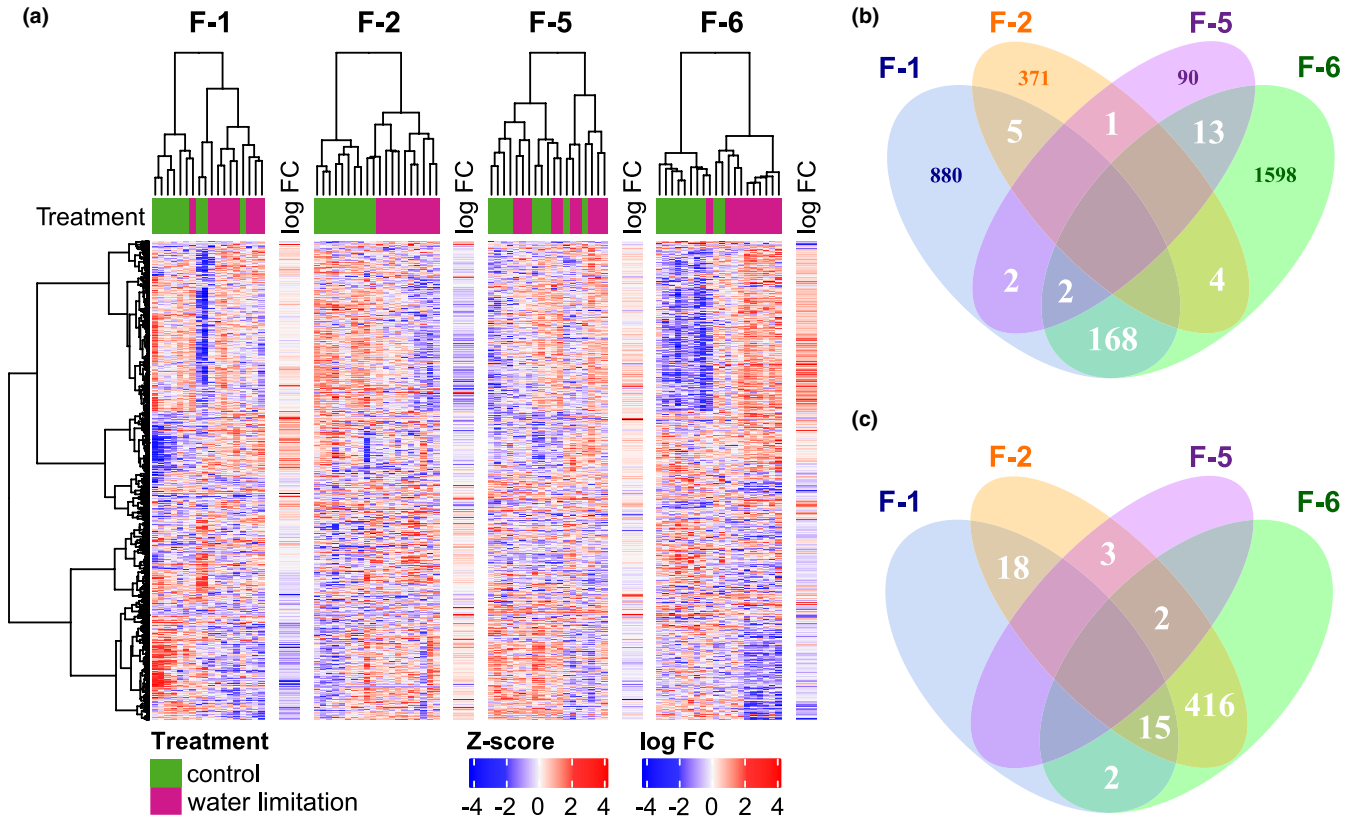

FIGURE 4 Divergent within-family transcriptomic responses of *Melitaea cinxia* to early development water limitation. (a) A heatmap of differential gene expression in which different panels refer to different larval families with columns and rows representing individuals and transcripts, respectively. The Z-score values in the heatmap (standardized within each family) refer to expression of each transcript in each individual, and average expression differences between control and early development water limitation treatment in each transcript in each family are illustrated as log fold-change values (log FC). (b) Venn diagram for the numbers of transcripts that are differentially expressed in the same direction between control and drought for each family (i.e. either up- or downregulated within the set of families). (c) Venn diagram for the numbers of differentially expressed transcripts that respond in opposite directions in at least one of the families

geographical range can sometimes be considerable, with even opposite responses between families.

Our study differs from the two previous ones in the sense that, despite different host plant desiccation histories at our sampling localities (Figure S2), local adaptation is not expected to be the main driving force behind the among-family variability in the *M. cinxia* metapopulation. This is because the classical metapopulation dynamics of the system are characterized by high dispersal, frequent extinction–(re)colonization events, rapid population turnover and transient population genetic structures, allowing for few opportunities for local adaptation to emerge (Blanquart et al., 2013; DiLeo et al., 2018; Fountain et al., 2017; Orsini et al., 2008; Van Nouhuys & Hanski, 1999). Instead, it has been suggested that the metapopulation dynamics maintain genetic variability in plastic responses via complex trade-offs between dispersal ability and other life-history characteristics (Kvist et al., 2013; Niitepõld & Saastamoinen, 2017). Indeed, the Åland islands *M. cinxia* metapopulation contains ample variation in behavioural and developmental responses to different environmental variables (e.g. temperature) across different life-history stages (Kvist et al., 2013; Niitepõld & Saastamoinen, 2017; Verspagen et al., 2020). Although not explicitly the focus of the current study, it is possible that similar trade-offs are also associated with the observed variability in responses to water-stressed host plants.

4.2 | Parallel transcriptomic and developmental responses reveal a stress response to suboptimal nutrition

Full transcriptome sequencing (RNA-seq) of 77 female larvae belonging to four of the nine studied families revealed a transcriptomic response parallel to the divergent phenotypic responses. RDA highlighted divergent transcriptomic responses to water-limited plants between the larval families performing better on control and water-limited plants (Figure 3a). A similar divergent pattern was also observed in a transcriptwise differential gene expression analysis conducted within each of the families (Figure 4a,c).

Although *M. cinxia* larval families exhibited opposite transcriptomic responses to feeding on water-limited plants, the fact that

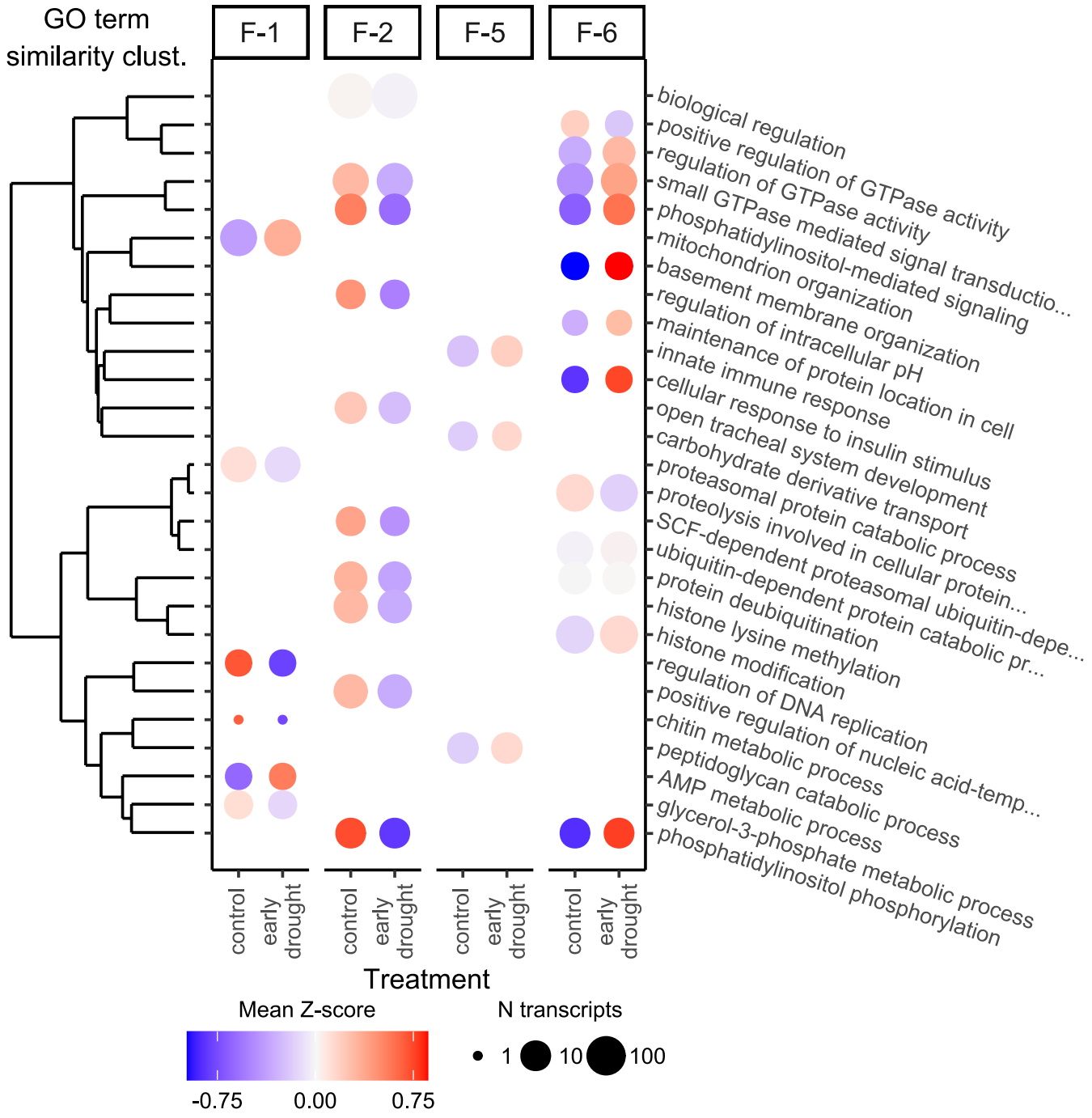

FIGURE 5 Biological processes associated with divergent within-family responses of *Melitaea cinxia* to early development water limitation. For each family, only the statistically significantly (p <.01) enriched BP GO terms are included. Z-score standardization of expression levels was conducted within families

individuals exhibiting reduced developmental performance responded quite similarly with several transcripts points towards a stress response that is shared among families (Figures 2–4). This view is strengthened by the fact that the transcripts associated with the divergent responses were involved in growth-related processes, with larvae exhibiting reduced developmental performance having lower expression of transcripts coding for intracellular signalling proteins involved in endocytosis and the serine/theroninen-protein kinase TOR (TOR) pathway (Figures 3b and 5; Tables S7, S10–S12) (Corvera et al., 1999; Flinn et al., 2010; X. Li et al., 2013).

The TOR pathway is associated with increased cell proliferation and growth during good nutrient conditions, and suppression of genes associated with TOR signalling indicates a stress response to a suboptimal nutrient environment (Betz & Hall, 2013; N. Li et al., 2019; Scott et al., 2004). Indeed, a recent study discovered that inhibiting the expression of TOR slowed down the growth rate of the

larvae of the lepidopteran *Maruca virata* and further verified that the expression of TOR varied in concert with growth rate on different alternative host plants that differ in nutritional quality (Al Baki et al., 2018).

4.3 | Transcriptomic differences between families underlie divergent responses to host plant water stress

Once intrapopulation variability in plastic responses are found, the next natural step is to uncover the mechanisms creating variability between individuals. Examining overall transcriptomic differences between families can be useful in identifying candidate processes and pathways associated with the differing responses. To this end, we detected that among-family variability in the *M. cinxia* larval transcriptomes was considerable, with most of the variability (34.6%) being explained by differences between families (Figure 3a). Notably, the greatest differences between the transcriptomes were observed when comparing families exhibiting opposite developmental responses (Figures 2 and 3a; Table S4).

The transcripts associated with the among-family variability revealed differences regarding metabolizing and storing nutrients. Individuals of the family F-2, in which performance was better on control treated plants, had higher baseline expression of transcripts involved in the production of storage proteins and fat accumulation, indicating that individuals in this family allocated more resources to nutrient storage than individuals in other families. This potentially indicates that individuals in this family were preparing for diapause and/or were experiencing nutritional stress (Exil et al., 2010; Hahn & Denlinger, 2011; Sonoda et al., 2006) (Table S9). The diapause body masses of individuals in family F-2 were generally lower than those of individuals in other sequenced families, which may be an indication that the increased nutrient storage may have come at the expense of growth (Figure 2c; Table S4). Alternatively, individuals of the family F-2 may have experienced generally more stressful conditions, which led to lower diapause body mass and increased storage protein production.

In families F-5 and F-6, individuals benefitted from feeding on water-limited plants. They had higher average expression of transcripts associated with catabolizing amino acids and allantoin, a purine metabolism product (Bursell, 1967) (Figure 3; Table S7). Increased allantoin catabolism may be an indication of more efficient breakdown of amino acids allowing for better nitrogen intake when feeding on the amino acid-rich water-limited plants (Figure 1). Also, in several plant species, allantoin is enriched in plant tissues during water limitation, and better allantoin catabolism may have directly enabled increased nitrogen availability when feeding on drought-stressed hosts (Bowne et al., 2012; Casartelli et al., 2019; Coleto et al., 2014; Oliver et al., 2011). Unfortunately, we cannot explicitly single out accumulation of allantoin in our drought-stressed plants due to overlapping peaks in its ^{1}H-NMR chemical shift range. Alternatively, allantoin metabolism may be linked to direct effects of reduced water availability as observed in the lepidopteran moth *Orthaga exvinacea* (Kuzhivelil & Mohamed, 1998). However, this explanation is not supported by the observation that the relative water contents did not differ between water-limited and control plants (Table 1).

Families F-5 and F-6 also had higher average expression of transcripts associated with intraciliary and microtubule-based movement (Figure 3; Table S7). This could be related to the observed allantoin and purine catabolism, because these processes typically occur in the peroxisome (Islinger et al., 2010), which are transported along microtubules (Hancock, 2014). Furthermore, purinosomes (enzyme clusters involved in purine synthesis) have recently been shown to be tightly associated with microtubule-based movement (Chan et al., 2018). However, as the intracellular transport system is central to a range of cellular processes, the connection between higher baseline allantoin catabolism and intracellular transportation in families benefitting from water-limited plants should be interpreted with caution (Berbari et al., 2009; Finetti et al., 2019; Hancock, 2014; Hua & Ferland, 2018).

4.4 | A butterfly in a changing world

Like so many natural systems, the Åland islands *M. cinxia* metapopulation is currently threatened by human-induced climate change. A long-term time series of over 25 years of demographic records have revealed marked changes in the metapopulation dynamics of the system, with increasing fluctuations in abundance elevating the extinction risk (van Bergen et al., 2020; Hanski & Meyke, 2005; Kahilainen et al., 2018; Tack et al., 2015). Although we know that regional population growth rates are linked to precipitation across larval stages (van Bergen et al., 2020; Kahilainen et al., 2018; Tack et al., 2015), a detailed understanding of individual-level responses to water limitation (and variability therein) is required to predict the behaviour of the system and, if necessary, plan conservation actions.

The observed intrapopulation variability in responses to host plant water limitation warrants cautious optimism about the ability of the Åland islands *M. cinxia* metapopulation to produce different responses to changing water availability. Provided that the variability is realized also in the wild, it could buffer the demographic effects of environmental fluctuations (Forsman & Wennersten, 2016; Wennersten & Forsman, 2012) and, if genetically inherited, allow for adaptive evolutionary responses to *Plantago lanceolata* water stress (Gotthard & Nylin, 1995; Price et al., 2003; Via & Lande, 1985). However, although we tried to minimize potential transgenerational effects by allowing the parental generation to spend their diapause period and postdiapause development in controlled laboratory conditions, we cannot completely rule out their effect, as the parental generation spent their prediapause conditions in natural conditions. Thus, the relative contributions of environmental and genetic effects to the intrapopulation variability remain untested.

5 | CONCLUSIONS

We observed divergent performance and transcriptomic responses across different *Melitaea cinxia* larval full-sib families. The observed among-family variability suggests intrapopulation variability in insect water stress responses can be considerable even within small geographical scales and provides evidence for variation in phenotypic plasticity within the Finnish *M. cinxia* metapopulation, potentially improving its chances of persisting in the changing climate. Our results highlight the importance of unravelling the magnitude and mechanisms behind intrapopulation variability for understanding and predicting the abilities of natural populations to respond to climate change.

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AUTHOR CONTRIBUTIONS

A.K. and M.S. developed the study questions and study design, A.K. conducted the experiment, A.K., V.O., P.S. and G.M. analysed the data, and A.K. wrote the first version of the manuscript to which all authors contributed.

DATA AVAILABILITY STATEMENT

Processed *P. lanceolata* ^{1}H-NMR metabolite data and *M. cinxia* developmental data are available at Data Dryad (https://doi.org/10.5061/dryad.nk98sf7ss). RNA-seq reads, raw and processed read count tables and transcriptome assembly are available at NCBI's Gene Expression Omnibus, with the accession no. GSE159376

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