INTRODUCTION

Most temperate insects survive winter by entering a developmental arrest, known as diapause (Tauber et al., 1986). Diapause is an obligate part of the life cycle for some species (Danilevskii, 1965; Stålhandske et al., 2015), while in others it is facultative, with the induction of diapause being dependent upon environmental stimuli such as photoperiod and temperature (Danilevskii, 1965; Nylin et al., 1989; Pruisscher et al., 2018). Typically, its initiation involves endocrine mechanisms, which result in a decreased metabolic rate, a cessation of development, and an increase in cold and stress tolerance (Hahn & Denlinger, 2010; Koštál, 2006; Lehmann et al., 2016; 2018b). Once diapause is initiated it must be maintained throughout the winter period and subsequently terminated so that development can be restarted when conditions again are permissible (Lehmann et al., 2017; Lindestad et al., 2020). Although the induction, maintenance and termination of diapause all are vital aspects of insect life cycle timing, there is no general understanding of the how these
processes are regulated by alternative gene expression across the developmental period on a transcriptome wide level (Koštál et al., 2017).

Among insect species, diapause can be expressed in all stages of development, but for a given species it is usually limited to a single development stage (Clark & Platt, 1969; Forsberg & Wiklund, 1988; Söderlund & Nylin, 2011; Tougeron et al., 2018). While diapause in different life-stages likely has varying unique constraints, there might exist a common “tool-kit” of diapause adaptations shared among species (Amsalem et al., 2015; Poelchau et al., 2013; Sim & Denlinger, 2013), that may be generally important for diapause across species and diapause phenotypes. Therefore, studies of additional species, with diverse diapause phenotypes, are needed to understand if mechanistic commonalities in the regulation of diapause indeed do exist. One concern for such endeavours regards how comparisons among diapause transcriptomes are made, as diapause versus direct transcriptomes are expected to vary dramatically over the time-course of development. Therefore, comparing single time points per developmental pathway will necessarily miss much of the important dynamics within each pathway. Still comparisons using more detailed, whole-genome sampling across these stages are largely missing (but see Koštál et al., 2017; Dowlie et al., 2020).

A comparative and general understanding of diapause becomes complicated by the fact that diapause is made up of several distinct physiological phases (Koštál, 2006). These different phases may have alternative selection pressures acting upon them, and could therefore be due to different genes or pathways that are active at a given phase in diapause. While several studies have sampled across the different phases, investigating early versus late diapause in the flesh fly Sarcophaga crassipalpis (Ragland et al., 2010), early versus late versus later developmental stages in the apple maggot fly Rhagoletis pomonella (Ragland et al., 2011), or across a range of different phases of diapause in the drosophilid fly, Chymomyza costata (Koštál et al., 2017), these were limited to a subset of the transcriptome using 1,000s of preselected genes on custom microarrays. Recent studies have generated a more comprehensive approach by using the whole transcriptome via RNA-Seq analysis of two time points during embryonic diapause (Hao et al., 2017), and among different larval developmental stages of direct (i.e., nondiapause) versus diapause development destined larvae (Leal et al., 2018). More focused investigations have revealed diapause-specific expression of candidate genes, including heat shock proteins (Goto & Kimura, 2004; Yocum et al., 1998), circadian clock proteins (Doležel et al., 2005; Koštál & Shimada, 2001; Tauber et al., 2007; Zhu et al., 2017), genes involved in hormonal regulation (Xu & Denlinger, 2003), and genes regulating energy metabolism (Levin et al., 2003; Uno et al., 2004). While these advances have been important, we still lack insights into how much the whole transcriptome changes during diapause, and whether the patterns observed are species-specific or part of a general diapause “transcriptional phenotype”.

Here, we investigate the transcriptome of a Swedish population of Pieris napi butterflies across several stages of its pupal diapause, using RNA-Seq to more fully characterize the dynamic landscape of gene transcription in diapause. P. napi larvae primarily use photoperiod, and to a lesser extent temperature, to determine whether or not to induce diapause. The exact photoperiod thresholds that induce diapause varies among populations at differing latitudes, indicating local adaptation in the induction mechanisms (PruiSScher et al., 2017). The decision to switch to the diapause pathway is made during the larval stage, and can be initiated already from the third instar, after which this decision will continually be updated until the fifth and last instar, where the decision becomes fixed (Friberg et al., 2014). The larvae will then enter the pupal stage, which is where diapause is expressed. Pupae of the direct development and diapause pathways show differences in brain development, hinting at possible tissue-specificity of diapause-related pathways and regulation. Brain development could affect the ability to reliably process external or internal information during diapause, and likely influences transcriptional dynamics (Lehmann et al., 2017). Diapause termination is uniquely well-characterized in P. napi, which occurs after an obligate period in cold temperatures, as a lack of exposure to low temperatures will result in indefinite maintenance of the pupal diapause state (Lehmann et al., 2017b). Further, the duration of endogenous diapause is known to vary in a latitudinally dependent manner, becoming longer at higher latitudes, further documenting the extent to which this trait is under selection in the wild (Posledovitch et al., 2015). Recent work has characterized the lipidomic and metabolomic profile of pupal diapause in P. napi (Lehmann et al., 2016, 2018), revealing dynamic fluctuations in specific metabolites that matched the termination of endogenous diapause, which appeared independent of photoperiodic or temperature related cues (Lehmann et al., 2018). Using a whole transcriptome approach on two key body parts during different time points in diapause and direct development, we attempt to answer several questions: (i) How different are diapausing and directly developing pupae at the start of pupal development? (ii) What are the temporal patterns of divergence in expression of diapause and direct developmental pathways? (iii) Are there dynamic changes in expression during diapause, independent of changes in environmental conditions? (iv) Do the adults differ between the two different pathways?

2 | MATERIALS AND METHODS

2.1 | Insect collection and rearing

Pieris napi eggs were obtained from wild plants in Skåne, southern Sweden (Kullaberg; 56°18’N, 12°27’E and Vejbystrand; 56°18’N, 12°46’E), and reared in a laboratory as described previously (Lehmann et al., 2016, 2018). Briefly, larvae were reared using a split brood design, and subjected to conditions inducing either direct development (Light:dark 22h:2 h, 20°C), or diapause development (L:D 10 h:14 h, 20°C), where they were sampled at specific time points during the pupal and adult stages (Figure 1a). Temperature was lowered to 10°C on day 10, and to 2°C on day 17, as to simulate a decrease in temperature as winter approaches. Diapause pupae were kept in constant darkness from day 17 until day 144. On day 144 temperature was
increased to 10°C, and to 20°C on day 151. Direct developing pupae were sampled at day 0, 3, and 6 of pupation, and as 2-day old adults. The pupal period lasted about 10 days in the direct developing pupae. Diapause pupae were sampled at day 0, 3, 6, 24, 114, 144, 155 after pupation, and as 2-day old adults. Sampling was done on females only to exclude an effect of sex in the analysis. Individuals were sampled at the desired time points by first collecting the sampling individuals from the rearing cabinets and then placing each individual into a 1.5 ml tube, which were subsequently submerged into liquid nitrogen and stored in –80°C. All sampling was done at the same time during the day, between 10:00 and 13:00, to avoid effects of circadian variability on the expression profiles. Adults were kept with access to water but not food during the 2-day period before sampling.

2.2 | RNA extraction and sequencing

After sampling was done for all time points, head and abdomen body parts were extracted from four individuals per sample point, per pathway, for a total of 96 samples. Total RNA was isolated from pupae and adult butterflies by homogenizing the head, or first abdominal segment (counted from the anal side) in TRIzol (Thermo Fisher Scientific). RNA was purified with the Direct-zol RNA MiniPrep (Zymo) as per manufacturer’s instructions. Quality and quantity of the total RNA purified were determined using the Experion equipment (Bio-Rad) and the Qubit instrument (Thermo Fisher Scientific) respectively. Library preparation, sequencing and data processing of the RNA was performed at the National Genomics Infrastructure Sweden (NGI Stockholm) using strand-specific Illumina TruSeq RNA libraries with poly-A selection (Illumina HiSeq HO mode v4, paired-end 2 × 125 bp).

2.3 | Read quality trimming

For the scripts and commands used in the analysis, see Supporting Information Materials. Adapter sequences and low-quality bases were trimmed in the raw fastq data using bbduk v37.31 bbduk.sh with the parameters: ref=truseq.fa.gz,nextera.fa.gz ktrim=r k=23 mink=11 hdist=1, and bbduk2.sh with the parameters: ref=phix174_ill.ref.fa.gz k=27 hdist=1 qtrim=r trimq=20 minlen =40 qout =33. Due to technical error the abdomen of one individual failed to produce quality sequences, therefore this sampling point only has three replicates (Direct development abdomen pupal day 6).

2.4 | Mapping to the genome

All cleaned reads were mapped to the Pieris napi genome v1.1 (Hill et al., 2019), using HISAT2 v2.1.0 (Kim et al., 2015) with the parameters: --dta --mp 4.1. The resulting SAM file was sorted and output as BAM file using samtools sort v1.3 (Li, 2009).

2.5 | Estimating transcript abundance

The mapped reads were assembled into transcripts using StringTie v1.3.4 (Pertea et al., 2015) with the Pieris napi v1.1 annotation file in GTF format. Assembled transcripts for each sample were merged using StringTie v1.3.4 -merge, creating an updated GTF annotation file for the P. napi genome. Transcript abundances were estimated for each sample using StringTie v1.3.4 with the parameters -e -B, and the merged transcript file as input. A
gene-level read count matrix was generated using the prepDE.py script provided as part of the StringTie package, using an average read length of 125. Genes were only kept when having at least five reads per million in at least three replicates. This ensured the removal of genes with low levels of transcription that could introduce noise into the analysis.

2.6 | Differential expression analysis

Pairwise comparisons between all samples were conducted using DESeq2 at the gene level (Love et al., 2014). Genes were determined to be significantly differentially expressed when having an adjusted p-value of .05 or lower, representing a false discovery rate of 5% on a p-value of .05. Genes were not filtered on fold-change, as it could potentially affect the topology used for finding gene modules in downstream analyses. However, this assumption was not tested. Furthermore, filtering at a fold change is something that can help reducing the number of differentially expressed genes, but we do not necessarily need a large fold-change to affect downstream processes in diapause. Furthermore, we are looking at a heterogeneous collection of cells (e.g., heads with containing multiple types of tissue, rather than one specific cell type; if it is a specific and small group of cells that is expressing the genes that matter, this would potentially show up as a low signal in our data, which is nonetheless important). We do not know all the genes that are important in our phenotype, and are hesitant to exclude any candidates based on fold change alone.

2.7 | GO term enrichment

Gene set enrichment analysis (GSEA) for significantly differentially expressed genes was performed for each pairwise comparison using topGO on default settings (Alexa & Rahnenfahrer, 2018), comparing the set of differentially expressed genes against the rest of the annotated genes. The original annotation file gene ID’s were linked to the new annotation file containing stringtie gene ID’s, and existing gene annotations were lifted over from the original gene set. Thus, the genes considered in the GSEA were those existing GO annotations in the annotation of the genome assembly (Hill et al., 2019).

2.8 | Time-series clustering of diapause samples

Clustering of expression profiles in the time-series data was performed for the pupal diapause samples separately for heads and abdomens. The day 0 and day 155 samples were excluded as preliminary analysis showed that virtually all clusters were grouped based on the up- or downregulation of expression at these two time points; since diapause day 0 is so similar to direct development day 0, the main signal was downregulation of genes not found in the other diapause time points. For day 155, development had resumed (see also PCA in Figure 1b,c).

The number of clusters present in the data was determined by comparing the results of two methods: average silhouette width clustering and SSE clustering using K-means (Jain & Dubes, 1988), after which fuzzy c-means clustering was performed by estimating the “fuzzifier” needed for c-means clustering (Kumar & Futschik, 2007), and then using the function cmeans of the R package e1071 (Pal et al., 1996). The combination of these methods allowed for the detection of supported clusters inherent in the data. The raw expression data of the genes that were significant in the pairwise comparisons between day 3 until day 144 were variance stabilizing transformation (vst) transformed using DESeq2, and subsequently centered and scaled using the function “scale” in edgR (Robinson et al., 2010). This was done in order to be able to compare between genes and to identify clusters with similar expression profiles independent of expression levels. After scaling, the four replicates of each sample were averaged within each gene to one sample mean. These scaled mean values were used in the cluster estimation and subsequent clustering. Finally, GSEA was performed as described above on each cluster, using the genes with a cluster similarity score of >0.6 in order to obtain the most representative estimate of the biological processes representing each cluster. This similarity score cutoff was chosen as in fuzzy c-means clustering each gene is technically a representative of each cluster, and a cutoff of 0.6 then indicates that of all clusters this gene belongs most to this particular cluster.

3 | RESULTS

3.1 | Sample relationships

Investigation of the overall transcriptome profile used a principal component analysis (PCA), revealing a tight clustering of replicate samples within PCs 1 and 2, which together accounted for nearly 50% of sample variance and separated samples by body part and pathway (Figure S1). Expression clustering analysis revealed an identical pattern where samples were grouped by body part and pathway, and replicates within each sample clustered together (Figure S2).

We next performed a PCA only for the pupal samples, separately for the head and abdomen body parts (Figure 1b,c). This revealed that direct development day 0 clustered close to diapause day 0. Transcriptome changes in both pathways then diverged during their chronological progression, with the direct pathway traversing a narrower set of the PC space compared to diapause. The diapause development samples, day 3 until day 144, occurred in close proximity of each other (Dia_003:Dia_144; Figure 1b,c), and in PC space that was very different from all direct pathway samples. Diapause day 155 was separated from the other diapause samples and clustered with direct development samples day 3.
3.2 | Divergence between the pathways

In order to assess differences between pathways in the beginning of pupal development, we performed pairwise comparisons between the pathways for day 0, 3, and 6. A substantial number of differential expressed genes (DEGs) between the pathways were already present at pupal day 0 ($n_{\text{mean-of-body-part-DEGs}} = 409$), and the number of DEGs increased between the pathways through days 3 ($n_{\text{mean-of-body-part-DEGs}} = 2,184$) to day 6 ($n_{\text{mean-of-body-part-DEGs}} = 2,695$; Table 1). GSEA revealed that in the diapause pathway these were mostly related to maintenance of organism functions and oxidation reduction processes, as determined by GO term enrichment analysis. The direct development pathway saw significant enrichment for expression changes in energy metabolism and DNA replication functional categories (Table 1; Tables S1–S12).

More specifically, in the head samples, divergence between pathways at day 0 was dominated by an upregulation of oxidation reduction processes in the diapause samples, while in the direct development pathway the DEGs were predominantly involved in protein biosynthesis. Divergence increased between pathways at day 3, and DEG function was dominated by metabolic processes in the diapause samples, while direct development samples continued to show protein biosynthesis along actin cytoskeleton organization. At day 6 DEGs were mostly involved in metabolic and biological processes for diapause, and the generation of precursors of metabolites and energy in direct development samples (Table 1; SM tables). In the abdomen samples, divergence between pathways at day 0 was dominated by oxidation reduction processes in diapause samples, and ATP metabolic processes in direct development samples. At day 3 divergence increased between the pathways similar to the head samples, and diapause samples showed continued upregulation of oxidation reduction processes in the DEGs, while direct development abdomen DEGs were dominated by functions related to DNA replication and regulation of cellular processes. At day 6 DEGs upregulated in diapause were mostly involved in regulation of transcription, while in direct development there was significant upregulation of organonitrogen compound metabolism (Table 1).

3.3 | Diapause expression dynamics

The dynamic changes in expression during diapause were quantified by estimating DEGs between each pair of pupal diapause time points between day 0 and day 155 for each body part. In the head samples, DEGs ranged from 2601 between day 6 and day 24, down to 20 between day 114 and 144, with a similar progression in the abdomen samples, albeit the total number of DEGs were lower in this body part (Figure 2).

In order to further understand the transcriptome dynamics during diapause, we investigated the head tissue samples for the number of genes expressed above 10 and 100 cpm (read counts per million), and compared this to the library size (number of reads
after filtering for the whole sample, as well as the total amount of extracted RNA (Figure 3). While the library size was the same for all samples (Kruskal-Wallis: $X^2 = 12.483$, df = 9, $p$-value = .1874), there were significant differences in the number of genes expressed at >10 cpm ($X^2 = 29.06$, df = 9, $p$-value < .001), and at >100 cpm ($X^2 = 30.353$, df = 9, $p$-value < .001). Furthermore, the total amount of RNA present in the samples differed significantly ($X^2 = 25.322$, df = 9, $p$-value < .003). The amount of RNA follows the pattern observed in metabolic rate (Lehmann et al., 2016), which is used here to represent their metabolic activity level. Thus, while the amounts of RNA sequenced and investigated are the same, the total amount of RNA in an individual that is in diapause is half of what is present in a directly developing animal (mean Dia_24-114 = 7525 μg; mean Dir_03-06 = 15150 μg).

We next investigated transcriptome dynamics during diapause using a cluster analysis for each body part on all genes that were found to differ significantly in expression in any reciprocal pairwise comparison between samples from day 3 up to day 144. To correct for the difference in RNA levels of the original samples (Figure 3), and any potential biases arising from this, we performed the following analyses only on the samples ranging from day 3–144 going forward, as these are comparable to each other with respect to RNA amounts used to make their respective libraries.

Starting with heads, estimation of the number of expression clusters revealed seven clusters (Figures S3, S4) differing in their expression trajectory over time (Figure 4 and Table 2). Clusters contained between 85 and 433 DEGs with a similarity score of 0.6 or higher, which indicates their similarity to the overall cluster profile. Cluster 1 showed a high expression at Day 3, which steadily

![Figure 2](image2.png)

**Figure 2.** The number of differentially expressed genes (DEG) between pupal diapause time points in *P. napi*, for head and abdomen tissue. Within boxes, the number of DEGs upregulated (top) and downregulated (bottom) for each time-point comparison (i.e., 833 DEGs were upregulated between days 3 and 6 in heads), for head (left column) and abdomen (right column) parts. Genes were considered as differentially expressed when having an adjusted $p$-value <.05

![Figure 3](image3.png)

**Figure 3.** Summary statistics in the head tissue samples. From top to bottom: the top red graph shows the number of expressed genes for each sample at >10 cpm. In yellow the number of expressed genes at >100 cpm is shown. Library sizes are shown in green. The total amount of RNA extracted is shown in blue, and finally the MR (metabolic rate) is shown in purple (MR data obtained from Lehmann et al., 2016).
decreased over time until Day 114, and GSEA on DEGs with a similarity score >0.6 predominantly showed processes related to regulation of metabolic and cellular processes. Using the same approach (and hereafter for all clusters), cluster 2, which showed a downregulation on day 24, was dominated by (nucleotide) biosynthetic processes. Cluster 3, showing the inverse pattern of cluster 2, was enriched for protein biosynthesis and cellular metabolism. Cluster 4, characterized by low expression on day 3 and 6, with a linear increase through day 24 to 114, contained genes with functions related to tRNA aminoacylation for protein biosynthesis, cellular metabolism, methylation and histone modification. Cluster 5, which was similar to cluster 4 but with high expression on days 24–144, was enriched for macromolecule and nitrogen compound metabolism. Cluster 6 was the inverse of cluster 5, and was characterized by regulation of metabolism, mRNA processing and protein transport. Finally, cluster 7, which showed high gene expression early (days 3, 6, and 24) and then was downregulated on days 114 and 144, showed predominantly catabolic processes.

Analysis of DEGs in the abdomen samples of diapause day 3 until day 144 revealed the seven clusters (Figure S5, S6), with distinct expression profiles (Figure 5 and Table 3). Cluster 1 showed a downregulated expression in day 3 and 6, with higher expression in day 24–144. GSEA on the DEGs with a similarity score >0.6 represented proteolysis and responses to oxidative stress. Cluster 2 showed the inverse expression pattern of cluster 1, showing enrichment for biological processes involved in mitochondrial transport and cellular ketone metabolism. Cluster 3 showed gene expression downregulated in day 114 and 144, and was enriched for oxidation-reduction processes and metabolism. Cluster 4 showed a downregulation at day 24 that slightly increased again at day 114 and 144, and processes dominating this cluster were ncRNA metabolism, as well as cellular processes. Cluster 5 showed the inverse pattern of cluster 4, and significant biological processes included cellular metabolism and tRNA aminoacylation. Cluster 6 showed low gene expression at day 3 and day 6 that gradually increased until day 144, which was dominated by protein biosynthesis, cellular processes, and cellular amide processes. Cluster 7 showed high gene expression that decreased linearly until day 114, representing negative regulation of gene expression.

3.4 | Differences between adults

Differential expression analysis between the adults revealed a low number of DEGs between the pathways (Table 4). Upregulated DEGs in the heads of diapause development adults were involved in DNA recombination and metabolism, while the DEGs upregulated in the direct development adults all related to monocarboxylic acid and lipid biosynthesis. In the abdomens, DEGs upregulated in diapause development adults were related to cellular amide metabolism and protein biosynthesis, while DEGs in the direct development adults were related to protein phosphorylation.

4 | DISCUSSION

In this study we performed a whole transcriptomic profiling of pupal development during both diapause and nondiapause development in the butterfly *P. napi* for two different body parts in order to characterize the landscape and dynamics of the transcriptome during these developmental programs. Divergence between pathways started immediately and increased quickly in both body parts. Diapause itself seemed to follow a specific program with a highly dynamic transcriptional landscape in the first four weeks of pupal diapause. Cluster analysis showed distinct and diverse transcription profiles, as well as a sudden reduction in transcriptional dynamics during the last half of diapause, which is when endogenous diapause is typically terminated in overwintering *P. napi*. Interestingly, the transcriptional profile of diapause pupae when exposed to warm conditions after 155 days of pupal development seems to converge on the direct development pathway of day 3. In line with this similarity towards the end of both pathways' diapause development, the adults that emerged out of both pathways showed a large overlap in their transcription.

Direct and diapause developmental pathways showed substantial transcriptome differences already at the very start of pupal development. Rather than a downregulation in the diapause samples, the DEG between the day zero points were equally distributed between up and downregulation, representing alternative development trajectories rather than a simple transcriptional suppression in the diapause developmental trajectory. Presumably as preparation for diapause, GSEA identified an increased expression in protection against oxidative stress in both the heads and abdomen samples. This downregulation of energy consumption is supported by findings in *D. melanogaster* (Zhao et al., 2016). Likewise, signatures of suppression of development and metabolism were seen in the downregulation of processes related to protein biosynthesis in heads and ATP metabolism in the abdomens. The pathways continued to diverge drastically in both body parts from day three onwards, reflecting a continued divergence of the transcriptome phenotype, despite being subjected to identical conditions.

Early on during diapause there is a dynamic change in gene expression, both in the heads and abdomen samples, which is concurrent with the idea that insects initiate many transcriptional changes to prepare for diapause (Denlinger, 2002). This suggests an initial process early on in the pupal stage that continues despite the act of entering “winter conditions” at day 17, as there are still substantial transcriptional changes at day 24 and 114. Nevertheless, it seems clear that the sudden decrease in temperature influences the transcriptional profile, as several clusters in both tissues show conspicuously strong responses at day 24.

At some point between day 24 and day 114 the dynamic change of the transcriptional profile ceases, and the expression profile continues to stay the same through day 144. As endogenous diapause is terminated (i.e., the point when an individual pupa becomes sensitivity to ontogenetic-development-promoting high temperatures).
around 3–4 months in this population (Posledovich et al., 2015), sample day 114 and day 144 presumably represent quiescent individuals (consistent with their low MR and RNA concentrations [Figure 3]). This coincides with large numbers of DEGs between diapause time points, until the comparison between day 114 and 144. This pattern of change between days 24 and 114 and a lack of it during days 114 and 144, potentially represent that the building of the post-diapause phenotype is completed somewhere between day 24 and day 114, and the individuals are ready and awaiting the resumption of beneficial developmental conditions. A similar pattern of expression is noted in a microarray study on Chymomyza costata where the number of DEGs detected during diapause maintenance was much lower than during the initial induction and initiation phase (Koštál et al., 2017). As post-termination quiescence can take a long time, very strong dynamics in expression is perhaps not expected during this time. These findings highlight the idea of diapause development as a dynamic physiological process leading to the termination of endogenous diapause (Koštál, 2006), which is followed by a more static, potentially very long (up to several years in some species) period of post-diapause quiescence.

Recent work characterizing the metabolomic profile of pupal diapause in P. napi revealed dynamic fluctuations in specific metabolites that matched the termination of endogenous diapause, which occurred independent of photoperiodic or other external cues.
This matches the dynamic transcriptional profiles of several gene expression clusters in the head and abdomen samples. Still, it is important to note that we do miss the “peak” of the metabolites that were shown to be dynamic (Lehmann et al., 2018), as these occurred somewhere between the day 24 and day 114 sampling points.

**FIGURE 5** DEG clusters during diapause in the abdomen samples day 3 until day 144. Expression was standardized so it was possible to compare the shape of expression between each gene. The shading indicates a similarity score from 0 (blue) to 1 (red), with higher values indicated a higher similarity to the cluster mean.

**TABLE 3** The number of differentially expressed genes (DEG) in each cluster, and their associated most significant GO term for the abdomen samples of day 3 until day 144. See Tables S20–S26 for the full list.

| Cluster | DEGs | DEG score >0.6 | Top GO category | p-value |
|---------|------|----------------|-----------------|---------|
| 1       | 999  | 283            | Cellular protein catabolic process | .0036   |
| 2       | 624  | 101            | Aromatic amino acid family metabolic process | .038    |
| 3       | 989  | 297            | Metabolic process | .001    |
| 4       | 847  | 279            | ncRNA metabolic process | .00014  |
| 5       | 967  | 346            | tRNA aminoacylation for protein translation | 8.5e−06 |
| 6       | 608  | 152            | Protein biosynthesis | 6.1e−11 |
| 7       | 1,058| 292            | Negative regulation of biosynthetic process | .0084   |

**TABLE 4** DEGs between adults from the diapause and direct developmental pathways with associated most significant GO term. For full list see Tables S27–S30.

| Up     | Head | Top GO category | p-value | Thorax | Top GO category | p-value |
|--------|------|-----------------|---------|--------|-----------------|---------|
|        | DEG  | DNA recombination |         | DEG    | Translation     |         |
| Diapause| 40   | DNA recombination | .014    | 68     | Translation     | 1.1e−13 |
| Direct | 32   | Small molecule biosynthetic process | .0002   | 20     | Protein phosphorylation | .0013   |
Earlier research has shown dispersal polymorphism (Zera et al., 1994, 1997) and differences in other life history parameters in adult insects having developed via the two pathways (Mousseau & Roff, 1989), including *P. napi* (Karlsson & Johansson, 2008). However, while the pupa shows dramatic plasticity, there are relatively low numbers of DEGs between adults in our study, representing a canalization of the adult life stage. These DEGs could be responsible for numerous differences, such as previously observed differences in flight performance and fecundity between the adults. However, such phenotypic differences could also arise due to potential differences in accumulated resources, or changes happening before reaching the adult stage, while the overall transcriptional needs of a resting adult are similar. More investigation is needed to reveal the relation between the DEGs observed and the phenotypic differences in the adults.

It is striking that in both body parts the overall patterns of expression dynamics follow the same trends throughout diapause, even though there are very different processes active in these two body parts. This difference in gene expression between tissues has also been observed in D. melanogaster, where the diapause response was not uniform between multiple tissue types (Zhao et al., 2016). This complicates attempts to understand the regulation of diapause, as processes in one tissue could be driven by signals coming from other tissues instead of being independent of each other, or, more likely, a combination of local and global signals. Moreover, characterization of the transcriptional control of diapause development using whole body parts may be difficult, as important signals can be very localized.

One caveat revealed in this study is the correlation between the patterns observed in the data and the actual activity levels of the animals studied. We show that there are certain numbers of DEGs between time points, however this represents their relative expression for the amount of RNA that is sequenced. As the total amount of RNA in an individual that is in diapause is half of what is present in a directly developing animal, the number of DEGs would change drastically if this was considered. It would probably change the interpretation of the data and reveal a much more substantial downregulation of genes in diapause, going from diapause day 0 onwards. This is a known problem in RNA-Seq analysis (Evans et al., 2018), which does not appear to be considered in experimental studies involving dramatic differences in metabolic rate between groups, such as those involving diapause.

In summary, during diapause there are strong gene expression dynamics, revealing a preprogrammed transcriptional landscape that is active during the winter. Different biological processes appear to be active in the two body parts. Hence, in terms of transcriptomics the formation of a diapause phenotype cannot be described as a pause. Future studies would benefit from even higher temporal sampling during the elusive diapause termination period. A realistic ecophysiological perspective would take into account the various demands that thermal stress, energetic stress and timing stress puts on the organism, and this requires further partitioning of the temperature-dependence of the clusters. Still, despite the substantial differences present between direct and diapause developmental programmes, adults emerging from either pathway show a large overlap in transcription, indicating strong canalization of the adult phenotype on the transcriptome level. The current study shows a number of key clusters containing both down and upregulated genes that require further partitioning and in-depth study in order to clarify the mechanisms operating during the physiologically dynamic pupal diapause of *P. napi*.

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**AUTHOR CONTRIBUTIONS**

Peter Pruisscher, Philipp Lehmann, Sören Nylin, Karl Gotthard and Christopher W. Wheat designed research; Peter Pruisscher and Philipp Lehmann performed research; Peter Pruisscher analysed data; and wrote the manuscript with input of all the other authors.

**DATA AVAILABILITY STATEMENT**

Sequencing data are available at NCBI Bioproject PRJNA684967.

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**REFERENCES**

Alexa, A., & Rahnenfuhrer, J. (2018). topGO: Enrichment analysis for gene ontology. R package version 2.34.0.

Amsalem, E., Galbraith, D. A., Cnaani, J., Teal, P. E. A., & Grozinger, C. M. (2015). Conservation and modification of genetic and physiological toolkits underpinning diapause in bumble bee queens. *Molecular Ecology*, 24, 5596–5615. [https://doi.org/10.1111/mec.13410](https://doi.org/10.1111/mec.13410)

Clark, S. H., & Platt, A. P. (1969). Influence of photoperiod on development and larval diapause in the viceroy butterfly, Limenitis archippus. *Journal of Insect Physiology*, 15, 1951–1957. [https://doi.org/10.1016/0022-1910(69)90024-9](https://doi.org/10.1016/0022-1910(69)90024-9)

Danilevskii, A. S. (1965). *Photoperiodism and seasonal development of insects.* Oliver and Boyd.

Denlinger, D. L. (2002). Regulation of diapause. *Annual review of entomology*, 47, 93–122.

Doležel, D., Vaněčková, H., Šauman, I., & Hodkova, M. (2005). Is period gene causally involved in the photoperiodic regulation of reproductive diapause in the linden bug, Pyrrhocoris apterus? *Journal of Insect Physiology*, 51, 655–659. [https://doi.org/10.1016/j.jinsphys.2005.01.009](https://doi.org/10.1016/j.jinsphys.2005.01.009)

Evans, C., Hardin, J., & Stoebel, D. M. (2018). Selecting between-sample RNA-Seq normalization methods from the perspective of their assumptions. *Briefings in Bioinformatics*, 19, 776–792. [https://doi.org/10.1093/bib/bbx008](https://doi.org/10.1093/bib/bbx008)
Tougeron, K., Blanchet, L., van Baaren, J., Le Lann, C., & Brodeur, J. (2018) Effect of diapause on cold-resistance in different life-stages of an aphid parasitoid wasp. bioRxiv, 1, 489427.

Uno, T., Nakasuji, A., Shimoda, M., & Aizono, Y. (2004). Expression of cytochrome c oxidase subunit 1 gene in the brain at an early stage in the termination of pupal diapause in the sweet potato hornworm, Agrius convolvuli. Journal of Insect Physiology, 50, 35–42. https://doi.org/10.1016/j.jinsphys.2003.09.011

Xu, W. H., & Denlinger, D. L. (2003). Molecular characterization of prothoracicotropic hormone and diapause hormone in Heliothis virescens during diapause, and a new role for diapause hormone. Insect Molecular, 12, 509–516. https://doi.org/10.1046/j.1365-2583.2003.00437.x

Yocum, G. D., Joplin, K. H., & Denlinger, D. L. (1998). Upregulation of a 23kDa small heat shock protein transcript during pupal diapause in the flesh fly, Sarcophaga crassipalpis. Insect Biochemistry and Molecular Biology, 28, 677–682. https://doi.org/10.1016/S0965-1748(98)00046-0

Zhao, X., Bergland, A. O., Behrman, E. L., Gregory, B. D., Petrov, D. A., & Schmidt, P. S. (2016). Global transcriptional profiling of diapause and climatic adaptation in Drosophila melanogaster. Molecular Biology and Evolution, 33(3), 707–720.

Zhu, L., Liu, W., Tan, Q.-Q., Lei, C.-L., & Wang, X.-P. (2017). Differential expression of circadian clock genes in two strains of beetles reveals candidates related to photoperiodic induction of summer diapause. Gene, 603, 9–14. https://doi.org/10.1016/j.gene.2016.12.004

SUPPORTING INFORMATION

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