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Monarch butterflies use an environmentally sensitive, internal timer to control overwintering dynamics

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Abstract

The monarch butterfly (*Danaus plexippus*) complements its iconic migration with diapause, a hormonally controlled developmental programme that contributes to winter survival at overwintering sites. Although timing is a critical adaptive feature of diapause, how environmental cues are integrated with genetically-determined physiological mechanisms to time diapause development, particularly termination, is not well understood. In a design that subjected western North American monarchs to different environmental chamber conditions over time, we modularized constituent components of an environmentally-controlled, internal diapause termination timer. Using comparative transcriptomics, we identified molecular controllers of these specific diapause termination components. Calcium signalling mediated environmental sensitivity of the diapause timer, and we speculate that it is a key integrator of environmental condition (cold temperature) with downstream hormonal control of diapause. Juvenile hormone (JH) signalling changed spontaneously in diapause-inducing conditions, capacitating response to future environmental condition. Although JH is a major target of the internal timer, it is not itself the timer. Epigenetic mechanisms are implicated to be the proximate timing mechanism. Ecdysteroid, JH, and insulin/insulin-like peptide signalling are major targets of the diapause programme used to control response to permissive environmental conditions. Understanding the environmental and physiological mechanisms of diapause termination sheds light on fundamental properties of biological timing, and also helps inform expectations for how monarch populations may respond to future climate change.

KEYWORDS
diapause, ecdysone, insulin signalling, juvenile hormone, monarch butterfly

1 | INTRODUCTION

Organisms subject to seasonally variable environments have evolved myriad adaptations to translate environmental condition into accurately timed behavioural and physiological responses. The monarch butterfly (*Danaus plexippus*) has evolved migration and diapause to survive inhospitable winters across its temperate North American range. Each fall, millions of monarchs across the US and southern Canada migrate to specific locations in central Mexico if in the eastern North American population, or along the Pacific Coast if in the western North American population, where they overwinter in reproductive diapause. In spring, they mate, remigrate northwards, and repopulate their breeding grounds over three to four successive generations. Previous work has revealed environmental cues and sensory modalities required to accurately interpret environmental condition for both migration and diapause initiation (Gegear, Casselman, Waddell, & Reppert, 2008; Goehring & Oberhauser, 2002, 2004; Guerra, Gegear, & Reppert, 2014; Guerra & Reppert,

2013; Merlin, Gegear, & Reppert, 2009; Zhu et al., 2008), as well as hormonal mechanisms that control monarch diapause development (Barker & Herman, 1973; Dallmann & Herman, 1978; Herman, 1975; Herman & Lessman, 1981). The missing link, however, is how monarchs, and other diapausers, integrate external cues with internal, genetically-controlled responses to achieve specifically timed seasonal responses (Hand, Denlinger, Podrabsky, & Roy, 2016). Here, we use monarch diapause termination as a model to understand molecular control of environmental response and seasonal timing.

Diapause development proceeds through stereotypic ecophysiological phases: initiation, maintenance, and termination (Andrewartha, 1952; Koštál, 2006). Together, initiation and termination define the specific time interval of diapause maintenance, the environmentally insensitive refractory period during which organisms experience suppressed metabolic rate, bolstered stress resistance, and halted reproductive development (Herman, 1973). Upon termination, individuals either immediately resume nondiapause development if prevailing conditions are permissive (e.g., warm), or as is more often the case, enter post-diapause quiescence, an environmentally sensitive dormancy, in adverse conditions (e.g., cold). Monarch diapause, like that of many other temperate species, is primarily induced by low and decreasing photoperiod, and is also enhanced by low temperature and host plant quality (Goehring & Oberhauser, 2002). Termination, on the other hand, is much more variable among insects and is rarely under photoperiodic control in hibernal diapauses (Hodek, 2002; Koštál, 2006; Liedvogel & Lundberg, 2014; Tauber, Tauber, & Masaki, 1986). Eastern and western North American monarch populations terminate diapause before the winter solstice (Herman, 1981; Herman, Brower, & Calvert, 1989), proving that increasing photoperiod is not a relevant termination cue, although not excluding any photoperiodic involvement. Counterintuitively, cold temperature often hastens spontaneous diapause termination in overwintering diapausers (reviewed in Tauber et al., 1986). How cold temperature controls diapause timing in insects is unknown. More generally, how nonphotoperiodically controlled developmental timing occurs is a little explored and open problem.

Hormonal signalling plays critical roles in insect diapauses. In preadult diapauses, 20-hydroxyecdysone (20-HE) is most often recognized as the diapause terminator (reviewed in Denlinger, Yocum, & Rinehart, 2011). The role of 20-HE in adult reproductive diapause is more variable. 20HE plays opposing roles in different organisms, promoting diapause termination and reproduction in fruit flies (*Drosophila melanogaster*), locusts (*Locusta migratoria*), and European firebugs (*Pyrrhocoris apterus*), while being associated with diapause maintenance in Colorado potato beetles (*Leptinotarsa decimlineata*) (reviewed in Denlinger et al., 2011). Insulin/insulin-like peptide signalling (IIS) has been shown to influence adult reproductive diapause phenotypes in fruit flies and mosquitoes (*Culex pipens*) (Sim & Denlinger, 2008; Williams et al., 2006). The consistent controller of adult reproductive diapause across insects is juvenile hormone (JH) (reviewed in Tauber et al., 1986). Increased JH titre is associated with reproductively active monarchs in spring and summer (Lessman et al., 1989). JH signalling is required for reproductive development in nonmigrating monarchs, and exogenous JH analogue application is sufficient to terminate diapause and induce reproductive development in normally nonreproductive migrant monarchs in summer-like conditions (Barker & Herman, 1973; Herman, 1975). While these studies consistently associate JH signalling with reproductive state, they leave open the specific mechanism by which JH controls diapause because they do not follow JH signalling dynamics with sufficient temporal resolution across diapause development. Despite knowing that these hormonal pathways are associated with particular diapause states, it is not clear how these pathways actually function in diapause development. Are they involved in transducing the environmental signal? Do they engender maintenance phenotypes? Do they specify termination? Moreover, JH, ecdysteroid, and IIS pathways interact to achieve coherent responses in different contexts. For example, in *Drosophila*, JH does not control metamorphosis timing as it does in *Manduca sexta* (Nijhout & Williams, 1974; Riddiford, Truman, Mirth, & Shen, 2010). Rather, JH indirectly regulates larval growth rate through controlling ecdysone concentration, versus its timing, whereby influencing IIS pathway activity, which is a well known controller of larval growth rate (Mirth et al., 2014). How do these interactions change in different contexts, and what role, if any, does diapause play in influencing interactions between JH, ecdysteroid, and IIS?

Here, we examined diapause termination in the western North American monarch population in order to understand (a) the relative influence of external environmental cues versus internal physiological mechanisms for terminating diapause; (b) what molecular mechanisms mediate environmental sensitivity during diapause development; and (c) what controls diapause timing. We took a comparative transcriptomics approach to investigate the molecular basis of termination because this allowed us to simultaneously assay genome-wide responses in situ without molecular manipulation of the system.

2 | MATERIALS AND METHODS

2.1 | Experimental design

Herman (1981) previously demonstrated that female monarchs from California undergo true diapause from September to December as measured by decreased sensitivity of reproductive organs to artificial summer-like conditions. We conducted a nearly identical experiment in which we tested responsiveness of wild-caught female monarchs ("Natural" cohort) to simulated summer conditions in an environmental chamber over the course of the overwintering season (November 2015–January 2016). We extended the Herman analysis by adding a treatment in which monarchs that were collected in November were held over the course of the overwintering season in an environmental chamber that approximated conditions at the overwintering site in November ("Chamber" cohort). Female overwintering monarch butterflies were collected from private property (with appropriate permissions granted) near Pismo Beach, CA.

Monarchs were batch collected from roosts and kept in cool, moist conditions until overnight shipment to the University of Chicago (within 48 hr).

Natural cohort females were collected from the overwintering site three times throughout the season and initially acclimated for 4 days in "fall" conditions: 10 hr light at 17°C, 14 hr dark at 10°C (Figure 1a). The initial collection in November was large in order to establish the "Chamber" cohort. Chamber cohort females remained in the chamber for the duration of the experiment. After 4 days in fall conditions, we switched a random sample of Chamber cohort females, as well as newly acclimated Natural cohort females, into "summer" conditions (16 hr day at 25°C, 8 hr night at 18°C) to test for reproductive development after 10 days. At the same acclimation day 4, we evaluated ovary development for a random sample of five females from both Chamber and Natural cohorts without transfer to summer conditions. Samples sizes for each group are indicated in Figure 1b. We set minimum target sample sizes of n = 5 individuals per mature oocytes (MO) count group and n = 3 individuals per group for sequencing. We met these targets for all but the Natural December summer cohort for which we obtained four MO counts and sequenced transcriptomes for two individuals. Individual numbers were largely determined by the number of individuals that survived shipment (Natural cohort), extended containment in the fall chamber (Chamber cohort), and experimental (summer condition) treatment. With these sample sizes, we saw similar variation to that observed by Herman (1981), suggesting that these sizes were sufficient to capture variation in the trait.

Chamber cohort individuals in fall conditions were fed butterfly nectar twice per week. Natural cohort individuals were fed once the day after arrival at the University of Chicago. Chamber cohort individuals were fed on the same day. Therefore, all individuals were fed 3 days prior to removal from fall conditions for dissection or for transfer to summer conditions. All individuals were fed every other day in the summer condition.

We chose to analyze MO in females after 10 days in summer conditions as a measure of reproductive maturity. MOs were defined as fully-chorionated oocytes with ridges appearing along the length. In a small number of individuals (n = 4 total; n = 2 in each December and January Natural cohorts), only a single ovary was significantly developed to the stage of containing MOs. We only recorded MOs for those females in which both ovaries were distinctly visible, indicating normal oocyte development. We chose to measure diapause response in females because Herman (1981) found that male diapause response measurement was less robust and potentially more variable. Female MO number showed the strongest response to diapause of all the reproductive phenotypes, male or female, measured in the study. Female reproductive organs also responded in similar degree to one another, while male reproductive organs showed variability in their responses. Despite this differential response, Herman (1981) did demonstrate that a clear diapause period also exists in male monarchs. This all suggests that the measure of diapause in males is variable and not necessarily the diapause itself.

In no case did a female show significant ovary development (presence of mature or immature oocytes; evidence of vitellogenesis) in fall conditions. Therefore, butterflies transferred to summer conditions throughout the experiment almost certainly contained no MOs when initially placed in summer conditions. All females from all time points showed significant ovary development and contained at least one MO upon analysis at 10 days in summer conditions.

2.2 | Statistics

Comparisons of group central tendencies were made using the corrected Mann–Whitney/Wilcoxon rank-sum W test statistic (to

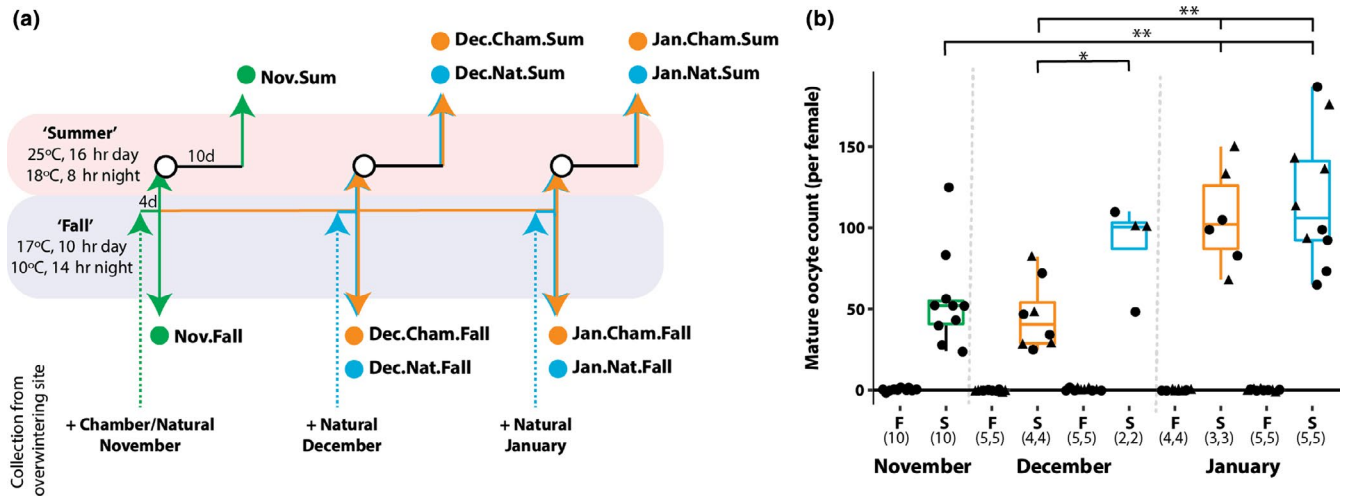


FIGURE 1 Environmental chamber modularizes internal and external components of diapause termination. (a) Overview of the design of the environmental chambers experiment. "Fall" and "summer" conditions are as noted. d, days. (b) Natural (blue) and Chamber (orange) cohorts display different diapause termination dynamics over the course of the overwintering season. Each point/shape represents an individual monarch. Triangles represent individuals included in sequence analysis. Numbers in parentheses present sample sizes. Where two numbers presented, the first is the number of individuals sequenced. F, Fall conditions; S, Summer conditions. *p < .05; **p < .005 [Colour figure can be viewed at wileyonlinelibrary.com]

account to MO count non-normality) unless otherwise noted as implemented in R version 3.5.0 (R Core Team, 2018).

2.3 | RNA extraction, library preparation and sequencing

Half of the butterflies within a cohort, time point, and treatment were selected for sequencing. Butterflies were acclimated at room temperature for approximately 1 hr prior to tissue collection. Heads were collected with antennae and proboscises removed, immediately flash frozen in liquid nitrogen, and stored at −80°C until further processing. Total RNA was extracted from single heads in two separate fractions ("large": >200 nt bases and "small": <200 nt) using the MACHEREY-NAGEL NucleoSpin miRNA kit (Cat #740971). Large RNA fractions (1 μg) were processed for ribosomal RNA depletion using a custom protocol adapted from the RNaseH hybrid degradation method described by Morlan, Qu, and Sinicropi (2012). Single-stranded synthetic DNA probes (Integrated DNA Technologies) were designed to include sequences complementary to the *D. plexippus* 5S, 18S and 28S rRNAs. Probes were 50 bases in length and designed to start 100 bases apart such that every 50 bases of each sequence were covered. Probes were resuspended in water and pooled at a final concentration of 0.5 μM for each probe. With this monarch-specific probe mix, the Morlan et al. (2012) protocol was followed, and depleted RNA was purified with the MACHEREY-NAGEL RNA Clean-up XS kit (Cat #740903).

Libraries were prepared using the KAPA Stranded RNA-Seq kit (Cat #KK8401). Samples were quality controlled by BioAnalyzer and confirmed to have no significant adapter peaks. Samples were individually indexed and sequenced as a single pool on three lanes of the Illumina HiSeq4000 (single end 50 bp reads).

2.4 | Read mapping statistics

RNA sequencing generated 639,995,826 raw reads from 33 monarch head libraries. Quality filtering resulted in 573,340,723 uniquely mapped reads (average 89.6% uniquely mapping reads). Reads were mapped to the *D. plexippus* genome assembly version 3 (Zhan & Reppert, 2012) using STAR version 2.5.2b (Dobin et al., 2012) and gene read counts were generated by htseq2 within STAR (mapped to OGS 2.0). 349,776,743 unique reads (61% of uniquely mapped reads, 54.7% of total reads) mapped to exons. This exon sequence coverage met expected coverage values given the use of an rRNA depletion protocol versus mRNA enrichment (Zhao et al., 2014).

2.5 | Data quality control, differentially expressed gene identification, and functional annotation

We used DESEQ2 version 3.8 (Love, Huber, & Anders, 2014) to identify differentially expressed genes (DEGs). To test the general effect of each experimental variable across all levels of the remaining two, we used likelihood ratio testing to identify DEGs. For individual group comparisons, a grouping variable was created that combined the cohort "source" (Chamber vs. Natural), month, and experimental condition (fall vs. summer). This variable was used to define the linear model of the counts and contrasts of individual groups that were used to identify specific groups of DEGs via the Wald test. We used clustering functions within DESEQ2 and PCAEXPLORER version 2.8.0 (Love et al., 2014; Marini & Binder, 2017) to assess variability within the data set. We used the top 5,000 genes with most variable (normalized) expression across all samples to cluster samples. For visualization and clustering, gene counts were normalized using a variance stabilizing transformation and blinded dispersion estimation as implemented in DESEQ2 by the versus *t*() function. Genes with adjusted *p*-value (padj) < .05, baseMean > 10 and |log2FoldChange| > 0.5 were considered as significantly differentially expressed. Gene set enrichment analysis was conducted in BLAST2GO version 5.2.0 (Conesa & Götz, 2008; Conesa et al., 2005; Götz et al., 2008, 2011) using default parameters. Fisher's exact test was performed using a reference set containing 10,599 genes comprising the head transcriptome (genes with baseMean > 10).

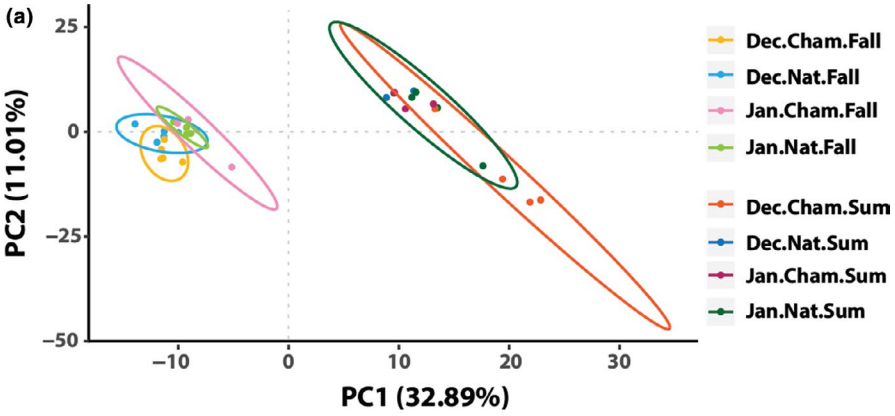
3 | RESULTS

3.1 | Chamber fall conditions modularize environmental versus internal control of diapause timing

To dissociate diapause dynamics that were a response to natural environmental cues experienced at the overwintering site versus dynamics controlled by internal physiological mechanisms, we compared reproductive development in wild-caught females ("Natural" cohort) versus those kept in an environmental growth chamber ("Chamber" cohort; Figure 1a). First, we found that Natural cohort diapause dynamics upon exposure to summer conditions are consistent with those found by Herman (1981). In the Natural cohort, MO count increased from November (mean = 55.5, s.e.m. = 5.8) to December (mean = 89.8, s.e.m. = 5.2; $W = 9$, $p = .1358$) and from December to January (January mean = 117.70, s.e.m. = 13.2; $W = 25$, $p = .5395$) (Figure 1b) in summer conditions. Comparison of November and January counts reveals a significant increase in MO count ($W = 8$, $p = .001679$; Figure 1b), while intermediate comparisons did not. In November, we recorded individuals with 125 and 83 MOs and interpret these points as females that had already terminated diapause and entered post-diapause quiescence before transfer to summer conditions. In December, a single outlier of comparatively low MO count (MO = 48; Figure 1b) was recorded among females with at least 100 MOs, probably indicating that this individual was in diapause when initially transferred to summer conditions. Herman (1981) found similar variation in this population. Altogether, these data show that the majority of individuals in the Natural cohort terminated diapause between November and December.

The Chamber cohort showed different diapause dynamics compared to the Natural cohort. Chamber cohort MO count was unchanged from November to December (December mean = 45.6, s.e.m. = 7.5; $W = 49.5$, $p = .4227$; Figure 1b) and significantly

lower than the Natural cohort in December ($W = 29.5$, $p = .02698$; Figure 1b) in summer conditions. This indicates that Chamber cohort monarchs persist in diapause in December while Natural cohort monarchs terminate diapause over the same time period. This reveals environmental regulation of diapause termination dynamics and suggests that some aspect of natural conditions promotes early diapause termination. We confirm that increasing photoperiod is not the relevant condition because photoperiod declined between the 21 November and 13 December time points, during which the Natural cohort terminated diapause. We can also reject absolute number of short photoperiod days because both Natural and Chamber cohorts experienced the same number of short days. These data leave open the possibility that decreasing photoperiod is an environmental controller because photoperiod was constant in the fall chamber. However, we do not believe decreasing photoperiod controls diapause termination because decreasing photoperiod increases incidence of diapause induction in monarchs (Goehring & Oberhauser, 2002), and variation in day length is small during this interval. Based on day length calculations at Pismo Beach, CA, photoperiod decreases an average of 59 s per day (maximum 86 s), and the average rate of change of photoperiod reduction is 2.6 s per day. Therefore, we conclude that photoperiod



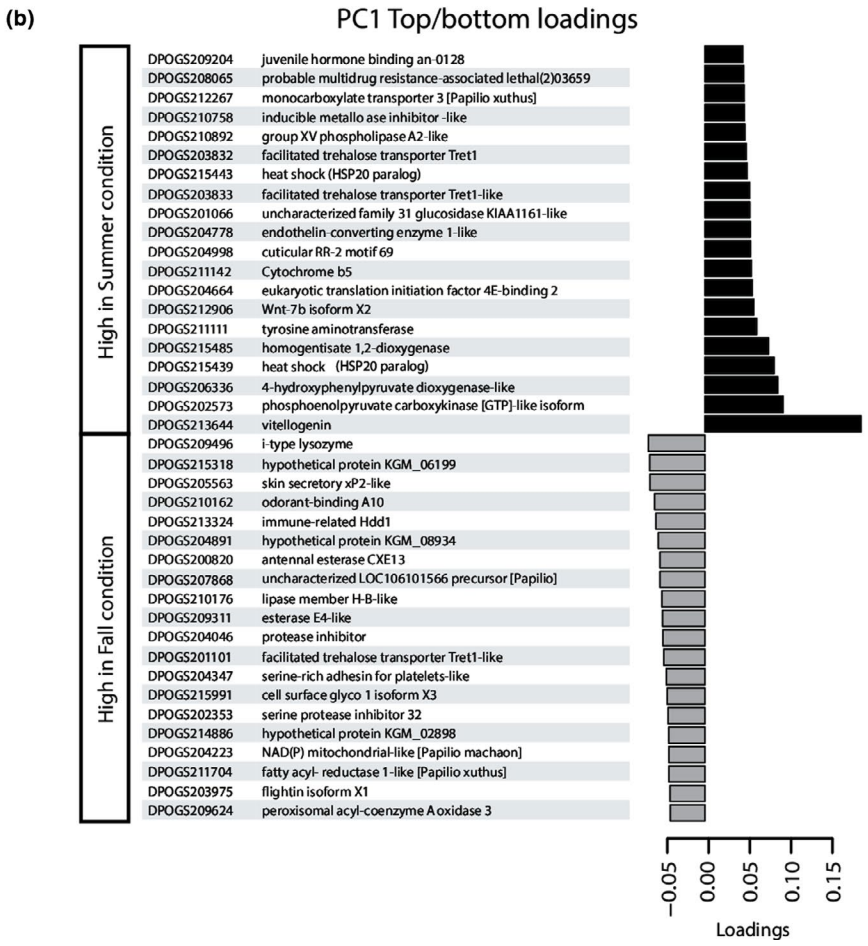


FIGURE 2 PCA analysis of head transcriptional profiles distinguishes reproductive status and diapause development. (a) The results of PCA analysis of head transcriptomes of individuals from eight indicated groups. Ellipses represent 95% confidence intervals. (b) Top 20 loadings (both positive and negative) for PC1 [Colour figure can be viewed at wileyonlinelibrary.com]

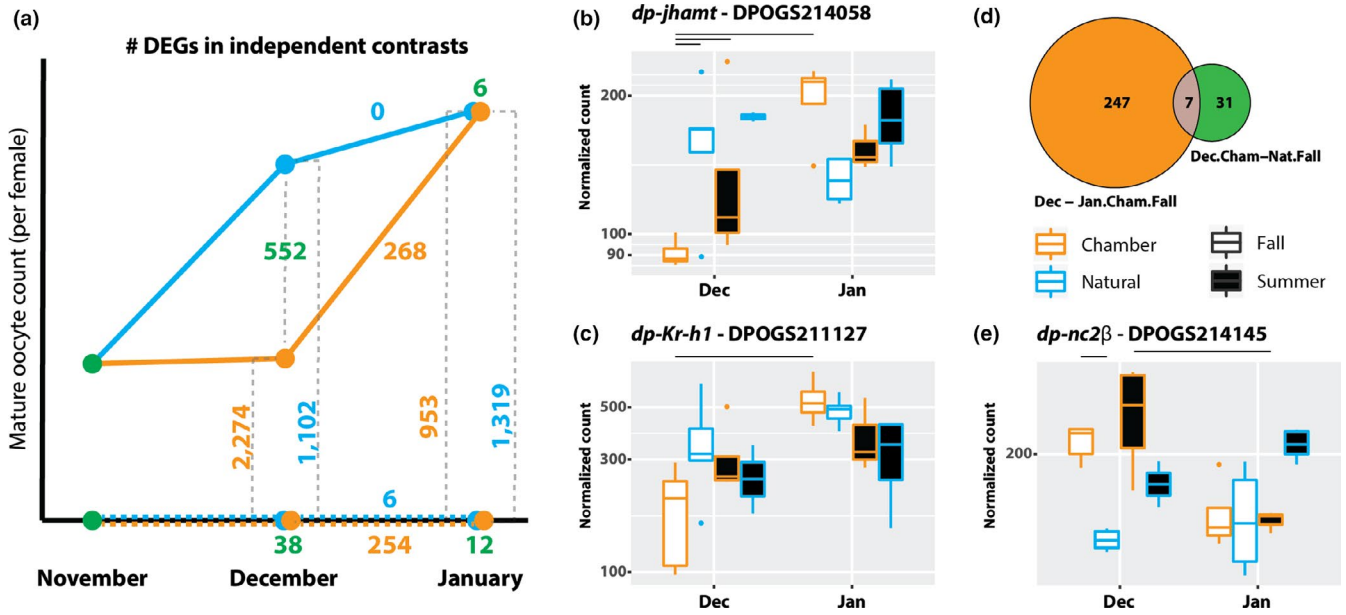


FIGURE 3 Differential termination dynamics in Chamber and Natural cohorts reveal genetic controllers of component termination processes. (a) Summary of DEG numbers across tested groups and indicated comparisons. Number of DEGs in comparisons between Chamber and Natural cohorts, Chamber cohorts, and Natural cohorts for the indicated months and conditions are indicated in green, orange, and blue, respectively. (b and c) Normalized counts of *dp-jhamt* and *dp-Kr-h1*. (d) Venn diagram of DEGs found in fall conditions in two diapause versus nondiapause comparisons (1. December vs. January Chamber cohort and 2. Chamber vs. Natural cohorts in December). (e) Normalized counts of *dp-nc2β* transcription factor. Lines under gene names indicate pairwise comparisons for which normalized counts significantly differ (FDR adjusted *p*-value < .05) [Colour figure can be viewed at wileyonlinelibrary.com]

does not play a significant role in modulating the diapause termination timer in monarchs.

Chamber cohort MO count increased significantly in January in summer conditions (mean = 106.3, s.e.m. = 12.5; W = 46, p = .002664; Figure 1b), showing that this cohort does not persist perpetually in diapause under fall conditions, as do some species (reviewed in Andrewartha, 1952; Tauber et al., 1986). Chamber cohort monarchs eventually terminate diapause between December and January despite being in a constant fall chamber without receiving natural cues. This demonstrates that diapause termination is controlled by a physiological timer. Surprisingly, Chamber cohort MO count was indistinguishable from the Natural cohort in January (W = 26.5, p = .7447; Figure 1b) in summer conditions. Nearly identical reproductive development between Natural and Chamber cohorts in January suggests that the physiological timer controls a spontaneous termination response. Taken together, these results demonstrate that an environmentally-sensitive, internal physiological timer controls spontaneous diapause termination.

3.2 | Head transcriptomes capture molecular profiles of reproductive development and diapause development

To generate hypotheses for molecular determinants of the internal timer and infer mechanisms that mediate the distinct diapause responses observed between the Chamber and Natural cohorts, we generated head transcriptomes for Chamber and Natural cohort individuals in December and January in fall (diapause/post-diapause quiescence) and summer (reproductive) conditions. We focused on heads in order to uncover putative neurohormonal and neuroendocrine mechanisms of diapause development. To get a general description of gene expression variation in the data set, we first assessed the main factors grouping the head transcriptomes via PCA on all samples. Individuals were most strongly differentiated by reproductive development (diapause/post-diapause quiescence vs. reproductive) as indicated by distinct clustering along PC1, which explained 33% of the variance in the data (Figure 2a). The number of genes differentially expressed between fall and summer conditions ranged from 953 to 2,274 (9%–21.5% of the head transcriptome; Figure 3a; Tables S2a–d). These broad transcriptomic shifts are consistent with previous transcriptome profiling of diapause development in other insects (Koštál, Štětina, Poupardin, Korbelová, & Bruce, 2017; Ragland, Denlinger, & Hahn, 2010), even though these studies were conducted with whole animals. The strongest loading on PC1 was vitellogenin (DPOGS213644; Figure 2b), whose expression has been related to diapause status in monarchs (Pan & Wyatt, 1976) and other insects (Okuda & Chinzei, 1988) because it reliably reflects the state of vitellogenesis and oocyte maturation (reviewed in Roy, Saha, Zou, & Raikhel, 2018). More generally, PC1 captures the increased metabolic activity, protein translation, and morphogenetic events related to the nondiapause reproductive state (Figure 2b). PC1 also reflects increased immune-related transcription (e.g., DPOGS209496, a predicted lysozyme, DPOGS213324, a predicted

HDD1-reltaed protein, and DPOGS202353, *Drosophila* Serpin 77Ba homolog; Figure 2b) that is characteristic of insect diapause states (Denlinger, 2002; Ragland et al., 2010). From December to January, there is a slight shift of both Chamber and Natural diapause/post-diapause quiescence cohorts along PC1 towards the reproductive expression profiles. This suggests that post-diapause quiescence is not only a distinct state from diapause, but also may involve the initial steps towards the reproductive transcriptional profile, even in fall conditions.

We next identified differentially expressed genes (DEGs) for each of the three experimental variables (month: December vs. January, source population: Chamber vs. Natural, and condition: Fall vs. Summer) across all levels of the remaining two variables. Relatively few genes were differentially expressed due to month (n = 54 DEGs) and source (n = 33 DEGs) (Tables S1a,b). No GO terms were significantly enriched, even at the highly permissive FDR value of 0.2, in either of these gene sets. In contrast, rearing condition generated the greatest changes in gene expression (n = 1,870 DEGs; Table S1c). Significant GO terms reflect increased metabolic activity (carbohydrate, chitin, amino acid, trehalose, and protein metabolism), redox activity and neurotransmission (Table 1).

3.3 | Diapause termination converges on similar transcriptional profiles in Chamber and Natural cohorts

Diapause termination is often classified as occurring naturally over an extended period of time ("horotelic") or being induced in an accelerated period of time under laboratory settings ("tachytelic"; Hodek, 2002, 2012; Koštál, 2006; Tauber et al., 1986). In our experiment, transfer to summer conditions constitutes tachytelic termination, while both the Chamber and Natural cohorts terminate diapause and enter post-diapause quiescence via horotelic termination in fall conditions. Therefore, we expected transcriptional profiles of the Chamber and Natural cohorts, in both fall and summer conditions, to be similar if termination in the two cohorts reflects the same underlying processes. Indeed, we found that head transcriptomes from Chamber and Natural cohorts in January formed overlapping clusters in the PCA in both fall and summer conditions (Figure 2). Only 12 and six genes (0.11% and 0.06% of head transcriptome, respectively) were differentially expressed between Chamber and Natural cohorts in fall and summer conditions, respectively (Figure 3a; Table 2e–f). This is despite the fact that 254 genes (2.4% of head transcriptome) were differentially expressed in the Chamber cohort between December and January (Figure 3a; Table S2g), consistent with the major transcriptomic change expected of a transition from diapause to post-diapause quiescence (Koštál et al., 2017; Ragland et al., 2010). Natural cohort transcription changed much less between December and January, showing only six DEGs (0.06% of head transcriptome; Figure 3a, Table S2h). This is consistent with the interpretation that most Natural cohort individuals have already exited diapause and entered post-diapause quiescence by December. These results demonstrate that natural and chamber diapause termination mechanisms converge upon common transcriptional profiles both in fall and summer conditions. Furthermore, they indicate that the chamber condition represents naturally-relevant diapause mechanics, revealing modular controls of diapause termination

TABLE 1 "Biological Process" GO terms overrepresented in 1,870 DEGs that differ due to rearing condition among all source populations and months

GO ID	GO Name	FDR adjusted *p*-value
GO:0006022	Aminoglycan metabolic process	3.79E−04
GO:0006030	Chitin metabolic process	9.36E−04
GO:0055114	Oxidation-reduction process	.002
GO:1901071	Glucosamine-containing compound metabolic process	.002
GO:0006040	Amino sugar metabolic process	.003
GO:0006508	Proteolysis	.003
GO:0005975	Carbohydrate metabolic process	.007
GO:0007218	Neuropeptide signalling pathway	.012
GO:0005991	Trehalose metabolic process	.033
GO:0005984	Disaccharide metabolic process	.033
GO:0042133	Neurotransmitter metabolic process	.033
GO:0006720	Isoprenoid metabolic process	.041
GO:0008299	Isoprenoid biosynthetic process	.041
GO:0042391	Regulation of membrane potential	.042
GO:0060078	Regulation of postsynaptic membrane potential	.042
GO:0060079	Excitatory postsynaptic potential	.042
GO:0099565	Chemical synaptic transmission, postsynaptic	.042
GO:0003008	System process	.042
GO:0009123	Nucleoside monophosphate metabolic process	.042
GO:0050877	Nervous system process	.047
GO:0009127	Purine nucleoside monophosphate biosynthetic process	.047
GO:0009168	Purine ribonucleoside monophosphate biosynthetic process	.047
GO:1901605	Alpha-amino acid metabolic process	.049
GO:0044262	Cellular carbohydrate metabolic process	.051
GO:0009124	Nucleoside monophosphate biosynthetic process	.058

TABLE 2 "Biological Process" GO terms overrepresented in 38 DEGs between Chamber and Natural cohorts in fall conditions in December

GO ID	GO Name	FDR adjusted *p*-value
GO:0051282	Regulation of sequestering of calcium ion	.004
GO:0051283	Negative regulation of sequestering of calcium ion	.004
GO:0051208	Sequestering of calcium ion	.004
GO:0051209	Release of sequestered calcium ion into cytosol	.004
GO:0007204	Positive regulation of cytosolic calcium ion concentration	.004
GO:0097553	Calcium ion transmembrane import into cytosol	.004
GO:0051480	Regulation of cytosolic calcium ion concentration	.004
GO:0060402	Calcium ion transport into cytosol	.004
GO:0072503	Cellular divalent inorganic cation homeostasis	.009
GO:0072507	Divalent inorganic cation homeostasis	.009
GO:0006874	Cellular calcium ion homeostasis	.009
GO:0060401	Cytosolic calcium ion transport	.009
GO:0055074	Calcium ion homeostasis	.009

dynamics (i.e., environment vs. internal timing). We propose that in natural conditions, the internal physiological timer exerts ultimate control over diapause termination and environmental signals modulate the timer.

3.4 | Calcium signalling mediates environmental modulation of diapause termination timing

Natural cohort monarchs exit diapause earlier than those of the Chamber cohort, indicating that specific environmental conditions at the overwintering site modulate diapause termination timing. In order to gain insight into what these environmental factors might be, we identified potential functional differences between the Chamber and Natural cohorts in December in fall conditions. A total of 38 DEGs were found in this comparison (Table S2i). Cellular calcium ion regulation is significantly overrepresented among these genes (Table 2). Calcium signalling mediates environmental and stress response in many organisms, and specifically influences cold sensing in insects (Teets, Yi, Lee, & Denlinger, 2013). Chilling advances, and in many cases is required for, diapause termination in many temperate insect species (reviewed in Tauber et al., 1986). Therefore, our data suggest that temperatures in the natural overwintering environment were, at periods, colder than the fall environmental chamber, and this, in turn, led diapause to terminate earlier in the Natural cohort than in the Chamber cohort.

3.5 | JH signalling is a general marker of post-diapause quiescence and a key timer target, but is not itself the timer

Monarchs in diapause are characterized by low haemolymph JH titre, while reproductive monarchs in permissive conditions have high JH titre (Lessman et al., 1989). We found in the Chamber cohort that JH signalling increased between December and January, during the diapause to post-diapause quiescence transition, in fall conditions. Two key genes regulating JH biosynthesis and downstream activity were significantly upregulated in January: juvenile hormone acid O-methyltransferase (*dp-jhamt*; DPOGS214058) and Krüppel homolog 1 (*dp-Kr-h1*; DPOGS211127) (Figure 3b–c; Table S2g). JHAMT is expressed in the corpora allata in insects and has been shown to be the rate-limiting enzyme in JH biosynthesis in *Bombyx mori* (Shinoda & Itoyama, 2003). Kr-h1 is expressed in peripheral tissues and is an early-response transcription factor target and effector of JH signalling (Kayukawa et al., 2012; Minakuchi, Zhou, & Riddiford, 2008). These results show that in January, under fall conditions, JH biosynthesis is upregulated, leading to increased JH activity and *dp-Kr-h1* transcription. Furthermore, of the 38 genes differentially expressed between Natural and Chamber cohorts in fall conditions in December (Figure 3a; Table S2i), seven genes were found in the intersection of the two diapause versus post-diapause quiescence comparisons (December vs. January in Chamber cohort in Table S2l). Among these seven was *dp-jhamt*, whose expression is significantly upregulated in the Natural cohort. This suggests that increased JH biosynthesis is a general marker of post-diapause quiescence and is a key controller of environmental response.

JH promotes diapause termination through 20-HE signalling in some insects (Denlinger, 1979; Zdárek & Denlinger, 1975). Surprisingly, no regulators or downstream transcriptional targets of ecdysteroid signalling were found to be differentially expressed from December to January in the Chamber cohort in fall conditions (Table S2g). The lack of 20-HE-dependent transcriptional changes indicates that JH signalling does not activate ecdysteroid signalling in fall conditions, and further suggests that 20-HE does not control diapause termination timing in fall conditions in monarchs.

Administration of high amounts of JH promotes reproductive development in permissive conditions in diapause monarchs (Herman, 1981). However, development is significantly reduced in the JH-induced diapause monarchs compared to post-diapause monarchs. Herman (1981) hypothesized that this dampened response was a result of reduced effective JH titre. We did not find evidence that JH clearing (via esterases or epoxide hydrolases) or sequestration (via hexamerins) are increased in diapause butterflies compared to nondiapause cohorts (Table S2g,i). This implies that additional mechanisms beyond low JH titre act to suppress response to permissive environmental conditions. Furthermore, it indicates that JH signalling, in and of itself, is not the timer. This leaves two open questions: what additional mechanisms during diapause control response to inductive conditions and what is the primary internal timing mechanism?

The monarch homolog of the *Negative Cofactor 2β* (dp-NC2β; also referred to as "Dr1") transcription factor presents a compelling candidate for a controller of environmental response during diapause and the proximate diapause timer. Although only a modest number of DEGs differentiated Chamber and Natural cohorts in fall conditions in December (38 DEGs; Figure 3a), the Chamber cohort transcriptome showed substantially greater change in summer conditions than did the Natural cohort (2,274 vs. 1,102 DEGs; Figure 3a). This suggests that one or more of the 38 DEGs may be a factor with broad control of transcription. We found that *dp-nc2β* (DPOGS214145) was significantly upregulated in the Chamber cohort compared to the Natural cohort in fall conditions in December (Table S2i). *dp-nc2β* was also upregulated in December compared to January in the Chamber cohort in summer conditions (Table S2j). NC2β is a conserved protein that contributes to histone acetyltransferase activity and activates downstream promoter element binding motifs and represses TATA promoters (Willy, Kobayashi, & Kadonaga, 2000). It influences global transcriptional dynamics in response to heat or stress in yeast, *Drosophila*, and humans (de Graaf et al., 2010; Honjo, Mauthner, Wang, Skene, & Tracey, 2016; Spedale et al., 2011; Wang, Faiola, Xu, Pan, & Martinez, 2008). These results are consistent with the hypothesis that *dp-nc2β* expression renders monarchs insensitive to inductive summer conditions and may also directly control diapause termination timing via histone acetylation.

3.6 | Diapause state controls environmental sensitivity through regulating hormonal and neuroendocrine pathways

Genes that differ in expression in the inductive summer environmental conditions as a consequence of diapause state are the effective targets of diapause. To identify these genetic pathways that mediate environmental response with respect to diapause state, we identified DEGs between December and January Chamber cohorts in summer conditions. Hundreds of genes are differentially expressed between December and January in the Chamber cohort in summer conditions (n = 268 DEGs; Table S2j). Redox chemistry functions differed significantly between these months as evidenced by the single GO term (GO:0055114, oxidation-reduction process) to reach the

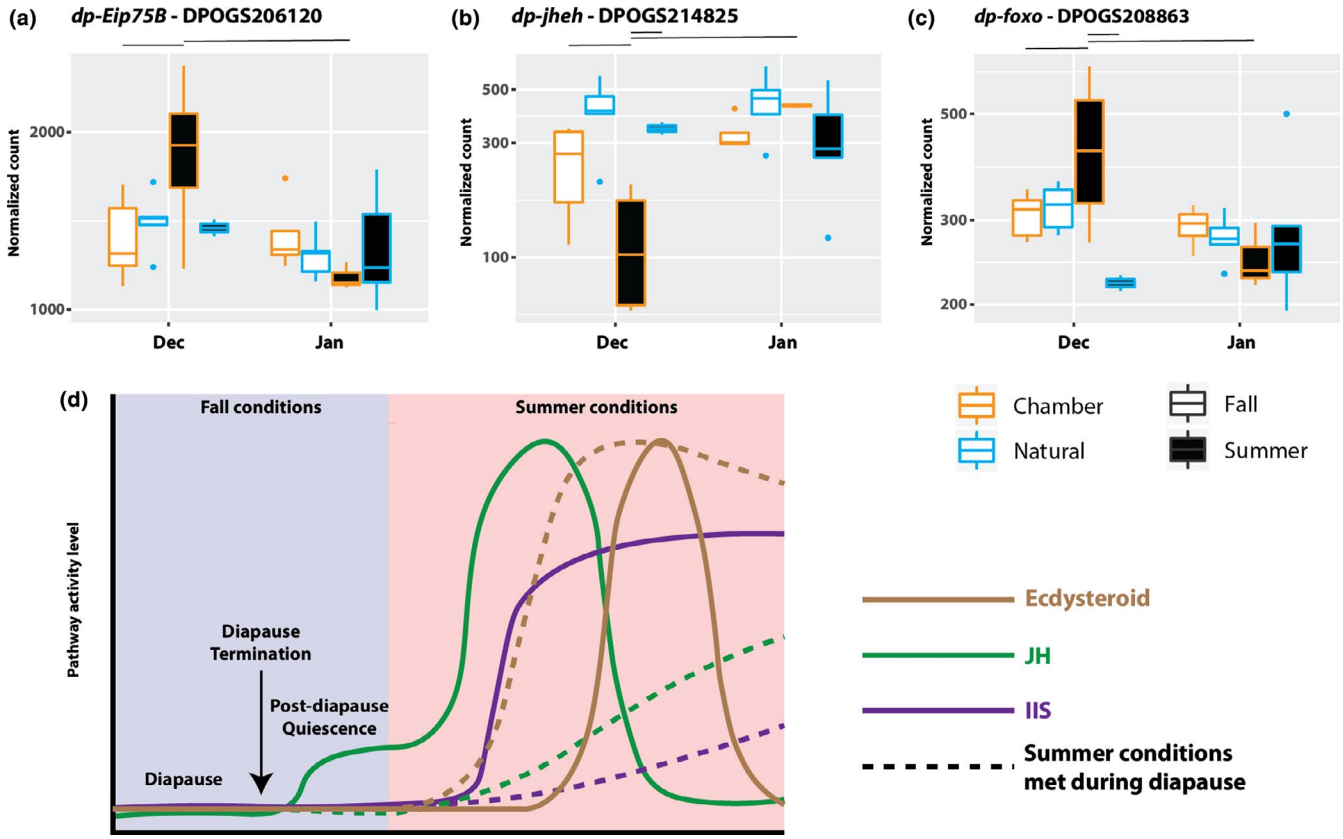


FIGURE 4 Hormonal gene expression is targeted by diapause programme. Normalized counts of (a) *dp-e75* transcription factor (DPOGS206120), (b) *dp-jheh* homolog (DPOGS214825), and (c) *dp-foxo* (DPOGS208863) in ecdysteroid, JH and IIS pathways, respectively. Lines under gene names indicate pairwise comparisons for which normalized counts significantly differ (FDR adjusted p-value < .05). (d) Putative model of ecdysteroid (brown), JH (green), and IIS (purple) pathway activity in fall conditions (blue background) and summer conditions (red background). Solid lines indicate pathway activity under normal diapause development in which case individuals experience summer conditions after having transitioned to post-diapause quiescence. Dashed lines indicate pathway activity in the case when individuals are still in diapause upon experiencing summer conditions. Diapause development labels apply to solid lines, only. See main text (Discussion) for detailed explanation of model interpretation [Colour figure can be viewed at wileyonlinelibrary.com]

significance threshold (FDR = 0.052). These results suggest that energy metabolism operates differently depending on diapause state at the time that summer conditions are entered.

Hormonal pathways previously associated with insect diapause were changed. Ecdysteroid signalling, which involves a cascade of oxidation-reduction reactions mediated by cytochrome P450 enzymes, plays a role in differential response. Several components related to ecdysone and 20-HE metabolism and transcriptional regulation are differentially expressed (Table S2j; *dp-Eip75B* (DPOGS206120) shown as example in Figure 4a), demonstrating that diapause alters ecdysteroid response in summer conditions. A strongly expressed JH epoxide hydrolase (*dp-jheh*) isoform (DPOGS214825), which degrades JH, shows significantly low expression in December in the Chamber cohort in summer conditions (Figure 4b). This result suggests that diapause alters JH clearance dynamics in summer conditions. Finally, the transcription factor *dp-foxo* (DPOGS208863), a central effector and negative transcriptional regulator of the IIS pathway, is expressed at significantly high levels in the Chamber cohort in December compared to January. In fact, the Chamber cohort in December presents the single exception where *dp-foxo* expression in summer conditions remains high, mirroring relative reproductive development among groups (Figure 4c). This suggests that diapause causes repression of IIS activity upon experiencing summer conditions. It appears that diapause forces specific regulation of these pathways in order to control environmental response.

4 | DISCUSSION

4.1 | Diapause termination as a model of long-term timekeeping

Diapause has been studied for over a century in scores of species across class Insecta (Andrewartha, 1952) not least of which because it exemplifies fundamental properties of biological systems including the recording and storage of historical information over long, specific time periods. Although resolution of both of these properties requires elucidation of a diapause termination mechanism, termination remains poorly understood. Lehmann et al. (2018) outlined particular challenges of studying termination that are primarily a result of the complex interaction of environment and internal physiological control. By studying a combination of different source populations in different environmental chamber conditions over time, we were able to modularize individual components of termination and identify molecular controllers of specific termination processes. While the focus of this work was on head transcription in order to understand neurohormonal controllers of diapause timing, other tissues, including the reproductive organs and abdominal fat body, are associated with the diapause response. In subsequent work it will be interesting to investigate how these organs communicate with the diapause timer to coordinate overall diapause response.

Our data suggest that epigenetic mechanisms play a critical role in the recording and storage properties of biological timing. Epigenetic regulation of diapause development in other insects has previously been hypothesized (reviewed in Reynolds, 2017). Moreover, seasonal photoperiodic timing is controlled epigenetically through histone modification in plants and mammals. In a classic example of seasonal timing, histone modifications at FLOWERING LOCUS C control vernalization and flowering time in *Arabidopsis thaliana* (reviewed in Whittaker & Dean, 2017). Histone methylation controls hormonal regulation of a winter refractory period in Siberian hamsters (Stevenson & Prendergast, 2013). Interestingly, both of these cases involve timing of cold-mediated refractory periods such as in monarch adult reproductive diapause. Validation of this mechanism in monarchs would add to the mounting evidence that epigenetics is a common way across diverse biological systems to achieve long-term timekeeping (e.g., Stevenson & Prendergast, 2013; Whittaker & Dean, 2017).

Although we found that canonical neurohormonal (ecdysteroid, JH, and IIS) pathways are important for mediating environment-dependent response, our work suggests that histone modification and JH signalling, specifically, work in concert to control the specific timing of diapause dynamics. Interestingly, in the few cases in which epigenetic regulation of JH signalling has been studied, histone acetylation, in particular, has been shown to control transcription of JH-response genes (Roy & Palli, 2018; Xu, Roy, & Palli, 2018). We hypothesize that JH signalling, via *dp-jhamt*, is one of several direct transcriptional targets of *dp-nc2β* that are required for a full termination of diapause.

Furthermore, we show that environmental conditions (we predict cold temperature in this case) modulate the timer and propose that this may occur through cryoprotectant accumulation. Accumulation of small molecule cryoprotectants (e.g., sorbitol, alanine, etc) in response to cold exposure has been observed in diapause and implicated in diapause termination in other insects (e.g., Leal et al., 2018; Lehmann et al., 2018; Michaud & Denlinger, 2007; Wang, Egi, Takeda, Oishi, & Sakamoto, 2014). Calcium signalling mediates osmotic stress due to small molecule cryoprotectant accumulation in insects (Storey, 1990). Interestingly, Ca^{2+} stimulates JH III production from the corpora allata in in vitro culture in *Gryllus bimaculatus* (Woodring & Hoffman, 1994). This leads us to speculate that cold hastens monarch diapause through promoting accumulation of small molecule cryoprotectants that increase calcium signalling, and in turn prompts JH biosynthesis, whereby promoting diapause termination. It is possible that this mechanism may operate more generally across insects to control diapause dynamics. While well documented that cold mediates epigenetic regulation of transcriptional states in plants (reviewed in Banerjee, Wani, & Roychoudhury, 2017), this relationship is not well established in animals. It will be interesting to determine if in monarchs cold-mediated effects on diapause through cryoprotectant accumulation and calcium signalling, and effects due to epigenetic regulation, are integrated or independent responses.

Altogether, this work illuminates molecular mechanisms underlying diapause termination and provides important insights into the mechanisms of broader biological properties represented by the termination model. We believe this represents one of the first accessible models to study the molecular basis of long-term photoperiod-independent timekeeping, which is pervasive (e.g., obligate, low-latitude, or underground diapauses, Denlinger, Hahn, Merlin, Holzapfel, & Bradshaw, 2017).

4.2 | Model for the evolution of hormonal pathway interactions

The defining feature of diapause is that it fundamentally changes organismal response to environment, rendering individuals insensitive to normally permissive environmental cues. Much previous work has been dedicated to understanding the molecular mechanisms behind the maintenance of environmental insensitivity (e.g., Lehmann et al., 2016), or rather how gene expression differs between diapause and either nondiapause or post-diapause quiescence states (e.g., Poelchau, Reynolds, Elsik, Denlinger, & Armbruster, 2013; Ragland & Keep, 2017). Here we are able to address an equally important but open problem: through what genetic mechanisms is diapause acting to change response to environmental conditions? Our evidence points to canonical hormonal pathways (ecdysteroid, JH, and insulin) as playing significant roles. These pathways mediate environment-dependent growth, development, and reproduction in many different cases such as seasonal polyphenism in *Bicyclus anynana* (Koch, Brakefield, & Kesbeke, 1996), colouration in *M. sexta* (Suzuki & Nijhout, 2006), and reproductive division of labour in ants (Chandra et al., 2018).

Importantly, our approach allows us to make hypotheses about how regulation of and interaction between these pathways changes as a result of diapause (Figure 4d). We propose that the critical change that controls subsequent pathway dynamics is reduced JH signalling in fall conditions (*dp-jhamt*; Figure 3b). Given this change, our data suggest key hypotheses about monarchs that experience summer conditions while still in diapause. First, JH pathway activity increases slowly in summer conditions (*dp-jhamt*; Figure 3b) while being less efficiently cleared (*dp-jheh*; Figure 4b). Second, while 20-HE is generally expressed as a pulse, ecdysteroid signalling activity in summer conditions rises earlier due to initially reduced and only slowly increasing JH levels. Ecdysteroid signalling remains high after extended time in summer conditions because less efficient clearance of JH leads to less efficient clearance of 20-HE, hence an elevated *dp-Eip75B* signal (Figure 4a). JH clearance has been shown to be necessary for full ecdysone response in some cases (e.g., in *G. bimaculatus*, Espig & Hoffmann, 1985). Third, IIS activity remains low in summer conditions due to increased *dp-foxo* transcription (Figure 4c). Previous work exploring the relationship between JH signalling and IIS in other insects provides clues into how JH may control IIS dynamics. JH promotes IIS in *Tribolium castaneum* (Xu, Sheng, & Palli, 2013), which may provide a mechanism by which IIS is reduced via *dp-foxo*. FOXO has been shown to regulate JH degradation in *B. mori* (Zeng et al., 2017), which suggests an additional, orthologous mechanism by which JH accumulates slowly in summer conditions when met during diapause. This model highlights how extensive crosstalk between these developmental hormones can render this integrated network vulnerable to a single perturbation, leading to a completely reorganized hormonal landscape that changes organismal function.

These hormonal pathways have been repeatedly coopted to generate a diversity of phenotypes by controlling major life history traits and coordinating them with environment (Finch & Rose, 1995; Flatt, Tu, & Tatar, 2005). Monarch diapause presents a compelling model to study the evolution of regulatory connections among these pathways. Global monarch populations show a range of variation in seasonally-controlled traits, presenting an attractive model for pursuing evolutionary studies. Some traits show a certain degree of conservation among populations (Freedman et al., 2018). In contrast, Australian monarchs do not show a full diapause but rather undergo a seasonally-controlled oligopause (James, 1982; James & Hales, 1983).

4.3 | Diapause timing and monarch overwintering dynamics

This work highlights the importance of the overwintering period for monarch biology. Diapause timing has important implications for monarch survival (Herman & Tatar, 2001) and mating at overwintering sites. Interestingly, according to Herman (1981), male monarch diapause lasts for shorter duration (September–November) than does female diapause (September–December), potentially suggesting that diapause timing in females may have a stronger impact on population dynamics. In subsequent studies, it will be interesting to determine if similar or different molecular mechanisms control male monarch diapause timing and determine what mechanisms lead to sex differences in diapause timing.

Environmental sensitivity of diapause dynamics means that monarchs will act as an important sentinel species for monitoring environmental change and disturbance at overwintering sites. Spatial structure and temporal dynamics of the overwintering colonies change within and across years (Vidal & Rendón-Salinas, 2014). It is possible that these movements at least partially reflect a strategy to optimize diapause timing. Changes in diapause dynamics may be considered alongside ecological modelling (e.g., Oberhauser & Peterson, 2003) to understand distribution at overwintering sites. Indeed, understanding how diapause dynamics are affected by environmental and anthropogenic factors at their overwintering sites may be critical for understanding North American monarch population decline (Agrawal & Inamine, 2018) and guiding future conservation efforts, a point highlighted by the record low number of monarchs recorded in the western North American monarch population in 2018 (The Xerxes Society Western Monarch Thanksgiving Count).

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AUTHOR CONTRIBUTIONS

D.A.G. conceived the project, D.A.G. and M.R.K. designed the analysis, D.A.G. collected and analyzed the data, and D.A.G. wrote the manuscript with input from MRK.

DATA AVAILABILITY STATEMENT

All raw sequence reads from this study have been uploaded to NCBI Sequence Read Archive to BioProjectID PRJNA548105 and mature oocyte count data uploaded to Dryad (https://doi.org/10.5061/dryad.fd517kq).

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SUPPORTING INFORMATION

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