Comparative Biochemistry and Physiology, Part A 256 (2021) 110937

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Overwintering conditions impact insulin pathway gene expression in diapausing *Megachile rotundata*

Lizzette D. Cambron [a], George D. Yocum [b], Kathleen M. Yeater [c], Kendra J. Greenlee [a,*]

[a] North Dakota State University, Fargo, ND, United States of America
[b] USDA-ARS, Fargo, ND, United States of America
[c] USDA-ARS, Plains Area Office of the Director, Fort Collins, CO, United States of America

ARTICLE INFO

Keywords:
Insect
Insulin signaling
Diapause
Alfalfa leafcutting bee
Development
Differential gene expression
Metabolism
Pollinator
Temperature
NanoString
nCounter

ABSTRACT

Diapause is a non-feeding state that many insects undergo to survive the winter months. With fixed resources, overall metabolism and insulin signaling (IIS) are maintained at low levels, but whether those change in response to seasonal temperature fluctuations remains unknown. The focus of this study was to determine 1) how genes in the insulin signaling pathway vary throughout diapause and 2) if that variation changes in response to temperature. To test the hypothesis that expression of IIS pathway genes vary in response to temperature fluctuations during overwintering, alfalfa leafcutting bees, *Megachile rotundata,* were overwintered at either a constant 4 °C in the lab or in naturally fluctuating temperatures in the field. Expression levels of genes in the IIS pathway, cell cycle regulators, and transcription factors were measured. Overall our findings showed that a few key targets of the insulin signaling pathway, along with growth regulators, change during overwintering, suggesting that only cell cycle regulators, and not the IIS pathway as a whole, change across the phases of diapause. To answer our second question, we compared gene expression levels between temperature treatments at each month for a given gene. We observed significantly more differences in expression of IIS pathway targets, indicating that overwintering conditions impact insulin pathway gene expression and leads to altered expression profiles. With differences seen between temperature treatment groups, these findings indicate that constant temperatures like those used in agricultural storage protocols, lead to different expression profiles and possibly different diapause phenotypes for alfalfa leafcutting bees.

1. Introduction

To survive winter months, insects living in temperate zones undergo diapause, a state of developmental dormancy (Denlinger 2002). For many insects, diapause is a non-feeding state, meaning all feeding to collect sufficient energy reserves for the winter and subsequent period of development must be done prior to diapause initiation (Denlinger 2002; Hahn and Denlinger 2007, 2011; Lehmann et al. 2016). Energy reserves not only help insects survive the winter, but also provide the initial energy to insects coming out of post-diapause quiescence. Insects emerging from diapause need to have fuel to replenish reserves and prepare for further development or reproduction (Hahn and Denlinger 2011). Energy expenditure during overwintering is minimized by a decrease in metabolic rate and cell-cycle arrest, key characteristics indicating the onset of diapause (Danks 1987; Denlinger 2002; Hahn and Denlinger 2011). The suppression of metabolism during diapause is crucial for conserving energy reserves and enables insects to survive long periods without feeding. Because diapause can span from months to years (Hahn and Denlinger 2007, 2011), both the collection of initial reserves and subsequent reduction of energy use are important.

Many studies point to the insulin signaling pathway as a possible mediator of nutrient regulation and sensing (Arsic and Guerin 2008; Defferrari et al. 2016a; Defferrari et al. 2016b; DiAngelo et al. 2009; Li et al. 2016; Taguchi and White 2008). The insulin/IGF-1 signaling (IIS) pathway is well known to regulate growth, development, and both carbohydrate and lipid metabolism in mammals (Bates et al. 2013; Dupont and Holzenberger 2003; Dupont and Scaramuzzi 2016; Saltiel and Kahn 2001). Research on insect insulin-like peptides (ILP) has shown they play an important role in diapause phenotypes, such as halted reproduction (Sim and Denlinger 2009a), reserve accumulation (Satake et al. 1997), metabolic depression (Hahn and Denlinger 2011), and enhanced stress-tolerance (Matsunaga et al. 2016; Sim and

* Corresponding author.
E-mail address: kendra.greenlee@ndsu.edu (K.J. Greenlee).

https://doi.org/10.1016/j.cbpa.2021.110937
Received 19 November 2020; Received in revised form 5 March 2021; Accepted 6 March 2021
Available online 15 March 2021

Denlinger 2013; Wu and Brown 2006). Closing the gap in knowledge about how the IIS pathway is involved in diapause regulation is crucial for understanding the physiological and biochemical changes that occur in insects during this sensitive period. This knowledge could also be used to improve the care of agriculturally important pollinators such as *Megachile rotundata*.

To understand how the IIS pathway is involved in regulating diapause, one starting point would be correlating changes in gene expression of the IIS pathway during this developmental stage. Traditionally, this is done using quantitative polymerase chain reaction (qPCR), which has limitations, such as variations in reverse transcription amplification and primer efficiencies, as well as inconsistent use of control genes (Huggett et al. 2005; Nolan et al. 2006). In addition to general limitations, finding control genes for non-model organisms can be expensive and time-consuming (Bustin 2004; Huggett et al. 2005; Nolan et al. 2006). Despite these limitations, the use of qPCR continues, because lack of evidence and support for new techniques can be risky in non-model organisms. A multiplex approach, nCounter analysis (NanoString), can overcome some of the limitations of qPCR, but only a handful of studies have used this method with insect samples (Ammeux et al. 2016; Choi et al. 2014; Epstein et al. 2017; Fulga et al. 2015; Keith et al. 2019; Lai et al. 2019; Sandler and Stathopoulos 2016; Schell et al. 2017; Shiao et al. 2015; Shiao et al. 2013; Sun et al. 2018; Sung et al. 2013; West and Silverman 2018; Winkler et al. 2017; Zheng et al. 2018).

The focus of this study is to determine 1) how the expression of insulin signaling pathway genes varies throughout diapause and 2) if the insulin signaling pathway genes change in response to temperature. We hypothesize that IIS pathway genes vary over the course of diapause indicating a role in allocating energy reserves in response to temperature. To test this hypothesis, we measured mRNA expression of key players in the IIS pathway and some of its targets in the alfalfa leafcutting bee, *Megachile rotundata*.

2. Materials and methods

2.1. Experimental design

Samples collected during a previously published experiment (Yocum et al. 2018) were used for nCounter analysis. Briefly, *Megachile rotundata* were reared in an on-farm facility in Utah, USA in the summer of 2010. Adults were released into an alfalfa field (Logan, UT, 41°47′37.04″N; 112°8′18.35″W), and offspring were placed into treatment groups in October of 2010. Offspring were collected between June 30th and July 19th and placed into one of two overwintering treatment groups, a laboratory setting at a constant 4–5 °C in darkness (constant) or a field setting where insects were exposed to naturally fluctuating temperatures (fluctuating), as described in Yocum et al. (2018). Temperatures outside and inside the field shelter were recorded with a HOBO Datalogger (Onset Computer Corp., Bourne, MA, USA). Prepupae overwintered in the field were exposed to temperatures ranging from −18 °C (January 2011) to 35 °C (May 2011) (Fig. S1; Yocum et al. 2018). Each month, individual bees were chosen from both constant and fluctuating temperature treatments, flash-frozen in liquid nitrogen, and stored in −80 °C until RNA extraction.

2.2. Sample preparation

Frozen prepupae were ground in liquid nitrogen, and RNA was extracted with TRIzol (Invitrogen, Life Technologies, Grand Island, NY, USA) according to manufacturer's instructions. RNA pellets were stored in −80 °C under absolute ethyl alcohol until used in this study. RNA samples from all months (November, December, January, February, March, April, May, June) and temperature treatments were used. RNA pellets were taken from −80 °C, and ethanol was removed. Pellets were washed in 70% ethanol and dried before being resuspended in 30 μl of DEPC-treated water. RNA concentration was quantified using both a NanoDrop 1000 Spectrophotometer (V3.8 Thermo Fisher Scientific, Wilmington, DE, USA) and a Qubit 4 Fluorometer (Invitrogen, Life Technologies, Grand Island, NY, USA). Four bees per treatment group were used for nCounter analysis.

2.3. nCounter analysis

A custom probe code set was designed and manufactured for measuring gene expression in *M. rotundata* (NanoString Technologies Inc., Seattle, WA, USA). Because nCounter is a multiplex technique, we measured expression level of a total of 30 genes, including those from the insulin pathway, proto-oncogenes that are downstream of the insulin pathway, and genes involved in cell cycle regulation to monitor development during overwintering (Table S1). *Insulin-like receptor* and *insulin receptor-like* are predicted mRNA transcripts for receptor proteins, but it is unclear whether these are isoforms. Transcripts showed no significant similarity when aligned with BLAST, and predicted proteins only had a 39% identity. RNA pellets from November through June were resuspended as described above, diluted to a target concentration of 20–50 ng/μL, and loaded into a 96-well skirted plate. Plates were shipped on dry ice to the University of Minnesota Genomics Center (Minneapolis, MN) for processing with nCounter Analysis System (NanoString Technologies Inc.). Resulting copy numbers were normalized to the geometric mean of 10 reference genes selected from a previous Illumina study (Yocum et al. 2018).

2.4. Statistical analysis

JMP Pro software (v.14.0, SAS Institute Inc., 2018, Cary, NC, USA) and SAS (v.9.4 SAS/STAT 15.1, SAS Institute Inc., 2018, Cary, NC, USA) were used for statistical analyses. Hierarchical cluster analyses with a Ward-linkage method were performed with JMP Pro (v.14.0, SAS Institute Inc., 2018, Cary, NC, USA). Initial exploration of the data showed that there was a strong correlation between transcript copy number and month. Even though the samples were independent observations, the month-to-month expression levels showed a strong correlation pattern that needed to be properly assessed in the statistical model. To do this, a random coefficient growth curve model (RCGCM) was used to model the linear relationship between copy number (gene expression level) and the interaction of month and temperature for each gene (Vonesh 2012). Each gene's regression line was determined by the gene's random intercept and random slope, with separate population intercept and slope also fit to the constant and fluctuating temperatures for each gene, respectively. In the MIXED procedure, the MODEL statement contains the response variable, Copy number, equal to the fixed main effect of Temperature and the Month as a continuous regressor, and their interaction. The RANDOM statement is utilized to instruct the procedure to treat the intercept and slope of the Month (as a continuous numeric) as random effects, thereby determining a regression for each gene. Several variance-covariance types were compared within the RCGCM, and based on the Information Criteria, the AR(1) structure was observed to provide the most parsimonious fit across all genes. The ESTIMATE statement was utilized to calculate differences in intercepts (Main Temperature contrast) and differences in slopes (interaction of Temperature and Month/Time). Differences for intercepts and slopes between groups were calculated by subtracting estimates for the constant treatment group from estimates for the fluctuating treatment group. The delta values for slope and intercept estimates are reported. Negative values indicate that the fluctuating temperature treatment group were lower than the constant temperature group, and positive values indicate that they were higher than the constant temperature group. A difference in intercept indicates a significant difference between temperature treatments overall, while a difference in slope indicates a significant difference between temperature treatments over time. Post-hoc analyses of intercepts and slopes were conducted by comparing upper and lower confidence limits

between treatment groups at each month. Hierarchical cluster analyses with a Ward-linkage method and constellation plots were performed with JMP Pro (v.14.0, SAS Institute Inc., 2018, Cary, NC, USA). The line length values are meaningful only with respect to each other, and the axis scaling, orientation of points, and angles of the lines are arbitrary. Limits that did not overlap were considered significantly different with 95% confidence. Copy number means +/− SEM and 95% CI are reported.

3. Results

Of the genes that were measured, 56% varied significantly, in either the slope, intercept, or both (Table 1). Hierarchical cluster analysis clearly showed that expression profiles for fluctuating temperature group (Fig. 1A, B) were different than that of bees overwintered at a constant temperature (Fig. 1C, D). When placed together, hierarchical cluster also showed that monthly expression patterns clustered

Table 1
Estimates table for nCounter results.

Gene	Label	Estimate	StdErr	DF	tValue	Probt
(AKH) gonadotropin-releasing hormone II receptor (AKH)	Intercept	45.79178571	33.3379	64	1.37356	0.17437
	Slope	−7.475744048	6.6019	64	−1.1324	0.26171
3-phosphoinositide-dependent protein kinase 1 (PDK1)	Intercept	−23.18596047	35.7668	33.8378	−0.6483	0.5212
	Slope	−13.85865023	9.33107	63.9154	−1.4852	0.1424
AKT-interacting protein-like (AKT-IP)	Intercept	−43.85837489	10.8387	42.229	−4.0464	**0.00022**
	Slope	11.04902498	2.38727	62.5661	4.6283	**1.9E-05**
GRB2-associated-binding protein 2 (GAB2)	Intercept	12.35568048	27.649	43.3936	0.44688	0.65719
	Slope	−23.22836656	5.64971	62.8628	−4.1114	**0.00012**
GTP-binding protein Rheb homolog (RHEB)	Intercept	−14.82613217	17.6783	35.0201	−0.8387	0.40735
	Slope	0.992221936	4.48531	63.8347	0.22122	0.82563
Samui	Intercept	239.7792857	330.243	64	0.72607	0.47044
	Slope	−170.303869	65.3979	64	−2.6041	**0.01144**
Adipokinetic prohormone type 2-like (AKH2)	Intercept	−2.964464286	2.65994	64	−1.1145	0.26924
	Slope	−0.103035714	0.52675	64	−0.1956	0.84554
Cyclin-D	Intercept	7.658303571	11.3524	64	0.6746	0.50236
	Slope	−6.590178571	2.24811	64	−2.9314	**0.00467**
Cyclin-E	Intercept	13.59029365	17.4312	40.0143	0.77965	0.44018
	Slope	−22.53686747	3.73643	63.6005	−6.0317	**9.1E-08**
Cyclin-G	Intercept	−102.1923618	20.4443	63.9392	−4.9986	**4.8E-06**
	Slope	1.843043323	6.76212	63.1752	0.27255	0.78608
Cyclin-K	Intercept	−13.10182438	10.5206	33.6174	−1.2453	0.22162
	Slope	2.688142921	2.50577	63.977	1.07278	0.2874
Dual specificity mitogen-activated protein kinase kinase dSOR1 (ERK)	Intercept	−14.83131043	8.33239	44.2098	−1.78	0.08196
	Slope	4.469989935	1.68386	62.6266	2.65461	**0.01005**
Forkhead box protein O (FOXO)	Intercept	−1.235	66.4256	64	−0.0186	0.98522
	Slope	−47.39229167	13.1542	64	−3.6028	**0.00062**
Glycogen synthase kinase-3 beta-like (GSK3B)	Intercept	−7.066339286	15.0586	64	−0.4693	0.64048
	Slope	−4.142619048	2.98205	64	−1.3892	0.16959
Insulin receptor substrate 1 (IRS)	Intercept	−6.707568265	24.0376	43.313	−0.279	0.78154
	Slope	−5.050862319	4.91715	62.8848	−1.0272	0.30826
Insulin receptor-like	Intercept	103.956875	182.749	64	0.56885	0.57145
	Slope	−82.610625	36.1897	64	−2.2827	**0.02578**
Insulin-like growth factor I (IGF1)	Intercept	−4.943381538	2.14636	23.5827	−2.3031	**0.03041**
	Slope	2.314064502	0.81295	62.8864	2.84649	**0.00596**
Insulin-like receptor	Intercept	1.643392857	16.5877	64	0.09907	0.92139
	Slope	−8.05985119	3.28486	64	−2.4536	**0.01688**
Mitogen-activated protein kinase 1 (MAPK1)	Intercept	−11.62571429	8.03486	64	−1.4469	0.1528
	Slope	3.373422619	1.59114	64	2.12013	**0.03788**
Mitogen-activated protein kinase 14B-like (MAPK14B)	Intercept	33.48422931	12.3141	28.2158	2.71917	**0.01107**
	Slope	−9.67307648	3.67719	56.165	−2.6306	**0.01098**
Phosphatidylinositol 3-kinase catalytic subunit type 3 (PI3Kc)	Intercept	−23.02781929	8.20151	33.4646	−2.8078	**0.00826**
	Slope	2.528402643	2.15867	63.9346	1.17128	0.24583
Phosphatidylinositol 3-kinase regulatory subunit alpha (p85)	Intercept	90.64870891	38.7744	37.4714	2.33785	**0.02484**
	Slope	−41.2715591	8.63805	63.9022	−4.7779	**1.1E-05**
Phosphatidylinositol 3/4/5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase (PTEN)	Intercept	−12.93742548	5.63165	31.8353	−2.2973	**0.02833**
	Slope	3.325966349	1.3925	63.875	2.38849	**0.01988**
Protein L-Myc-1b	Intercept	−19.12116071	37.9513	64	−0.5038	0.61611
	Slope	−13.31758929	7.51548	64	−1.772	0.08115
Protein kinase C (PKC)	Intercept	−8.645178571	4.51415	64	−1.9151	0.05995
	Slope	1.300386905	0.89394	64	1.45468	0.15065
Protein son of sevenless (SOS)	Intercept	−1.832841902	5.67282	35.6865	−0.3231	0.74851
	Slope	−0.197954078	1.41831	63.7754	−0.1396	0.88944
Ras-like protein 1 (RAS1)	Intercept	−2.204411893	11.6861	32.4029	−0.1886	0.85155
	Slope	3.031749535	2.85529	63.9151	1.0618	0.29232
Ras-like protein 2 (RAS2)	Intercept	−65.89658571	12.8324	30.6111	−5.1352	**1.5E-05**
	Slope	10.42929581	3.27178	63.7647	3.18766	**0.00222**
Serine/threonine-protein kinase mTOR	Intercept	−8.340877081	6.84603	29.8493	−1.2184	0.23263
	Slope	−2.081295327	1.78793	63.6827	−1.1641	0.24873
Tether containing UBX domain for GLUT4 (ASPSCR1)	Intercept	−4.454910714	9.67512	64	−0.4605	0.64675
	Slope	3.712410714	1.91596	64	1.93762	0.05708

Estimates table for the effects of temperature treatment (intercepts) and temperature over time (slopes) of each gene tested with nCounter. Statistics include estimates, standard error (StdErr), Kenward-Roger adjusted degrees of freedom (DF), tValue, and *p*-value for the two-tailed test (Probt) for each gene's slope and intercept. *p*-values in bold are statistically significant.

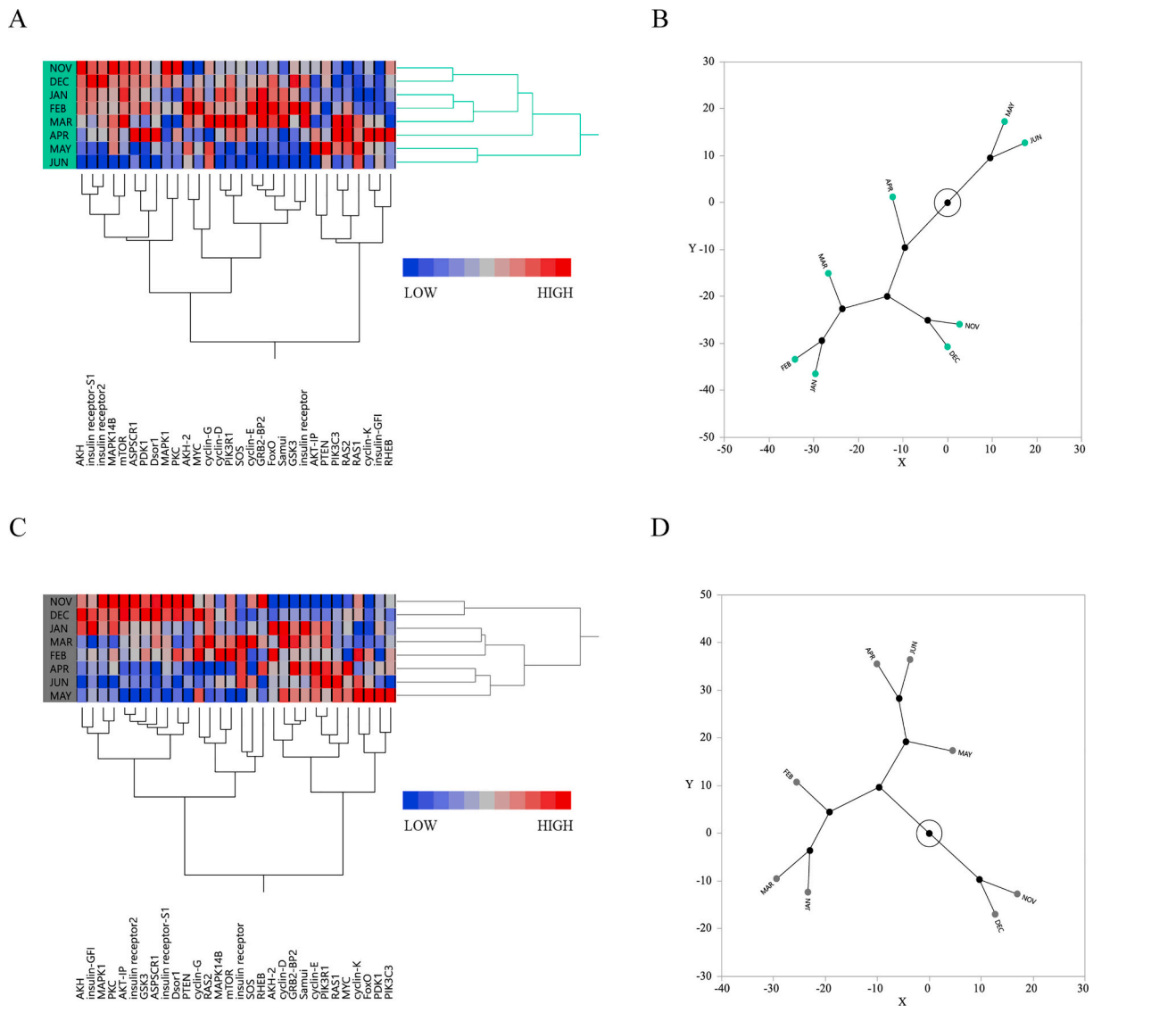


Fig. 1. Different expression profiles of bees overwintered in field (fluctuating temperatures) and lab (constant temperature) conditions. Gene expression for individual genes over time are shown in the heat maps for the A) fluctuating treatment group and C) constant treatment group. Constellation plots for B) fluctuating treatment group and D) constant treatment group show how monthly expression values cluster in relation to each other. Constellation plots arrange the individual months as endpoints and each cluster join as a new point, with lines drawn that represent membership. The circle identifies the root of the tree and the longer lines represent greater distance between clusters, meaning most dissimilarity.

differently by treatment group (Fig. 2). For the fluctuating temperature treatment group, gene expression separated into three clusters consisting of 1) Nov-Dec, 2) Jan-Mar, and 3) Apr-Jun. However, the constant temperature treatment group only had two clusters, 1) Nov-Dec and 2) Jan-Jun. Additionally, monthly variation in expression levels in the constant temperature treatment group was much higher as shown by March being more similar to June than April (Fig. 2).

3.1. IIS during overwintering

To determine how gene expression changes during overwintering, we used bees overwintered in field conditions with naturally fluctuating temperatures and compared monthly gene expression levels to T_0 (November). Mean copy numbers of *cyclin D*, *cyclin E*, *cyclin G*, *GAB2*, *insulin-like receptor*, *MAPK14B*, and *samui* varied significantly throughout overwintering (Table S2). As overwintering progressed to spring, some cell cycle genes and the insulin receptor decreased in expression levels. *Cyclin D* mean copy number decreased by 44% from November to June (Fig. 3A; Nov: 36.39 ± 5.5; Jun: 20.23 ± 3.73). *Cyclin G* showed a similar pattern with a 34% decrease in mean copy number throughout overwintering (Fig. 3C; Nov: 280.73 ± 15.90; Apr: 183.54 ± 14.47). Expression of *insulin-like receptor* decreased by over half from November to June (Fig. 4B; Nov: 158.50 ± 18.49; May: 53.20 ± 5.89; Jun: 47.59 ± 16.05), as did *MAPK14B* expression (Fig. 5D; Nov: 246.75 ± 6.04; Jun:135.60 ± 33.03). Other differentially expressed genes increased expression levels throughout the overwintering period. *Cyclin E* and *GAB2* expression levels increased by nearly 50% from November to January, and continued to show a 25% increase until spring (Fig. 3B; November: 32.49 ± 5.20; January: 52.82 ± 4.47; February: 66.14 ± 7.63) (Fig. 5B; Nov: 68.37 ± 6.06; Dec: 100.55 ± 10.18; Jan: 137.15 ± 10.17; Feb: 139.13 ± 30.59; Mar: 140.76 ± 35.13). *Samui* doubled every month until January and then remained high until April when it dropped 10-fold (Fig. 5F; Nov: 371.14 ± 44.17; Dec: 664.62 ± 21.59; Jan: 1363.43 ± 205.52; Feb: 1464.55 ± 138.57; Mar: 1800.33 ± 463.10; Apr: 181.21 ± 26.73).

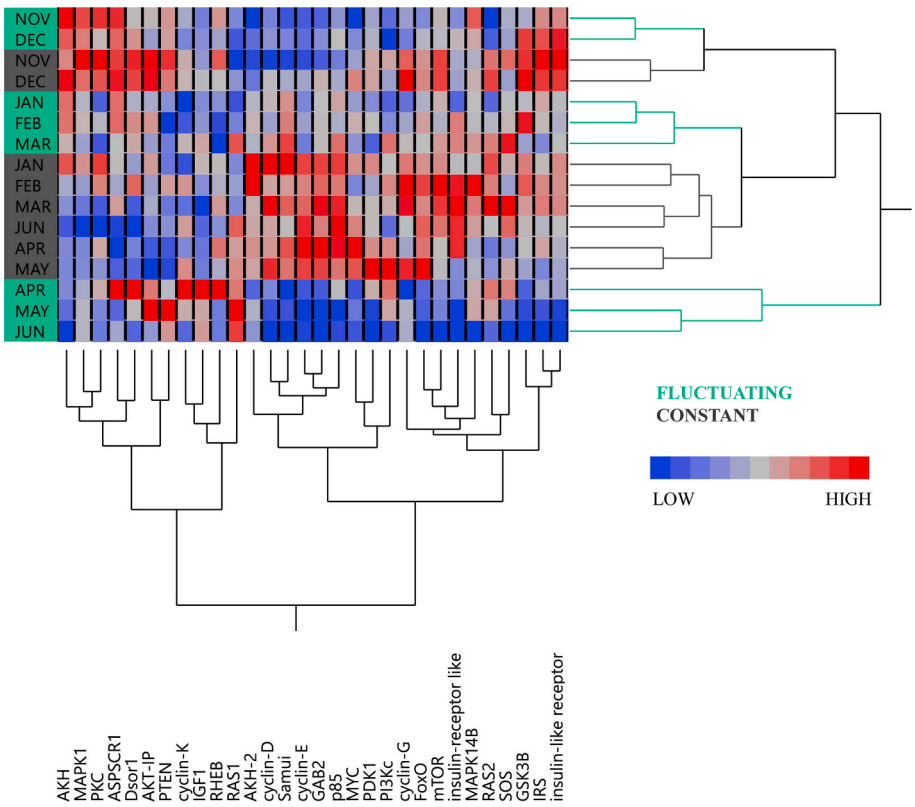


Fig. 2. Hierarchical cluster of nCounter results. Two-way hierarchical cluster of nCounter results showing gene by month clustering. Blue to red indicates low to high expression levels.

3.2. Effects of temperature on IIS

Next, we looked at whether temperature affected gene expression by comparing monthly expression levels between temperature treatment groups. Our results showed that *cyclin D*, *cyclin E*, *cyclin G*, *FOXO*, *GAB2*, *insulin-like receptor*, *insulin receptor-like*, *MAPK14B*, *PI3K* regulatory subunit, *RAS2*, and *samui* all varied between temperature treatments at a given month or over time within a treatment (Table 1).

The slope for *cyclin D* expression levels in the fluctuating treatment group was significantly lower than that of the constant temperature group (Table 1; slope: estimate = −6.59, DF = 64, $p < 0.01$) In November, *RAS2* was significantly higher in the constant treatment group (Fig. 5E; intercept: estimate = −65.90, DF = 30.61, $p < 0.0001$). Temperature treatment significantly affected expression levels of cyclins E and G during the middle months of overwintering. In January, bees in fluctuating temperatures had approximately 30% lower mean copy number for *cyclin G* compared to bees in constant temperature (Constant: 348.95 ± 39.18; Fluctuating: 240.26 ± 21.02). Bees in the fluctuating temperature group showed overall lower expression of *cyclin E*, and its expression was statistically lower than the constant temperature group from January through June (Fig. 3B; slope: estimate = −22.54 DF = 63.60, $p < 0.001$).

During the end of the overwintering period, April, May, and June, several genes showed significant differences between temperature treatments. Expression levels of several genes were lower in fluctuating temperatures compared to the constant temperature groups, including *FOXO* (Fig. 3C; slope: estimate = −47.39, DF = 64, $p < 0.001$), *GAB2* (Fig. 5B; slope: estimate = −23.23, DF = 62.86, $p < 0.001$), *samui* (Fig. 5F; slope: estimate = −170.30, DF = 64, $p = 0.01$), *cyclin D* (Fig. 3A; slope: estimate = −6.59, DF = 64, $p < 0.01$), *insulin-like receptor* (Fig. 4B; slope: estimate = −8.06, DF = 64, $p = 0.02$), *insulin receptor-like* (Fig. 4A; slope: estimate = −82.61, DF = 64, $p = 0.03$), *MAPK14B* (Fig. 5D; slope: estimate = −9.67, DF = 56.16, $p = 0.01$), and *PI3K* regulatory subunit (Fig. 4D; slope: estimate = −41.27, DF = 63.90, $p < 0.001$).

4. Discussion

This study is the first to use nCounter Analysis (Nanostring) in a Hymenopteran and to investigate IIS pathway gene expression in an overwintering solitary bee. Our results showed that a few key targets of the insulin signaling pathway along with growth regulators change during overwintering, suggesting that only cell cycle regulators, and not the insulin signaling pathway as a whole, change across the phases of diapause. Of the genes that varied significantly over time, only 3 were targets of the insulin signaling pathway, not supporting our hypothesis. The other genes, cyclins and *samui*, responded over time as expected based on their roles in regulating development (Fig. 6). To answer our second question, whether temperature impacted IIS gene expression, we compared gene expression levels between temperature treatments at each month for a given gene. When looking at the effects of temperature on gene expression, we observed significantly more differences in expression of target genes of the insulin signaling pathway. The results showed that all three cyclins (D, E, G), *FOXO*, *GAB2*, *insulin-like receptor*, *insulin-receptor like*, *MAPK14B*, *PI3K* regulatory subunit, and *samui* varied between constant and fluctuating temperature groups at several time points (Fig. 7). Our findings indicate that temperature and not diapause development itself is the primary regulator of insulin pathway gene expression in overwintering *M. rotundata*. Interestingly, a key finding of our study is that overwintering conditions significantly impacted gene expression profiles; bees overwintered in field conditions were physiologically different than those kept under constant laboratory conditions.

Overwintering consists of different phases of diapause (initiation, maintenance, termination) and post-diapause quiescence, and thus we expected the gene expression levels to reflect these different stages (Koštál 2006; Kostal et al. 2017). Key genes in the IIS pathway did not

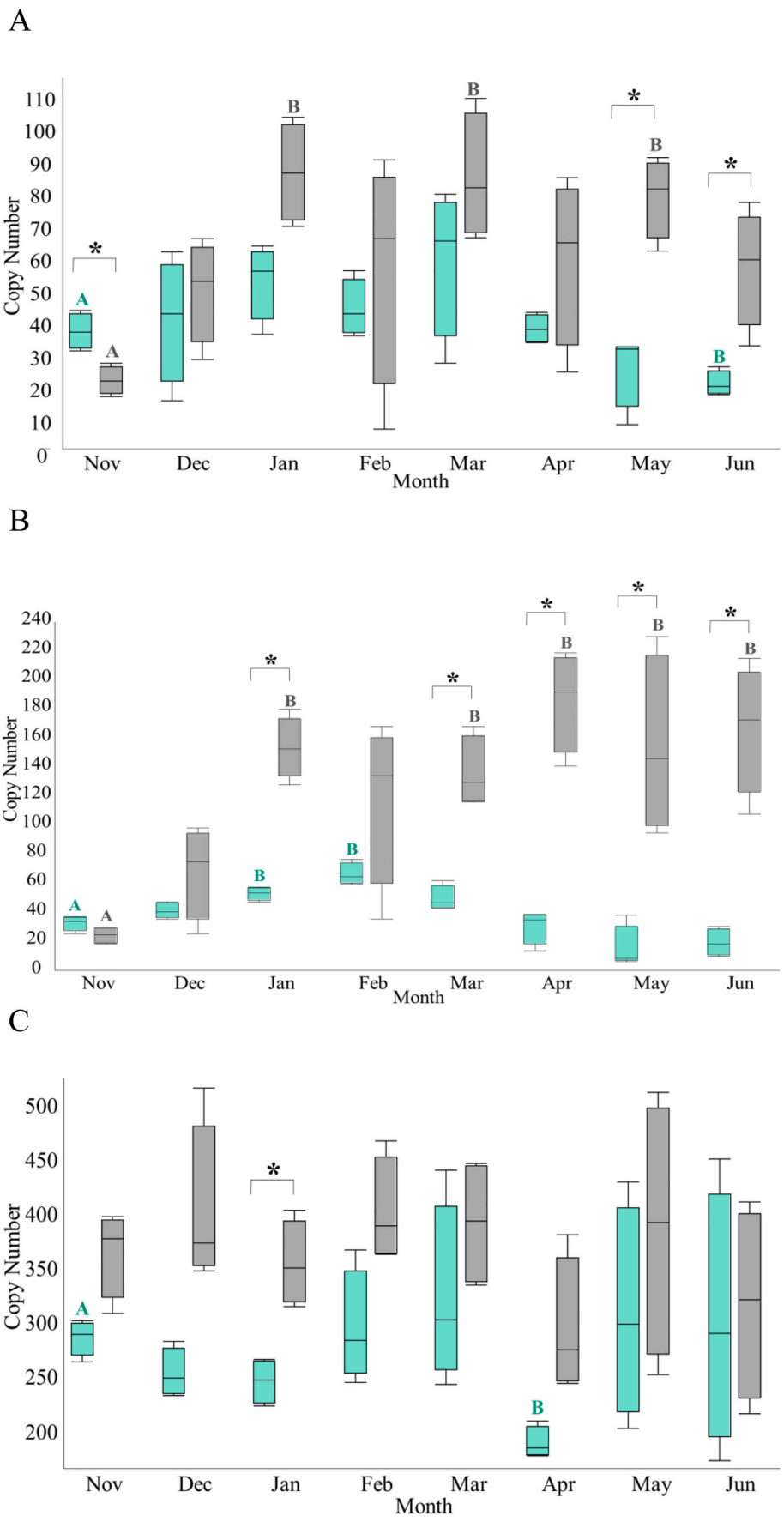


Fig. 3. Expression of cyclin genes. Cell cycle genes, A) cyclin D, B) cyclin E, and C) cyclin G, were significantly different by temperature treatment and over time. Fluctuating temperature treatments are shown in light teal and constant is shown in gray. Months with different letters are significantly different. Months without letters were not significantly different from any other month. Letter colors correspond to temperature treatment. Asterisks indicate significant differences between temperature treatments in a given month.

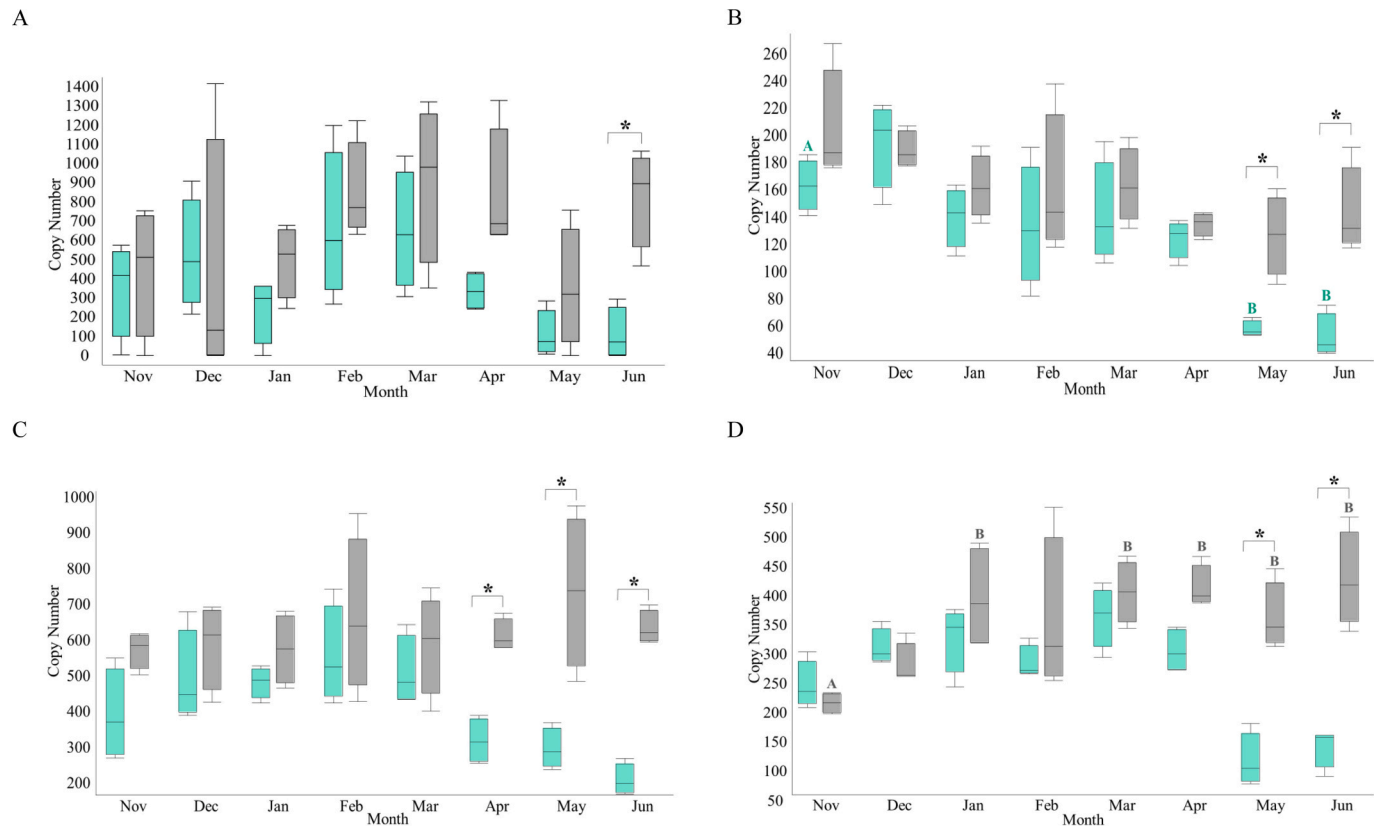


Fig. 4. Expression of genes in the insulin pathway. Genes in the insulin signaling pathway that showed significant differences included A) insulin receptor-like B) insulin-like receptor, C) FOXO, and D) PI3K regulatory subunit. Fluctuating temperature treatments are shown in light teal and constant is shown in gray. Months with different letters are significantly different. Months without letters were not significantly different from any other month. Letter colors correspond to temperature treatments. Asterisks indicate significant differences between temperature treatments at a given month.

differ between temperature treatment groups in earlier months. This was expected since bees had already entered diapause in November. When overwintered in naturally fluctuating temperatures, hierarchical clustering results showed separation of gene expression into three clusters that reflect the phases of diapause: 1) November and December, 2) January through March, and 3) April through June (Fig. 2). Diapause maintenance for *M. rotundata* is predicted to be from November to December (Yocum et al. 2018), corresponding to the first month cluster (Fig. 2), and insects in diapause maintenance are not typically stimulated by external factors (Kostal et al. 2017). Therefore, the lack of differences between temperature treatments in earlier months made sense since diapause maintenance is endogenously regulated. It is not until the post-diapause quiescence stage, around January for *M. rotundata*, that processes are exogenously regulated.

The major differences seen occurred during spring months, which is most likely associated with increasing temperatures and resumption of development, was not seen in the constant low temperature group. This is shown in the expression profile for the constant temperature group where monthly expression clusters did not have a third cluster like the fluctuating temperature group did (Fig. 2). Most differences between temperature treatments occurred during April, May, and June, the months when the transition from post-diapause quiescence to direct development would occur. Direct development is initiated when temperatures are greater than 18 °C (Undurraga and Stephen 1980). Average temperatures for the fluctuating temperature treatment group increased during April (7.4 °C), to May (13 °C) and June (22 °C) (Fig. S1, Yocum et al. 2018), whereas bees in the constant temperature group did not receive appropriate thermal cues to progress to direct development. Similar to our hierarchical results (Fig. 2), different developmental phases of diapause for the drosophilid fly, *Chymomyza costata,* were distinguishable by characteristic patterns of gene expression for each phase (Kostal et al. 2017). In diapause-destined larvae, gene expression profiles showed inhibition of developmental hormone signaling and as a result, down-regulation of transcriptional factors leading to cell cycle arrest (Kostal et al. 2017). Cell cycle regulators like the cyclins and transcription factors such as *samui* were differentially expressed over time, similar to results in the current study. Although our study was not focused on searching for genes involved with diapause regulation, these results may be a starting point for identifying characteristic patterns of gene expression for diapause developmental phases in *M. rotundata.*

With previous studies showing that suppression of the IIS pathway is important for diapause regulation, it is not unreasonable to consider genes from this pathway as a starting point for characterizing expression profiles for diapause developmental stages. Our results showed that IIS pathway gene expression as a whole pathway did not change over time (Figs. 4 and 6), confirming previous studies in adult diapause. For both *Culex pipiens* and *Drosophila melanogaster*, insulin signaling has been strongly tied to regulating adult diapause (Kubrak et al. 2014; Kubrak et al. 2016; Nassel et al. 2013; Schiesari et al. 2016; Sharma et al. 2019; Sim and Denlinger 2009b; Zhang et al. 2017). IIS pathway targets like FOXO have been shown to regulate adult diapause, and suppression of the whole cascade signaling has shown to produce a diapause phenotype in *Cx. pipiens* (Sim and Denlinger 2007, 2008; Sim et al. 2015). Other targets such as *phosphatidylinositol 3-kinase* (PI3K) and even the insulin receptor have been key regulators of adult diapause in *D. melanogaster* (Kubrak et al. 2016; Williams et al. 2006). This suggests that in response to unfavorable conditions like those during diapause, flies alter their metabolic homeostasis, which in turn alters insulin signaling. Additionally, mutant strains of *D. melanogaster* that were insulin-deficient showed more ovarian development arrest, indicating a strong

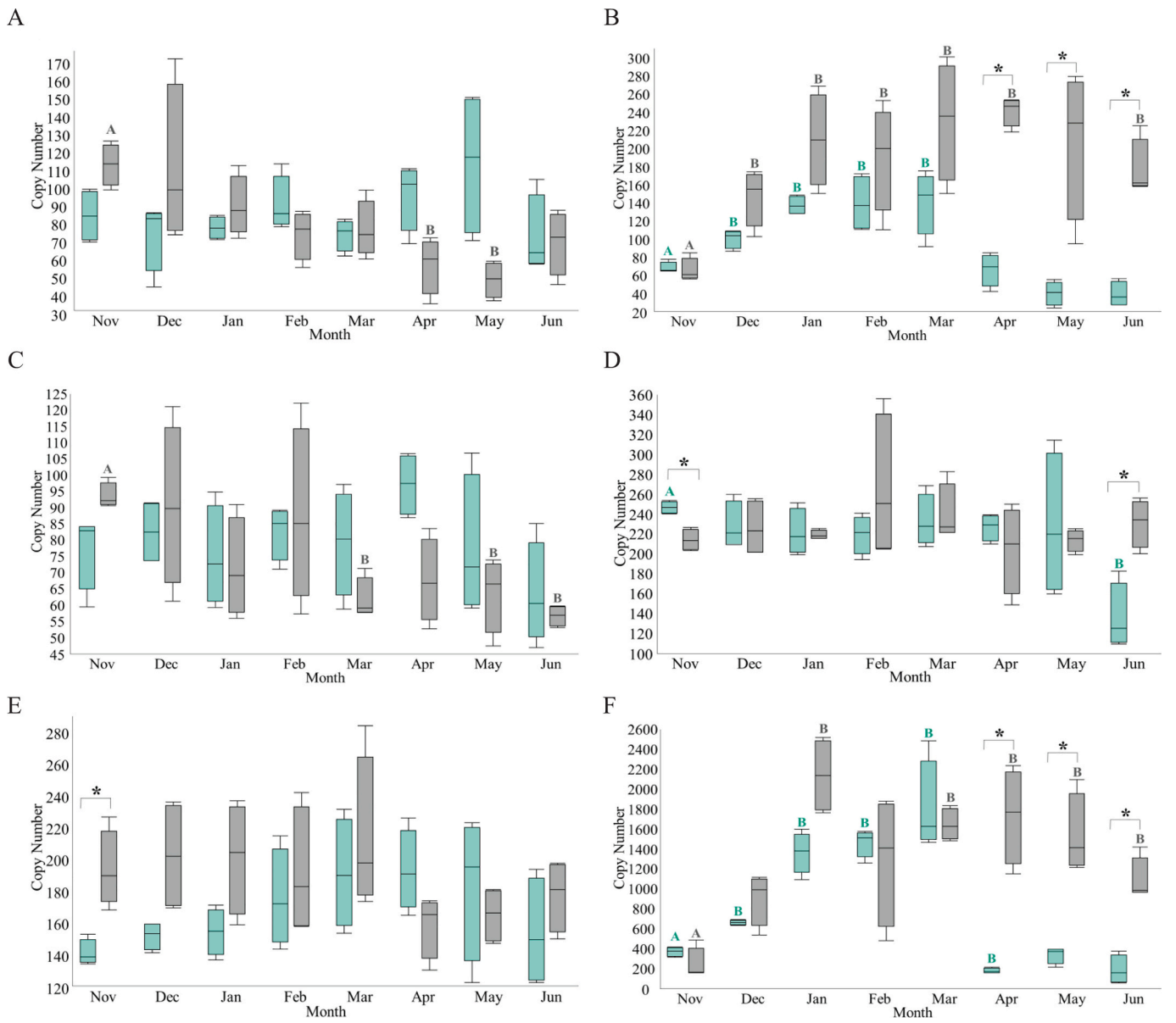


Fig. 5. Expression of regulatory genes. Regulatory genes such as A) AKT-interacting protein, B) GAB2, C) ERK, D) MAPK14B, E) RAS2, and F) Samui were significantly different by location and over time. Fluctuating temperature treatments are shown in light teal and constant is shown in gray. Months with different letters are significantly different. Letter colors correspond to temperature treatment. Asterisks indicate significant differences between temperature treatments at a given month.

connection between diapause induction and reduced insulin signaling (Kubrak et al. 2014). Our findings of prepupal overwintering support those of adult diapause studies, however few studies have investigated the complex relationship between insulin signaling and diapause regulation in insects overwintering at different developmental stages.

The IIS pathway is clearly involved in diapause regulation, but how this pathway is utilized seems to vary. A recent meta-analysis of diapause transcriptomic data sets compared how diapause responses varied across species and throughout ecophysiological phases of diapause development (Ragland and Keep 2017). With data sets representing three orders (Diptera, Lepidoptera, and Hymenoptera) and various life stages from larva to adult, results showed that a core set of differentially regulated genes included targets of the insulin signaling pathway (Ragland and Keep 2017). However, these genes interacted with several other pathways such as JNK and TOR to modulate insulin signaling (Ragland and Keep 2017), possibly indicating plasticity in how the insulin signaling pathway is regulated during diapause since both pathways interconnect with IIS at different points. With insufficient studies covering more species and different life stages in which insects diapause, much remains to be understood about the regulatory role of insulin signaling in diapause, especially in solitary bees.

Although previous studies to determine diapause regulatory mechanisms in solitary bees have been inconclusive in elucidating a complete mechanism (Forrest et al. 2019; Kemp and Bosch 2001), the role of temperature has been shown to be important (Kemp and Bosch 2001; Tepedino and Parker 1986; Yocum et al. 2018; Yocum et al. 2005, 2006). Several transcriptome studies have scratched the surface on diapause regulation in *M. rotundata* (Torson et al. 2015; Yocum et al. 2018; Yocum et al. 2015). However, these studies only provide a macro-scale view of what is occurring. Our results for bees that were overwintered in the fluctuating temperatures were expected (Fig. 6), however, those kept in a constant temperature showed drastically different outcomes (Fig. 7). When looking at expression profiles side by side (Fig. 1), it is very clear that the constant temperature group showed similar expression patterns

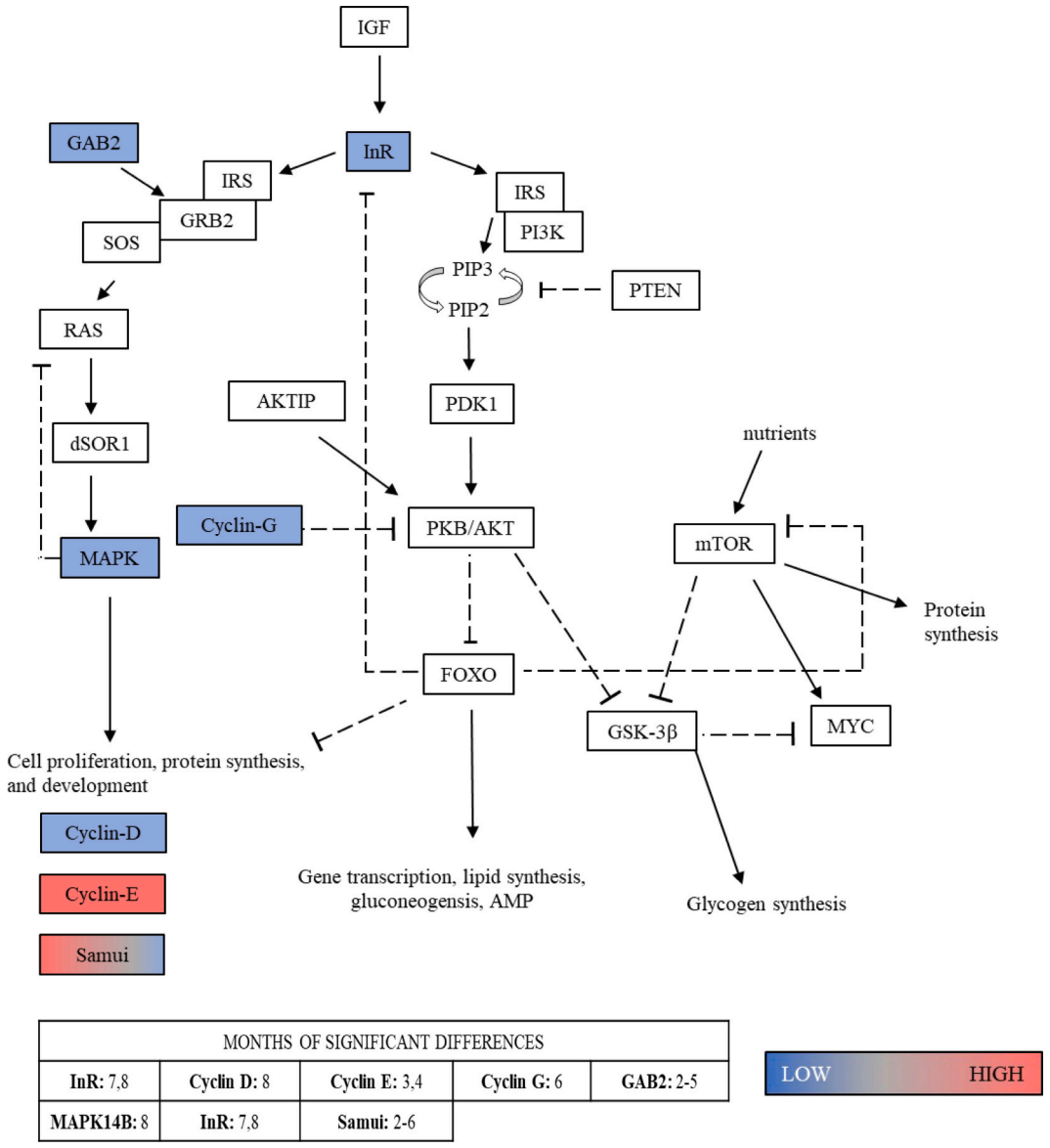
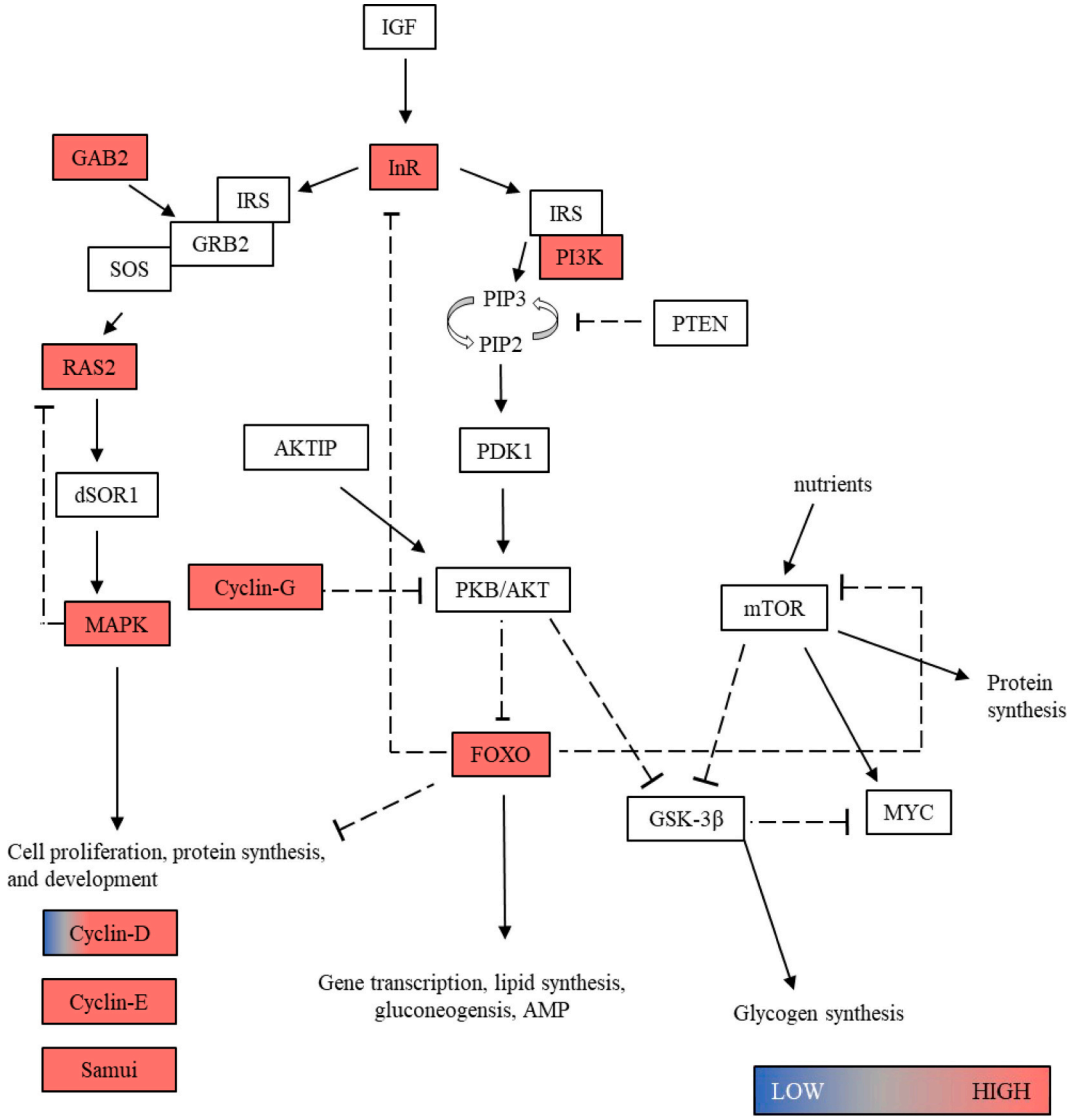


Fig. 6. Gene expression of IIS pathway over time during overwintering months for fluctuating temperature group. Genes that were significantly different relative to November are shown in blue (lower) or red (higher). The months that these differences were seen are indicated in the table. Months include November (1), December (2), January (3), February (4), March (5), April (6), May (7), and June (8).

to the fluctuating temperature group in the beginning (Nov-Dec), but by January differences arose. Differences between low and high expression levels in the constant group are more distinctly visible as most red colour is shown moving from top left down to bottom right (Fig. 1C). However, in the fluctuating treatment group, there is much more variability between low and high expression levels as shown by red and blue colors being more intermixed (Fig. 1A). These differences are even more distinct in the constellation plots where monthly variability in expression causes different orientations (Fig. 1B, D). This result of different expression patterns was also shown in the geometric dendrogram in Yocum *et al.* (2018, fig. 4B), providing strong evidence of environmental history impacting expression profiles. Several other studies in *M. rotundata* have also shown differential effects between constant and fluctuating temperatures (Rinehart et al. 2016; Rinehart et al. 2013; Rinehart et al. 2011; Torson et al. 2015; Yocum et al. 2018; Yocum et al. 2005, 2006; Yocum et al. 2012; Yocum et al. 2010). Because insects are ectotherms, their metabolic rate is directly correlated with temperature. This could explain why the fluctuating temperature group showed much more variation in expression over time (Fig. 1A). This supports previous studies where solitary bees exposed to a rapid pulse of high temperature caused metabolic rates to increase (Yocum et al. 2011) and genes in metabolic pathways to be upregulated (Torson et al. 2015). Our study showed similar results with several genes of the insulin signaling pathway, such as *PI3K*, *FOXO*, and *insulin receptors*, differing between temperature treatments, indicating a response of IIS pathway gene expression to temperature (Fig. 7).

Our samples originated from a previous study examining the effects of overwintering in laboratory or field conditions (Yocum et al. 2018). Although a subset of those samples were used for an RNAseq study, comparing the results of our current investigation with the previous work has some limitations. Due to logistical and financial constraints, the previous study only screened bees from four of the available 9 months of samples. Also, the previous work focused on the impact of environmental history on diapause development using a multivariate approach and not on specific pathways as in this investigation. Regardless of the differences in techniques, and different statistical approaches, the results presented here align with our previous work showing that the bees overwintering in the field are physiologically



MONTHS OF SIGNIFICANT DIFFERENCES				
InR: 8	**PI3K Reg:** 5,6	**FOXO:** 6-8	**GAB2:** 6-8	**RAS2:** 1
MAPK14B: 8	**Cyclin D:** 1,7, 8	**Cyclin E:** 1, 3, 5-8	**Cyclin G:** 3	**Samui:** 7, 8

Fig. 7. Gene expression of IIS pathway between temperature treatments. Gene expression relative to that of the fluctuating temperature treatment group is shown as either lower (blue) or higher (red) for the constant temperature treatment group. The months that these differences were seen are indicated in the table. Months include November (1), December (2), January (3), February (4), March (5), April (6), May (7), and June (8).

distinct from their laboratory-maintained cohorts.

Information about the environmental requirements for diapause, such as temperature, humidity, and photoperiod, has helped establish optimal storage conditions for commercially managed insects (Bloch et al. 2010; Goldsmith et al. 2005; MacKenzie et al. 1997; Pitts-Singer and James 2009; Rinehart et al. 2011). Although answering questions about diapause requirements are being studied in a variety of insects, it is still vastly understudied in agriculturally important pollinators. This study adds to the growing knowledge of *M. rotundata* physiology. The alfalfa leafcutting bee is the most commercially-managed solitary pollinator, and, in the USA, efforts for developing a sustainable production protocol for this bee have been unsuccessful. With a wide range of environments due to high use in agricultural fields across the US, it is unclear how environmental factors, such as temperature, impact diapause regulation and colony sustainability in *M. rotundata.* Many molecular tools are not yet optimized for answering these questions in non-model organisms like *M. rotundata.* However, our study is the first to use this new multiplex technique in a solitary bee, and it showed promising results for use in future studies. To elucidate the mechanism (s) behind diapause regulation, more studies of diapause and insulin signaling at different developmental stages are desperately needed.

Funding

This research was funded, in part, by grants from the National Science Foundation Graduate Research Fellowship and a Graduate Research Internship with the USDA-ARS Fargo, ND, to LDC, an NSF EPSCoR-1826834 to KJG, and USDA-ARS funding 3060-21220-032-00D to GDY.

Declaration of competing interest

We declare no conflict of interest. Kendra Greenlee is a member of the editorial board of CBP.

Acknowledgements

We would like to thank Dr. Theresa Pitts-Singer at the USDA-ARS Logan, Utah for supplying the bees used in this study. We would like to thank the two anonymous reviewers for their insightful comments and feedback on this manuscript. Special thanks to our laboratory technician Marnie Larson for her guidance and help on sample processing. Thank you to the Greenlee laboratory members for their help, the NDSU Department of Biological Sciences, Dr. James Kopco, and the Insect Genetics and Biochemistry Unit at the USDA-ARS Edward T. Schafer Agricultural Research Center.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2021.110937.

References

Ammeux, N., Housden, B.E., Georgiadis, A., Hu, Y., Perrimon, N., 2016. Mapping signaling pathway cross-talk in *Drosophila* cells. Proc. Natl. Acad. Sci. 113 (35), 9940–9945, 201610432.

Arsic, D., Guerin, P.M., 2008. Nutrient content of diet affects the signaling activity of the insulin/target of rapamycin/p70 S6 kinase pathway in the African malaria mosquito *Anopheles gambiae*. J. Insect Physiol. 54, 1226–1235.

Bates, P.W., Liang, Y., Shingleton, A.W., 2013. Growth regulation and the insulin signaling pathway. Netw. Heterog. Media 8, 65–78.

Bloch, G., Francoy, T.M., Wachtel, I., Panitz-Cohen, N., Fuchs, S., Mazar, A., 2010. Industrial apiculture in the Jordan valley during biblical times with Anatolian honeybees. Proc. Natl. Acad. Sci. U. S. A. 107, 11240–11244.

Bustin, S.A., 2004. AZ of Quantitative PCR. International University Line La Jolla, CA.

Choi, Y.-J., Lai, W.S., Fedic, R., Stumpo, D.J., Huang, W., Li, L., Perera, L., Brewer, B.Y., Wilson, G.M., Mason, J.M., Blackshear, P.J., 2014. The *Drosophila* Tis11 protein and its effects on mRNA expression in flies. J. Biol. Chem. 289, 35042–35060.

Danks, H.V., 1987. Insect Dormancy: An Ecological Perspective. Biological Survey of Canada (Terrestrial Arthropods), Ottawa.

Defferrari, M.S., Orchard, I., Lange, A.B., 2016a. Identification of the first insulin-like peptide in the disease vector *Rhodnius prolixus*: involvement in metabolic homeostasis of lipids and carbohydrates. Insect Biochem. Mol. Biol. 70, 148–159.

Defferrari, M.S., Orchard, I., Large, A.B., 2016b. An insulin-like growth factor in *Rhodnius prolixus* is involved in post-feeding nutrient balance and growth. Front. Neurosci. 10.

Denlinger, D.L., 2002. Regulation of diapause. Annu. Rev. Entomol. 47, 93–122.

DiAngelo, J.R., Bland, M.L., Bambina, S., Cherry, S., Birnbaum, M.J., 2009. The immune response attenuates growth and nutrient storage in *Drosophila* by reducing insulin signaling. Proc. Natl. Acad. Sci. U. S. A. 106, 20853–20858.

Dupont, J., Holzenberger, M., 2003. Biology of insulin-like growth factors in development. Birth Defects Res. 69, 257–271.

Dupont, J., Scaramuzzi, R.J., 2016. Insulin signalling and glucose transport in the ovary and ovarian function during the ovarian cycle. Biochem. J. 473, 1483–1501.

Epstein, Y., Perry, N., Volin, M., Zohar-Fux, M., Braun, R., Porat-Kuperstein, L., Toledano, H., 2017. miR-9a modulates maintenance and ageing of *Drosophila* germline stem cells by limiting N-cadherin expression. Nat. Commun. 8, 600.

Forrest, J.R.K., Cross, R., CaraDonna, P.J., 2019. Two-year bee, or not two-year bee? How voltinism is affected by temperature and season length in a high-elevation solitary bee. Am. Nat. 193, 560–574.

Fulga, T.A., McNeill, E.M., Binari, R., Yelick, J., Blanche, A., Booker, M., Steinkraus, B.R., Schnall-Levin, M., Zhao, Y., DeLuca, T., Bejarano, F., Han, Z., Lai, E.C., Wall, D.P., Perrimon, N., Van Vactor, D., 2015. A transgenic resource for conditional competitive inhibition of conserved *Drosophila* microRNAs. Nat. Commun. 6, 7279.

Goldsmith, M.R., Shimada, T., Abe, H., 2005. The genetics and genomics of the silkworm, *Bombyx mori*. Annu. Rev. Entomol. 50, 71–100.

Hahn, D.A., Denlinger, D.L., 2007. Meeting the energetic demands of insect diapause: nutrient storage and utilization. J. Insect Physiol. 53, 760–773.

Hahn, D.A., Denlinger, D.L., 2011. Energetics of insect diapause. Annu. Rev. Entomol. 56 (56), 103–121.

Huggett, J., Dheda, K., Bustin, S., Zumla, A., 2005. Real-time RT-PCR normalisation; strategies and considerations. Genes Immun. 6, 279–284.

Keith, S.A., Eutsey, R., Lee, H., Solomon, B., Oliver, S., Kingsford, C., Hiller, N.L., McCartney, B.M., 2019. Identification of microbiota-induced gene expression changes in the *Drosophila melanogaster* head. bioRxiv 561043.

Kemp, W.P., Bosch, J., 2001. Postcocooning temperatures and diapause in the alfalfa pollinator *Megachile rotundata* (Hymenoptera: Megachilidae). Ann. Entomol. Soc. Am. 94, 244–250.

Koštál, V., 2006. Eco-physiological phases of insect diapause. J. Insect Physiol. 52, 113–127.

Kostal, V., Stetina, T., Poupardin, R., Korbelova, J., Bruce, A.W., 2017. Conceptual framework of the eco-physiological phases of insect diapause development justified by transcriptomic profiling. Proc. Natl. Acad. Sci. U. S. A. 114, 8532–8537.

Kubrak, O.I., Kucerova, L., Theopold, U., Nassel, D.R., 2014. The sleeping beauty: how reproductive diapause affects hormone signaling, metabolism, immune response and somatic maintenance in *Drosophila melanogaster*. PLoS One 9.

Kubrak, O.I., Kucerova, L., Theopold, U., Nylin, S., Nassel, D.R., 2016. Characterization of reproductive dormancy in male *Drosophila melanogaster*. Front. Physiol. 7.

Lai, W.S., Arvola, R.M., Goldstrohm, A.C., Blackshear, P.J., 2019. Inhibiting transcription in cultured metazoan cells with actinomycin D to monitor mRNA turnover. Methods 155, 77–87.

Lehmann, P., Pruisscher, P., Posledovich, D., Carlsson, M., Kakela, R., Tang, P., Nylin, S., Wheat, C.W., Wiklund, C., Gotthard, K., 2016. Energy and lipid metabolism during direct and diapause development in a pierid butterfly. J. Exp. Biol. 219, 3049–3060.

Li, F.C., Hu, J.S., Tian, J.H., Xu, K.Z., Ni, M., Wang, B.B., Shen, W.D., Li, B., 2016. Effects of phoxim on nutrient metabolism and insulin signaling pathway in silkworm midgut. Chemosphere 146, 478–485.

MacKenzie, K., Javorek, S., Rogers, D., 1997. The alfalfa leafcutting bee, *Megachile rotundata* Fabr: an alternative managed pollinator of lowbush blueberry. In: Yarborough, D.E., Smagula, J.M. (Eds.), Sixth International Symposium on Vaccinium Culture, pp. 87–90.

Matsunaga, Y., Honda, Y., Honda, S., Iwasaki, T., Qadota, H., Benian, G.M., Kawano, T., 2016. Diapause is associated with a change in the polarity of secretion of insulin-like peptides. Nat. Commun. 7.

Nassel, D.R., Kubrak, O.I., Liu, Y., Luo, J., Lushchak, O.V., 2013. Factors that regulate insulin producing cells and their output in *Drosophila*. Front. Physiol. 4.

Nolan, T., Hands, R.E., Bustin, S.A., 2006. Quantification of mRNA using real-time RT-PCR. Nat. Protoc. 1, 1559–1582.

Pitts-Singer, T.L., James, R.R., 2009. Prewinter management affects *Megachile rotundata* (Hymenoptera: Megachilidae) prepupal physiology and adult emergence and survival. J. Econ. Entomol. 102, 1407–1416.

Ragland, G.J., Keep, E., 2017. Comparative transcriptomics support evolutionary convergence of diapause responses across Insecta. Physiol. Entomol. 42, 246–256.

Rinehart, J.P., Yocum, G.D., West, M., Kemp, W.P., 2011. A fluctuating thermal regime improves survival of cold-mediated delayed emergence in developing *Megachile rotundata* (Hymenoptera: Megachilidae). J. Econ. Entomol. 104, 1162–1166.

Rinehart, J.P., Yocum, G.D., Kemp, W.P., Greenlee, K.J., 2013. A fluctuating thermal regime improves long-term survival of quiescent prepupal *Megachile rotundata* (Hymenoptera: Megachilidae). J. Econ. Entomol. 106, 1081–1088.

Rinehart, J.P., Yocum, G.D., Kemp, W.P., Bowsher, J.H., 2016. Optimizing fluctuating thermal regime storage of developing *Megachile rotundata* (Hymenoptera: Megachilidae). J. Econ. Entomol. 109, 993–1000.

Saltiel, A.R., Kahn, C.R., 2001. Insulin signalling and the regulation of glucose and lipid metabolism. Nature 414, 799–806.

Sandler, J.E., Stathopoulos, A., 2016. Quantitative single-embryo profile of *Drosophila* genome activation and the dorsal-ventral patterning network. Genetics 202, 1575–1584.

Satake, S., Masumura, M., Ishizaki, H., Nagata, K., Kataoka, H., Suzuki, A., Mizoguchi, A., 1997. Bombyxin, an insulin-related peptide of insects, reduces the major storage carbohydrates in the silkworm *Bombyx mori*. Comp. Biochem. Physiol. B-Biochem. Mol. Biol. 118, 349–357.

Schell, J.C., Wisidagama, D.R., Bensard, C., Zhao, H., Wei, P., Tanner, J., Flores, A., Mohlman, J., Sorensen, L.K., Earl, C.S., Olson, K.A., Miao, R., Waller, T.C., Delker, D., Kanth, P., Jiang, L., DeBerardinis, R.J., Bronner, M.P., Li, D.Y., Cox, J.E., Christofk, H.R., Lowry, W.E., Thummel, C.S., Rutter, J., 2017. Control of intestinal stem cell function and proliferation by mitochondrial pyruvate metabolism. Nat. Cell Biol. 19, 1027.

Schiesari, L., Andreatta, G., Kyriacou, C.P., O'Connor, M.B., Costa, R., 2016. The insulin-like proteins dILPs-2/5 determine diapause inducibility in *Drosophila*. PLoS One 11.

Sharma, A., Nuss, A.B., Gulia-Nuss, M., 2019. Insulin-like peptide signaling in mosquitoes: the road behind and the road ahead. Front. Endocrinol. 10.

Shiao, M.-S., Fan, W.-L., Fang, S., Lu, M.-Y.J., Kondo, R., Li, W.-H., 2013. Transcriptional profiling of adult *Drosophila* antennae by high-throughput sequencing. Zool. Stud. 52, 42.

Shiao, M.-S., Chang, J.-M., Fan, W.-L., Lu, M.-Y.J., Notredame, C., Fang, S., Kondo, R., Li, W.-H., 2015. Expression divergence of chemosensory genes between *Drosophila sechellia* and its sibling species and its implications for host shift. Genome Biol. Evol. 7, 2843–2858.

Sim, C., Denlinger, D.L., 2007. RNA interference of the insulin receptor in *Culex pipiens* arrests ovarian development and simulate diapause. Am. J. Trop. Med. Hyg. 77, 67.

Sim, C., Denlinger, D.L., 2008. Insulin signaling and FOXO regulate the overwintering diapause of the mosquito *Culex pipiens*. Proc. Natl. Acad. Sci. U. S. A. 105, 6777–6781.

Sim, C., Denlinger, D.L., 2009a. A shut-down in expression of an insulin-like peptide, ILP-1, halts ovarian maturation during the overwintering diapause of the mosquito *Culex pipiens*. Insect Mol. Biol. 18, 325–332.

Sim, C., Denlinger, D.L., 2009b. Transcription profiling and regulation of fat metabolism genes in diapausing adults of the mosquito *Culex pipiens*. Physiol. Genomics 39, 202–209.

Sim, C., Denlinger, D.L., 2013. Insulin signaling and the regulation of insect diapause. Front. Physiol. 4.

Sim, C., Kang, D.S., Kim, S., Bai, X., Denlinger, D.L., 2015. Identification of FOXO targets that generate diverse features of the diapause phenotype in the mosquito *Culex pipiens*. Proc. Natl. Acad. Sci. U. S. A. 112, 3811–3816.

Sun, W., Samimi, H., Gamez, M., Zare, H., Frost, B., 2018. Pathogenic tau-induced piRNA depletion promotes neuronal death through transposable element dysregulation in neurodegenerative tauopathies. Nat. Neurosci. 21, 1038–1048.
Sung, H.W., Spangenberg, S., Vogt, N., Grosshans, J., 2013. Number of nuclear divisions in the *Drosophila* blastoderm controlled by onset of zygotic transcription. Curr. Biol. 23, 133–138.
Taguchi, A., White, M.F., 2008. Insulin-like signaling, nutrient homeostasis, and life span. Annu. Rev. Physiol. 70, 191–212.
Tepedino, V.J., Parker, F.D., 1986. Effect of rearing temperature on mortality, second-generation emergence, and size of adult in *Megachile rotundata* (Hymenoptera: Megachilidae). J. Econ. Entomol. 79, 974–977.
Torson, A.S., Yocum, G.D., Rinehart, J.P., Kemp, W.P., Bowsher, J.H., 2015. Transcriptional responses to fluctuating thermal regimes underpinning differences in survival in the solitary bee *Megachile rotundata*. J. Exp. Biol. 218, 1060–1068.
Undurraga, J.M., Stephen, W.P., 1980. Effect of temperature on development and survival in post-diapausing leafcutting bee pupae (*Megachile rotundata* (F.)). II. Low temperature. J. Kansas Entomol. Soc. 53, 677–682.
Vonesh, E.F., 2012. Generalized Linear and Nonlinear Models for Correlated Data: Theory and Applications Using SAS. SAS Institute.
West, C., Silverman, N., 2018. p38b and JAK-STAT signaling protect against invertebrate iridescent virus 6 infection in *Drosophila*. PLoS Pathog. 14, e1007020.
Williams, K.D., Busto, M., Suster, M.L., So, A.K.C., Ben-Shahar, Y., Leevers, S.J., Sokolowski, M.B., 2006. Natural variation in *Drosophila melanogaster* diapause due to the insulin-regulated PI3-kinase. Proc. Natl. Acad. Sci. U. S. A. 103, 15911–15915.
Winkler, F., Kriebel, M., Clever, M., Gröning, S., Großhans, J., 2017. Essential function of the serine hydroxymethyl transferase (SHMT) gene during rapid syncytial cell cycles in *Drosophila*. G3 (Bethesda) 7, 2305–2314.
Wu, Q., Brown, M.R., 2006. Signaling and function of insulin-like peptides in insects. Annu. Rev. Entomol. 51, 1–24.
Yocum, G.D., Kemp, W.P., Bosch, J., Knoblett, J.N., 2005. Temporal variation in overwintering gene expression and respiration in the solitary bee *Megachile rotundata*. J. Insect Physiol. 51, 621–629.
Yocum, G.D., Kemp, W.P., Bosch, J., Knoblett, J.N., 2006. Thermal history influences diapause development in the solitary bee *Megachile rotundata*. J. Insect Physiol. 52, 1113–1120.
Yocum, G.D., Rinehart, J.P., West, M., Kemp, W.P., 2010. Interrupted incubation and short-term storage of the alfalfa pollinator *Megachile rotundata* (Hymenoptera: Megachilidae): a potential tool for synchronizing bees with bloom. J. Econ. Entomol. 103, 234–241.
Yocum, G.D., Greenlee, K.J., Rinehart, J.P., Bennett, M.M., Kemp, W.P., 2011. Cyclic CO_2 emissions during the high temperature pulse of fluctuating thermal regime in eye-pigmented pupae of *Megachile rotundata*. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 160, 480–485.
Yocum, G.D., Rinehart, J.P., Kemp, W.P., 2012. Duration and frequency of a high temperature pulse affect survival of emergence-ready *Megachile rotundata* (Hymenoptera: Megachilidae) during low-temperature incubation. J. Econ. Entomol. 105, 14–19.
Yocum, G.D., Rinehart, J.P., Horvath, D.P., Kemp, W.P., Bosch, J., Alroobi, R., Salem, S., 2015. Key molecular processes of the diapause to post-diapause quiescence transition in the alfalfa leafcutting bee *Megachile rotundata* identified by comparative transcriptome analysis. Physiol. Entomol. 40, 103–112.
Yocum, G.D., Childers, A.K., Rinehart, J.P., Rajamohan, A., Pitts-Singer, T.L., Greenlee, K.J., Bowsher, J.H., 2018. Environmental history impacts gene expression during diapause development in the alfalfa leafcutting bee, *Megachile rotundata*. J. Exp. Biol. 221.
Zhang, X.-S., Wang, T., Lin, X.-W., Denlinger, D.L., Xu, W.-H., 2017. Reactive oxygen species extend insect life span using components of the insulin-signaling pathway. Proc. Natl. Acad. Sci. U. S. A. 114, E7832–E7840.
Zheng, W., Rus, F., Hernandez, A., Kang, P., Goldman, W., Silverman, N., Tatar, M., 2018. Dehydration triggers ecdysone-mediated recognition-protein priming and elevated anti-bacterial immune responses in *Drosophila* Malpighian tubule renal cells. BMC Biol. 16, 60. https://doi.org/10.1186/s12915-018-0532-5.