ROS and hypoxia signaling regulate periodic metabolic arousal during insect dormancy to coordinate glucose, amino acid, and lipid metabolism

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Metabolic suppression is a hallmark of animal dormancy that promotes overall energy savings. Some diapausing insects and some mammalian hibernators have regular cyclic patterns of substantial metabolic depression alternating with periodic arousal where metabolic rates increase dramatically. Previous studies, largely in mammalian hibernators, have shown that periodic arousal is driven by an increase in aerobic mitochondrial metabolism and that many molecules related to energy metabolism fluctuate predictably across periodic arousal cycles. However, it is still not clear how these rapid metabolic shifts are regulated. We first found that diapausing flesh fly pupae primarily use anaerobic glycolysis during metabolic depression but engage in aerobic respiration through the tricarboxylic acid cycle during periodic arousal. Diapausing pupae also clear anaerobic by-products and regenerate many metabolic intermediates depleted in metabolic depression during arousal, consistent with patterns in mammalian hibernators. We found that decreased levels of reactive oxygen species (ROS) induced metabolic arousal and elevated ROS extended the duration of metabolic depression. Our data suggest ROS regulates the timing of metabolic arousal by changing the activity of two critical metabolic enzymes, pyruvate dehydrogenase and carnitine palmitoyltransferase I by modulating the levels of hypoxia inducible transcription factor (HIF) and phosphorylation of adenosine 5′-monophosphate-activated protein kinase (AMPK). Our study shows that ROS signaling regulates periodic arousal in our insect diapasue system, suggesting the possible importance ROS for regulating other types of of metabolic cycles in dormancy as well.

ROS | hypoxia signaling | periodic arousal | diapause | hibernation

Many organisms have evolved dormancy strategies that allow them to synchronize their lifecycles with favorable times for growth and reproduction as well as to mitigate stressful times, such as the cold of winter and periods of drought. Diapause is a programmed dormancy strategy entered into by many insects in anticipation of stress (1, 2). Metabolic suppression is one of the hallmarks of dormancy from seed banks in the soil to mammalian hibernators to insects in diapause. The majority of insects do not feed during diapause and must use nutrient reserves accumulated prior to dormancy to fuel the long dormant period as well as the catabolic and anabolic demands of resuming the lifecycle after dormancy (3, 4). Understanding how organisms naturally switch between high active metabolic rates and severe metabolic depression has a wide range of implications from building models for how climate change will affect dormant organisms to inducing or terminating dormant states artificially in whole organisms, recovery from ischemia in live tissues, maintenance of ex vivo organs for transplant, or even metabolic manipulation of specific groups of cells (e.g., cancer) (5–7). Thus, the mechanisms regulating metabolic suppression during dormancy have been an active source of study for decades. While some dormant organisms maintain relatively constant lowered metabolism, others including a few mammalian hibernators and a few diapausing insects regularly fluctuate between strong metabolic depression and short bursts of higher metabolic activity, termed periodic arousal (5, 8). The flesh fly *Sarcophaga crassipalpis* Macquart (Diptera: Sarcophagidae) shows dramatic metabolic suppression during their multiple-month pupal diapause with less than 10% of the CO_2 output of nondiapausing pupae (9). However, this strong metabolic depression predictably alternates with periods of arousal wherein the metabolic rates of diapausing pupae approximate those of nondiapause pupae (9), akin to the periodic arousal cycles observed in some hibernating mammals (5).

In mammalian hibernators, periodic arousal has been associated with multiple nonmutually exclusive functions including: replenishing key metabolic substrates, restoring the energetic state, purging metabolic waste, protein homeostasis, repairing damage incurred during depression, sleep, and a number of other physiological and biochemical events (5, 6). Even though changes in the abundances of many transcripts, proteins, and metabolites (10–13) are associated with periodic arousal, the molecular signals that

Significance

Organisms from bacterial spores, plant seeds, and invertebrate cysts to diapausing insects and mammalian hibernators dramatically suppress metabolism to save energy during dormant periods. Understanding regulatory mechanisms controlling metabolic depression provides insights into fundamental control of energy use during dormancy and offers perspectives that may allow artificial induction and breaking of dormancy. Some insects and mammalian hibernators show periodic arousal from metabolic depression during dormancy. We show that insects follow similar metabolic tactics during torpor and periodic arousal as mammalian hibernators, characterized by anaerobic metabolism during depression and aerobic metabolism during arousal, emphasizing fundamental similarities in metabolic regulation. Physiological levels of ROS regulate the switch from metabolic depression to periodic arousal thereby controlling substrate flow into the tricarboxylic acid cycle.

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trigger metabolic arousal from depression have not yet been elucidated for mammalian hibernators. It has been suggested that mitochondria are activated during the periodic arousal, and cyclic changes in key metabolites associated with mitochondrial oxidative phosphorylation may be a fundamental driving force for the metabolic cycles (14). Studies from other systems, such as the budding yeast metabolic cycle (YMC), have shown cyclic changes in abundance of molecules involved in redox signaling, such as reduced nicotinamide-adenine dinucleotide phosphate (NADP[H]) and reactive oxygen species (ROS) can regulate metabolic cycles (15–19).

Redox signaling has long been recognized as playing roles in both insect diapause (20) and mammalian hibernation (21–23) with a recent study showing ROS regulates insect diapause through insulin signaling (24). Cyclic changes in ROS have been associated with periodic arousal cycles in mammalian hibernators (22, 25), and it has been proposed that ROS may even play a regulatory role (24). It is well recognized that an excess of ROS

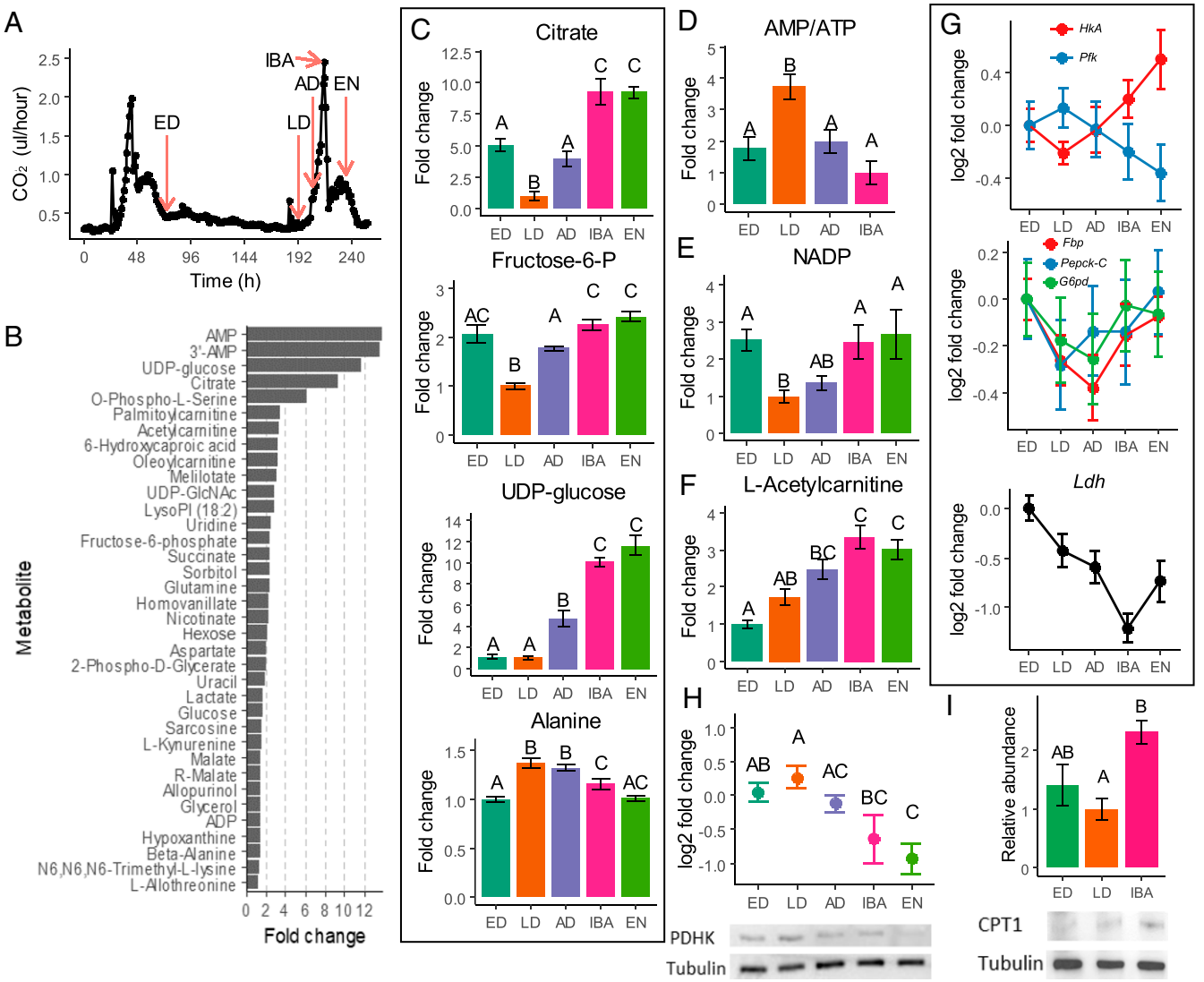

Fig. 1. Metabolite and transcript abundances fluctuate with metabolic depression and arousal in a pattern that suggests anaerobic glycolysis in depression and aerobic metabolism during arousal. (*A*) A representative metabolic trajectory (CO_2) from an individual diapausing pupa showing the metabolic cycle and defining our sampling points as arousal from metabolic depression (AD) was sampled ~6 h after the initial uptick in CO_2 production; interbout of metabolic arousal (IBA) was sampled ~15 h after the initial uptick in CO_2 production; EN (entering depression) was sampled ~30 h after the initial uptick in CO_2 production; early depression (ED) was sampled ~48 h after the initial CO_2 uptick in production and after CO_2 levels came back down; late depression (LD) was sampled 5 to 6 d after entering metabolic depression. (*B*) Fold-change differences in metabolites that differed the most across any two of the five sampling points across the metabolic cycles. (*C*) Representative temporal patterns of key metabolites across the cycle. (*D*) AMP:ATP ratio increases during depression and ATP is recharged during arousal. (*E*) NADP reducing equivalents were depleted during depression and recharged during arousal. (*F*) L-acetylcarnitine increases from early depression and peaks during arousal. (*G*) Temporal expression patterns of glycolysis/gluconeogenesis genes. *Hexokinase-A* (*Hka*) was low during metabolic depression but high during metabolic arousal, while *phosphofructokinase* (*Pfk*) was high during metabolic depression but decreased during metabolic arousal. *Fructose bis-P phosphatase* (*Fbp*), *Zw*, and *phosphoenolpyruvate carboxykinase* (*Pepck-C*) were low during the metabolic depression but high during metabolic arousal. *Hka*; *Pfk*; *Fbp*; *glucose 6-P dehydrogenase* (*G6pd*); *Pepck-C*, the cytosolic form of phosphoenolpyruvate carboxykinase; *lactate dehydrogenase kinase* (*Ldh*). (*H*) the PDHK was high during the metabolic depression and low during metabolic arousal. (*I*) CPT1 was low during the metabolic depression and high during metabolic arousal. Statistical significance was determined by one-way ANOVA with $P < 0.05$ after false discovery rate correction for multiple testing. Tukey's HSD post hoc test was used to compare different time points within a metabolite. Distinct letters in graphs show groups that are significantly different from each other after Tukey's HSD. Statistical results for *G* are in *SI Appendix*, Table S1.

compromises cellular function and causes oxidative stress, however, ROS have also been found to be essential cell signaling molecules to regulate cellular energy metabolism by interacting with central regulators of cellular energy metabolism, such as hypoxia-inducible transcription factor (HIF) and adenosine 5′-monophosphate-activated protein kinase (AMPK) in both insects and mammals (26, 27). Yet in the context of mammalian hibernation periodic arousal cycles, ROS have largely been considered to be a stress-inducing by-product, and the role of ROS in regulating mammalian and insect periodic arousal cycles has not been tested.

Over the course of diapause, flesh fly pupae rely on a combination of aerobic lipid catabolism and anaerobic glycolysis to support energetic demand (20, 28), but we do not know the degree that aerobic lipid catabolism and anaerobic glycolysis each, respectively, contribute to periodic arousal. We previously hypothesized that diapausing flesh fly pupae alternate between: 1) anaerobic glycolysis with the tricarboxylic acid (TCA) cycle shut down during metabolic depression, and 2) strong aerobic metabolism and flux of metabolites through the TCA cycle during arousal (20). Given the importance of ROS signaling in other insects that diapause as pupae (24), we further hypothesized that ROS signaling regulates periodic arousal through interactions with hypoxia signaling and activation of mitochondrial respiration via flux of metabolites into the TCA cycle. Using a combination of global metabolomics and ^{13}C-labeled isotope based metabolic flux analysis, we show that diapausing flesh fly pupae do indeed rely primarily on anaerobic glycolysis with TCA cycle shutdown during metabolic depression and combine glycolysis and aerobic metabolism of glycolytic end products, lipids, and amino acids during arousal. Key anaerobic metabolites, including lactate and alanine, are cleared during arousal, and key energetic metabolites to be used during anaerobic metabolic depression are regenerated, including adenosine 5′-triphosphate (ATP) and trehalose. We also show that hypoxia signaling and AMP:ATP ratios are low during arousal and increase during metabolic depression, whereas ROS levels are high during arousal and early in metabolic depression but decline with time spent in metabolic depression. Using pharmacological manipulations, we demonstrate that low ROS levels and high levels of hypoxia signaling both trigger arousal, likely via regulation of the entry of pyruvate into the TCA cycle through the activity of the critical gatekeeping metabolic enzymes pyruvate dehydrogenase (PDH) and carnitine palmitoyltransferase 1 (CPT1).

Results and Discussion

Key Glycolytic and TCA Metabolites as Well as Their Associated Enzymes Cycle with Periodic Arousal. Our observations agree with previous work showing the metabolic rate of diapausing pupae is not constant, flesh fly diapause is characterized by periods of metabolic depression that show as little as 10% of the CO_2 production of a nondiapause pupa with regular periodic bouts of high metabolic activity (Fig. 1*A*) (9). This pattern is similar to the periodic cycles of metabolic arousal observed in some mammalian hibernators (5) and some other diapausing insects (8). At 18 °C, the metabolic depression bout in flesh fly pupae lasts 3 to 4 d at the beginning of diapause and can stretch to 8 to 9 d in the middle of diapause, but becomes shorter again near the end of diapause (Fig. 1*A*) (9). In contrast to the changing duration of metabolic depression from early to mid-diapause, the flesh fly metabolic arousal phase lasts 36–48 h throughout diapause at 18 °C (Fig. 1*A*), and each arousal bout follows a predictable structure that has been previously described (9, 29).

Studies from mammalian hibernators showing similar periodic arousal cycles have suggested these cycles play several critical and nonmutually exclusive roles, including but not limited to, replenishing key metabolites depleted during the metabolic depression, sleeping, regenerating the organism's energetic state (i.e., AMP:ATP), protein homeostasis, and purging anaerobic by-products that accumulated during metabolic depression (5, 6). To uncover the metabolic strategies used by diapausing flesh flies to regulate periodic arousal, we first obtained global metabolomic profiles from diapausing pupae at five time points across different phases of the depression and arousal cycles (Fig. 1*A*). A total of 142 metabolites were identified from liquid chromatography-tandem mass spectrometry (LC-MS/MS) metabolomics scans (Dataset S1). Most metabolites that changed significantly across the phases of periodic arousal were related to carbohydrate, amino acid, and fatty acid metabolism; particularly intermediates of glycolysis/gluconeogenesis and the TCA cycle (Fig. 1*B*, *SI Appendix*, Fig. S1, and Dataset S1). We found two dominant abundance patterns of these metabolites across the cycle: 1) metabolites associated with oxidative phosphorylation and glycolysis/gluconeogenesis were depleted during metabolic depression and restored during arousal (e.g., citrate and fructose-6-phosphate) (Fig. 1*C*), and 2) metabolites associated with anaerobic metabolism accumulated during metabolic depression (e.g., alanine and lactate) but were depleted after arousal (Fig. 1*C* for alanine and *SI Appendix*, Fig. S2*A* for lactate). The contrasting patterns between oxidative phosphorylation and anaerobic metabolites suggest that diapausing pupae switch between aerobic metabolism during arousal and anaerobic metabolism during the depression period of the metabolic cycles.

Metabolites associated with the adenosine metabolic pathway, AMP and 3′-AMP, showed the biggest change across the cycles (Fig. 1*B* and *SI Appendix*, Fig. S2*A*). Both were very high during metabolic depression and very low during arousal, suggesting the energy supply was suppressed during metabolic depression. This was confirmed by a separate targeted study showing the AMP:ATP ratio was significantly higher during late depression (ANOVA, $F_{3,10} = 8.07$, and $P < 0.01$) (Fig. 1*D*), while many other high-energy molecules, such as guanosine triphosphate and uridine 5′-triphosphate did not change across the cycle (Dataset S2). This targeted assay also found NADP was significantly higher during early depression, interbout arousal, and entry into depression than during late depression ($F_{4,15} = 8.07$ and $P < 0.005$) (Fig. 1*E*). NADPH is a major source of cellular reducing equivalents and is a substrate for many biosynthetic reactions protecting cells against oxidative stress. Thus, accumulation of NADP during early depression, metabolic arousal, and upon entering depression also suggests high oxidative-radical load during these phases.

Two intermediates associated with flux through glycolysis/gluconeogenesis rather than through the TCA cycle, uridine 5′-diphosphate (UDP) glucose and citrate also showed massive changes across the metabolic arousal cycle. UDP glucose ($F_{4,40} = 74.32$ and $P < 0.001$), a precursor of glycogen and trehalose (30), was low during both early depression and late depression but increased more than 12-fold during arousal (Fig. 1*C*). This suggests that metabolic arousal cycles are critical for regeneration of glycogen and trehalose that can be used to support anaerobic glycolysis during metabolic depression. Previous work that did not consider the metabolic cycles showed diapausing flesh fly pupae use glycogen and trehalose as primary energy sources for anaerobic metabolism under anoxia (31), further supporting a switch from aerobic metabolism during arousal to anaerobic metabolism during depression. Citrate decreased approximately fivefold from early depression to late depression and increased ∼10-fold from late depression to interbout arousal (Fig. 1*C*, $F_{4,40} = 30.30$, and $P < 0.001$), indicating that the TCA cycle was activated during metabolic arousal but not during depression. Similarly, L-acetylcarnitine, which is associated with fatty acid metabolism, increased significantly during metabolic arousal ($F_{4,40} = 15.28$ and $P < 0.001$), further suggesting a shift to aerobic lipid metabolism at arousal (Fig. 1*F*).

Our interpretation that diapausing flesh flies are primarily using glycolysis during metabolic depression and running the TCA cycle during arousal was also supported by changes in transcript abundance of key metabolic enzymes across the cycles (Fig. 1*G* and *SI Appendix*, Table S1). Transcript abundance for glycolytic enzymes *Pfk* and *Ldh* were high during metabolic depression but decreased during arousal (Fig. 1*G*). However, another important glycolytic enzyme *hexokinase*-A (*HkA*) showed the opposite pattern with transcripts low during metabolic depression and higher during arousal (Fig. 1*G*). The opposite transcript abundance pattern between *HkA* and *Pfk* points to glucose being directed to branching pathways of glycolysis (*SI Appendix*, Fig. S2*B*), such as trehalose/glycogen synthesis (suggested by UDP glucose, Fig. 1*C*), the pentose phosphate shunt (suggested by NADP, Fig. 1*E*), and hexosamine synthesis (suggested by UDP GlcNAc, *SI Appendix*, Fig. S2*A*). Two gluconeogenesis-related genes, *Fbp* and the cytosolic form of *Pepck-C* as well as a key enzyme related to generation of NADH via the pentose phosphate shunt, *G6pd* had lower transcript abundance during metabolic depression but higher transcript abundance during arousal (Fig. 1*G*), further suggesting that glucose is used to support the branching pathways of glycolysis during arousal and then used to support anaerobic glycolysis during depression. The protein abundance of PDH kinase (PDHK), which decreases activity of the PDH complex that converts pyruvate into acetyl-CoA, a critical early step in the TCA cycle, was high during metabolic depression but much lower during metabolic arousal (Fig. 1*H*). This pattern of high PDHK during depression and low PDHK during arousal suggests that regulation of PDH activity may affect the timing of metabolic arousal and, thus, the duration of depression.

Further supporting the shift to aerobic lipid metabolism during arousal, the abundance of CPT1, an enzyme that controls the first rate-limiting step of β-oxidation of fatty acids, was significantly higher during metabolic arousal (ANOVA, $F_{2,15} = 15.28$, and $P < 0.005$) than in late depression and marginally higher during arousal than early depression ($P = 0.063$) (Fig. 1*I*). With regard to other key TCA cycle enzymes, we found no changes in transcript abundance of citrate synthase or succinate dehydrogenase kinase B across the cycle (*SI Appendix*, Table S1). This observation is in line with our previous study showing transcript levels of most TCA cycle enzymes were not changed when entering diapause in flesh flies (20). Thus, our working hypothesis is that diapausing flesh flies keep the internal machinery to run the TCA cycle steady across the metabolic cycles and that the activity of the TCA cycle during depression and arousal is regulated by steps that shunt in intermediates, such as PDH.

Taken together, our metabolite and transcript/protein abundance data are consistent with a shift from glycolytic anaerobic metabolism during metabolic depression to aerobic metabolism during arousal leading to regeneration of ATP, reducing equivalents, gluconeogenesis, and conversion to storage carbohydrates, such as trehalose, as well as clearance of anaerobic byproducts. However, static snapshots are not always representative of metabolic flux. Thus, we used carbon-13 NMR-based isotopomer analysis to estimate flux through glycolysis, gluconeogenesis, TCA cycle turnover, and substrate selection across metabolic depression and arousal.

Metabolic Flux Using Isotopic Tracers Reinforces Our Observations of High TCA Cycle Activity during Arousal but Anaerobic Glycolysis in Depression. Diapause-destined flesh fly larvae accumulate substantial lipid reserves that are catabolized during the pupal diapause period (20, 28), but they also appear to rely heavily on glycolysis during metabolic depression (20). Thus, we first performed a double-tracer experiment with [1,6-$^{13}C_2$]glucose and [U-^{13}C]palmitate to measure the competitive oxidation and/or anaplerotic flux of both glucose and fatty acids into the TCA cycle to recharge key metabolic intermediates across different points of the metabolic depression and arousal cycles (Fig. 2*A*).

We detected C4-C5–labeled glutamate during metabolic arousal by analysis of the proton-decoupled ^{13}C-NMR spectrum, suggesting [U-^{13}C]palmitate is oxidized via acetyl-CoA only during arousal (Fig. 2*B* and *SI Appendix*, Fig. S3). We did not detect C3-C4–labeled glutamate, suggesting no oxidation of [1,6-$^{13}C_2$]glucose. However, we cannot completely eliminate the possibility of glucose oxidation with this experiment alone because it is possible that an unbalanced quantity of ^{13}C-labeled glucose and palmitate were injected into the insect, causing the C4-C5–labeled glutamate spectrum to dominate and reduce our ability to detect C3-C4–labeled glutamate. Thus, we conducted another single [U-^{13}C]glucose-tracer experiment (Fig. 2*C*). With the single [U-^{13}C]glucose tracer we found that glucose-derived pyruvate is anaplerosed via PC flux to refill the TCA cycle intermediates by pathways other than pyruvate oxidation (i.e., PDH flux) during arousal. This conclusion is supported by the fact that only C2-C3 ^{13}C-labeled glutamate, but not C4-C5–labeled glutamate, was detected (Fig. 2*D* and *SI Appendix*, Fig. S4). However, the observed anaplerosis could also be assigned to flux through malic enzyme because both produce the same labeling patterns.

The single [U-^{13}C]glucose tracer also suggested high glycolytic flux into lactate and alanine during metabolic depression (Fig. 2*D*), reinforcing the pattern of anaerobic metabolism during depression, while the lactate and alanine accumulated during depression was metabolized during arousal (Fig. 2*E*). We also found that glucose was important for anabolic pathways besides its use as fuel during metabolic arousal. Glucose was used to synthesize trehalose to some degree in all cycle phases, but glucose flux into trehalose was substantially greater during arousal than during early depression (Fig. 2*D*; $F_{2,9} = 7.137$ and $P < 0.05$) and levels of total trehalose were higher during arousal than in early depression (Fig. 2*E*). We also detected ^{13}C-labeled serine and glycine during arousal, suggesting high glucose flux into biosynthesis of serine and glycine during metabolic arousal, but not during depression (*SI Appendix*, Fig. S5). Serine and glycine are essential precursors for synthesis of proteins and nucleic acids. Increased serine and glycine synthesis during arousal is consistent with the finding in a previous study that protein synthesis occurs during arousal, but not during depression, in diapausing flesh flies (32). Perhaps the high glucose demand for these biosynthetic pathways explains why fatty acids are favored over glucose as a metabolic fuel during arousal.

Overall, these results suggest that during metabolic arousal high β-oxidation of palmitate, and presumably other fatty acids, fueled aerobic metabolism while glucose catabolism replenished TCA cycle intermediates. In contrast, palmitate oxidation was not detected during metabolic depression, supporting the importance of anaerobic glycolysis during depression.

Another important hypothesized function of periodic arousal during dormancy is purging anaerobic by-products produced during metabolic depression. Thus, we infused [U-^{13}C]alanine into pupae during early depression, late depression, and arousal stages to examine the fate of anaerobic by-products (Fig. 2*F*). We detected both C2-C3 and C4-C5 ^{13}C-labeled glutamate isotopomers from [U-^{13}C]alanine (Fig. 2*G* and *SI Appendix*, Fig. S6) as well as C1-C2 ^{13}C-labeled trehalose (Fig. 2*G* and *SI Appendix*, Fig. S6), but only during metabolic arousal. These patterns of ^{13}C labeling in glutamate showed that [U-^{13}C]alanine-derived pyruvate was both anaplerosed and oxidized via PC and PDH fluxes into the TCA cycle, respectively. ^{13}C labeling in trehalose suggested that pyruvate is recycled through PEPCK flux and is used for gluconeogenesis and storing glucose in the form of trehalose, suggesting high gluconeogenesis as well as trehalose-6-phosphate synthase complex activation during metabolic arousal. We also detected ^{13}C-labeled lactate in all cycle phases, but significantly more ^{13}C-labeled alanine was converted

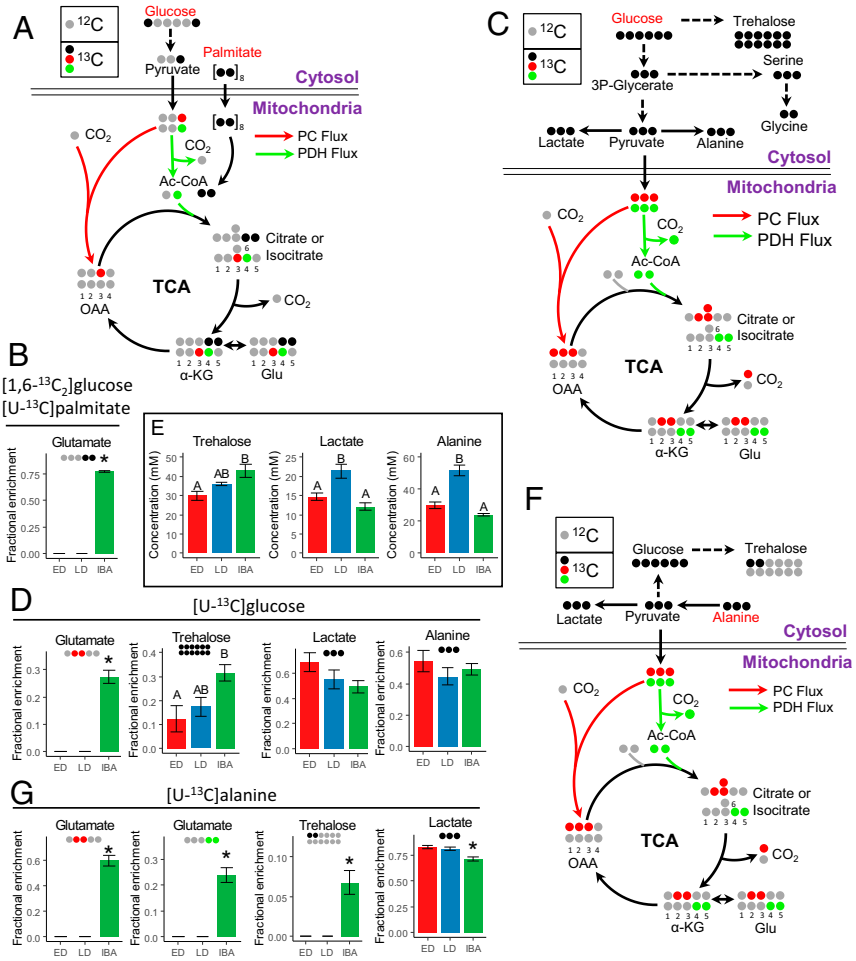

Fig. 2. Determination of metabolic flux of key metabolites by injection of ^{13}C-labeled metabolites into pupae in early depression, late depression, or early metabolic arousal and sampled 15 h after injection. (*A*) Predicted ^{13}C-labeling pattern of the metabolites derived from double-tracer [1,6-^{13}C2]glucose and [U-^{13}C]palmitate. Oxidation of palmitate is suggested if C4-C5–labeled glutamate is detected. ^{13}C-labeled glucose can enter the TCA cycle through pyruvate carboxylase (PC) (red dots) or PDH flux (green dots). Glucose oxidation through PDH flux is suggested only when C3-C4–labeled glutamate is detected. (*B*) Fractional enrichment of ^{13}C-labeled glutamate. Black dots in glutamate suggest that ^{13}C-labeled carbon from palmitate. (*C*) Predicted ^{13}C-labeling pattern of the metabolites derived from [U-^{13}C]glucose. Glucose can enter the TCA cycle through PC (red dots) or PDH flux (green dots). If glucose is metabolized through PC flux then C2-C3–labeled glutamate should be detected, and C4-C5–labeled glutamate should be detected if glucose is metabolized through PDH flux. (*D*) Fractional enrichment of ^{13}C-labeled metabolites. Red dots in glutamate suggest ^{13}C-labeled carbon from PC flux. (*E*) Total concentrations of lactate and alanine were significantly higher during late depression, suggesting these anaerobic byproducts were accumulated during metabolic depression but purged during metabolic arousal. Trehalose levels increased significantly from metabolic depression to metabolic arousal. (*F*) Predicted ^{13}C-labeling pattern of metabolites derived from [U-^{13}C]alanine. Alanine can enter the TCA cycle through PC (red dots) or PDH flux (green dots). If alanine is metabolized through PC flux C2-C3–labeled glutamate should be detected, and C4-C5–labeled glutamate should be detected if alanine is metabolized through PDH flux. (*G*) Fractional enrichment of ^{13}C-labeled metabolites. Red dots in glutamate suggest ^{13}C-labeled carbon from PC flux and green dots suggest ^{13}C-labeled carbon from PDH flux. Statistical significance was determined by one-way ANOVA followed by Tukey's HSD post hoc test. Distinct letters or "*" show groups that are significantly different from each other ($P < 0.05$).

to lactate during both early and late depressions compared to during metabolic arousal (Fig. 2*G*), further suggesting greater reliance on anaerobic metabolism during depression.

The differences in our results between the glucose and the alanine tracers regarding PDH flux suggests that there may be tissue-specific substrate uptake, and we propose that alanine may act as a shuttle to move fuel between tissues during metabolic arousal. Because glucose is in heavy demand for glycolysis and its branching pathways, circulating alanine can potentially help effectively decouple glycolysis and the TCA cycle across tissues, allowing independent tissue-specific regulation of metabolic processes. Similar mechanisms have been found in starved mice that use lactate as the primary circulating TCA substrate to decouple glycolysis and the TCA cycle across tissues (33) as well as insects that use alanine and other amino acids as critical metabolic intermediates in flight (34, 35). Our results showed that palmitate and alanine were used as energy sources for metabolic arousal, while glucose was mainly used for biosynthetic purposes during arousal as well as for anaerobic glycolysis during depression.

Studies of mammalian periodic arousal cycles have similarly shown that fat is the major energy source during metabolic arousal and glucose is primarily used for glycolysis during metabolic depression (36, 37). Interestingly, shifts in fuel use from aerobic metabolism of fat during periodic arousal to anaerobic glycolysis during metabolic depression cycles have also been suggested to be regulated by substrate entry into the TCA cycle through PDH in mammalian periodic arousal cycles (34). Yet, this role for PDH in regulating periodic arousal had not previously been manipulatively tested in insects or mammals.

PDH Activation Induces Metabolic Arousal. It has been hypothesized that cyclic changes in key metabolites associated with mitochondrial oxidative phosphorylation may be a fundamental driving force of mammalian hibernation cycles (14) and manipulation of metabolic intermediates has previously been shown to affect pupal diapause duration (38). Therefore, we injected palmitate, pyruvate, alanine, and lactate into pupae in metabolic depression. Increasing levels of these metabolites did not trigger metabolic arousal. However, our metabolic flux analysis clearly suggested that oxidation of alanine and fatty acids was associated with metabolic arousal, suggesting the regulation of metabolic rate may occur by mediating critical enzymes controlling substrate oxidation. Transcript levels of TCA cycle enzymes did not change across the cycle; thus, regulation of metabolic rate may be due to controlling the entry of substrates into the TCA cycle.

We first focused on two important enzymes that control substrates entering the TCA cycle; PDH, a critical regulator of the conversion of pyruvate into acetyl-CoA to enter the TCA cycle, and CPT1, a critical regulator of fatty acid transport into the mitochondrion for catabolism. We expected increasing the activity of PDH or CPT1 could induce the transition from metabolic depression to arousal. Because PDH activity is negatively regulated by the PDHK, inhibition of PDHK can increase PDH activity. Thus, we injected a classic PDHK inhibitor dichloroacetate (DCA) into pupae in early metabolic depression. DCA injection increased PDH activity (Fig. 3*A*, $F_{1,8} = 5.28$, and $P < 0.01$) and induced metabolic arousal within 24 h (Fig. 3*B* and *SI Appendix*, Fig. S6). However, injection of a fatty acid oxidation activator C75 that should stimulate CPT1 activity (39) did not induce metabolic arousal (ANOVA, treatment effect: $F_{1,6} = 2.88$ and $P > 0.05$). These results showed that metabolic arousal can be induced by increasing PDH activity through inhibition of PDHK, consistent with associations between PDH activity and metabolic arousal observed in mammalian hibernator periodic arousal cycles (34).

Hypoxia Signaling Is Involved in Regulating Metabolic Cycles. PDHK is transcriptionally regulated by the HIF in *Drosophila* and mammals (26, 40). Consistent with hypoxic regulation of PDHK in flesh fly metabolic cycles, we identified two hypoxia-response elements (HREs) consensus sites (5′-ACGTG-3′) in the PDHK promotor region of a closely related flesh fly species *Sarcophaga bullata* that has an available genome sequence (*SI Appendix*, Fig. S8*A*) (41). HIF has been previously implicated in regulating energy metabolism in insect diapause (42) and mammalian hibernation (43). Thus, we tested HIF protein levels across the metabolic cycle. We found that HIF protein abundance was low in early depression and significantly increased by late depression. HIF protein abundance showed a trend toward decreasing during metabolic arousal, although this trend did not reach statistical significance and should be viewed cautiously (Fig. 3*C*). Injection of a HIF inhibitor Ech (44) during metabolic depression shortened the time to arousal (Fig. 3*D* and *SI Appendix*, Fig. S9), and levels of *Pdhk* were significantly decreased after injection of Ech (*SI Appendix*, Fig. S7*B*), consistent with HIF regulation of PDHK. Another well-known HIF mediated gene *Ldh* showed significantly decreased transcript abundance after injection of Ech (*SI Appendix*, Fig. S8*B*). These data suggest that HIF is involved in regulating periodic arousal in diapause. If HIF regulates flesh fly periodic arousal, we expected that stabilizing HIF during depression should lengthen the duration of time until metabolic arousal. However, injection of neither of two known HIF protein stabilizers cobalt (45) nor FG-4592 (46) stretched the length of metabolic depression (ANOVA, treatment effect for cobalt: $F_{1,4} = 0.04$ and $P > 0.05$; treatment effect for FG-4592: $F_{1,4} = 1.19$ and $P > 0.05$). These data suggest that HIF was not sufficient to regulate the metabolic cycle by itself, and, thus, there must be other central players upstream of HIF.

ROS Signaling Regulates Metabolic Cycles. ROS are a well-known upstream regulator that can stabilize HIF levels by preventing HIF degradation or increasing HIF gene expression (26, 47). A study in *Caenorhabditis elegans* has shown that ROS affects metabolic rate by regulating HIF protein levels (48). Redox signaling has long been recognized as playing roles in both insect diapause (20) and mammalian hibernation (21–23) with a recent study showing ROS regulates insect diapause through insulin signaling (24). In the context of metabolic cycles, studies in other

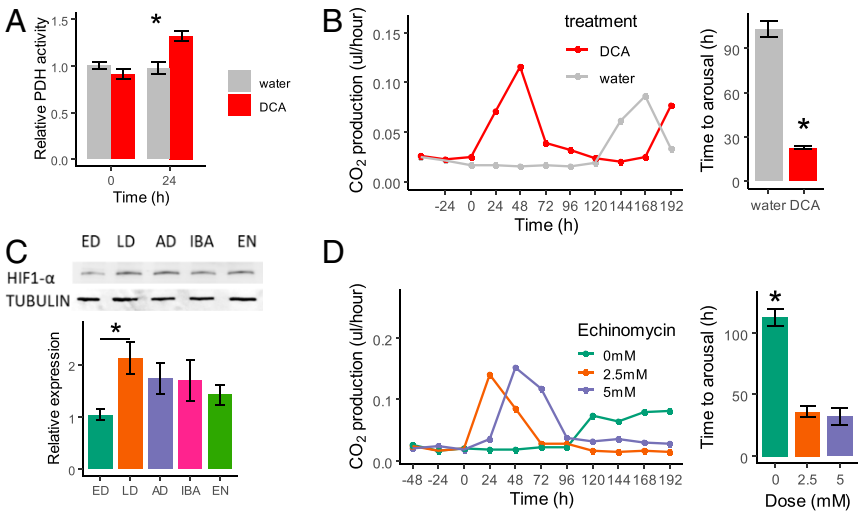

Fig. 3. Injection of inhibitors of PDHK and HIF accelerated the time to metabolic arousal, suggesting that PDH and HIF are involved in regulating transitions between metabolic depression and periodic arousal. (*A*) PDH activity was significantly increased 24 h after injection of the PDHK inhibitor DCA. (*B*) CO_2 production was increased within 24 h of injection of DCA, effectively accelerating the timing of periodic arousal. Time 0 on the x axis represents the time of injection. (*C*) Relative levels of HIF were significantly higher in LD than in ED and gradually decreased during metabolic arousal, consistent with functional hypoxia during metabolic depression and reversal of that hypoxia during arousal. (*D*) Injection of HIF inhibitor echinomycin (Ech) increased relative CO_2 production in a pattern consistent with acceleration of periodic arousal. Time 0 on the x axis represents the time of injection. Statistical significance was determined by one-way ANOVA with Tukey's HSD post hoc test. *$P < 0.05$.

systems, such as circadian rhythms, mammalian hibernator torpor-arousal cycles (23, 25, 49), and the budding yeast metabolic cycle (YMC) (19) all suggested that redox signals, including ROS, are involved in metabolic cycle regulation. Perhaps the most compelling results originate from the study of YMCs showing that a strain with pentose phosphate pathway disruption and, thus, insufficient production of reducing equivalents (e.g., NADPH), did not undergo metabolic cycles (19), indicating the importance of redox signaling to metabolic cycle regulation. Prior studies of mammalian periodic arousal in hibernation considered changes in ROS levels to be a result of mitochondrial metabolism without testing the role of redox signaling in regulating periodic arousal (23, 49).

In our study, the clear decrease in NADP from early depression to late depression followed by a rapid increase in NADP with arousal (Fig. 1*E*) suggests redox signaling may affect metabolic cycling in diapausing flesh flies. We, thus, tested ROS levels at different time points across the cycles. We found ROS were high in early metabolic depression and low in late metabolic depression, but ROS increased during metabolic arousal in both brain tissue (Fig. 4*A*) and whole-body extracts (*SI Appendix*, Fig. S8*C*), a pattern concordant with NADP levels. To experimentally test the extent to which ROS levels affect periodic arousal cycles, we first injected the ROS scavenger *N*-acetyl-cysteine (NAC) into pupae in early metabolic depression (24–48 h after entering metabolic depression) to decrease ROS levels. A dose of 300 μg NAC decreased ROS levels 24 h after injection in both brain tissue (Fig. 4*B*) and whole-body homogenates (*SI Appendix*, Fig. S8*D*) and induced metabolic arousal in pupae that were expected to be in metabolic depression (Fig. 4*C* and *SI Appendix*, Fig. S10). Decreasing ROS effectively shortened the duration of the depression stage and accelerated the timing of arousal. However, if ROS regulate the metabolic cycles, we expect that increasing ROS during depression should lengthen the duration of time until metabolic arousal. We injected the ROS generator paraquat (PQ) into pupae late during metabolic depression (24–48 h before predicted metabolic arousal based on monitoring individual pupae). PQ injection increased ROS levels for 24 h, but ROS levels dropped back to the preinjection levels after 48 h (Fig. 4*D* and *SI Appendix*, Fig. S8*E*). Even increasing ROS levels for just 24 h by PQ injection detectably delayed the timing of metabolic arousal (Fig. 4*E* and *SI Appendix*, Fig. S11). Taken together, these results suggest redox signaling via ROS causally regulates the cycles of periodic metabolic arousal during flesh fly diapause.

Given our results above, we tested whether ROS regulate periodic arousal cycles by affecting activity of PDH through HIF (26, 40). We found that PDH activity (Fig. 5*A*) was significantly increased 24 h after ROS levels declined from NAC injection as would be expected if greater quantities of pyruvate were fluxing into the TCA cycle. Furthermore, transcript levels of the negative regulator of PDH, *Pdhk* (*SI Appendix*, Fig. S8*F*) were decreased and significantly lower in NAC-treated diapausing pupae than in the vehicle injection treatment. Similar to the expression pattern found during natural metabolic arousal, we also found the gluconeogenic gene *Pepck-C* was up-regulated during the induced metabolic arousal by injection of NAC (*SI Appendix*, Fig. S8*F*), while transcript levels of citrate synthase (*SI Appendix*, Fig. S8*F*) did not change. Overall, these results suggest that ROS may regulate PDH activity through a HIF-dependent pathway during diapause periodic arousal cycles.

Even though injection of the CPT1 activator C75 did not induce metabolic arousal, fatty acid oxidation was associated with metabolic arousal in our metabolic flux analysis. Thus, we tested whether fatty acid oxidation was regulated by ROS signaling. Indeed, we found CPT1 protein levels increased after ROS levels were decreased by NAC injection during the early metabolic depression phase (Fig. 5*B*), suggesting that fatty acid oxidation

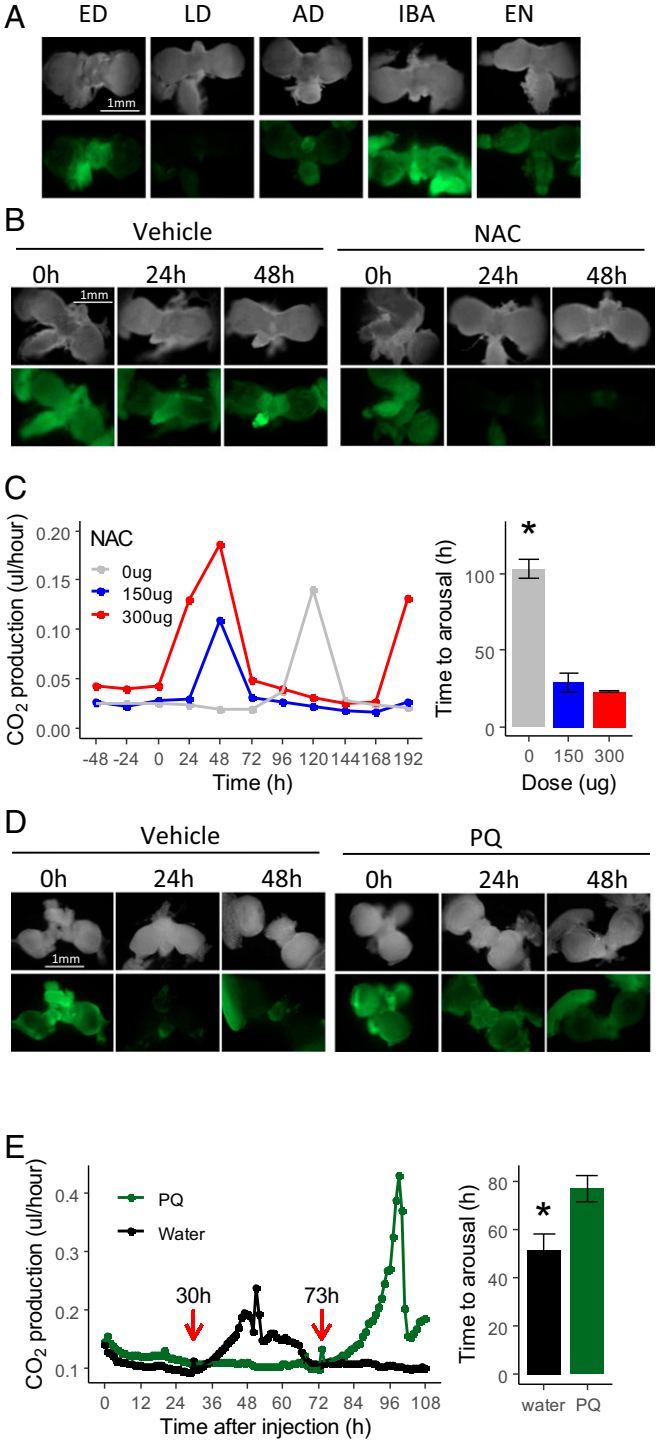

Fig. 4. Reducing ROS levels accelerated time to metabolic arousal while increasing ROS delayed the time to arousal. (*A*) ROS levels in the brain were decreased from ED to LD but increased again during metabolic arousal. (*B*) ROS levels in the brain were decreased after injection of NAC. (*C*) Injection of the ROS scavenger NAC increased relative CO_2 production in a pattern consistent with acceleration of periodic arousal. Time 0 on the x axis represents the time of injection. (*D*) ROS levels in the brain were maintained after injection of the ROS generator paraquat (PQ) rather than falling as in controls. (*E*) Injection of PQ caused a delay in the timing of periodic arousal. Time 0 on the x axis represents the time of injection. Statistical significance was determined by one-way ANOVA with Tukey's HSD post hoc test. $*P < 0.05$.

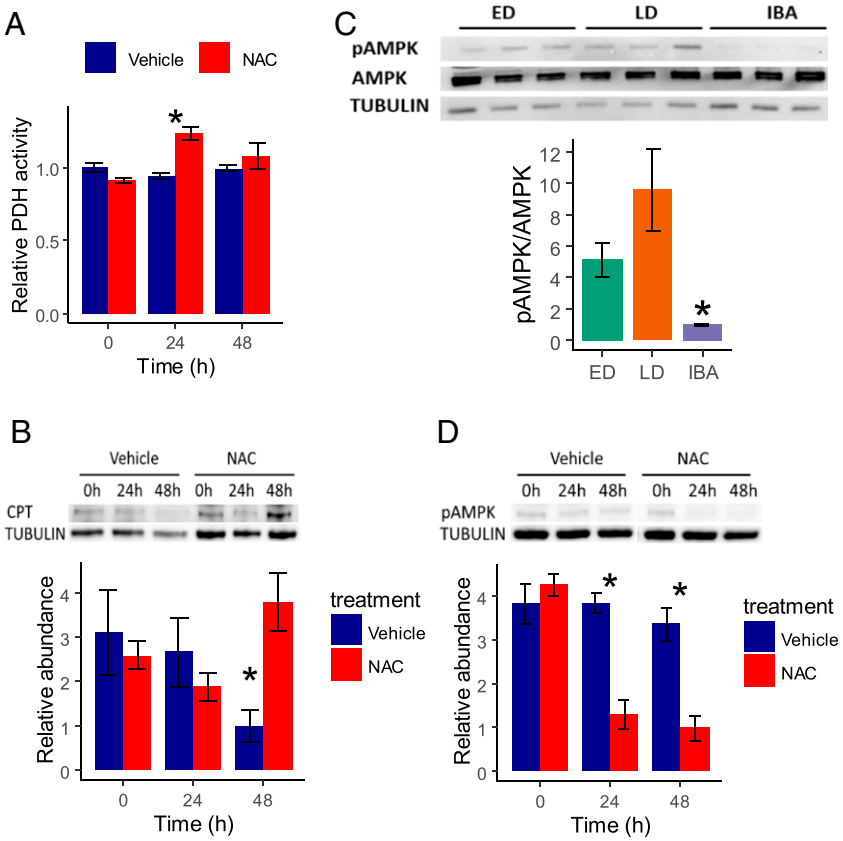

Fig. 5. Lowering ROS levels by injecting NAC also changed the activity and abundance of key metabolic proteins. (*A*) PDH activity was increased 24 h after NAC injection. (*B*) Levels of CPT1 increased 48 h after NAC injection applied early in metabolic depression. (*C*) The ratio of pAMPK/AMPK increased during metabolic depression, suggesting AMPK activation. (*D*) pAMPK was decreased after NAC injection applied early in metabolic depression. Statistical significance was determined by one-way ANOVA with Tukey's HSD post hoc test. $*P < 0.05$.

could be regulated by ROS signaling. We also expected the well-known lipid metabolism regulator, AMPK could be involved in the ROS signaling cascade by regulating CPT1 expression. As expected, we found that abundance of the active phosphorylated form of AMPK significantly increased from early metabolic depression to late depression and decreased during metabolic arousal (Fig. 5*C*) while total AMPK levels remained steady across the cycle. Similarly, the phosphorylation of AMPK also dropped significantly when metabolic arousal was induced by injection of the ROS scavenger NAC (Fig. 5*D*), suggesting AMPK is involved in ROS signaling regulation of periodic arousal in flesh flies. In contrast, a study in hibernating 13-lined ground squirrels showed the opposite pattern, AMPK activity was high during metabolic arousal but decreased during the metabolic depression (50). AMPK activity is known to respond to AMP:ATP ratios with high AMP:ATP ratios increasing AMPK activity (51). Our paper shows the ratio of AMP:ATP was significantly increased from early to late metabolic depression and significantly decreased during flesh fly metabolic arousal (Fig. 1*D*). However, in hibernating 13-lined ground squirrels the level of ATP during torpor was elevated because low body temperature slows down the degradation of ATP while ATP was heavily used during arousal to support thermogenesis (10, 52, 53). The opposite pattern of AMPK activity in periodic arousal cycles between 13-lined ground squirrels and flesh flies suggests changes in the energetic state may be a result rather the driver of dormancy metabolic cycles.

In conclusion, we found that ROS play key regulatory roles in periodic arousal from metabolic depression during flesh fly pupal diapause by controlling substrates or metabolic intermediates entering the TCA cycle through mediating the activity of PDH and CPT1. With respect to what triggers the timing of periodic arousal, our current working hypothesis is that as metabolism falls from early to late depression ROS levels eventually reach a lower threshold that ultimately precipitates metabolic arousal. However, there are still a number of important questions remaining. Even though we found that ROS may mediate the activity of metabolic enzymes through HIF and AMPK, mitochondrial membranes, proteins, and DNA are also particularly sensitive to oxidative damage. ROS can directly modify mitochondrial proteins, leading to their inactivation or alter their function (54). Thus, future work should focus on identifying the proximal targets of ROS as well as providing an understanding of how upstream signals impinge on mitochondrial ROS production. In addition, previous studies in flesh fly diapause linked periodic arousal cycles with juvenile hormone (JH) (55), but the mechanisms by which JH may affect periodic arousal cycles are still unknown. Could JH be regulated by ROS as well, or is JH signaling somehow regulating ROS and downstream metabolic control? JH has been associated with energy metabolism in many other insects through interactions with the fat body and direct effects on mitochondria (56–58). JH is also an important messenger between the brain and other tissues that play critical roles in regulating insect development and reproduction. Ultimately, understanding the extent to which JH regulates energy metabolism could provide insights into mechanistic crosstalk between the brain and other tissues, such as fat body, in regulating insect diapause as well as other biological processes in insects more broadly. That diapausing flesh flies employ a shift between aerobic lipid metabolism and gluconeogenesis

during periodic arousal to anaerobic glycolysis during metabolic depression that is similar to the metabolic shifts in substrate use that have been previously observed in mammalian hibernator period arousal cycles suggests deeper conservation of, at least, some aspects of metabolic depression-periodic arousal cycles between insects and mammals (5, 7, 36, 37). However, more work is needed to test the extent to which regulatory mechanisms for these characteristic shifts in substrate use are shared between diapausing insects and hibernating mammals.

Materials and Methods

We characterized flesh fly periodic arousal cycles by measuring CO_2 production from individual diapausing *S. crassipalpis* pupa hourly (Fig. 1*A*). We sampled at five specific time points based on the time of the initial uptick in CO_2 production that signaled the start of a metabolic arousal bout: arousal from AD was sampled ~6 h after the initial uptick in CO_2 production, the perk of the IBA was sampled ~15 h after the initial uptick in CO_2 production; EN was sampled ~30 h after the initial uptick in CO_2 production while metabolic rates were declining from their peak but remained well above depression levels; ED was sampled ~48 h after the initial CO_2 uptick in production, a time after CO_2 levels came back down to the low levels characteristic of metabolic depression; and LD was sampled 5 to 6 d after the pupa had been stably in metabolic depression. We used these five clearly identifiable points from metabolic depression through periodic arousal to characterize changes in the metabolome as well to perform targeted studies on specific metabolites, transcripts, or enzymes. To test our hypotheses about the relative roles of anaerobic glycolysis and aerobic metabolism in metabolic depression and periodic arousal, we used ^{13}C-labeled isotopes to track the flux of specific metabolites using NMR-based isotopomer analysis. Pharmacological manipulations were performed at specifically defined times during metabolic depression and periodic arousal to test hypotheses about the regulation of diapause metabolic cycling in pupae. Detailed methods for each of these approaches are available in the *SI Appendix, SI Methods*.

Data Availability. All study data are included in the article and supporting information.

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