Insect Biochemistry and Molecular Biology 127 (2020) 103493

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

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

The oncometabolite L-2-hydroxyglutarate is a common product of dipteran larval development

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ARTICLE INFO

Keywords:
L-2-hydroxyglutarate
Oncometabolite
Hypoxia
Drosophila

ABSTRACT

The oncometabolite L-2-hydroxyglutarate (L-2HG) is considered an abnormal product of central carbon metabolism that is capable of disrupting chromatin architecture, mitochondrial metabolism, and cellular differentiation. Under most circumstances, mammalian tissues readily dispose of this compound, as aberrant L-2HG accumulation induces neurometabolic disorders and promotes renal cell carcinomas. Intriguingly, *Drosophila melanogaster* larvae were recently found to accumulate high L-2HG levels under normal growth conditions, raising the possibility that L-2HG plays a unique role in insect metabolism. Here we explore this hypothesis by analyzing L-2HG levels in 18 insect species. While L-2HG was present at low-to-moderate levels in most of these species (<100 pmol/mg; comparable to mouse liver), dipteran larvae exhibited a tendency to accumulate high L-2HG concentrations (>100 pmol/mg), with the mosquito *Aedes aegypti*, the blow fly *Phormia regina*, and three representative *Drosophila* species harboring concentrations that exceed 1 nmol/mg – levels comparable to those measured in mutant mice that are unable to degrade L-2HG. Overall, our findings suggest that one of the largest groups of animals on earth commonly generate high concentrations of an oncometabolite during juvenile growth, hint at a role for L-2HG in the evolution of dipteran development, and raise the possibility that L-2HG metabolism could be targeted to restrict the growth of key disease vectors and agricultural pests.

1. Introduction

The field of cancer metabolism has become increasingly focused on how small molecule metabolites regulate cell proliferation and promote cancer progression (Martinez-Reyes and Chandel, 2020). In this regard, a number of compounds have emerged as oncometabolites - pro-growth molecules that enhance tumor growth by interfering with gene expression, epigenetic modifications, mitochondrial physiology, and signal transduction cascades (Mullen and DeBerardinis, 2012; Yang et al., 2013; Ye et al., 2018). These compounds, however, are not simply cancer-causing molecules, but also serve essential roles in normal metabolism and physiology. For example, the first molecules implicated as oncometabolites were the tricarboxylic acid intermediates fumarate and succinate (Raimundo et al., 2011), both of which play ancient and essential roles in energy production. Similarly, the oncometabolite D-2-hydroxyglutarate (D-2HG), which is perhaps best known for promoting glioblastoma (Ye et al., 2018), also serves normal metabolic roles in bacteria, yeast, and even humans (Becker-Kettern et al., 2016; Struys et al., 2005; Zhang et al., 2017). Such examples illustrate how oncometabolites act in diverse and important metabolic mechanism across all kingdoms of life and suggest that studying normal oncometabolite function can advance our understanding of how these molecules induce human disease.

Among known oncometabolites, the compound L-2HG stands out as unusual because eukaryotes lack enzymes dedicated to L-2HG production. Mammalian cells synthesize L-2HG as a result of promiscuous

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https://doi.org/10.1016/j.ibmb.2020.103493
Received 13 July 2020; Received in revised form 6 October 2020; Accepted 19 October 2020
Available online 3 November 2020

Lactate Dehydrogenase (Ldh) and Malate Dehydrogenase (Mdh) activity and degrade this compound via the enzyme L-2HG dehydrogenase (L2HGDH) (Ye et al., 2018). Most mammalian tissues, with the exception of the mouse testis, maintain low L-2HG concentrations and inappropriate L-2HG accumulation can induce dramatic changes in epigenetic modifications, central carbon metabolism, and growth factor signaling (Ma et al., 2017; Teng et al., 2016; Ye et al., 2018). As a result, nearly all *in vivo* studies of L-2HG focus on the detrimental effects of this compound in neurological disorders and renal cell carcinoma, thus the question remains as to whether L-2HG, like the other oncometabolites, serves a normal physiological role (Ma et al., 2017; Shim et al., 2014; Ye et al., 2018). In this regard, several studies have observed that cultured mammalian cells accumulate excess L-2HG in response to oxidative stress, with hypoxia, acidic pH, and elevated NADH levels enhancing L-2HG synthesis and accumulation (Intlekofer et al., 2015, 2017; Mullen et al., 2014; Nadtochiy et al., 2016; Oldham et al., 2015; Reinecke et al., 2012; Teng et al., 2016). These cell culture studies suggest that L-2HG metabolism could act as a metabolic signaling molecule that helps cellular physiology adapt to redox stress; however, little is known about L-2HG function *in vivo*.

One of the only examples of a healthy animal accumulating high L-2HG levels in a regulated and predictable manner is during larval development of the fruit fly *Drosophila melanogaster* (Li et al., 2017). While the exact reason for why *Drosophila melanogaster* larvae accumulate L-2HG remains to be elucidated, this observation raises the possibility that L-2HG serves a unique role in insects and suggests that comparative studies of insect metabolism could illuminate the endogenous function of this oncometabolite. Towards this goal, we used gas chromatography-mass spectrometry to quantify L- and D-2HG levels in 18 species of insects. Our analysis revealed that representative species from order Diptera seem particularly adept at accumulating very high L-2HG concentrations during larval development. Moreover, we demonstrate that while dipteran larvae can generate excess L-2HG in response to hypoxia, larvae generate high concentrations of this compound regardless of oxygen concentration. This finding indicates that one of the largest and most diverse animal orders on the planet commonly produces high concentrations of an oncometabolite in a developmentally-regulated manner and suggests that further studies of dipteran L-2HG metabolism could help elucidate the endogenous functions of this compound. In addition, our study suggests the L-2HG metabolism serves a unique role in the dipteran development, thus raising the possibility that production of this compounds could be used to control populations of common disease vectors and agricultural pests.

2. Methods

2.1. Insect husbandry

Aedes aegypti: RexD (Puerto Rico) derived Higgs White Eye (HWE) strain were maintained in an insect incubator (Percival Model I-36VL, Perry, IA, USA) at 28 °C and 75% relative humidity with a 12 h:12 h light:dark cycle. Larvae were reared in freshwater (dH2O) at a density of 200 larvae/L of water. Water was changed every other day. Each larval cup was fed a 4:1 mixture of finely ground fish pellets to baker's yeast a day. Adult mosquitos were fed 10 mL of 10% sucrose daily via cotton balls. Samples contained 10–20 mg of third instar larvae.

Apis mellifera: Bee samples were collected from a colony established and maintained by Dr. Irene Newton's lab at Indiana University-Bloomington. Samples were collected in 2018 during the months of June, July, and August. For both larvae and adults, each sample contained an individual animal. Adult samples consisted of workers.

Drosophila species: All *Drosophila* species were maintained on standard Bloomington *Drosophila* Stock Center (BDSC) media at 25 °C. The *Drosophila melanogaster* strain w^{1118} was used for all experiments. *Drosophila hydei* and *Drosophila busckii* cultures, which were kindly provided by Dr. Irene Newton's lab (Indiana University-Bloomington, USA), are derived from wild isolates collected in Brown County, Indiana.

For all species, males flies were collected immediately following eclosion and aged for 3 days on BDSC media prior to collection or treatment. For larval analyses, embryos were collected on molasses agar with covered with yeast paste as previously described (Li and Tennessen, 2018). Larvae were allowed to develop for 60 h (*D. melanogaster* and *D. busckii*) or 84 h (*D. hydei*) prior to collection. For hypoxic and hyperoxic treatments, larvae were placed in 35 mm plates with Whatman filter paper at the bottom that was wetted with phosphate buffer saline (PBS; pH 7.4) and contained approximately 500 mg of yeast paste in the center. Regardless of treatment or age, larval samples were collected by placing ~20 mg of larvae in a 1.5 ml microfuge tube on ice. Larvae were washed at least three times using ice-cold PBS to remove all yeast and debris from the sample. Following the final wash, all PBS was removed from the sample and the tube was flash frozen in liquid nitrogen.

Galleria mellonella: Larvae were purchased from Carolina Biological Supply (Item # 143928; Burlington, North Carolina) and raised on the provided media according to the distributors care sheet. All samples contained a single individual.

Libellulida species *and Enallagma* species.: Nymphs were purchased from Carolina Biological Supply (*Libellulida* species, 143526; *Enallagma* species, 143520) and maintained following the supplier's recommendation. For collections, individual nymphs were removed from the culture, briefly patted dry, placed in a pre-tared 2 mL tube containing 1.4 mm ceramic beads, massed, and flash frozen in liquid nitrogen.

Phormia regina: All flies were collected from a laboratory colony (>5 generations) that was generated from wild-caught *P. regina* (collected from Military Park, Indianapolis, IN, USA) and maintained in a 30 × 30 × 30 cm cage (Bioquip, Rancho Dominguez, CA) within the IUPUI "fly room." The colony was reared at ~25 °C ambient temperature, 60% ambient humidity and 24 h light and were provided sugar and water ad libitum. Chicken liver was provided to the colony ~1 week post-eclosion for ovary maturation. 2–4 days following ovary maturation, chicken liver (25 g) was provided as the egg oviposition substrate for a period of 4–6 h. Following oviposition, the cup containing the chicken liver and eggs were placed in a one-quart glass jar half-filled with fine pine shavings (Lanjay Inc., Montreal, QC). Larvae were allowed to develop under ambient conditions. For sample collection, an individual larva was placed in a 1.5 ml microfuge tube on ice. Samples were washed as described for the *Drosophila* species (see above). For adult analysis, adult flies were collected 3–5 days post-emergence from the lab colony.

Oncopeltus fasciatus: Milkweed bugs were obtained from Carolina Biological Supply (Item # 143800) and maintained on organic sunflower seeds.

Hermetia illucens: Larval cultures were shipped from Dr. Jeffery Tomberlin's lab (Texas A&M University; College Station, Texas, United States) and collected upon arrival. Each sample contained an individual larva. Cultures were maintained at ambient temperature and adults were collected upon emergence.

Musca domestica: Larvae were purchased from Carolina Biological Supply (Item #144410) and raised on Instant House Fly Medium (Item # 144424) at 25 °C. Individual larva were collected in a 1.5 ml microfuge tube and immediately placed on ice. Samples were washed as described for the *Drosophila* species (see above). All samples contained a single individual.

Manduca sexta: Larvae were obtained from Carolina Biological Supply (#143886) and maintained at room temperature on hornworm diet (Carolina Biological Supply; #143910). Animals were collected throughout the L3 stage. Each sample consisted of an individual animal. For larvae with a mass greater than 50 mg, individual animals were homogenized in 800 μl of methanol extraction buffer as described below (see *Sample collection and L-2HG Quantification*) and the homogenate was immediately diluted 1:2 or 1:4 with additional extraction buffer, depending on the mass of the larvae.

Onthophagus taurus: Adults were collected from cow dung pads at

Busselton, Western Australia (−33° 39′ 8" S, 115° 20′ 43" E) in January 2016 and shipped to Bloomington, IN, for rearing. All beetles were maintained as a single colony in the laboratory at 24 °C on a 16 L: 8 D cycle, and fed homogenized cow dung ad libitum following an established protocol (Moczek et al., 2002). In order to obtain offspring, beetles were allowed to breed in plastic containers (25 cm tall x 20 cm in diameter) and filled ~75% with a moist sand:soil mixture. For each round of breeding, six female and three male beetles were added to one breeding container and provisioned with ~0.5 L of homogenized cow dung. Beetles were allowed to breed for one week, at which point they were recaptured and brood balls containing offspring were collected and placed in plastic containers. All samples contained a single individual.

Tribolium castaneum: Cultures were maintained on King Arthur whole wheat flour supplemented with active dry yeast at 28 °C and 55–65% humidity. For larval analysis, 3rd and 4th instar larvae were collected. For adult hypoxia experiment, recently emerged adults were collected and adults were kept on new flour for the duration of the treatment. The strain *Vermillion* ^{white} was used for all the studies in this report.

Gryllodes sigillatus: Nymphs were obtained from Carolina Biology (item #143558). Upon arrival, cultures were maintained at ambient temperature and fed a diet of dried dog food, lettuce, and apple slices. All samples contained a single individual.

Tenebrio molitor: Larvae (~100–200 mg; Item #144274 and ~20 mg; Item #144287) and adults (Item #144270) were purchased from Carolina Biological Supply and fed whole wheat flour and apple slices, as per the distributors care sheet. All samples contained an individual animal. For larvae of a mass >100 mg, individual animals were homogenized in 800 μl of methanol extraction buffer as described below (see *Sample collection and L-2HG Quantification*) and the homogenate was immediately diluted 1:2 or 1:4 with additional extraction buffer, depending on the mass of the larvae. All samples contained a single individual.

Vanessa cardui: Larvae were obtained from Carolina Biology (Item # 144076) and maintained on the culture media provided by the distributor at ambient temperature. All samples contained a single individual.

3. Method for mouse liver harvest

Liver tissue was harvested from male and female C57BL/6 J control mice and a previously described *L2hgdh* knockout (KO) mutant (Brinkley et al., 2020). All mice were aged 2–4 months and given normal chow (Labdiet) ad libitum. For mouse starvation, chow was removed in the evening, 14 h prior to tissue harvest. At time of harvest, mice were anesthetized using isoflourane (Vetone) and blood was collected. Mice were then euthanized. Tissues were briefly washed in chilled DPBS (Corning), dried using kimwipes (Kimberly-Clark) and snap frozen using liquid nitrogen. All animal studies were approved by institutional animal care and use committee (IACUC) as previously described (Brinkley et al., 2020).

3.1. Sample collection and L-2HG quantification

For pooled samples that contained multiple individuals, samples were collected in 1.5 ml microfuge tubes and flash frozen in liquid nitrogen. Prior to homogenization, tubes were removed from liquid nitrogen, the cap end swiftly pounded against the desktop to dislodge the sample, and the pellet was transferred into a pre-tared 2 ml screwcap tube containing 1.4 mm ceramic beads. The sample mass was immediately measured with an analytical balance, the tube was flash frozen in liquid nitrogen, and samples were stored at −80 °C. For samples that contained an individual insect, the animal was collected and immediately transferred into a pre-tared 2 ml screwcap tube containing 1.4 mm ceramic beads. The sample mass was measured using an analytical balance, the tube was flash frozen in liquid nitrogen, and samples were stored at −80 °C.

Homogenization and metabolite extractions were conducted as previously described (Li and Tennessen, 2019). Briefly, samples were removed from the −80 °C freezer and placed into a benchtop enzyme cooler pre-chilled to −20 °C. 800 μl of pre-chilled methanol extraction buffer (−20 °C; 90% methanol diluted with HPLC-grade water + 8 μg (RS)-2-Hydroxy-1,5-pentanedioate-2,3,3-d3) was added to individual sample tubes. Sample were homogenized in an Omni Beadruptor 24. Homogenized samples were returned to a −20 °C benchtop enzyme cooler and incubated in a −20 °C freezer for 1 h. Samples were then centrifuged at ~20,000×*g* in a refrigerated centrifuge for 5 min to pellet insoluble debris. 600 μl of the supernatant was removed and transferred to a 1.5 ml microfuge tube and dried overnight in a vacuum centrifuge. Samples were then derivatized using a two-step method involving R-2-butanol and acetic anhydride.

Derivatized samples were injected (1.5 μL, 1:5 split ratio) into an Agilent GC6890-5973i instrument using a Gerstel MPS autosampler. Separation of compounds was achieved by gas chromatography (GC) with a Phenomex ZB5-5 MSi column. The GC was programmed to increase temperature as follows: (1) Inlet temperature was set to 250 °C and initial temperature was set to 95 °C with a 1-min hold. (2) Temperature was increased at a rate of 40 °C per minute until it reached 110 °C with a 2-min hold. (3) Temperature was increased a rate of 5 °C per minute to 200 °C. (4) Temperature was increased at a rate of 5 °C per minute to 330 °C followed by a 3-min hold. Selected ion monitoring (SIM mode) was programmed to record m/z ion values 173 for endogenous D-/L-2HG, and 176 for the deuterated D-/L-2HG internal heavy standard. Concentration in each sample was calculated by comparison to the internal standard and normalized to the sample mass.

3.2. Hypoxia and hyperoxia treatments

For all manipulations of atmospheric oxygen concentration, samples were placed in an air sealed plexiglass chamber equipped with a pressure release valve that was located within a 25 °C temperature-controlled room. A Sable systems ROXY-4 gas regulator (Las Vegas, Nevada) was used to control oxygen concentration within the chamber. Desired oxygen concentrations were maintained by the ROXY-4 system by injecting either N_2 or O_2 gas into the chamber.

3.3. Statistical analysis

All data analyses were conducted using JMP v. 14. Prior to analysis, variables were evaluated for normality and homoscedasticity using Shapiro-Wilk and Levene's tests, respectively. Where these assumptions were met, we used two-tailed pooled t-tests or ANOVA followed by Tukey-Kramer HSD test to determine differences among treatment groups. Where these assumptions were not met, we conducted non-parametric Wilcoxon rank sum or Kruskal-Wallis tests. To compare L-2HG titers across larval insect species with those of mouse samples (see Figs. 1 and 2), we conducted Dunnett's post hoc test with a Bonferroni adjustment for multiple comparisons.

4. Results

4.1. Several dipteran species accumulate high L-2HG levels during larval development

Recent findings that *D. melanogaster* larvae accumulate high L-2HG levels motivated us to determine if this molecule is abundant in other insects. Towards this goal, we used a chiral derivatization method coupled with gas chromatography-mass spectrometry (GC-MS) to quantify L- and D-2HG levels in a diversity of insect species, C57BL/6 J mouse liver, which possesses L-2HG levels that are comparable to other mammalian tissues and served as a baseline control in our study, as well as liver tissue from *L2hgdh*$^{-/-}$ mutant mice that accumulates abnormally high L-2HG levels (Brinkley et al., 2020; Ma et al., 2017; Rzem et al., 2015). We first set out to determine if adult insects of selected species

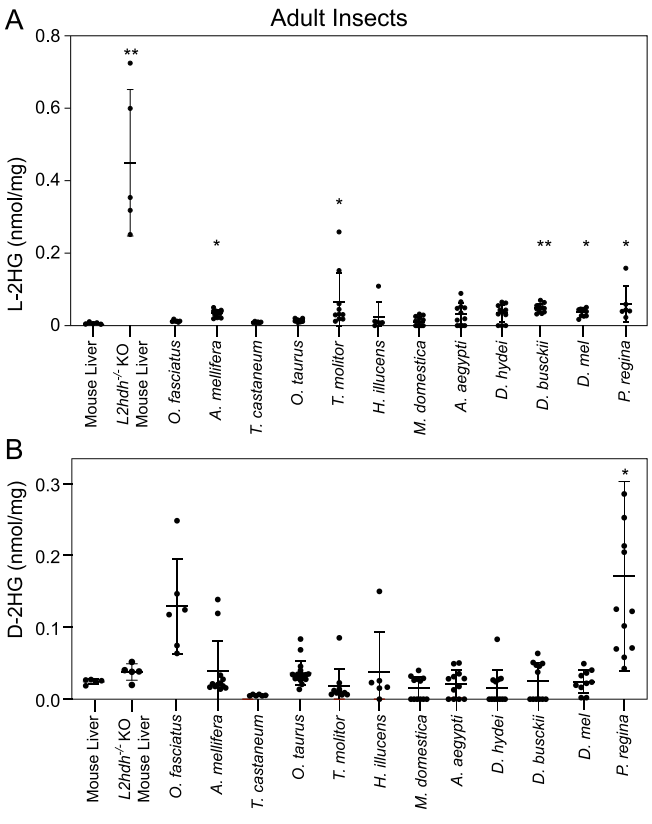

Fig. 1. Adult insects have low levels of L- and D-2HG. L-2HG (A) and D-2HG (B) levels were measured in adult insects as well as mouse liver samples harvested from wild-type controls and *L2ghdh* KO mutants. Asterisks indicate that L- or D-2HG levels are significantly higher than those measured in mouse liver. Data are presented in scatter plots with mean ± SD and *P-values* represent significance relative to wild-type mouse liver samples. $*P < 0.05$. $**P < 0.01$. $***P < 0.001$. *P*-values calculated using a Dunnett's post hoc test with a Bonferroni adjustment for multiple comparisons.

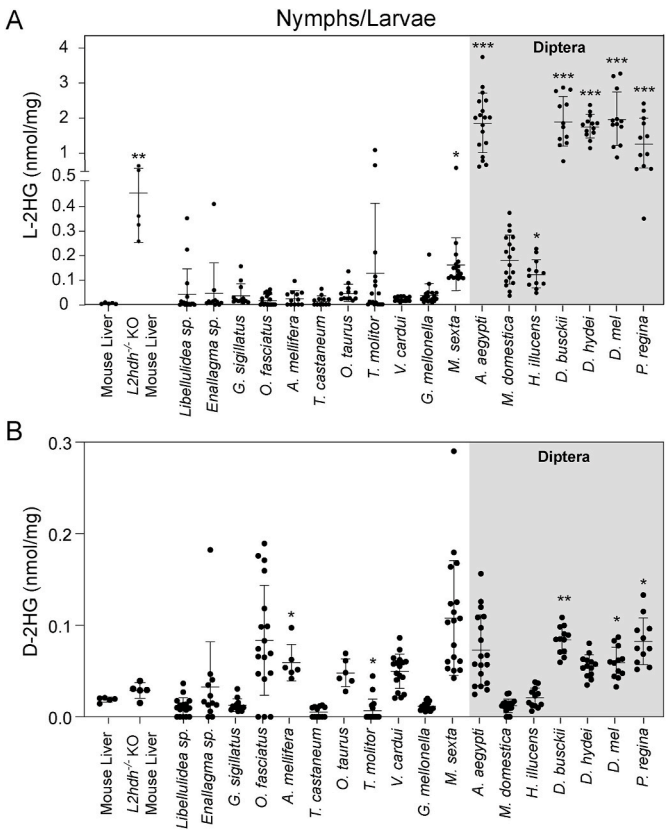

Fig. 2. Dipteran larvae accumulate high L-2HG levels. L-2HG (A) and D-2HG (B) levels were measured in juvenile insects as well as mouse liver samples harvested from wild-type controls and *L2ghdh* KO mutants. Asterisks indicate that L- or D-2HG levels are significantly higher than those measured in mouse liver. Data are presented in scatter plots with mean ± SD. $*P < 0.05$. $***P < 0.001$. *P*-values calculated using a Dunnett's post hoc test with a Bonferroni adjustment for multiple comparisons.

harbored high concentrations of either 2HG enantiomer. Although several species accumulated L- or D-2HG levels that were significantly elevated compared with wild-type mouse liver (Fig. 1A and B), the concentration in these animals remained an order of magnitude less than the L-2HG concentration found in *D. melanogaster* larvae (~2 nmol/mg).

Considering that *D. melanogaster* only generates high L-2HG levels during larval development, we examined the possibility that insect metabolism is predisposed to generating these molecules during juvenile stages (Fig. 2A and B). Similar to adult insects, both L-2HG and D-2HG were present at low levels in nymphal stages of four hemimetabolous species (i.e., insects that do not undergo metamorphosis; Fig. 2A and B) and larval stages of holometabolous species including the European honey bee (*Apis mellifera*), representative species from the insect order Coleoptera (beetles), and two lepidopteran (moths and butterflies) species (*Vanessa cardui* and *Galleria mellonella*). Moreover, timecourse analyses of L-2HG concentrations within *Tenebrio molitor*, *Vanessa cardui* and *Galleria mellonella* suggest that L-2HG levels remain relatively low throughout the larval growth phase of these insects (Supplemental Figs. 1A–C). The only exception among members of these insect orders was the moth *Manduca sexta,* which accumulated L-2HG levels that were slightly, but significantly, higher than mouse liver (Fig. 2A).

In contrast to the insects described above, a majority of the dipteran species examined harbored notably elevated larval L-2HG levels, with the mosquito *Aedes aegypti*, the blow fly *Phormia regina*, and multiple members of genus *Drosophila* (*D. melanogaster*, *D. busckii*, and *D. hydei*) accumulating L-2HG levels that exceeded 1 nmol/mg. These L-2HG concentrations are comparable to those measured in both humans and mice lacking the enzyme L-2-hydroxyglutarate dehydrogenase, which is responsible for degrading L-2HG (Fig. 2A and Brinkley et al., 2020; Ma et al., 2017; Rzem et al., 2015; Rzem et al., 2004). In fact, of the seven dipteran species examined in this study, only the house fly, *Musca domestica*, maintained larval L-2HG levels that were not significantly higher than those observed in normal mouse liver tissue (Fig. 2A); however, we note that even these samples contained an average of >100 pmol/mg.

Our findings suggest that dipteran larval metabolism is predisposed towards generating L-2HG. To expand upon these observations, we measured L-2HG concentrations at multiple timepoints during *Aedes aegypti* and *Phormia regina* larval development. Consistent with previously studies of *Drosophila melanogaster* (Li et al., 2017), L-2HG levels in *Aedes aegypti* larvae remained at concentrations >0.5 nmol/mg at all stages surveyed (Fig. 3A). Similarly, L-2HG levels in three of four timepoints sampled during *Phormia regina* development exceeded 1 nmol/mg (Fig. 3B). However, *Phormia regina* samples from a single timepoint, 96 h after egg-laying, contained L-2HG concentrations ranging from ~0.2–0.8 nmol/mg – a level significantly below those concentrations measured in other larval timepoints (Fig. 3B). Such results raise the possibility that *Phormia regina* L-2HG levels fluctuate in a developmentally-regulated manner and should be the subject of future investigations. Regardless, even the lowest L-2HG level measured in 96 h *Phormia regina* larval samples (0.22 nmol/mg) exceeds the maximum L-2HG concentration measured in nearly all non-dipteran juvenile samples assayed in this study as well as all previously analyzed mammalian tissue samples (Fig. 2A and Brinkley et al., 2020; Ma et al., 2017).

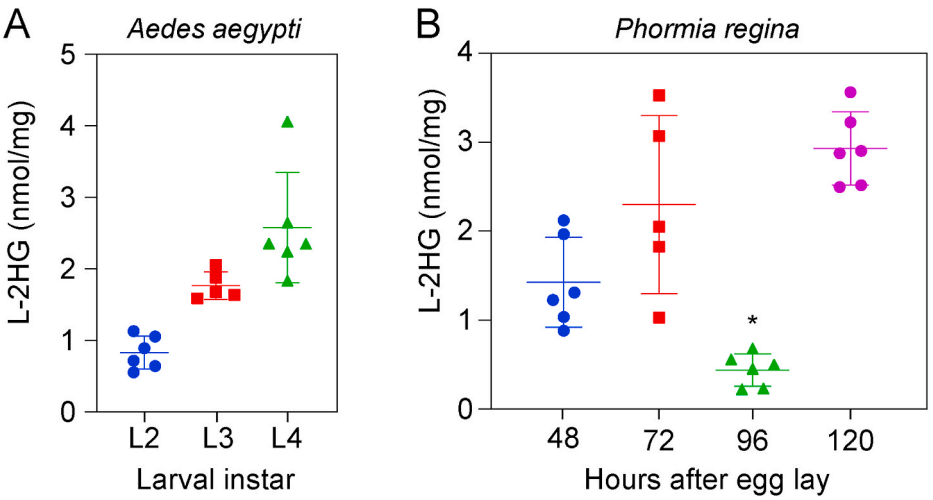

Fig. 3. L-2HG are elevated at multiple timepoints during *Aedes aegypti* and *Phormia regina* larval development. L-2HG levels were measured in (A) *Aedes aegypti* and (B) *Phormia regina* at the indicated timepoints during larval development. For *Phormia regina* (B), samples collected at the 48 h timepoint contained L2 larvae while those collected at 72 h, 96 h, and 120 h timepoints contained L3 larvae. *$P < 0.05$ for comparisons with all other individual timepoints. *P*-values calculated using a Dunnett's post hoc test with a Bonferroni adjustment for multiple comparisons.

When compared with L-2HG, D-2HG was present at relatively low levels in all dipteran samples (Fig. 2B and Supplemental Figs. 2C and D). However, it is noteworthy that three out of five species that have significantly higher D-2HG levels were members of the order Diptera (Fig. 2B). Overall, our observations suggest that dipterans exhibit a unique tendency to accumulate L-2HG during larval development.

4.2. Dipterans generate excess L-2HG in response to hypoxia

Dipteran larvae develop in moist environments. Since human cells accumulate L-2HG in response to hypoxia and decreased electron transport chain activity (Intlekofer et al., 2015; Mullen et al., 2014; Oldham et al., 2015), we examined the possibility that the dipteran species analyzed herein accumulate high L-2HG levels as the result of growing within a potentially hypoxic environment (e.g., yeast paste, water, and rotting chicken liver). To test this hypothesis, we first determined if hypoxia is capable of inducing L-2HG accumulation in adult males of these species, which normally harbor low L-2HG levels (Fig. 1A). Consistent with studies of mammalian cells, adult male *Aedes aegypti*, *D. melanogaster, D. hydeii*, and *Phormia regina* accumulated excess L-2HG when exposed to 1% O_2 (~1 kPa O_2) for 6 h (Fig. 4A). Intriguingly, we also observed a slight but significant increase in D-2HG production (Fig. 4B), suggesting that hypoxia-dependent production of these two molecules is linked by a yet to be determined mechanism.

We next examined if dipteran larvae, similar to adults, produce L-2HG when exposed to hypoxia. Indeed, third instar larvae of *Aedes aegypti*, *D. melanogaster, D. hydeii*, and *Phormia regina* generate significantly higher L-2HG levels than normoxic controls when exposed to 1% O_2 for 6 h (Fig. 4C). In fact, several individual blowfly larvae harbored L-2HG concentrations that exceeded 6 nmol/mg, which are among the highest levels ever recorded in animal tissues, even exceeding the L-2HG levels observed in *L2hgdh* mutant mouse brains and testis (Fig. 4C, Brinkley et al., 2020; Ma et al., 2017; Rzem et al., 2015). Moreover, we also observed a slight but significant increase in D-2HG in hypoxia treated *Aedes aegypti* and *Phormia regina* (Fig. 4D), reinforcing the possibility that D-2HG levels are sensitive to oxygen concentration. Overall, our observations demonstrate that, similar to mammalian cells, dipterans seem capable of accumulating relatively large quantities of L-2HG in response to hypoxia.

4.3. Influence of mild hypoxia and hyperoxia on larval L-2HG accumulation

Our findings that a low oxygen environment results in elevated L-2HG levels raises the question as to whether the high L-2HG concentrations observed in dipteran larvae are simply the result of transient exposure to hypoxia. We tested this possibility by measuring L-2HG levels in larvae following 6 h exposures to mild hypoxia (5%, an O_2 level that delays, but does not arrest larval development, Zhou et al., 2008), normoxia, or hyperoxia (30% O_2, 50% O_2). If the high L-2HG levels observed in these larvae simply result from hypoxic stress, we predict that L-2HG levels would inversely correlate with O_2 concentration. Instead, we observed that larval L-2HG concentrations in *Drosophila hydeii*, *Drosophila melanogaster*, and *Phormia regina* remained unchanged following exposure to mild hypoxia or hyperoxia (Fig. 5A). Moreover, a longer 24 h exposure to the same oxygen concentrations failed to induce changes in L-2HG concentration relative to normoxic controls (Supplemental Fig. 3A), suggesting that oxygen availability is not the primary driving force behind L-2HG accumulation in these species. Moreover, our finding that 1% O_2, but not 5% O_2, induces excess L-2HG accumulation supports previous observations in *Drosophila melanogaster* that larvae mount different physiological responses depending on the severity of hypoxic conditions (Lavista-Llanos et al., 2002).

In contrast to *Drosophila hydeii*, *Drosophila melanogaster*, and *Phormia regina*, L-2HG concentrations in *Aedes aegypti* were significantly lower in the 6 h hyperoxia treated samples as compared with controls (Fig. 5A), suggesting that L-2HG accumulation in these animals are due, in part, to limited oxygen availability within larval tissues. However, despite this difference, the amount present within hyperoxic larvae remain at high levels relative to non-dipteran species, indicating that L-2HG accumulation in *Aedes aegypti* is likely complex and involves both developmental and physiological causes. This hypothesis would be consistent with recent observations that the *Aedes aegypti* microbiome generates a hypoxic state within the larval intestine that influences growth and molting (Coon et al., 2017). Intriguingly, we observed no difference in L-2HG concentration among samples collected following 24 h exposure to either hypoxia or hyperoxia (Supplemental Fig. 3A), suggesting that mosquito larval metabolism adapts to the hyperoxic state over extended periods of time. Future studies should examine the significance of this difference as well as the potential role of the gut microbiome in regulating redox metabolism during larval development.

During the course of these experiments, we also observed that concentrations of D-2HG remained relatively unchanged at every O_2 condition tested (Fig. 5B and Supplemental Fig. 3B). Of all the oxygen concentrations measured, the only *Aedes aegypti* (hypoxia/normoxia vs hyperoxia) and *Phormia regina* (21% O_2 vs 50% O_2) displayed any O_2 dependent changes in D-2HG levels, and these changes were of modest amounts.

4.4. Manduca sexta L-2HG accumulation is sensitive to mild hypoxia

Our survey of 2HG metabolism reveals that *Manduca sexta* larvae

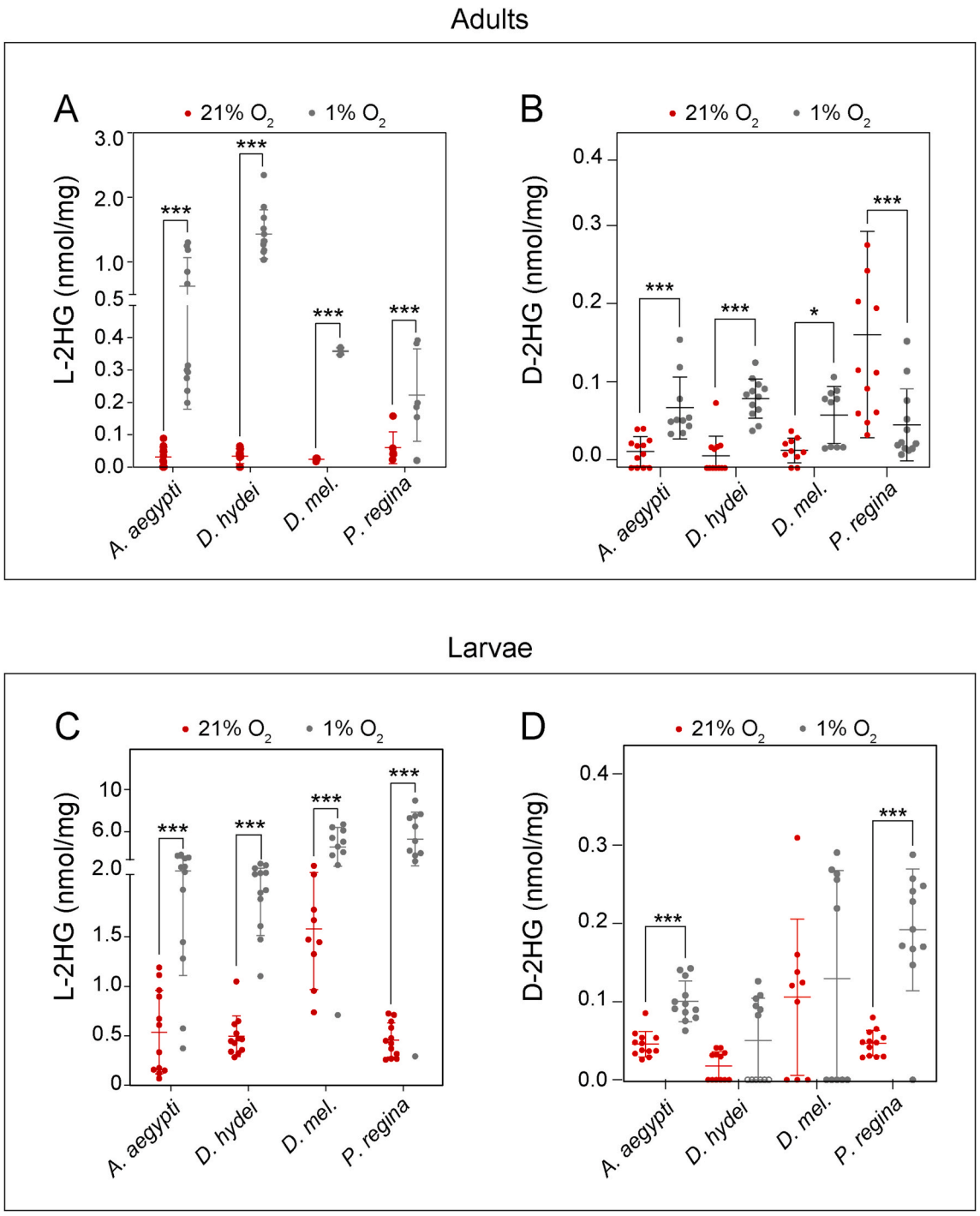

Fig. 4. Dipteran insects accumulate L-2HG in response to hypoxia. L-2HG and D-2HG levels were measured in select dipteran adults (A, B) or larvae (C, D) following a 6 h incubation in the presence of 1% O_2. Data are presented in scatter plots with mean ± SD. *$P < 0.05$. ***$P < 0.001$. *P*-values calculated using a Wilcoxon/Kruskal-Wallis tests except for *D. mel* in (C) and *A. aegypti* in (B) and (D), which were calculated using a pooled *t*-test.

also accumulate significant amounts of L-2HG when compared to mouse liver (Fig. 2A). Considering that *Manduca sexta* larvae become hypoxic during the instar, when growth outpace the tracheal systems ability to exchange oxygen (Callier and Nijhout 2012), L-2HG production in these animals could also be related to hypoxic stress. To determine if *Manduca sexta* larvae accumulate L-2HG due to growth-induced hypoxia, we first determined if these animals are capable of generating either 2HG enantiomer in response to low O_2 levels. Indeed, L3 larvae exposed to 5% O_2 for 24 h exhibited a dramatic 7-fold increase in L-2HG (Fig. 6A), as well as a modest, but statistically significant increase in D-2HG (Fig. 6B), thus demonstrating that *Manduca sexta* larvae generate these molecules in response to hypoxia. Based on this finding, we examined the possibility that *Manduca sexta* larvae accumulate L-2HG due to hypoxic stress resulting from increased body mass. Our analysis, however, revealed no correlation between L3 body mass and the concentrations of either 2HG enantiomer (Fig. 7A and B), suggesting that the L-2HG produced in these animals is likely not the result of changes in body size.

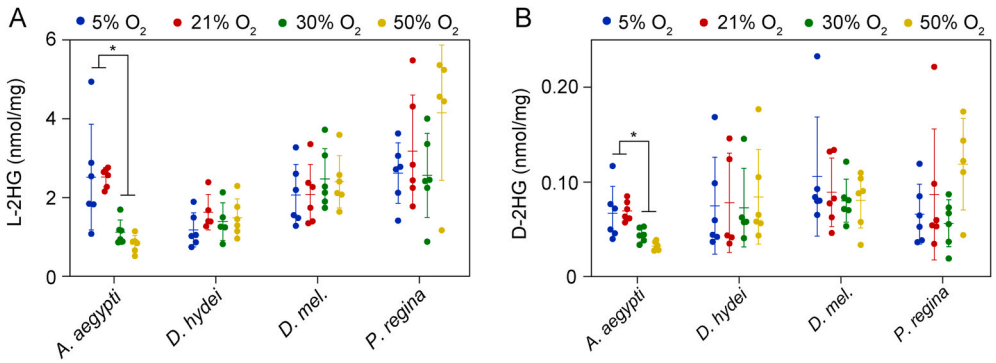

Fig. 5. The effects of mild hypoxia and hyperoxia on larval L-2HG accumulation. L-2HG (A) and D-2HG (B) levels were measured in select dipteran larvae following a 6 h incubation in the presence of 5%, 21%, 30% or 50% O_2. Data are presented in scatter plots with mean ± SD. *$P < 0.05$. **$P < 0.01$. *P*-values calculated using a Wilcoxon/Kruskal-Wallis tests followed by Tukey-Kramer HSD test in (A) and Dunn's test in (B).

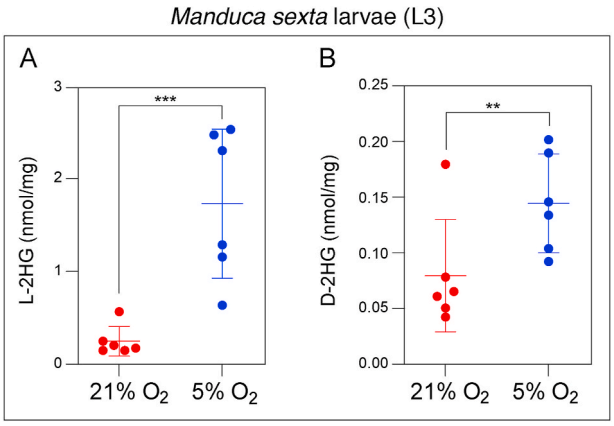

Fig. 6. *Manduca sexta* larvae accumulate L-2HG in response to mild hypoxia. Concentrations of L-2HG (A) and D-2HG (B) were measured in *M. sexta* larvae following a 24 h incubation in the presence of 5%, 21% O_2. Data are presented in scatter plots with mean ± SD. **$P < 0.01$. ***$P < 0.001$. *P*-values calculated using a Wilcoxon/Kruskal-Wallis test.

5. Discussion

Our results demonstrate that several dipteran species accumulate high levels of the oncometabolite L-2HG during normal larval development. While the endogenous functions of L-2HG within these insects remains to be elucidated, our observations raise important considerations. Insects are among the most diverse groups of animals on the planet, display complex life histories, and are adaptable to a wide range of environmental conditions. When considered in this context, our survey of L-2HG metabolism is small in both the number of species and life-stages surveyed. Despite this limitation, we uncovered several instances where larvae generated relatively high L-2HG concentrations - a result which implies that this compound is not simply an oncometabolite or a waste product of the TCA cycle, but rather accumulates during normal development of a potentially large number of animal species.

The amount of L-2HG found within dipteran larvae is striking, as similar L-2HG concentrations in mammals are associated with severe neurometabolic defects and renal tumors (Shim et al., 2014; Ye et al., 2018), suggesting that this compound serves a unique role in dipteran physiology when compared to other animals. One explanation for our observations is that developmentally regulated L-2HG accumulation acts as part of a metabolic program that protects dipteran larvae from unpredictable fluctuations in oxygen availability. L-2HG has been repeatedly observed to be produced in animal cells exposed to hypoxic conditions and the production of this molecule is thought to play a role in the cellular hypoxia response (Intlekofer et al., 2015, 2017; Nadtochiy et al., 2016; Oldham et al., 2015). Moreover, dipteran larvae have evolved to be exceptionally tolerant of hypoxia and anoxia, as evident by the ability of *D. melanogaster* larvae to remain motile for over 30 min under anoxic conditions (Callier et al., 2015). Based on these observations, we propose that dipteran larvae accumulate L-2HG in a developmentally-regulated oxygen-independent manner that preadapts larval development to survive transient exposure to mild hypoxic conditions that are common in the larval environment. This hypothesis is also supported by our finding that only other insect to accumulate significant L-2HG levels is *Manduca sexta* (Fig. 2A). During the larval stage of this insect, dramatic increases in body mass induces growth-induced bouts of hypoxia (Callier and Nijhout, 2012). Our studies, however, suggest that L-2HG production within this insect is not simply the result of changes in oxygen availability and raise the possibility that *Manduca sexta*, similar to dipteran larvae, generate L-2HG in preparation for inevitable bouts of hypoxic stress.

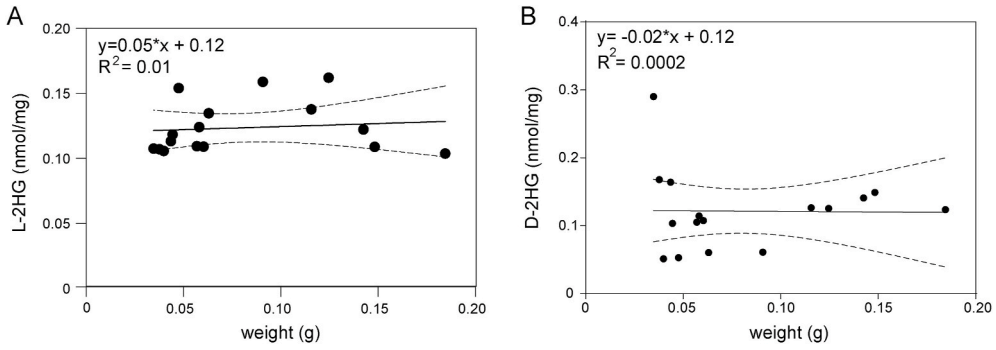

Fig. 7. 2HG concentrations remain constant during *Manduca sexta* L3 development. Concentrations of L-2HG (A) and D-2HG (B) plotted relative to body mass in *M. sexta* third instar larvae. Dashed lines represent the 95% confidence interval.

The outlier during our studies of O_2-dependent L-2HG accumulation was *Aedes aegypti*, wherein larval L-2HG levels significantly decreased following a 6 h bout of hyperoxia. Moreover, while this hyperoxia-induced decrease in L-2HG levels was significant, a very large L-2HG pool remained, suggesting *Aedes aegypti* L-2HG production results from both O_2-dependent and independent mechanisms within larval tissues. These finding are notable because bacteria within the *Aedes aegypti* larval gut induces a hypoxic microenvironment that regulates growth and molting (Coon et al., 2017). Future studies should explore this potential link between microbiome metabolism and L-2HG accumulation and signaling.

Our findings also raise the question as to how the dipteran species analyzed in this study have evolved to tolerate such high L-2HG levels. L-2HG is a potent inhibitor of enzymes that use α -ketoglutarate (α-KG) as a substrate and high concentrations of this molecule interfere with a diversity of α-KG-dependent processes (Chowdhury et al., 2011; Ye et al., 2018), which include mitochondrial metabolism, the removal of methyl groups for DNA and histones, and stabilization of the transcription factor HIF1α. Considering that the L-2HG concentration observed in *Aedes aegypti*, *Phormia regina*, and the three *Drosophila* species used in this study exceeds all previous reported K_i values for α-KG-dependent enzymes (Chowdhury et al., 2011), the cellular physiology of these systems must be uniquely adapted to the presence of this oncometabolite – a hypothesis that is partially supported by a recent study of DNA methylation in insects (Provataris et al., 2018). While the gene encoding the DNA methyltransferase enzyme DMNT1 was found to be present within representative species of nearly all insect orders and is thus assumed to have been present in the ancestor of all insects, Diptera was one of only three orders found to have lost this enzyme secondarily. This finding is consistent with the low levels of methylated DNA present within dipteran genomes and raises questions as to why flies largely abandoned this ancient mechanism of regulating gene expression (Bewick et al., 2016; Provataris et al., 2018; Zemach et al., 2010).

Considering that L-2HG interferes with the ability of cells to modify methylated nucleotides in DNA (Li et al., 2017; Shim et al., 2014; Ye et al., 2018), we speculate that the loss of *Dmnt1* during dipteran evolution and the resulting reduction in DNA methylation may have allowed larvae to accumulate excess L-2HG with minimal disruption of gene expression programs. One intriguing possibility is that this tradeoff would have provided early dipterans with the metabolic capacity to colonize hypoxic environments that were inaccessible to other insects that rely on DNA methylation as a means of regulating gene expression. Consistent with this model, the only other holometabolous order that seems to lack *Dmnt1* homologs are the Strepsiptera (Provataris et al., 2018), whose larvae develop, and female adults live, as endoparasites in diverse Hymenoptera (McMahon et al., 2011), and thus also have to contend with potentially low oxygen environments.

Our findings also raise questions as to how dipterans control L-2HG production in a developmentally-regulated manner and why is accumulation of this compound restricted to the larval stage? In regard to both mechanisms, work in *Drosophila melanogaster* indicates that the larval L-2HG pool is largely the result of two metabolic mechanisms: (1) LDH converts α-ketoglutarate to L-2HG in a NADH-dependent manner (Li et al., 2017). (2) Lactate inhibits the ability of L2HGDH to degrade L-2HG (Li et al., 2017, 2018). Since LDH is expressed at high levels during the larval stages and is down-regulated at the onset of metamorphosis, larval L-2HG accumulation within *Drosophila melanogaster* is likely due to developmental expression pattern of this enzyme. Future studies should determine if the elevated L-2HG levels observed in other dipterans is also results from larval-specific increases in LDH activity and lactate-induced inhibition of L2HGDH.

Regardless of why dipteran larvae accumulate L-2HG, we observed that all adult insects maintain low L-2HG levels under normoxic conditions, raising the possibility that this molecule can be detrimental to adult physiology. This hypothesis is supported by a recent study which suggests that L-2HG induces oxidative stress within the brain of adult *Drosophila melanogaster* (Hunt et al., 2019). Since L-2HG is known to inhibit activity of both ATP synthase and α-ketoglutarate dehydrogenase (Brinkley et al., 2020; Fu et al., 2015), inappropriate L-2HG production in adults might interfere with oxidative phosphorylation and ATP production. We hypothesize that larvae might generate L-2HG for exactly the same reasons - if L-2HG dampens larval mitochondrial function in a predictable and consistent manner, perhaps unexpected bouts of mild hypoxia will induce less oxidative stress in growing tissues. Moreover, such a larval function for L-2HG could also have a secondary effect of rewiring intermediary metabolism in a way that might promote biosynthetic reactions and support the rapid growth rate associated with dipteran larval development. Our model would also explain why L-2HG levels are reduced in adult flies, where the energetic demands of flight require high levels of oxidative phosphorylation. These possibilities should be tested by examining metabolic flux in both larvae lacking L-2HG and adult flies harboring excess L-2HG.

Finally, our limited survey of insect 2HG metabolism reveals that while D-2HG levels remain at relatively low levels at all life stages and environmental conditions surveyed, a clear correlation emerged between decreased oxygen availability and elevated D-2HG accumulation. While the significance of this change remains to be determined, future studies should investigate the potential relationship between the two 2HG enantiomers. We would note that unlike L-2HG, D-2HG is normally produced by the enzyme hydroxyacid-oxoacid-transhydrogenase (HOT) and as the result of noncanonical phosphoglycerate dehydrogenase (PHGDH) activity (Fan et al., 2015; Struys et al., 2005). Yet, despite the presence of both enzymes within dipteran genomes (Garapati et al., 2019), the function of D-2HG in flies or any other insect order remains largely unknown. There are indications, however, that unlike L-2HG, high levels of D-2HG are detrimental to *Drosophila melanogaster* larvae, as ectopic D-2HG production induces the formation of melanotic masses (Reitman et al., 2015). Why excess D-2HG is detrimental to larval development while L-2HG appears beneficial remains unknown, however, we note a controversial study in human cells suggests that D-2HG induces degradation of the transcription factor HIF1α while L-2HG stabilizes this protein (Koivunen et al., 2012). We are uncertain as to whether D-2HG and L-2HG regulate *Drosophila melanogaster* HIF1α stability in opposing manners, but this possibility leads to an interesting hypothesis where D-2HG acts in a negative feedback loop that dampens the hypoxia response by inhibiting HIF1α stability. Therefore, despite the relatively low levels of D-2HG found in our survey, future studies should examine the role of this molecule in the dipteran hypoxia response.

But regardless of the endogenous L-2HG function and the underappreciated role of D-2HG in the hypoxia response, our study highlights how the natural diversity of insects presents an underappreciated resource for discovering and exploring the metabolic mechanisms that support animal growth. And beyond the exploring the metabolic parallels between insect development and cancer, our findings demonstrate how studying dipteran development can identify unique metabolic features that could be targeted for controlling both agricultural pests and human disease vectors.

Acknowledgements

We thank the Newton, Zelhof, and Tomberlin labs for providing for samples and advice. S.S. is supported by NCI R01CA200653 and NCI F30CA232397. J.A.K. and JMT are supported by NSF 1726633. J.M.T. is supported by NIH 1R35GM119557.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2020.103493.

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