RESEARCH ARTICLE

Early-life hypoxia alters adult physiology and reduces stress resistance and lifespan in *Drosophila*

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ABSTRACT

In many animals, short-term fluctuations in environmental conditions in early life often exert long-term effects on adult physiology. In *Drosophila*, one ecologically relevant environmental variable is hypoxia. *Drosophila* larvae live on rotting, fermenting food rich in microorganisms, an environment characterized by low ambient oxygen. They have therefore evolved to tolerate hypoxia. Although the acute effects of hypoxia in larvae have been well studied, whether early-life hypoxia affects adult physiology and fitness is less clear. Here, we show that *Drosophila* exposed to hypoxia during their larval period subsequently show reduced starvation stress resistance and shorter lifespan as adults, with these effects being stronger in males. We find that these effects are associated with reduced whole-body insulin signaling but elevated TOR kinase activity, a manipulation known to reduce lifespan. We also identify a sexually dimorphic effect of larval hypoxia on adult nutrient storage and mobilization. Thus, we find that males, but not females, show elevated levels of lipids and glycogen. Moreover, we see that both males and females exposed to hypoxia as larvae show defective lipid mobilization upon starvation stress as adults. These data demonstrate how early-life hypoxia can exert persistent, sexually dimorphic, long-term effects on *Drosophila* adult physiology and lifespan.

KEY WORDS: Glucose, Glycogen, Akt, Lifespan, Starvation stress, Metabolism, Insulin, TOR, Lipids, Sexual dimorphism

INTRODUCTION

Animals often live in environments in which conditions such as temperature, food, oxygen and pathogen exposure can fluctuate. In many cases, these fluctuations constitute a stress that can impact fitness. The ability of animals to adapt their metabolism and physiology to these changing environments is essential for their survival.

Many adaptive responses occur immediately in response to environmental stressors (e.g. starvation, hypoxia, infection), to allow animals to survive while subjected to these stress conditions. It is also increasingly appreciated that acute, early-life environmental stresses can trigger longer-term responses that can influence later adult physiology and fitness (Gluckman and Hanson, 2004; Burdge and Lillycrop, 2014). In some cases, these early-life environmental

changes can confer subsequent beneficial effects on adult fitness. For example, starvation stress in larval honeybees leads to subsequent starvation tolerance as adults (Wang et al., 2016a,b). In a similar manner, anoxia exposure during the development of the Caribbean fruit fly confers later anoxia resistance in adults (Visser et al., 2018). Early-life mild heat stress in the zebra finch has also been shown to be associated with lower oxidative damage induced by heat stress in adult life (Costantini et al., 2012).

In contrast to these adaptive responses, in some situations, early-life environmental stress can have deleterious consequences on subsequent adult physiology. Examples of these types of responses have been described in rodents in which prenatal exposure to a deficient maternal diet subsequently leads to cardiovascular and metabolic defects, and shortened lifespan in adults (Langley-Evans et al., 1999; Aihie Sayer et al., 2001; Woods et al., 2001). These effects are examples of a concept known as the developmental origins of health and disease (DOHaD), which proposes that poor intrauterine conditions during fetal development (often caused by defective maternal nutrition) can subsequently increase risk of metabolic disease in adulthood (Bruce and Hanson, 2010; Hanson and Gluckman, 2014). This hypothesis is supported by many epidemiological studies in humans showing that low birth weight (a proxy for poor intrauterine environment) is associated with a number of metabolic diseases such as diabetes, obesity and heart disease (Gluckman et al., 2008). Together, these various reports emphasize the importance of investigating the mechanisms by which different early-life environmental stresses can alter adult physiology.

Drosophila has been an excellent model to study how environmental cues influence physiology, development and lifespan. In particular, several recent reports have described how modulation in environment during the larval period of the life cycle can subsequently influence adult physiology and aging. For example, it has been reported that when *Drosophila* larvae are raised on low nutrients they subsequently show an extension of adult lifespan (Stefana et al., 2017). These effects were mediated by secretion of lipid autotoxic pheromones in adults. In other studies, when *Drosophila* were subjected to mild oxidative stress only during the larval period, this led to microbiome remodeling and persistent epigenetic changes that led to an extension of adult lifespan (Borch Jensen et al., 2017; Obata et al., 2018). Together, these studies show that altered early-larval-life environmental conditions can cause persistent and long-lasting effects on adult *Drosophila* physiology.

An important environmental variable in the *Drosophila* life cycle is oxygen exposure. In their natural ecology, *Drosophila* larvae grow by burrowing into rotting, fermenting food that is rich in microorganisms (Markow, 2015). This environment is likely to be low in oxygen, and, as a result, *Drosophila* have evolved mechanisms to tolerate hypoxia. For example, when exposed to moderate (5–10% oxygen) hypoxia in the laboratory, larvae slow

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their growth and development, but can maintain their viability (Harrison and Haddad, 2011; Heinrich et al., 2011; Callier et al., 2015; Lee et al., 2019). These adaptive effects are mediated through several different changes in larval physiology, including increased tracheal branching, changes in cell–cell signaling and metabolic gene expression, and altered lipid metabolism (Wingrove and O'Farrell, 1999; Centanin et al., 2008; Zhou et al., 2008; Li et al., 2013; Zhou and Haddad, 2013; Wong et al., 2014; Lee et al., 2019). These changes have been shown to allow larvae to survive and maintain homeostasis while exposed to low oxygen. However, whether larval hypoxia exposure exerts any persistent, long-term effects on adult physiology is not entirely clear. We explore this question in this paper.

MATERIALS AND METHODS
Drosophila stocks
All experiments were performed using w^{1118} flies (*Drosophila melanogaster* Meigen 1830) obtained from the Bloomington *Drosophila* Stock Center. Flies were kept on medium containing 150 g agar, 1600 g cornmeal, 770 g Torula yeast, 675 g sucrose, 2340 g D-glucose and 240 ml acid mixture (propionic acid/phosphoric acid) per 34 l water and maintained at 25°C.

Hypoxia exposure
For all hypoxia experiments, *Drosophila* larvae were exposed to 5% oxygen. This was achieved by placing vials containing *Drosophila* into an airtight glass chamber into which a mix of 5% oxygen/95% nitrogen continually flowed. Flow rate was controlled using an Aalborg model P gas flow meter.

Measurement of Drosophila starvation stress and lifespan
Eggs were collected on grape plates for 3–4 h, and, the next day, hatched larvae were transferred to vials (50 larvae per vial). Newly hatched larvae were then placed into one of two experimental conditions (see Fig. 1): normoxic experimental condition – larvae maintained in normoxia until adulthood; hypoxic experimental condition – larvae maintained in hypoxia chambers for the duration of their larval period. They were then transferred back to normoxia as pupae and allowed to develop to adulthood. For both experimental conditions, eclosed flies were allowed to mate for 2 days, and then males and females were separated under light anesthesia into cohorts of 20 flies per vial. For the starvation stress experiments, flies were transferred at 5–6 post-eclosion into vials containing only 0.8% agar/PBS. Viability was then assessed twice daily until all flies had died. For the lifespan experiments, flies were transferred to fresh vials every 2–3 days, and the numbers of dead flies were counted until all flies had died.

Quantitative polymerase chain reaction (qPCR) analyses
Total RNA was extracted from groups of five adults using TRIzol reagent according to the manufacturer's instructions (Invitrogen; 15596-018). The RNA samples were treated with DNase (Ambion; 2238G) and then reverse transcribed using Superscript II (Invitrogen; 100004925). The complementary DNAs were used as a template for subsequent reverse transcription qPCR (qRT-PCR) assays using SyBr Green PCR mix and an ABI 7500 real-time PCR system. The PCR data were normalized to *Actin5C* mRNA levels. The following primers were used: *Actin5C* forward 5′-GAGCGC-GGTTACTCTTTCAC-3′, *Actin5C* reverse 5′-GCCATCTCCTGC-TCAAAGTC-3′; *dILP2* forward 5′-TCCACAGTGAAGTTGG-CCC-3′, *dILP2* reverse 5′-AGATAATCGCGTCGACCAGG-3′; *dILP3* forward 5′-AGAGAACTTTGGACCCCGTGAA-3′, *dILP3* reverse 5′-TGAACCGAACTATCACTCAACAGTCT-3′; *dILP5* forward 5′-GAGGCACCTTGGGCCTATTC-3′, *dILP5* reverse 5′-CATGTGGTGAGATTCGGAGCTA-3′.

Western blotting
Groups of five adult *Drosophila* were lysed in a buffer containing 20 mmol l^{-1} Tris-HCl (pH 8.0), 137 mmol l^{-1} NaCl, 1 mmol l^{-1} EDTA, 25% glycerol, 1% NP-40, 50 mmol l^{-1} NaF, 1 mmol l^{-1} phenylmethylsulfonyl fluoride, 1 mmol l^{-1} dithiothreitol, 5 mmol l^{-1} sodium ortho vanadate (Na$_3$VO$_4$), Protease Inhibitor
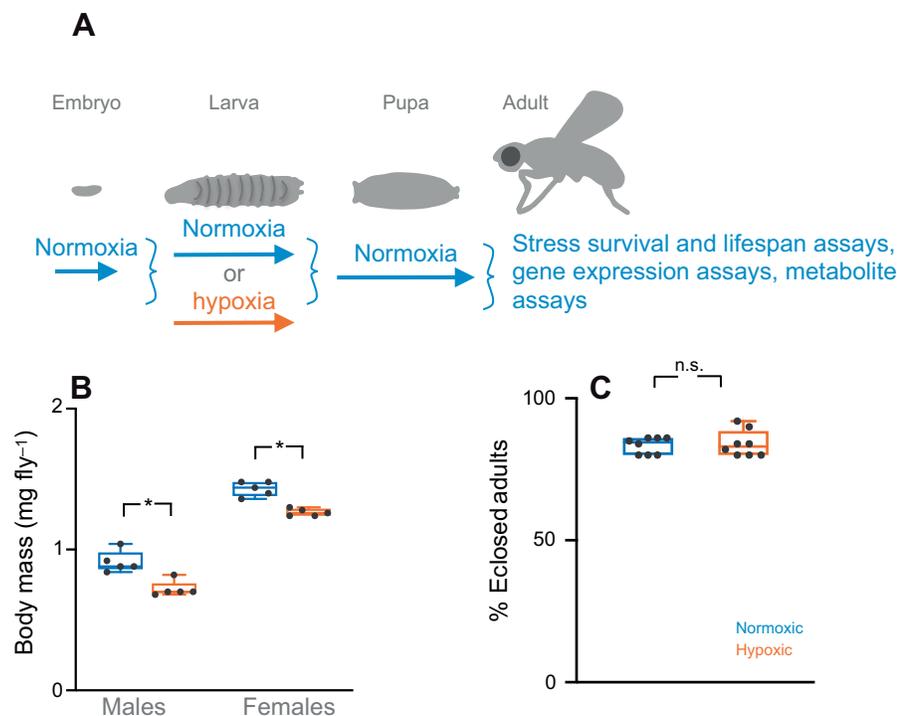

Fig. 1. Effects of larval hypoxia on adult body mass and survival in *Drosophila melanogaster*. (A) An outline of the experimental protocol for examining the effects of larval hypoxia on adult physiology in *D. melanogaster*. For all experiments, w^{1118} embryos were raised in normoxia. Upon hatching, they were transferred to food vials and kept in either normoxia or hypoxia (5% oxygen) for the duration of their larval period. The animals were then kept in normoxia throughout pupal development until they emerged as adults. Mated, 1-week-old adults were assayed for changes in their starvation stress survival, lifespan, gene expression and metabolite levels. (B) Male and female adult body masses from the normoxic and hypoxic groups. (C) Survival to adulthood of animals from the normoxic and hypoxic groups. Data were calculated as the percentage of eclosed adults from each group. Data are presented as box plots (25%, median and 75% values), with error bars indicating the minimum and maximum values, and individual data points shown as dots. *P<0.05; n.s., not significant; Student's *t*-test.

Cocktail (Roche; 04693124001) and Phosphatase Inhibitor Cocktail Tablets (Roche; 04906845001). Protein concentrations were measured using a Bio-Rad DC Protein Assay Kit II (5000112). Protein lysates (15–30 µg) were resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and electro transferred to a nitrocellulose membrane, and then subjected to western blotting with specific primary antibodies and horseradish peroxidase-conjugated secondary antibodies, before being visualized by chemiluminescence (enhanced ECL solution; Perkin Elmer). Primary antibodies used in this study were as follows: anti-phospho-S6K-Thr398 (1:1000; Cell Signaling Technology; 9209), anti-pAkt-T342 (1:1000; a gift from Michelle Bland; Roth et al., 2018), anti-pAkt-S505 (1:1000; Cell Signaling Technology; 4054) and anti-Actin (1:1000; Santa Cruz Biotechnology; sc-8432). Secondary antibodies were purchased from Santa Cruz Biotechnology (1:10,000; sc-2030, sc-2005, sc-2020).

Metabolite measurements

Groups of five flies were weighed and then frozen in Eppendorf tubes on dry ice. Total glycogen and triacylglyceride (TAG) levels were determined using colorimetric assays following the protocols described in detail in Tennessen et al. (2014). For TAG assays, animals were lysed, and lysates were heated at 70°C for 10 min. They were incubated first with triglyceride reagent (Sigma-Aldrich; T2449) and then mixed with free glycerol reagent (Sigma-Aldrich; F6428). Colorimetric measurements were made using absorbance at 540 nm, and TAG levels were calculated by comparing with a glycerol standard curve. Glycogen assays were performed by lysing animals in PBS and then heating lysates at 70°C for 10 min. For each experimental sample, duplicate samples were either treated with amyloglucosidase (Sigma-Aldrich; A1602) to break down glycogen intro glucose or left untreated, and then levels of glucose in both duplicates were measured by colorimetric assay following the addition of a glucose oxidase reagent (Sigma-Aldrich; GAGO-20). Levels of glycogen in each experimental sample were calculated by subtracting the glucose measurements of the untreated duplicate from the amyloglucosidase-treated sample. All experimental metabolite concentrations were calculated by comparison with glycogen and glucose standard curves. All calculated metabolite levels were then corrected for adult body mass and presented as percentage body mass (mg metabolite mg^{-1} body mass×100). In Fig. 6, data are presented as the percentage change in TAG levels upon starvation. These data were calculated by measuring TAG levels in normoxic and hypoxic flies that were either maintained on food or nutrient deprived for 16 h (by switching to 0.8% agar/PBS), and then using these values to calculate the percentage decrease in TAG levels upon starvation for each group.

Statistical analyses

Lifespan and stress survival data were analyzed using a log-rank test. Metabolite and qPCR data were analyzed by either Student's *t*-test or two-way ANOVA followed by *post hoc* Student's *t*-test. GraphPad Prism software was used to perform all statistical analyses and generate data plots.

RESULTS

The outline for all experiments is shown in Fig. 1A. For this study, we chose to examine the effects of 5% oxygen exposure during the larval stage on adult physiology. At this level of oxygen, larvae maintain normal feeding (Lee et al., 2019), but show reduced growth (Fig. 1B) and delayed development to the pupal stage by ~1 day (Lee et al., 2019). However, they show no defects in survival compared with normoxia-raised larvae and they develop into viable adults (Fig. 1C) (Lee et al., 2019), suggesting that this level of hypoxia is not exerting any pathological effects on survival. Indeed, it has been shown that the oxygen levels at the surface of the food in fly vials containing growing larvae is in the range of 5–10% (Callier et al., 2015), suggesting that larvae can tolerate low ambient oxygen levels as part of their normal development.

For all experiments, w^{1118} embryos were raised in normoxia, and when they hatched they were transferred to food vials and then maintained in either normoxia or hypoxia for the duration of their larval period (Fig. 1A). The animals were kept in normoxia throughout pupal development until they emerged as adults. Mated, 1-week old adults were then assayed for changes in their physiology caused by prior larval hypoxia (referred to as the hypoxic condition) compared with adults raised in normoxia (the normoxic condition).

Larval hypoxia has no effect on adult hypoxia tolerance

We first examined whether exposing larvae to hypoxia could subsequently alter their tolerance to hypoxia stress as adults. Adult *Drosophila* are generally more tolerant to low oxygen than larvae, so we chose to examine adult tolerance on 1% oxygen. We tested survival in response to exposure to hypoxia for either 16 h (which induces low level lethality) or 20 h (which induces higher lethality) to ensure that we could detect any potential increases or decreases in survival caused by prior larval hypoxia. However, we found that, compared with normoxic animals, the hypoxic condition adults showed no difference in hypoxia survival after either period of hypoxia exposure (Fig. 2A,B).
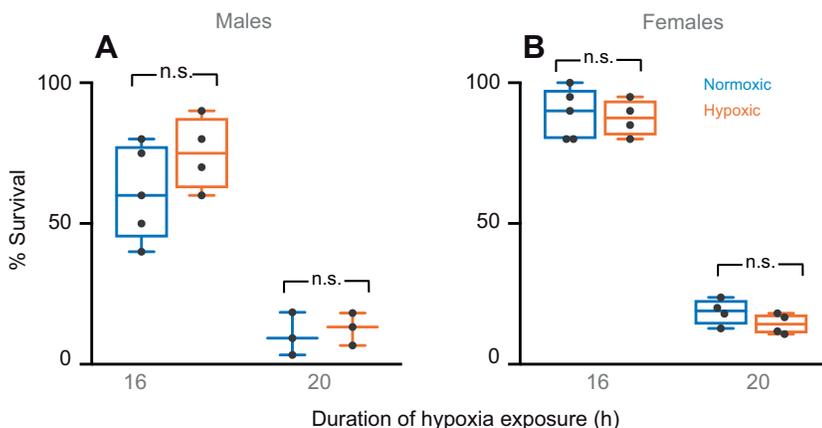

Fig. 2. Larval hypoxia has no effect on adult tolerance to hypoxia in *D. melanogaster*. (A,B) Hypoxia (1% oxygen) survival graphs for male (A) and female (B) adult *Drosophila* that had been exposed to either normoxia (blue lines) or hypoxia (brown lines) as larvae. Survival was measured after exposing adult animals to either a 16 h or 20 h hypoxia exposure. Data are presented as box plots (25%, median and 75% values), with error bars indicating the minimum and maximum values, and individual data points shown as dots. n.s., not significant; Student's *t*-test.

Larval hypoxia leads to reduced adult starvation stress tolerance and shorter lifespan

We next examined whether larval exposure to hypoxia could subsequently alter adult lifespan or responses to another stress. We first compared the ability of the hypoxic and normoxic adults to tolerate starvation stress. We found that, compared with normoxic animals, the hypoxic condition adults showed a significant reduction in viability when completely deprived of nutrients (Fig. 3A,B). This reduced starvation tolerance was more pronounced in males (29.4% decrease in median survival in hypoxic flies) than in females (5.6% decrease in median survival in hypoxic flies). We next examined the effects of larval hypoxia on adult lifespan. We found that hypoxic animals had a reduced lifespan compared with the normoxic animals (Fig. 3C,D). As with the starvation responses, these reductions in lifespan in hypoxic animals were stronger in males (17.6% decrease in median lifespan) than in females (6.9% decrease in median lifespan). Together, these results indicate that, when exposed to hypoxia as larvae, adult *Drosophila* have a reduced lifespan and a reduced ability to tolerate starvation.

Larval hypoxia leads to decreased adult insulin signaling but increased TOR signaling

The conserved insulin and TOR kinase signaling pathways are major regulators of systemic metabolism and physiology in *Drosophila*. In particular, both signaling pathways have been shown to control stress responses in adults. For example, genetic or pharmacological lowering of either insulin or TOR signaling has been shown to extend lifespan and to increase tolerance to different

stresses including starvation and oxidative stress (Katewa and Kapahi, 2011; Partridge et al., 2011). Given the effects of larval hypoxia on adult lifespan and stress tolerance that we observed, we examined whether larval exposure to hypoxia could lead to altered insulin or TOR signaling in adults.

We first measured mRNA levels of *Drosophila* insulin-like peptides (dILPs). *Drosophila* contain seven main dILPs that can bind the insulin receptor and activate a conserved downstream PI3K/Akt kinase pathway (Nässel et al., 2015). In particular, three dILPs (dILP2, dILP3 and dILP5) that are expressed and secreted from neurosecretory cells in the larval and adult brains have been shown to influence stress responses and lifespan in *Drosophila* (Nässel and Vanden Broeck, 2016). The expression and release of these brain-derived dILPs has been shown to be reduced in larvae upon acute hypoxia exposure (Texada et al., 2019). When we measured dILP levels by qPCR, we found that the hypoxic adults showed reduced expression of *dILP2*, *dILP3* and *dILP5* compared with the normoxic animals (Fig. 4A,B). We then measured phosphorylation of Akt in whole-body lysates as a read-out for systemic insulin signaling. When the insulin pathway is activated, Akt is phosphorylated at two sites, threonine 342 and serine 505. We found that phosphorylation at both these sites was reduced in the hypoxic adults compared with normoxic controls (Fig. 4C).

We next examined whether larval hypoxia exposure could alter adult TOR kinase signaling. One direct phosphorylation target of TOR is S6K. Using an antibody that recognizes the TOR phosphorylation site on S6K, we found that, in contrast to insulin signaling, TOR activity was elevated in hypoxic adult flies (Fig. 4C). Taken together, these data indicate that exposure of
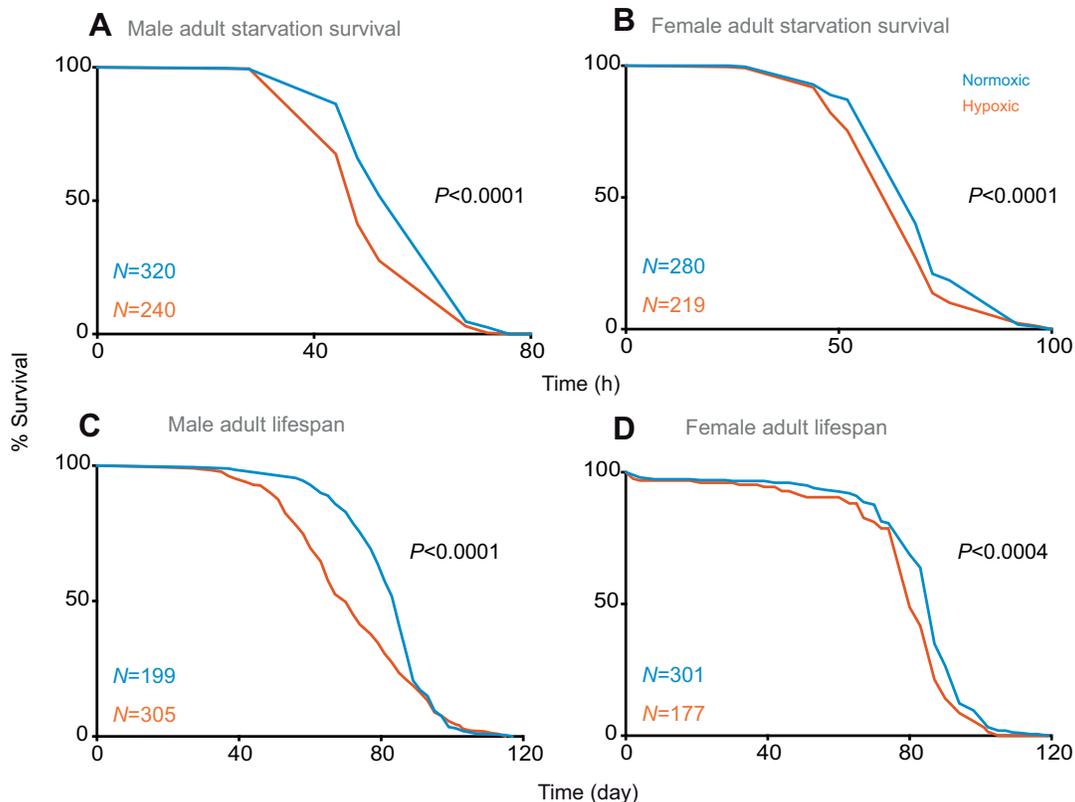

Fig. 3. Larval hypoxia leads to reduced adult tolerance to starvation stress and reduced adult lifespan in *D. melanogaster*. (A–D) Starvation survival curves (A,B) and survival curves (C,D) for male (A,C) and female (B,D) adult *Drosophila* that had been exposed to either normoxia (blue lines) or hypoxia (brown lines) as larvae. Data were analyzed using the Log-rank test.
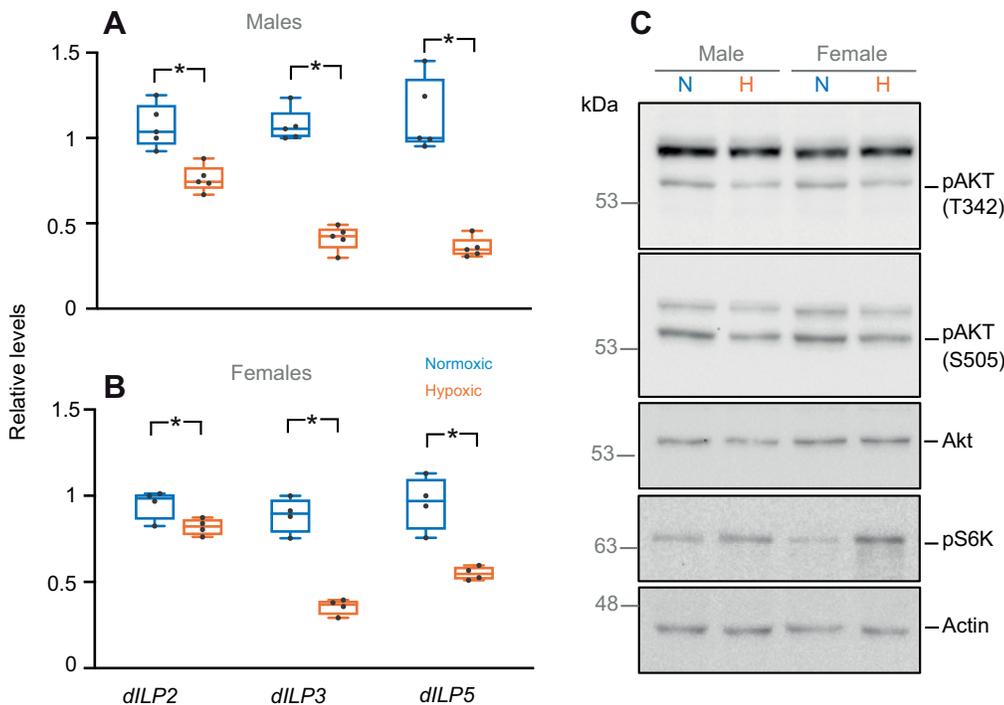
Fig. 4. Larval hypoxia leads to altered adult insulin and TOR signaling in *D. melanogaster*. (A,B) Levels of dILP mRNAs were measured by qRT-PCR in mated adult males (A) and females (B) after they had been exposed to either normoxia (blue plots) or hypoxia (brown plots) as larvae. Data are presented as box plots (25%, median and 75% values), with error bars indicating the minimum and maximum values, and individual data points shown as dots. *$P<0.05$; Student's *t*-test. (C) Western blot analysis of phosphorylated Akt (pAkt; threonine 342 and serine 505), total Akt, phosphorylated S6K (pS6K) and total Actin (loading control), in lysates from mated adult flies after they had been exposed to either normoxia (N) or hypoxia (H) as larvae.

larvae to hypoxia leads to a long-term persistent suppression of insulin signaling, but an increase in TOR signaling, in adults.

Larval hypoxia leads to sex-specific changes in adult nutrient storage

Animals often rely on mobilization of nutrient stores to fuel their metabolism during periods of stress, particularly nutrient deprivation. Studies in *Drosophila* have shown that genetic disruption of nutrient mobilization can reduce starvation tolerance and reduce lifespan (Mattila and Hietakangas, 2017; Heier and Kühnlein, 2018). Because we observed that larval hypoxia could exert effects on adult stress resistance and lifespan, we examined whether altered nutrient storage and mobilization might be involved. To do this, we analyzed adult whole-body levels of TAGs, the main lipid stores, and levels of glycogen and glucose, the main sugar stores, in the normoxic and hypoxic conditions. We found that, at the end of the larval period, the hypoxic animals had significantly higher levels of TAG, as previously reported (Lee et al., 2019), but no change in either glycogen or glucose levels (Fig. 5A). We then examined the effects of larval hypoxia exposure on adult metabolites. These studies revealed a sexually dimorphic effect of larval hypoxia on adult nutrient stores. We found that the hypoxic condition males had significantly elevated levels of both TAG and glycogen compared with normoxic males (Fig. 5B). By contrast, we found that TAG and glycogen levels in normoxic and hypoxic females were not significantly different (Fig. 5C). The findings that hypoxic animals have either normal (females) or elevated (males) levels of stored lipids and sugars are perhaps surprising given that these animals show reduced starvation tolerance compared with normoxic animals. However, one possibility is that, despite having high levels of stored nutrients, the hypoxic animals have defects in nutrient mobilization. We therefore examined whether nutrient mobilization might be different between the normoxic and hypoxic groups when they are subjected to nutrient starvation. We focused on looking at TAG levels because proper mobilization of lipid stores has been shown to be essential for

starvation tolerance in *Drosophila*. Both normoxic males and females showed a decrease in total TAG levels following starvation, with this effect being more pronounced in males, a previously reported result that is consistent with the mobilization of lipid stores in nutrient-deprived conditions (Grönke et al., 2007; Wat et al., 2020). By contrast, hypoxic animals displayed a sexually dimorphic response to starvation. Hypoxic males showed a significantly greater decrease in TAG levels upon starvation compared with normoxic males, as well as almost completely depleted (94% decrease) lipid stores (Fig. 6). By contrast, hypoxic females did not show any decrease in TAG levels (Fig. 6). Taken together, these data indicate that larval hypoxia exposure leads to abnormal nutrient storage and mobilization in adults.

DISCUSSION

In this study, we explored how early-life hypoxia affects adult physiology and homeostasis in *Drosophila*. In particular, we were interested in testing the possibility that early-life hypoxia might confer beneficial effects on adult fitness and stress tolerance. However, we found that larval hypoxia exerted no hormetic effect on hypoxia tolerance in adults. This finding contrasts with previous studies that showed that two other larval environmental manipulations – nutrient restriction and oxidative stress – could extend adult lifespan (Stefana et al., 2017; Obata et al., 2018). Similarly, genetic manipulations that trigger a pulse of mitochondrial stress in the early larval period can also lead to extended lifespan in adults (Owusu-Ansah et al., 2013; Borch Jensen et al., 2017). A similar beneficial effect of early-life mitochondrial stress has also been described in *Caenorhabditis elegans* (Dillin et al., 2002). One possibility is that the type of early-life stress dictates whether any beneficial effects are seen later in life. Thus, nutrient restriction and oxidative stress, but not oxygen limitation, may converge upon similar metabolic or regulatory processes to confer later effects on adult fitness. Alternatively, in the case of hypoxia, both the level and duration of larval low-oxygen exposure could be important in determining whether any potential
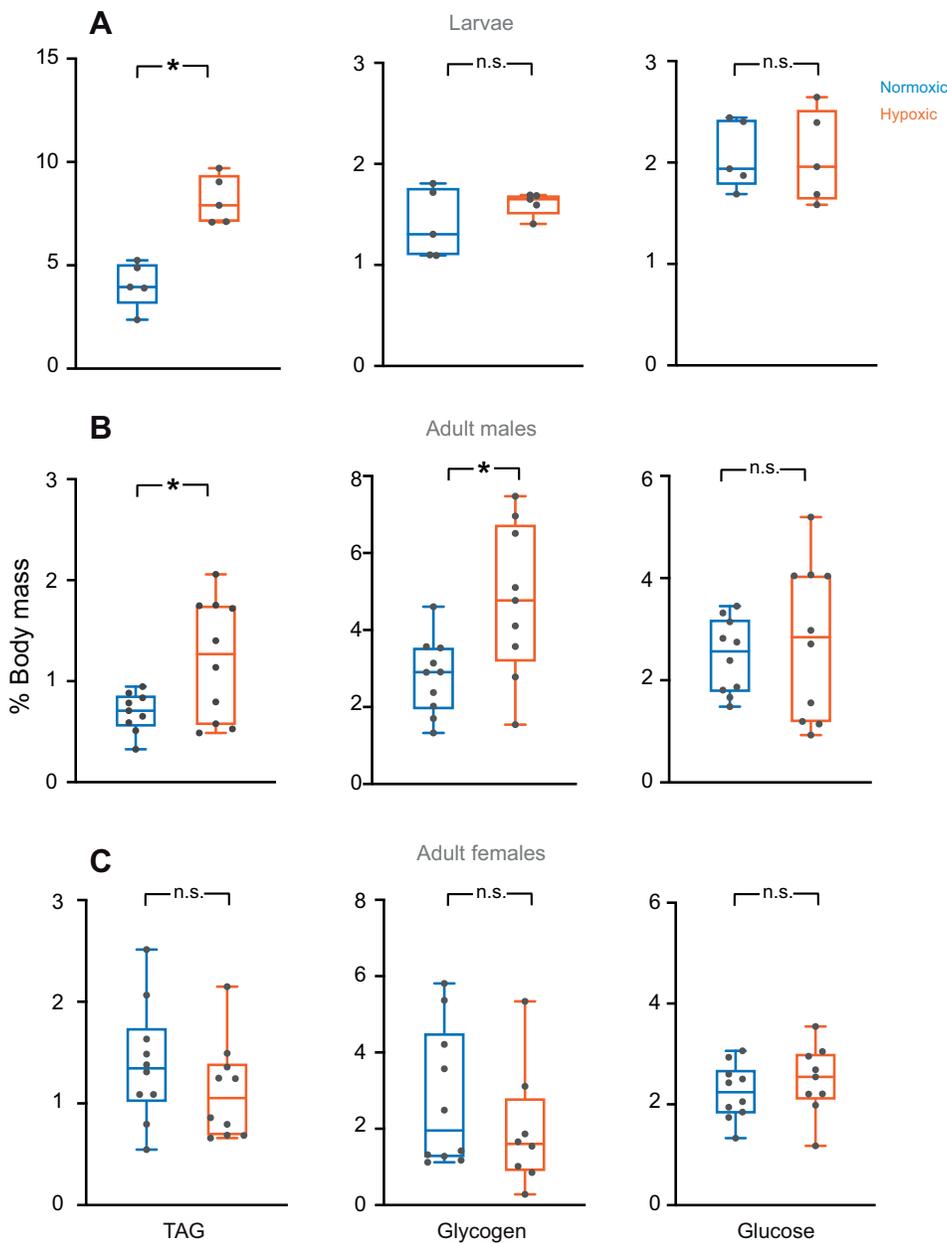
Fig. 5. Larval hypoxia alters adult male, but not female, nutrient storage in *D. melanogaster*. (A) Levels of triacylglyceride (TAG), glycogen and glucose in wandering third-instar larvae that had been exposed to either normoxia (blue plots) or hypoxia (brown plots) for the duration of their larval development. (B,C) Levels of TAG, glycogen and glucose in mated adult male (B) and female (C) animals after they had been exposed to either normoxia (blue plots) or hypoxia (brown plots) as larvae. Data are presented as box plots (25%, median and 75% values), with error bars indicating the minimum and maximum values, and individual data points shown as dots. *$P<0.05$; n.s., not significant; Student's *t*-test.

long-term effects in adults are beneficial or deleterious. A previous study showed that exposure of larvae to 10% oxygen also reduced adult lifespan (Rascon and Harrison, 2010). Thus, it could be the case that exposure to less severe hypoxia, possibly just a little lower than the 20% oxygen level in air, could exert beneficial effects. Alternatively, a pulse of transient, more severe, but non-lethal, hypoxia or anoxia could be important. This has been seen in *C. elegans*, in which short-term (36 h) exposure to 0.5% oxygen leads to a subsequent extension of adult lifespan (Schieber and Chandel, 2014).

Rather than seeing beneficial effects, we actually found that the effects of the larval hypoxia were deleterious to adult fitness – both male and female adults that had been exposed to hypoxia as larvae showed a reduced ability to tolerate nutrient starvation, and they had a shortened lifespan. Our metabolic analyses suggest that these effects could occur as a result of altered nutrient storage and mobilization caused by prior hypoxic exposure. Numerous studies have shown that the storage and mobilization of nutrient stores,

particularly lipids, are essential for *Drosophila* to tolerate starvation stress and to maintain normal lifespan (Heier and Kühnlein, 2018). This mobilization of nutrient stores is required to allow proper fueling of key metabolic processes required to allow animals to survive in the absence of food (Grönke et al., 2007; Palanker et al., 2009; Molaei et al., 2019; Wat et al., 2020). In this context, we identified alterations in both lipid storage and mobilization in *Drosophila* adults previously exposed to hypoxia as larvae. Interestingly, these alterations exhibited a sexual dimorphism. First, we saw that hypoxic males, but not females, had elevated levels of TAGs and glycogen. Given that nutrient stores are required for starvation survival, these high TAG and glycogen levels in hypoxic males might be expected to promote starvation survival rather than reduce starvation survival, which we actually observed. However, we also found that both male and female hypoxic animals showed a defect in lipid mobilization upon starvation, and that these effects were sexually dimorphic. In the case of males, the normoxic control group showed a reduction in total TAG levels following
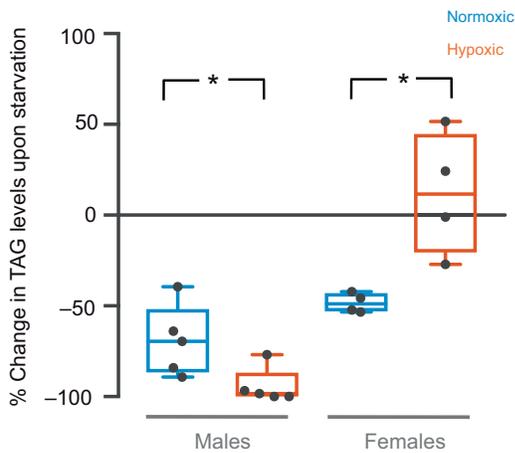
Fig. 6. Larval hypoxia leads to a sexually dimorphic effect on lipid mobilization during starvation stress in *D. melanogaster*. Changes in TAG levels upon 16 h of complete nutrient starvation in adult male and female animals after they had been exposed to either normoxia (blue plots) or hypoxia (brown plots) as larvae. Data were calculated as percentage change in TAG levels in starved animals compared with fed animals, and are presented as box plots (25%, median and 75% values), with error bars indicating the minimum and maximum values, and individual data points shown as dots. *$P<0.05$; two-way ANOVA followed by *post hoc* Student's *t*-test.

starvation – a phenotype that has been reported before and is consistent with the mobilization of lipid stores to support survival during nutrient starvation (Grönke et al., 2007; Palanker et al., 2009; Wat et al., 2020). However, we found that this decrease in TAG levels was significantly greater in the hypoxic group, which showed an almost complete depletion of their lipid stores. Thus, the increased starvation death in this group could be because they deplete their lipids too rapidly. By contrast, we saw an opposite phenotype in females. Whereas normoxic females showed lipid mobilization following starvation, the hypoxic females showed no depletion in TAGs upon starvation. Thus, in contrast to hypoxic males, the hypoxic females probably show decreased starvation survival owing to an inability to mobilize their lipid stores.

What mechanisms might explain this sexually dimorphic difference in metabolism? One possibility suggested by a recent study involves a sexually dimorphic regulation of the lipase, *brummer* (Wat et al., 2020). The authors reported that adult male flies showed higher starvation-mediated induction of *brummer* compared with females, and, as a result, they showed faster lipid depletion and decreased starvation survival (Wat et al., 2020). In our case, it is possible that larval hypoxia subsequently leads to male:female differences in *brummer* regulation that could explain why our hypoxic flies showed both sexually dimorphic changes in lipid mobilization and overall reduced starvation survival. Other studies have described how different transcriptional regulators and lipid metabolism genes coordinate lipid mobilization to promote starvation survival (Baker and Thummel, 2007; Heier and Kühnlein, 2018). In some cases, similar phenotypes to the ones we observed have been reported. For example, male flies mutant for the translational repressor 4E-BP display a similar phenotype to our male hypoxic flies in that they show an increased depletion of lipid stores and decreased survival upon starvation (Teleman et al., 2005). By contrast, flies mutant for the transcriptional regulator Sir4 display a phenotype similar to hypoxic females – they fail to mobilize lipid stores upon starvation and subsequently show reduced survival (Wood et al., 2018). Thus, it is possible that larval hypoxia can cause alterations in these regulatory genes to lead to adult metabolic phenotypes. However, most (almost

all) studies looking at the mechanisms of lipid mobilization during nutrient starvation have described results in only one sex. Hence, it is unclear whether any of the underlying mechanisms reported in these studies exhibit sexual dimorphisms that might explain the male:female differences that we see.

Other possible mediators of the effects of larval hypoxia on adult metabolism and survival are alterations in insulin and TOR signaling. In *Drosophila*, these pathways are normally both induced by nutrient and oxygen availability and have been shown to be suppressed by starvation and hypoxia (Grewal, 2009; Wong et al., 2014; Lee et al., 2019; Texada et al., 2019). Interestingly, we found diverging effects on TOR and insulin signaling – whole-body insulin signaling was lower, whereas TOR activity was elevated. These findings contrast with much of the previous work on TOR and insulin signaling that shows that both pathways tend to be regulated in a similar manner in larvae and adults – nutrient availability promotes both insulin and TOR signaling (Grewal, 2009), whereas hypoxia exposure suppresses them both (Lee et al., 2019; Texada et al., 2019; Barretto et al., 2020). One possibility is that larval hypoxia can lead to a long-lasting, but differential, alteration of the basal 'set point' for insulin versus TOR signaling in adults. The effects on insulin signaling could be explained by our observed hypoxia-dependent decrease in the expression of *dILP2*, *dILP3* and *dILP5*, three main dILPs secreted from the brain, the expression levels of which have previously been shown to control systemic insulin signaling. The elevation in TOR activity might be due to altered expression of upstream signaling components such as the TSC1/2 complex or the small G protein, Rheb. Do these alterations in insulin and TOR signaling explain why hypoxic animals have altered metabolic effects and reduced starvation survival and lifespan? Generally, lowering systemic insulin signaling confers extension of lifespan and tolerance to stress in *Drosophila* (Giannakou and Partridge, 2007). However, increased TOR signaling has been shown to reduce adult lifespan in *Drosophila* (Kapahi et al., 2004). Hence, in the case of early-life hypoxia exposure, the increased TOR might dominate to control survival in the hypoxic animals. Moreover, although reduced insulin and increased TOR was seen in both males and female flies, these changes could even potentially explain the dimorphism in lipid metabolic phenotypes that we observed. For example, a recent study showed that genetic suppression of insulin signaling in adult flies could alter sexually dimorphic differences in gene expression, including many metabolic genes (Graze et al., 2018). In addition, alterations in TOR signaling have also been shown to have sex-dependent effects on gene expression and on nutrient control of reproduction in *Drosophila* (Camus et al., 2019). Overall, our findings demonstrate how early-life hypoxia can exert persistent, sexually dimorphic, long-term effects on adult physiology and lifespan in *Drosophila*.

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