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Oxygen-induced changes in hemoglobin expression in *Drosophila*

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The hemoglobin gene 1 (*dmeglob1*) of the fruit fly *Drosophila melanogaster* is expressed in the tracheal system and fat body, and has been implicated in hypoxia resistance. Here we investigate the expression levels of *dmeglob1* and *lactate dehydrogenase* (a positive control) in embryos, third instar larvae and adult flies under various regimes of hypoxia and hyperoxia. As expected, mRNA levels of *lactate dehydrogenase* increased under hypoxia. We show that expression levels of *dmeglob1* are decreased under both short- and long-term hypoxia, compared with the normoxic (21% O_2) control. By contrast, a hypoxia/reoxygenation regime applied to third instar larvae elevated the level of *dmeglob1* mRNA. An excess of O_2 (hyperoxia) also triggered an increase in *dmeglob1* mRNA. The data suggest that *Drosophila* hemoglobin may be unlikely to function merely as a myoglobin-like O_2 storage protein. Rather, *dmeglob1* may protect the fly from an excess of O_2, either by buffering the flux of O_2 from the tracheoles to the cells or by degrading noxious reactive oxygen species.

The exchange of respiratory gases in insects is enabled by the tracheal system, which mediates diffusive gas transport to the inner organs [1,2]. In highly active organs, such as the insect flight muscle, tracheal protuberances can even enter cells and reach the mitochondria directly. Many insects are surprisingly resistant towards a low oxygen environment (hypoxia). Some species are exquisitely adapted to hypoxia due to their natural habitat: larvae of the horse botfly *Gasterophilus intestinalis*, living in the host's intestine, recover after 17 days of anoxia, and aquatic larvae of the midge *Chironomus plumosus* survive 200 days without O_2 [3]. The adult house fly (*Musca domestica*) survives 12–15 h without O_2 and recovers completely when re-oxygenated [4]. *Drosophila melanogaster* displays a remarkable resistance to hypoxia and anoxia as well. Embryonic, larval and adult *Drosophila* react to short-term O_2 deprivation by behavioral changes including paralysis, but recover completely when re-oxygenated [5–7]. Prolonged exposure to 6% O_2, however, stops embryonic development and is lethal [8]. In a stress-adaptive response, hypoxia influences the opening of spiracles and stimulates the growth and branching of tracheae [9] via induction of the nitric oxide/cyclic GMP pathway [7], the hypoxia-inducible factor (HIF)-dependent oxygen-sensing mechanism [10,11] and the fibroblast growth factor signaling pathway [12]. Thus, the genome-wide transcriptional response to hypoxia in *Drosophila* involves considerable expressional changes, particularly in known stress-inducible genes [13]. However, insects also seek to avoid cellular stress by an excess amount of tracheal O_2 (hyperoxia), which may generate noxious reactive oxygen species (ROS), for example, by a special rhythmic ventilatory behavior like

Abbreviations
Hb, hemoglobin; HIF, hypoxia-inducible factor; LDH, lactate dehydrogenase; ROS, reactive oxygen species; RPL17a, ribosomal protein L17a.

the discontinuous gas exchange cycle [14,15]. Exposure to 49% O_2 reduces fly longevity by half [16]. Microarray analyses of *Drosophila* adults treated with 100% O_2 or ROS-generating chemicals revealed a complex gene regulatory response, including the expected upregulation of antioxidant defense genes [17,18].

Many invertebrates harbor respiratory proteins that store or transport O_2, thereby enhancing their metabolic performance under low oxygen conditions [19]. Because of the highly efficient O_2 diffusion along the tracheal system, it has long been assumed that most insects do not need respiratory proteins [2]. Known exceptions were the aquatic larvae of the chironomids, aquatic backswimmers [*Buenoa confusa* and *Anisops pellucens* (Hemiptera)] and the parasitic larvae of *G. intestinalis* [19,20]. These species secrete hemoglobins (Hbs) from the fat body into their hemolymph (Chironomidae) or harbor intracellular Hb in specialized fat body-derived organs (*G. intestinalis*, backswimmers), apparently because Hb enhances their ability to deliver or store O_2 under hypoxic conditions. In addition, some basal insects have hemocyanin in their hemolymph, a copper-based respiratory protein which they apparently inherited from their crustacean ancestor [21,22].

Recently, we have shown that *D. melanogaster* encodes three Hb genes (*dmeglob1*, *dmeglob 2* and *dmeglob3*) [22–24]. While the closely related gene duplicates *dmeglob2* and *-3* are rather weakly expressed genes, *dmeglob1* constitutes the major Hb variant of *Drosophila*. It is expressed at substantial levels in the fat body and tracheae/tracheoles of all *Drosophila* developmental stages [23]. Dmeglob1 protein is a typical globin of 153 amino acids, which displays a characteristic 3-over-3 α-helical sandwich structure [25], and binds O_2 with a high affinity of P_{50} = 0.14 Torr [23]. Thus, both, expression patterns and ligand affinity of *dmeglob1* resemble other known insect Hbs. The available data suggest that *dmeglob1* may be involved in O_2 supply and, possibly, the hypoxia tolerance of *Drosophila*. However, the globin might also be instrumental in alleviating oxidative stress by detoxifying harmful ROS molecules. In any case, one might expect that hypoxic or hyperoxic stress should alter the expression levels of *dmeglob1* mRNA. For a better understanding of insect Hb function *in vivo*, we have therefore investigated the regulation of *dmeglob1* in different developmental stages under various hypoxia and hyperoxia regimes.

Results

Hemoglobin (*dmeglob1*) mRNA levels were measured employing quantitative real-time RT-PCR (qRT-PCR) in embryonic, larval and adult *D. melanogaster*, and quantities of the control gene *lactate dehydrogenase* (*LDH*) mRNA were determined in larvae and adult flies. The mRNA levels of these two genes were normalized according to the gene for ribosomal protein reference gene *RPL17A*. *RPL17A* was inferred to be unregulated during different hypoxia stress conditions in a pilot microarray study (B. Adryan and R. Schuh, unpublished results). RT-PCR on carefully standardized amounts of RNA and cDNA confirmed the unregulated expression of *RPL17A* (not shown). We measured and compared *dmeglob1* and *LDH* expression under various O_2 concentrations and exposure times relative to animals kept at normoxia (21% O_2), but otherwise identical conditions.

Globin expression in embryos under hypoxia

We tested *dmeglob1* mRNA expression levels in embryos after different exposure times to moderate hypoxia (~ 5% O_2). The level of *dmeglob1* mRNA decreased in a time-dependent manner to 63% after 1 h, 52% after 2 h and 36% after 6 h compared with normoxic control (Fig. 1A). Longer hypoxia regimes were not tested due to the known detrimental effects on embryonic cell cycle and protein expression [26].

Globin expression in larvae under hypoxia and hyperoxia

Moderate, long-term hypoxia (~ 5% O_2 for 24 h) was applied to third instar larvae. We observed a decrease in *dmeglob1* mRNA levels down to ~ 30% compared with the respective normoxic control (Fig. 1B). During long-term hypoxia treatment, larvae still moved, even though their motions were slowed compared with larvae kept under normoxic conditions. In L3 larvae kept under severe, short-term hypoxia (1% O_2 for 1, 3 and 5 h), a decrease in *dmeglob1* mRNA levels was detected to ~ 50% compared with the respective normoxic control (Fig 1C). Shortly after applying these severe hypoxia conditions, larvae movement slowed and finally stopped for the entire hypoxic phase.

The effect of hypoxia/re-oxygenation stress was investigated by keeping the larvae for 20 min at 5% O_2, subsequently returning them for 20 min to 21% O_2 before RNA extraction. These intermittent hypoxia conditions, repeated three times, caused *dmeglob1* mRNA expression to increase by ~ 70% compared with the normoxic control (Fig. 1D). Larvae exposed to intermittent hypoxia did not show any change in behavior.

The middle-term hyperoxia regime, which we applied to L3 larvae (95% O_2 for 12 h), caused the

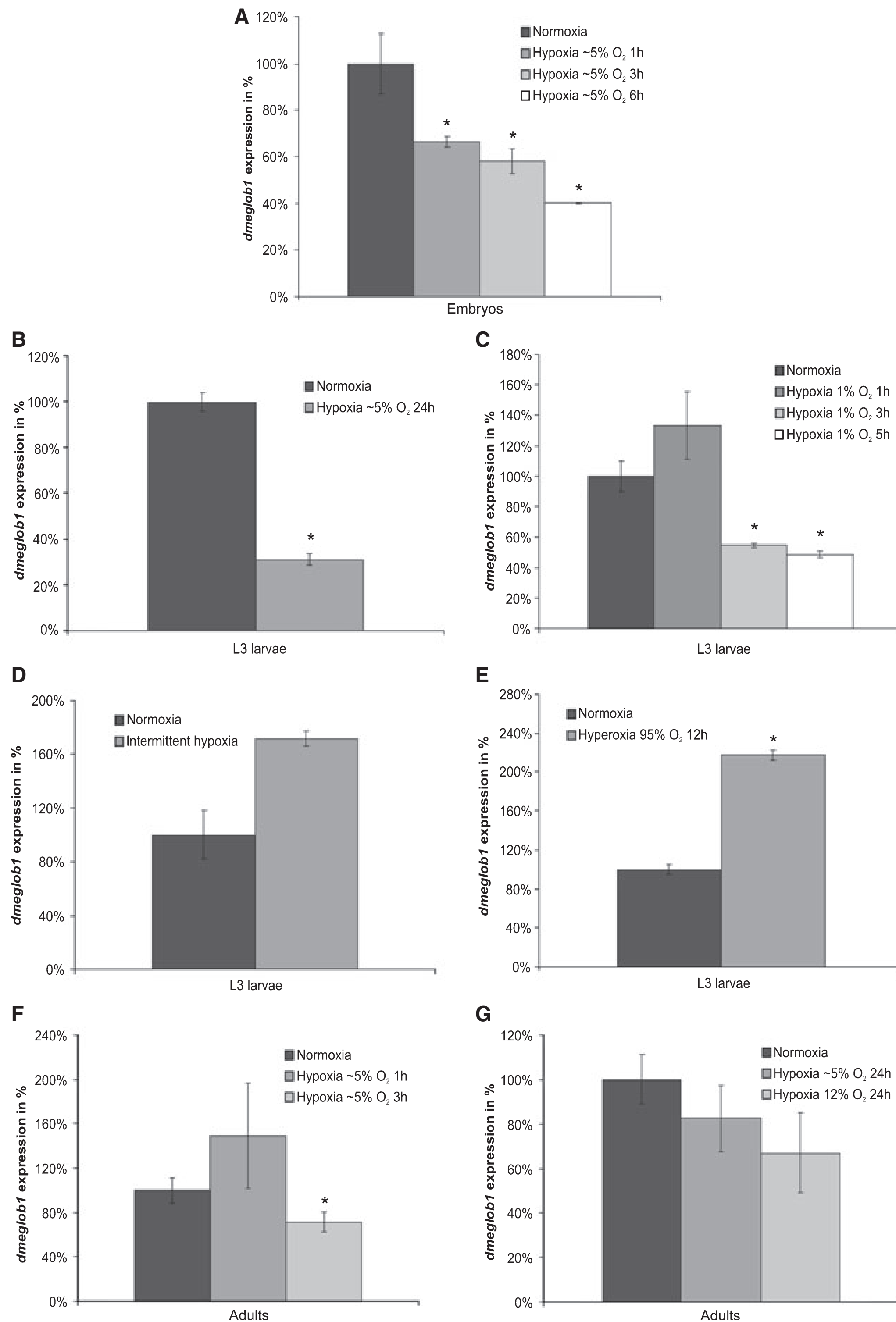

Fig. 1. Regulation of *dmeglob1* mRNA in *Drosophila melanogaster* developmental stages after hypoxia and hyperoxia stress. mRNA levels (bars) are shown relative to gene expression at normoxia (21%). The applied O_2 concentrations, exposure times and developmental stages are indicated. (A) Embryos, pooled stages, ~ 5% O_2 for 1, 3 and 6 h. (B) Third instar larvae, ~ 5% O_2 for 24 h. (C) Third instar larvae, 1% O_2 for 1, 3 and 5 h. (D) Third instar larvae, ~ 5% O_2 for 20 min alternating with 21% O_2 for 20 min, repeated three times. (E) Third instar larvae, 95% O_2 for 12 h. (F) adult flies, ~ 5% O_2 for 1 and 3 h. (G) Adult flies ~ 5% O_2 for 24 h and 12% O_2 for 24 h. *P < 0.05.

dmeglob1 mRNA levels to increase to $\sim 120\%$ compared with the respective normoxic control (Fig. 1E). Larvae exposed to hyperoxia showed normal behavior throughout the treatment.

Globin expression levels in adult flies under hypoxia

We applied both, long- and short-term moderate hypoxia regimes to adult flies. After 1 h at 5% O_2, *dmeglob1* mRNA levels first increased slightly by $\sim 50\%$, then declined to $\sim 70\%$ after 3 h compared to the normoxic control (Fig. 1F). Long-term moderate and mild hypoxia regimes were carried out for 24 h, applying 5 and 12% O_2, respectively. Here, we observed a tendency towards a slight downregulation of *dmeglob1* mRNA expression (Fig. 1G). During the entire hypoxia treatment, adult flies maintained normal behavior, apart from slightly decelerated movements.

Quantification of LDH expression as control for hypoxia

To confirm the observed changes in *dmeglob1* expression levels under hypoxia, we used *LDH* as a positive control for hypoxia-induced changes in gene expression. *LDH* expression in *Drosophila* cell culture is upregulated eightfold under O_2 deprivation (1% O_2) via the hypoxia-inducible factor 1 (HIF-1) pathway 2 [27].

Moderate, long-term hypoxia ($\sim$ 5% O_2 for 24 h) was applied to third instar larvae. We observed an increase in *LDH* mRNA levels in third instar larvae of $\sim$ 1.8-fold compared with the respective normoxic control (Fig. 2A). In larvae kept under severe, short-term hypoxia (1% O_2 for 1, 3 and 5 h) no alteration in *LDH* mRNA levels could be detected (Fig. 2B).

The intermittent hypoxia conditions, which were applied to third instar larvae caused the *LDH* mRNA levels to increase 2.95-fold compared with the respective normoxic control (Fig. 2C).

In adult flies, a 2.5-fold increase in *LDH* mRNA could be observed after 5% O_2 for 1 and 3 h (Fig. 2D). Long-term moderate to mild hypoxia regimes (5 and 12% O_2) were applied for 24 h, but no substantial changes of *LDH* mRNA levels could be detected after these prolonged exposures (Fig. 2E).

Discussion

Hypoxia-tolerance in insects

Drosophila and other insects have been shown to be surprisingly hypoxia resistant [4,6,7,28]. Genetic screens [6,29], differential gene expression analyses [13] and, very recently, experimental selection [8] have identified a number of genes involved in *Drosophila* hypoxia resistance. These include well-known candidates like antioxidant defense genes and electron transport genes, but also genes with widely disparate cellular functions. However, to date, none of these studies has listed *dmeglob1* as a primary gene candidate. This might be partly due to the observed decrease in *dmeglob1* expression under hypoxia, as analysis and interpretation of these studies appear to focus on genes showing upregulation under hypoxia.

As part of a metabolic transcriptional response to hypoxia, Gorr *et al.* [27] observed an eightfold increased expression of *LDH* in cell culture (SL2 cells), which is an enzyme that regenerates NAD^+ from NADH in the absence of O_2 by reducing pyruvate to lactate. Microarray data reported a 5- and 3.6-fold upregulation of *LDH* in *Drosophila* adults after 0.5 and 5% O_2 for 6 h, respectively [13]. Similar observations were reported for *LDH* gene regulation in other species [30]. In our study we could confirm a significant increase in *LDH* mRNA levels under hypoxia. Therefore, *LDH* can be used as a positive control to monitor hypoxia at the mRNA level in *Drosophila*.

Hemoglobins may confer hypoxia-tolerance to arthropods

The massive occurrence of Hb in insect species such as *Chironomus*, *Gasterophilus* and aquatic Hemiptera [19] can be easily associated with their hypoxic lifestyle. There is little doubt that these 'classical' insect Hbs enhance the availability of O_2 to the cells, either by facilitating O_2 extraction from the low-oxygen environment, by enhancing O_2 diffusion to the metabolically active organs, or by storing O_2 for hypoxic periods. Temporary induction of Hb synthesis upon hypoxia has been reported in the mud-dwelling, aquatic larvae of chironomid midges and in some brachiopod crustaceans [19,31]. The presence of Hb in *D. melanogaster* [22–24] and other insects [32,33] was unprecedented because, at first glance, these species appear to live under normal oxygen conditions throughout their life cycle. However, it should be considered that, especially during larval stages, *Drosophila* has to compete for O_2 with aerobic bacteria and fungi [7]. At this developmental stage, local O_2 levels may therefore be quite different from those available to the adult fly. In the context of hypoxia adaptation, the presence of a Hb, which enhances O_2 availability, might in fact be

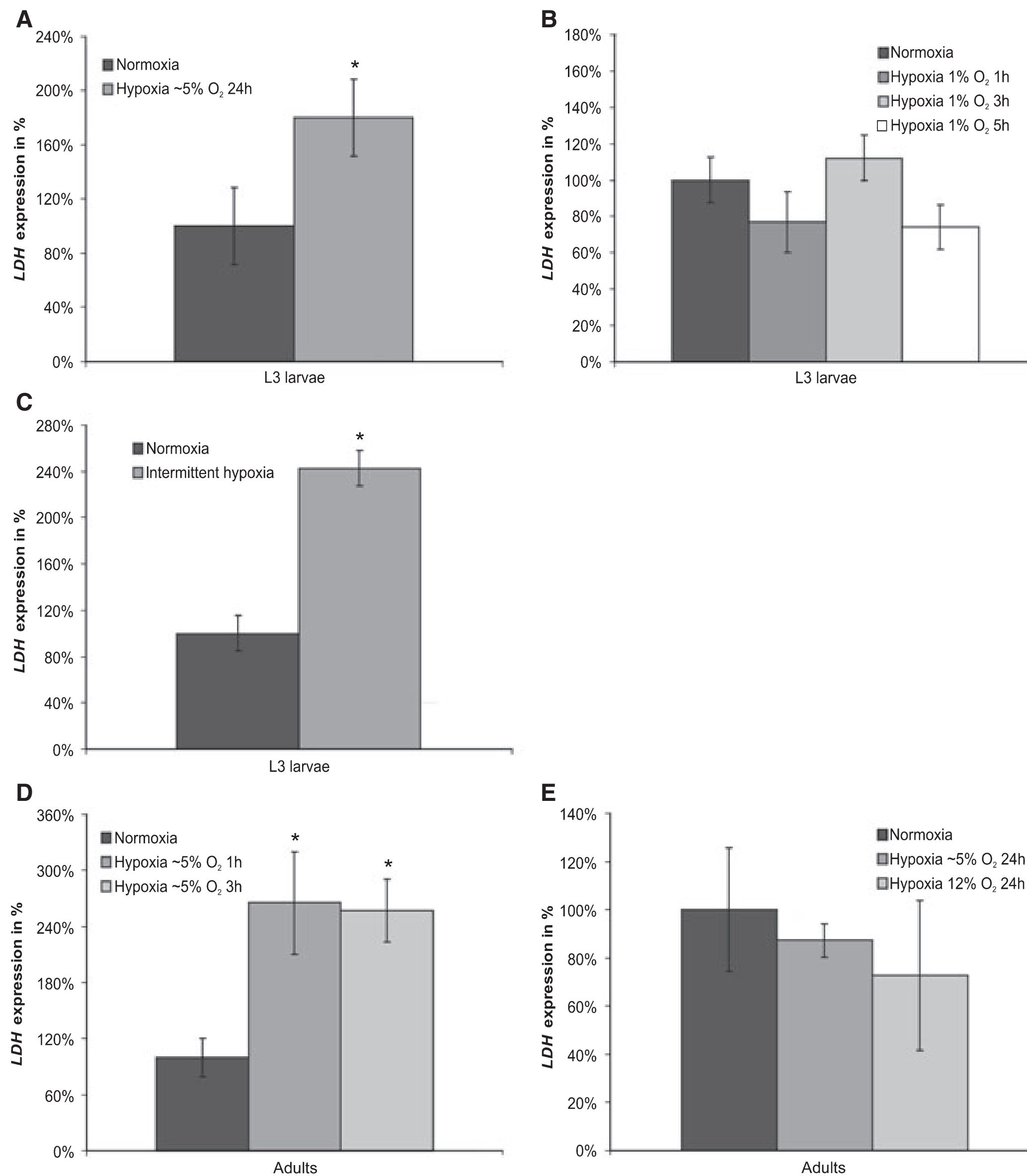

Fig. 2. Regulation of *LDH* mRNA in *Drosophila melanogaster* developmental stages after hypoxia stress. mRNA levels (bars) are shown relative to the gene expression at normoxia (21%). The applied O_2 concentrations, exposure times and developmental stages are indicated. (A) Third instar larvae, ~ 5% O_2 for 24 h. (B) Third instar larvae, 1% O_2 for 1, 3 and 5 h. (C) Third instar larvae, ~ 5% O_2 for 20 min alternating with 21% O_2 for 20 min, repeated three times. (D) Adult flies, ~ 5% O_2 for 1 and 3 h. (E) Adult flies ~ 5% O_2 for 24 h and 12% O_2 for 24 h. $*P < 0.05$.

advantageous, at least during certain developmental stages. The observation that *Drosophila* dmeglob1 protein exhibits ligand-binding properties and expression patterns that resemble those of other known insect globins has actually suggested a common, conserved function of the intracellular Hbs in O_2 supply [23]. However, our data on gene regulation under stress render this hypothesis rather unlikely, and it remains to be shown whether additional *dmeglob1* really confers increased hypoxia tolerance to *Drosophila*.

Dmeglob1 is downregulated under hypoxia, but upregulated under hyperoxia

Given the fact that increased levels of Hb under hypoxia have been observed, for example, in *Chirono-*

mus [34] and the crustacean *Daphnia magna* [31,35], one might assume that low-oxygen conditions also trigger an enhanced expression of *dmeglob1*. However, we have shown that hypoxia causes a decrease in *dmeglob1* mRNA levels in *Drosophila* embryos, larvae and adults. These results are in line with observations made by Gorr *et al.* [27], who demonstrated that in the *Drosophila* cell line SL2 hypoxia (16 h at 1% O_2) induces a downregulation of *dmeglob1* mRNA to ~ 15–20% compared with normoxia. In general, the changes we observed *in vivo* are less pronounced, possibly owing to the less stringent hypoxia regimes we applied.

Although the HIF signaling cascade is known to induce the expression of various genes involved in hypoxia tolerance [36], it has only recently become evident that mammalian HIF-1 and its *Drosophila* orthologs Sima/Arnt may also mediate the downregulation of certain target genes [27,37,38]. In fact, *dmeglob1* harbors several putative hypoxia response elements [23,27], of which some are conserved among distantly related *Drosophila* species [24]. It is, however, unknown which of the HRE motifs actually function in hypoxia-mediated downregulation.

In contrast to continuous short- or long-term hypoxia, the application of an intermittent hypoxia/normoxia regime and the exposure to elevated levels of O_2 both triggered an increase in *dmeglob1* mRNA by 1.7–2.2-fold in *Drosophila* larvae, which probably meet heavily fluctuating O_2 conditions *in vivo*. In agreement with our measurements, microarray data show a 2.3-fold upregulation of *dmeglob1* in *Drosophila* adults kept at 100% O_2 for 7 days [18], and a 2.2-fold increase after keeping adult males on the herbicide paraquat [17]. Because all these experimental conditions are known to produce oxidative stress via ROS, we interpret *dmeglob1* function in this context.

Implications for Drosophila hemoglobin function

Based on the predominant expression in the tracheal system we previously speculated that the presence of *dmeglob1* may facilitate O_2 diffusion across the tracheal walls [23]. However, this role may be considered unlikely because one would expect increased *dmeglob1* expression when O_2 availability is limited, and, in contrast, decreased expression at higher O_2 levels. In fact, we observed the opposite scenario. Thus, the actual pattern of O_2-dependent regulation of *dmeglob1* is not consistent with a simple myoglobin-like O_2-supply function of the protein. By contrast, the mRNA expression data are more compatible with the idea that dmeglob1 is involved in the protection from toxic ROS, which may damage proteins, DNA and lipids [39]. In recent years, ROS have been recognized as a major threat for cell survival, and toxic ROS effects have been attributed to aging and cell death [40,41]. The O_2 diffused via the tracheae is a potent source of ROS. Recently, it has been suggested that the insect tracheal system is well-adapted for efficient O_2 supply, but, under certain conditions, insects are forced to protect their inner cells from an excess of O_2 and thus ROS [14,15]. Therefore, it is certainly advantageous to keep cellular O_2 levels as high as necessary to mediate mitochondrial respiration, but as low as possible in order to minimise oxidative damage.

There are two conceivable hypotheses how dmeglob1 may be involved in such scenario. On the one hand, dmeglob1 may be directly involved in the enzymatic decomposition of ROS. Although at the moment we do not know any ROS-degrading enzyme reaction that dmeglob1 may carry out or in which it may be involved, a role of certain globins in ROS protection has repeatedly been proposed [42,43]. The fact that a hypoxia–normoxia regime also increases *dmeglob1* levels is fully compatible with this hypothesis, because reperfusion is known to enhance ROS production [44]. On the other hand, *Drosophila* dmeglob1 may serve as a buffer that does not facilitate but actually hampers O_2 diffusion from the tracheal air to the O_2-consuming cells. Such function may easily be associated with the observed gene regulation of *dmeglob1*: an excess of O_2 (hyperoxia) causes the increase in the putative buffer, whereas less O_2 brings about a decrease in the buffer capacity. Given the chief expression of *dmeglob1* in the tracheoles and tracheal terminal cells, we consider the latter scenario more likely at the moment.

Experimental procedures

Animals, hypoxia and hyperoxia regimes

Drosophila melanogaster wild-type strain Oregon R was maintained at 25 °C on standard yeast–soybean meal medium. We tested embryos (pooled, stages 0–17), third instar larvae (L3) and adult flies. Generally, approximately 25 larvae and adults were exposed to hypoxia/hyperoxia at 25 °C. In the Mainz laboratory, animals (larvae, adults) were kept in a hypoxia chamber (PRO-OX 110; BioSpherix Ltd, New York, NY, USA) at 25 °C at a given pre-adjusted O_2 concentration. Technical nitrogen and oxygen were obtained from Westfalen AG (Münster, Germany). The desired O_2 concentrations were obtained by mixing nitrogen with ambient air (hypoxia conditions) or by supplying pure oxygen (hyperoxia conditions) to the gas chamber. Gas concentrations were measured and kept constant by an oxygen

sensor (E-702; BioSpherix). During long-term hypoxia treatments larvae were prevented from desiccation by placing water-filled Petri dishes in the hypoxia chamber. In the Göttingen laboratory, a cell-culture chamber equipped with an oxygen sensor (Binder, CB 150, Tuttlingen, Germany) was used to treat embryos. After the desired time, animals were immediately collected and shock-frozen in liquid N_2. Tissues were stored at −80 °C until use.

Hypoxia conditions tested included moderate hypoxia (at 5 ± 1% O_2, depending on the hypoxia device used), short-term, severe hypoxia (at 1% O_2) and intermittent hypoxia (5% O_2 for 20 min alternating with 21% O_2 for 20 min, repeated three times). Severe hyperoxia was administered by exposure to 95% O_2. During hypoxia/hyperoxia treatments in the translucent PRO-OX chamber, animals were checked for vitality and the occurrence of phenotypic reactions, known to be caused by the applied O_2 concentrations [7].

RNA extraction

Total RNA from embryos and adult flies was extracted from samples of ~ 30 mg, employing the RNeasy Mini Kit by Qiagen (Hilden, Germany) according to the manufacturer's instructions. Total RNA from L3 larvae was extracted employing the SV Total RNA Isolation Kit by Promega (Mannheim, Germany) according to the manufacturer's instructions. RNA was eluted from the silica columns with DEPC-treated water. DNA contaminations were removed by 30 min incubation at 37 °C with RNase-free DNase I (Fermentas, St Leon-Rot, Germany). The quality and integrity of RNA was evaluated by reading the absorption ratio at 260 versus 280 nm and by agarose gel electrophoresis.

Quantitative real-time RT-PCR

For embryos and adult flies, reverse transcription was carried out with 500 ng total RNA per 20 µL reaction employing the Superscript II RNase H^- reverse transcriptase (Invitrogen, Karlsruhe, Germany) and an oligo-$(dT)_{18}$-primer (Biomers, Ulm, Germany). The real-time RT-PCR experiments were performed with an ABI Prism 7000 SDS (Applied Biosystems, Darmstadt, Germany). In each PCR we used the amount of cDNA equivalent to 50 ng of total RNA in a 20 µL reaction containing SYBR Green (*Power* SYBR Green PCR Master Mix, Applied Biosystems). We used the following oligonucleotide primer combinations: *dmeglob1*, 5′-GCTCAACTTGGAGAAGTTCC-3′ and 5′-TCGTCCAGCTTCTCCAGATC-3′; *L17A*, 5′-TAACCAGTCCGCGAGCAGC-3′ and 5′-AATAACCACGGCAGGCATGAC-3′; *LDH*, 5′-CTAACAGATCCATTCGCAACACC-3′ and 5′-ACTTGATGCTACGATTCGTGG-3′. The final primer concentrations during PCR were 0.19 µM each. After activation of the polymerase at 95 °C for 15 min, amplification was performed in a four-step protocol: 94 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s and 76.8 °C for 30 s for 40 cycles, measuring the fluorescence during the last step of each cycle. During the analysis of the larval stage, different oligonucleotide primer combinations were used, showing slightly improved PCR efficiencies: *dmeglob1*, 5′-GGAGCTAAGTGGAAATGCTCG-3′ and 5′-GCGGAATGTGACTAACGGCA-3′; *RPL17A*, 5′-TCGAAGAGAGGACGTGGAG-3′ and 5′-AACATGTCGCCGACACCAG-3′; *LDH*, 5′-CAAGCTGGTAGAGTACAGTCC-3′ and 5′-GACATCAGGAAGCGGAAGC-3′. Here, final primer concentrations were 0.4 µM each. All PCR experiments were followed by dissociation curves at a temperature range of 60–92 °C to analyze the specificity of the amplification reactions. No unspecific products or primer dimers were detected by melting curve analysis and gel electrophoresis of PCR amplificates.

Data analysis

Dmeglob1 and *LDH* expression levels were calculated by the standard-curve approach, measuring Ct-values. Data were normalized relative to expression of the ribosomal protein gene *L17A*a, which is unregulated according to microarray experiments (B. Adryan and R. Schuh, unpublished results). Factors of differential gene regulation were calculated relative to the normoxic condition (21% O_2). Statistical evaluation was performed by calculating the mean value of the factors of regulation and their standard deviation. Two independent experiments (biological replicates) were performed for each condition, and each assay was run in duplicate. The significance of the data was assessed by a two-tailed Student's *t*-test employing the Microsoft EXCEL spreadsheet program.

Acknowledgements

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