Oxygen-induced changes in hemoglobin expression in
Drosophila

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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 protu-
berances can even enter cells and reach the mitochon-
dria 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 Gasterophi-
lus 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 O₂ [3].
The adult house fly (Musca domestica) survives 12–15 h
without O₂ and recovers completely when re-oxygen-
ated [4]. Drosophila melanogaster displays a remarkable
resistance to hypoxia and anoxia as well. Embryonic,
larval and adult Drosophila react to short-term O₂
deprivation by behavioral changes including paralysis,
but recover completely when re-oxygenated [5–7]. Pro-
longed exposure to 6% O₂, 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 oxy-
gen-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]. How-
ever, insects also seek to avoid cellular stress by an
excess amount of tracheal O₂ (hyperoxia), which may
generate noxious reactive oxygen species (ROS), for
example, by a special rhythmic ventilatory behavior like

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% O₂) con-
trol. By contrast, a hypoxia/reoxygenation regime applied to third instar
larvae elevated the level of dmeglob1 mRNA. An excess of O₂ (hyperoxia)
also triggered an increase in dmeglob1 mRNA. The data suggest that
Drosophila hemoglobin may be unlikely to function merely as a myoglobin-
like O₂ storage protein. Rather, dmeglob1 may protect the fly from an
excess of O₂, either by buffering the flux of O₂ from the tracheoles to the
cells or by degrading noxious reactive oxygen species.

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% O₂ reduces fly longevity by half [16]. Microarray analyses of Drosophila adults treated with 100% O₂ 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 O₂, thereby enhancing their metabolic performance under low oxygen conditions [19]. Because of the highly efficient O₂ 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 (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 O₂ 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, dmeglob2 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 O₂ 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 O₂ 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 O₂ concentrations and exposure times relative to animals kept at normoxia (21% O₂), 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% O₂). 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 the respective 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% O₂ 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% O₂ 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% O₂, subsequently returning them for 20 min to 21% O₂ before RNA extraction. These intermittent hypoxia conditions, repeated three times, caused dmeglob1 mRNA expression to increase by ~70% compared with the respective 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% O₂ for 12 h), caused the
Fig. 1. Regulation of \(dmeglob1\) mRNA in \textit{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, \(\sim 5\%\) O\(_2\) for 1, 3 and 6 h. (B) Third instar larvae, \(\sim 5\%\) O\(_2\) for 24 h. (C) Third instar larvae, \(1\%\) O\(_2\) for 1, 3 and 5 h. (D) Third instar larvae, \(\sim 5\%\) 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, \(\sim 5\%\) O\(_2\) for 1 and 3 h. (G) Adult flies \(\sim 5\%\) O\(_2\) for 24 h and 12\% O\(_2\) for 24 h. \(* P < 0.05.\)
Hemoglobin expression in *Drosophila*

*dmeglob1* mRNA levels to increase to ~120% compared with the respective normoxic control (Fig. 1E). Larvae exposed to hyperoxic 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% O₂, *dmeglob1* mRNA levels first increased slightly by ~50%, then declined to ~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% O₂, 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 O₂ deprivation (1% O₂) via the hypoxia-inducible factor 1 (HIF-1) pathway 2 [27].

Moderate, long-term hypoxia (~5% O₂ for 24 h) was applied to third instar larvae. We observed an increase in *LDH* mRNA levels in third instar larvae of ~1.8-fold compared with the respective normoxic control (Fig. 2A). In larvae kept under severe, short-term hypoxia (1% O₂ 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% O₂ for 1 and 3 h (Fig. 2D). Long-term moderate to mild hypoxia regimes (5 and 12% O₂) 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 O₂ 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% O₂ 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 O₂ to the cells, either by facilitating O₂ extraction from the low-oxygen environment, by enhancing O₂ diffusion to the metabolically active organs, or by storing O₂ 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 O₂ with aerobic bacteria and fungi [7]. At this developmental stage, local O₂ 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 O₂ availability, might in fact be
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 O2 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-
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 controller.
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, Tuttingen, Germany) was used to treat embryos. After the desired time, animals were immediately collected and shock-frozen in liquid N₂. Tissues were stored at −80 °C until use.

Hypoxia conditions tested included moderate hypoxia (at 5 ± 1% O₂, depending on the hypoxia device used), short-term, severe hypoxia (at 1% O₂) and intermittent hypoxia (5% O₂ for 20 min alternating with 21% O₂ for 20 min, repeated three times). Severe hyperoxia was administered by exposure to 95% O₂. 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 O₂ 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’-GAGCTAAGTGGAATGCTCC-3’ and 5’-GCGGAAT GTGACTAACGGCA-3’; RPL17A, 5’-TCGAGAAGAGG ACCTGGAG-3’ and 5’-AACATGTCGCCGACACCAG -3’; LDH, 5’-CAAGCCTGCTAGGATCAGTCC-3’ and 5’- GACATCGAAGCGGAAGC-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 L17Aa, 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% O₂). 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.

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**References**


