Insect Biochemistry and Molecular Biology 72 (2016) 20–30

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

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

Knockdown of *Drosophila* hemoglobin suggests a role in O_2 homeostasis

Eva Gleixner [a], Fabian Ripp [a], Thomas A. Gorr [b, c], Reinhard Schuh [d], Christian Wolf [a], Thorsten Burmester [e, *], Thomas Hankeln [a, **]

[a] Institute of Molecular Genetics, University of Mainz, D-55099 Mainz, Germany
[b] Institute of Veterinary Physiology, Vetsuisse Faculty, University of Zurich, CH-8057 Zurich, Switzerland
[c] Regenerative Medicine Program, University and University Hospital Zurich, CH-8901 Zurich, Switzerland
[d] Max Planck Institute for Biophysical Chemistry, D-37077 Göttingen, Germany
[e] Institute of Zoology and Zoological Museum, Biocenter Grindel, University of Hamburg, D-20146 Hamburg, Germany

ARTICLE INFO

Article history:
Received 21 December 2015
Received in revised form
11 March 2016
Accepted 16 March 2016
Available online 18 March 2016

Keywords:
Hemoglobin
Oxygen
Hypoxia
Tracheae
Respiration
Reactive oxygen species

ABSTRACT

Almost all insects are equipped with a tracheal system, which appears to be sufficient for O_2 supply even in phases of high metabolic activity. Therefore, with the exception of a few species dwelling in hypoxic habitats, specialized respiratory proteins had been considered unnecessary in insects. The recent discovery and apparently universal presence of intracellular hemoglobins in insects has remained functionally unexplained. The fruitfly *Drosophila melanogaster* harbors three different globin genes (referred to as *glob1-3*). Glob1 is the most highly expressed globin and essentially occurs in the tracheal system and the fat body. To better understand the functions of insect globins, the levels of *glob1* were modulated in *Drosophila* larvae and adults by RNAi-mediated knockdown and transgenic over-expression. No effects on the development were observed in flies with manipulated *glob1* levels. However, the knockdown of *glob1* led to a significantly reduced survival rate of adult flies under hypoxia (5% and 1.5% O_2). Surprisingly, the *glob1* knockdown flies also displayed increased resistance towards the reactive oxygen species-forming agent paraquat, which may be explained by a restricted availability of O_2 resulting in decreased formation of harmful O_2^-. In summary, our results suggest an important functional role of glob1 in O_2 homeostasis, possibly by enhancing O_2 supply.

1. Introduction

Respiration in insects is usually sustained by a well-developed tracheal system that mainly functions by passive diffusion to supply the inner organs with sufficient O_2 (Kestler, 1985). In highly active tissues, such as the flight muscle, the terminal tips (tracheoles) may reach nearly every cell (Guillemin et al., 1996; Jarecki et al., 1999). While the tracheal system is crucial for O_2 delivery during phases of high activity, it also bears the risk of causing oxidative damage. O_2 partial pressures in the inner organs may be in the range of ~19 kPa, which is close to the atmospheric O_2 level (Hetz and Bradley, 2005) and much higher than the O_2 concentration in typical mammalian cells (Burmester, 2005). Therefore, for example, adult *Drosophila* open their spiracles during phases of high activity to allow continuous O_2 flow, but close them when the O_2 demand is low (Heymann and Lehmann, 2006; Lehmann, 2001).

Many insects are remarkably tolerant towards low O_2 conditions (Wegener, 1993). Adult *Drosophila* survives anoxic periods for hours without observable tissue damage (Haddad, 2006; Haddad et al., 1997b; Wingrove and O'Farrell, 1999) and even reproduce and develop with less than 8% O_2 (Zhou et al., 2007). Late-stage embryos and larvae withstand even day-long exposures to hypoxia (Wingrove and O'Farrell, 1999). This tolerance may be explained by

Abbreviations: da, daughterless; CDR, driver control flies (*da*-GAL4/w1118); CKD, knockdown control flies (RNAi-*glob1*/w1118); CNS, central nervous system; COE, overexpression control flies (UAS-*glob1*/w1118) flies; DIC, differential interference contrast; KD, glob1 knockdown flies (*da*-GAL4/RNAi-*glob1*); LPO, lipid peroxidation; OE, glob1 overexpressing flies (*da*-GAL4/UAS-*glob1*); qPCR, quantitative realtime reverse transcription polymerase chain reaction; ROS, reactive oxygen species; TUNEL, TdT-mediated dUTP-biotin nick end labeling.

* Corresponding author. Institute of Molecular Genetics, Johannes Gutenberg University Mainz, Johann Joachim Becher-Weg 30A, D-55128 Mainz, Germany.
** Corresponding author. Institute of Zoology, University Hamburg, Martin-Luther-King-Platz 3, D-20146 Hamburg, Germany
E-mail addresses: thorsten.burmester@uni-hamburg.de (T. Burmester), hankeln@uni-mainz.de (T. Hankeln).

http://dx.doi.org/10.1016/j.ibmb.2016.03.004
0965-1748/

the natural environment of *Drosophila* embryos and larvae, which dwell in fermenting fruits where they compete for O_2 with microorganisms. Adaptations of *Drosophila* to moderate and severe hypoxia (reviewed by Gorr et al., 2006) include a) reduced physical activity, loss of coordination and, eventually, emergence of a stupor-called state of complete immobility (Chadwick and Gilmour, 1940; Csik, 1939); b) fibroblast growth factor (FGF) and FGFR-driven ramification of tracheal terminal cells for improved O_2 diffusion capacity (Centanin et al., 2008, 2010; Jarecki et al., 1999); c) switch to reduced O_2-consumption rates (Haddad, 2000; Haddad et al., 1997b); d) onset of anaerobiosis (Grieshaber et al., 1994; Wegener, 1993); e) cell cycle arrest at G1/S and mitotic checkpoints (Lavista-Llanos et al., 2002; Wingrove and O'Farrell, 1999) and f) general, yet reversible chromatin condensation in fly embryos (Foe and Alberts, 1985). The genetic basis of the striking hypoxia tolerance of *Drosophila* has not been fully unraveled yet. Liu et al. (2006) found that genes from diverse stress-related pathways are upregulated by hypoxia and are possibly required for survival and recovery from low oxygen partial pressures (pO_2). Zhou et al (Zhou et al., 2008, 2011). identified the hypoxia-related signaling pathways and found hypoxia-induced down-regulation of genes regulating cell respiration and energy metabolism. On the other hand, *Drosophila* does not tolerate prolonged exposure to severe hyperoxic experimental conditions. At 95–100% O_2 a rapid decrease in the survival rate of adult flies was reported (Gruenewald et al., 2009; Walker and Benzer, 2004). The deleterious effects of hyperoxia are due to oxidative damage to proteins, nucleic acids and membranes, caused by an excess of O_2 overwhelming the antioxidant system (Walker and Benzer, 2004).

In many vertebrate and invertebrate species, O_2 supply is sustained by hemoglobin and myoglobin (Burmester and Hankeln, 2014; Weber and Vinogradov, 2001; Wittenberg and Wittenberg, 2003). In insects, however, the presence of respiratory proteins has long been thought to be limited to those few species living in persistently hypoxic environments (Law and Wells, 1989; Locke, 1997; Willmer et al., 2000). Prominent examples are the aquatic larvae of chironomid midges, the horse botfly *Gasterophilus intestinalis* and the backswimmers (Burmester and Hankeln, 2007; Wawrowski et al., 2012; Weber and Vinogradov, 2001). However, commencing with the discovery of a typical globin gene in *Drosophila melanogaster* (Burmester and Hankeln, 1999), globins have been identified in insects that live in normoxic environments, such as the honeybee *Apis mellifera* (Hankeln et al., 2006), the mosquitoes *Anopheles gambiae* and *Aedes aegypti* (Burmester et al., 2007), and the lepidopterans *Bombyx mori* and *Samia cynthia* (Kawaoka et al., 2009). These findings led to the conclusion that globins actually belong to the standard repertoire of insects and have an essential role in insect physiology (Burmester and Hankeln, 2007).

D. melanogaster harbors three distinct globin genes referred to as *glob1*, *glob2*, and *glob3* (Burmester and Hankeln, 1999; Burmester et al., 2006). *Glob2* and *glob3* are *Drosophila*-specific paralogs that are preferentially expressed at low levels in the testis (Gleixner et al., 2012). Little is known about their physiological role, which may be associated with alleviating oxidative stress during spermatogenesis. The conservation of the amino acids crucial for heme- and O_2 binding indicates functional globin proteins (Burmester et al., 2006). In contrast, *glob1* is expressed in relatively high amounts mainly in the tracheal system and fat body of embryos, larvae, and adults (Hankeln et al., 2002; Yadav et al., 2015). The glob1 protein displays a typical 3-over-3 α-helical sandwich structure, exhibits a hexacoordinate binding scheme in its deoxygenated state and binds O_2 reversibly with an affinity of $P50 = 0.12$ Torr, which is in the range of other insect globins (de Sanctis et al., 2005; Hankeln et al., 2002). The physiological function of glob1 is still uncertain. Its close evolutionary relationship, particularly to the O_2-storing globin of *G. intestinalis* (Burmester and Hankeln, 1999; Burmester et al., 2006), its O_2-binding kinetics and its prominent expression in the tracheal system (Hankeln et al., 2002), suggest a role of glob1 in O_2 supply. However, studies with *D. melanogaster* Schneider S2 cells (Gorr et al., 2004) and *D. melanogaster* embryos, larvae and adults (Gleixner et al., 2008) reveal a significant down-regulation of *glob1* mRNA by hypoxia, which is probably mediated by the hypoxia-inducible factor signaling pathway. By contrast, an excess of O_2 (hyperoxia) causes an increase of *glob1* transcripts in whole flies (Landis et al., 2004), adult brains (Gruenewald et al., 2009) and larvae (Gleixner et al., 2008). Thus a role of glob1 in the detoxification of reactive species (ROS), as proposed for vertebrate globins (see e.g. Flögel et al., 2008; Hendgen-Cotta et al., 2010), appears conceivable.

To better understand the function of *D. melanogaster* glob1, which is probably representative for other intracellular globins of insects (Burmester and Hankeln, 2007), we have modulated its expression by transgenic RNAi and overexpression, and analyzed the response of the flies towards changing O_2 conditions and oxidative stress. The results support the hypothesis of glob1 conferring an important homeostatic balance in insect respiration.

2. Material and methods

2.1. Fly strains

The UAS/Gal4-system (Brand and Perrimon, 1993) was used for the modulation of *glob1* expression. The responder strain for RNAi-mediated *glob1* knockdown was obtained from the Vienna *Drosophila* RNAi Center (transformant ID 101830). Transgenic flies for over-expression of *glob1* were generated by microinjection of a pUAST vector containing the *glob1* cDNA sequence. As a driver line for both RNAi-mediated knockdown and glob1 over-expression, *daughterless* Gal4 [w [1118]; P{*da*-GAL4.w [-]}] obtained from Bloomington was used. Control flies for experiments were generated by performing crosses between homozygous driver (*da*-GAL4) or responder (RNAi-*glob1* or UAS-*glob1*) strains, and the corresponding genetic background w [1118]. Resulting control genotypes were hemizygous for either RNAi-*glob1*, UAS-*glob1* or *da*-GAL4 constructs, thus mimicking the genotypes of flies with altered *glob1* expression. For simplification, the genotypes were named as follows: OE, glob1 overexpressing flies (*da*-GAL4/UAS-*glob1*); KD, glob1 knockdown flies (*da*-GAL4/RNAi-*glob1*); CDR, driver control (*da*-GAL4/w1118); CKD, knockdown control (RNAi-*glob1*/w1118); COE, overexpression control (UAS-*glob1*/w1118).

2.2. Quantitative real-time reverse transcription PCR

Total RNA was isolated from 3rd instar larvae and sexed adult flies. 25 3rd instar larvae or adult flies were collected, cleaned from remaining food and shock frozen in liquid nitrogen. Total RNA was isolated with the RNeasy mini kit (Qiagen, Hilden, Germany), including a DNase I digestion step according to the manufacturer's instructions. Total RNA was quantified spectrophotometrically with a Nanodrop ND 100 UV–Vis spectrometer (Thermo Scientific, Bonn, Germany). RNA integrity was checked by denaturing formaldehyde gel-electrophoresis. For cDNA synthesis by SuperScript III reverse transcriptase (Invitrogen, Darmstadt, Germany), equal amounts (1 μg) of total RNA were used. Quantitative real-time reverse transcription PCR (qPCR) experiments were carried out on the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Darmstadt, Germany). We used the amount of cDNA equivalent to 25 ng of total RNA in a 10 μl PCR reaction containing SYBR Green

(Power SYBR Green PCR Master Mix, Applied Biosystems). The final primer concentrations during PCR were 0.33 μM each. The following oligonucleotide primer combinations were used to detect glob1 knockdown: *Dmegb1_down_for* 5′-CAG CGA TGA GGT GCA ACT GAT-3'; *Dmegb1_down_rev* 5′-GAC CAT GTC TAC GGA ATC ATC-3'. qPCR primers used for verification of glob1 over-expression were *Dmeglob1_up_for* 5′-GGA GCT AAG TGG AAA TGC TCG-3′ and *Dmegb1_up_rev* 5′-TGC CGT TAG TCA CAT TCC GC-3'. After activation of the DNA polymerase (AmpliTaq Gold, Invitrogen) at 95 °C for 15 min, amplification was performed in a three-step protocol: 94 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s, measuring the fluorescence during the last step of each cycle. No unspecific products or primer dimers were detected by melting curve analysis and gel electrophoresis of PCR amplificates. mRNA expression levels were calculated by the standard-curve approach, measuring Ct-values. Normalization was done according to the total amount of RNA (Bustin, 2002). Factors of differential gene expression in double transgenic flies were calculated relative to the corresponding control fly lines. Two independent experiments (biological replicates) were performed for each condition, and each qPCR assay was performed in triplicate (technical replicates). The significance of the data was assessed by a two-tailed Student's *t*-test employing the Microsoft Excel spreadsheet program.

2.3. Western Blotting

Total protein extracts were obtained by homogenizing ~25 3rd instar larvae or adult, sexed flies in heated RIPA lysis buffer (10 mM Tris−HCl, 1 mM $CaCl_2$, 150 mM NaCl, 10 mM NaF, 25 mM β-glycerophosphate, 0.5% NP40, 0.5% deoxycholate, 0.1% SDS, pH 7.4). Debris was removed by centrifugation and protein concentration was determined by a Bradford assay. 30 μg total protein extracts were diluted in 2x Laemmli buffer, separated by SDS-PAGE and transferred to a PVDF membrane for 1 h at 100 V using the Mini Trans-Blot Electrophoretic Transfer Cell (BioRad, Munich, Germany). PVDF membranes were blocked in 5% non-fat dry milk and incubated with rabbit anti-glob1 antibodies (Hankeln et al., 2002) diluted 1:100 in PBS-T (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, 0.1% Tween-20) overnight at 4 °C. Membranes were subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (Biozym, Hessisch-Oldendorf, Germany; 541088) diluted 1:15,000 in PBS-T for 1 h at room temperature. Antibody binding was visualized by the ECL™ Western Blotting Detection Reagent (GE Healthcare, Freiburg, Germany) and exposure of the membrane to an X-ray film (Kodak BioMax). To confirm that equal amounts of protein were loaded in each lane, membrane were stripped, blocked and incubated with mouse monoclonal anti-actin antibodies (Sigma−Aldrich, Hamburg, Germany; A2228), diluted 1:2000 in PBS-T overnight at 4 °C. Secondary antibody incubation was done with horseradish peroxidase-conjugated sheep anti-mouse secondary antibodies (GE Healthcare, Freiburg, Germany; NA931-1ML) and signal detection were performed as described above. Quantification of Western Blot signals was performed with the program ImageJ (http://imagej.nih.gov/ij/) by measuring the area under the curve of the specific signals and the loading control.

2.4. Immunofluorescence studies

Brains were isolated from 3rd instar larvae that had been dissected in cold PBS and fixed in 4% paraformaldehyde/PBS for 30 min. The brains were washed in PBS +0.1% Triton X, blocked in blocking solution containing 0.5% cold-water fish gelatin (Sigma−Aldrich), 0.1% ovalbumin (Sigma−Aldrich) in PBS. Brains were incubated with anti-glob1 antibodies (Hankeln et al., 2002) (diluted 1:10 in PBS-T) overnight at 4 °C. Larval brains were washed with PBS-T and incubated with secondary antibodies conjugated to Alexa® 568 (Thermo Scientific, Schwerte, Germany; A-11011) diluted 1:400 in blocking solution for 2 h at room temperature in the dark. Washed brains were mounted in Mowiol 4.88 (Hoechst, Frankfurt, Germany) containing 2% n-propyl gallate. Light microscopy analyses of immunofluorescence were performed with a Leica DM 6000 B (Leica Microsystems, Bensheim, Germany) and images were processed with Adobe Photoshop CS (Adobe Systems, San Jose, CA, USA).

2.5. Hypoxia and hyperoxia treatment

Hypoxic and hyperoxic exposure conditions were obtained by using a translucent PRO-OX O_2 chamber (BioSpherix, Ltd., New York, USA). Technical O_2 and nitrogen were obtained from Westfalen AG (Münster, Germany). Gas concentrations were measured and kept constant by an O_2 sensor (E702, BioSpherix, Ltd., New York, USA). The constancy of temperature (22 °C) was regularly checked.

2.6. Phenotypic analyses

For lifespan determination, typically 180−200 newly eclosed flies per sex and genotype were collected, sexed and kept in batches of 50 flies per vial. Flies were kept under hypoxia (5% O_2) or hyperoxia (95% O_2) at 22 °C and were moved to vials containing fresh food every three days. Dead flies were scored every 24 h over a period of up to six weeks. To analyze the survival rates and times of young and old flies under severe hypoxic conditions, a total of 220−300 adult flies per genotype and sex were tested. Newly eclosed flies and flies aged for 21 days were collected, transferred to empty vials in batches of 50 flies and exposed to 1.5% O_2 for 6 h. Challenged flies became immobile shortly after hypoxia exposure (i.e. stupor). After hypoxic treatment, flies were exposed to normoxia. Recovery was recorded until 1 h after hypoxic treatment. Flies were counted as viable when they started climbing.

The tolerance of 3rd instar larvae to hypoxia was tested with 250 larvae from each genotype. Larvae were raised under non-crowding conditions and transferred to vials containing fresh food in batches of 50. Larvae were exposed to 1.5% O_2 for 7 h and were kept subsequently at normoxia until hatching. Emerging adult flies were counted. The recovery times from severe hypoxia were tested with 100 adult flies per genotype and sex. Flies were kept in batches of 20 in empty plastic vials and exposed to 1% O_2 for 2 h, following normoxia for recovery. Flies were scored as 'recovered' as soon as they started climbing.

The ability to tolerate complete O_2 deprivation (anoxia) was tested with a total of 100 adult flies per sex and genotype in batches of 20. Vials were capped with gauze and transferred into a sealed plastic box. Pure nitrogen, which had been bubbled through water, was flushed into the plastic box. The number of dropped flies was counted in 30 s intervals. Flies were scored as 'dropped' when they completely stopped moving.

For the analysis of developmental times, crosses were set up with 15 virgin females and 5 males each. Flies were allowed to settle for two days and were then moved to vials containing fresh food. Developmental times of progeny were recorded by observing the time it took for larvae to start wandering, pupate and finally hatch. To study the effect of hypoxia and hyperoxia, vials with embryos and newly hatched larvae were kept at either 5% or 95% O_2 at 22 °C. To prevent desiccation, water was added when necessary and a bowl filled with water was kept alongside in the O_2 chamber.

For analyzing the effects produced by exposure to ROS, adult flies (<3 days old) were kept in batches of 50 in plastic vials

containing filter paper soaked with 20 mM paraquat (Sigma–Aldrich) dissolved in 5% sucrose. Dead flies were counted three times a day over a period of four days. Larvae were kept in batches of 20–30 in plastic vials containing Formula 4-24 instant *Drosophila* medium (Carolina Biological Supply, USA) soaked with 20 mM paraquat in 5% sucrose. Larvae were placed directly in the mush and were regularly checked. The percentage of survivors was calculated by comparing the number of larvae to hatched adult flies.

2.7. Analyses of cell damage by ROS

Lipid peroxidation (LPO) measurement was performed with an LPO assay kit (Cayman Chemical, Ann Arbor, MI, USA), which detects ferric ions deriving from hydroperoxides reacting with ferrous ions. Larvae and flies were homogenized in 500 μl HPLC-grade water and centrifuged. Supernatant was deproteinized, and lipid hydroperoxides were extracted into degassed chloroform. For LPO measurement 50 μl FTS reagent 1 (4.5 mM ferrous sulfate in 0.2 M hydrochloric acid) and 50 μl FTS reagent 2 (3% methanolic solution of ammonium thiocyanate) were added. After incubation, absorbance at 500 nm was measured using a μQuant spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA). Each sample was measured in triplicate; LPO content in nmol was calculated relative to the amount of tissue used.

TdT-mediated dUTP-biotin nick end labeling (TUNEL) staining for observing apoptotic nuclei was performed with an *in situ* Cell Death Detection kit (Roche, Mannheim, Germany) which enzymatically labels free 3′OH-ends of fragmented DNA with fluorescein-labeled dUTPs that can be visualized by fluorescence microscopy. TUNEL was applied to 12 μm thick cryo-sections deriving from thoraces of female flies either reared under normoxia or treated with 20 mM paraquat for 24 h or 95% O_2 for 96 h. Cryo-sections were fixed with 4% paraformaldehyde, followed by TUNEL staining according to the manufacturer's instructions. Analyses of TUNEL-stained cryo-sections were performed with a Leica DM 6000 B (Leica Microsystems, Bensheim, Germany) and images were processed with Adobe Photoshop CS.

2.8. Statistical analyses

All experiments were repeated at least three times and diagrams represent pooled data with error bars indicated. Two-sided analysis of variance (ANOVA) with Bonferroni posthoc test was performed to compare survival of flies during hypoxia with 5% O_2, recovery times of flies after exposure to 1% hypoxia for 2 h and flies treated with paraquat. For statistical analyses of all other experiments, two-tailed Student's t-test was applied.

3. Results

3.1. Modulation of glob1 expression in transgenic flies

The levels of *glob1* mRNA and protein in *D. melanogaster* were modulated employing the UAS/Gal4-system (Brand and Perrimon, 1993). *Glob1* knockdown and over-expression from embryos to adults was achieved by the *daughterless* (*da*)-GAL4 driver line, which mediates ubiquitous gene expression including the fat body and the tracheal system (http://www.flyatlas.gla.ac.uk/). Knockdown and over-expression of glob1 mRNA and protein in the double-transgenic animals were confirmed on mRNA level by means of qPCR, and on the protein level by Western blot analysis (Fig. 1; see also Supplementary Fig. S1). Compared to the controls with the same genetic background, *glob1* transcript levels were reduced in the KD flies by ~98% in larvae and female adults (Fig. 1A, E), and by ~94% in male flies (Fig. 1C). The impaired expression of glob1 was confirmed by Western blotting, which showed a reduction of glob1 protein levels below the detection level (Fig. 1B, D, F).

Overexpression of *glob1* (OE) resulted in a 3.7–6.6-fold increase of *glob1* mRNA levels (Fig. 1A, C, E) and an about twofold (male flies), fourfold (females) and twofold (larvae) increase of glob1 protein levels (Fig. 1B, D, F) compared to the corresponding controls. The ubiquitous over-expression pattern of glob1 protein and its knockdown was further confirmed by immunofluorescence studies using anti-glob1 antibodies. The larval central nervous system (CNS) with ectopic glob1 overexpression showed particularly strong staining in both central brain hemispheres (Fig. 2, upper panel), reflecting the published expression pattern of the *daughterless* driver (Cronmiller and Cummings, 1993). In line with earlier *in situ* hybridization and immunostaining data (Hankeln et al., 2002; Yadav et al., 2015), the tracheoles surrounding the ventral ganglion were stained (Fig. 2, upper panel, arrowheads), which likely reflects endogenous glob1 expression, as also seen in the control strain (Fig. 2, middle panel). The CNS from knockdown animals did not show any glob1-signal, proving the functionality of the *da*-driver (Fig. 2, lower panel).

3.2. Effect of glob1 knockdown and over-expression on lifespan and development at normoxia

We analyzed the effect of the *glob1* knockdown and overexpression of *glob1* on longevity and development without applying any stress regime. The lifespan of adult, sexed flies with either *glob1* knockdown or over-expression was monitored under normoxia with constant temperature, food supply, and breeding conditions (Supplementary Fig. S2). The median lifespan of KD flies (females 93 ± 2 days, males 68 ± 2 days) did not show significant differences compared to the control strains, CDR and CKD (females 86 ± 2 days and 79 ± 5 days; males 69 ± 2 days and 67 ± 2 days). Median lifespan of OE flies (females 85 ± 4 days, males 65 ± 3 days) also did not significantly deviate from the corresponding control strains (females 80 ± 4 days and 79 ± 5 days, males 61 ± 4 days and 67 ± 2 days). Similarly, no effect of KD or OE was observed on the lengths of the developmental stages (not shown).

3.3. Phenotypic analyses of glob1 knockdown and over-expression under hypoxia treatment

To study phenotypic effects under hypoxia, we first monitored the development of the flies with *glob1* knockdown and *glob1* overexpression relative to control strains at 5% O_2 at 22 °C, starting from embryos collected overnight (0–18 h). The number of embryos that reached the adult state did not differ between the strains. However, we observed a general decrease of the overall survival rate of embryos kept under hypoxia by about 20%, which agrees with previous studies (Zhou et al., 2007). Under hypoxia, developmental times from embryonic to pupal stage and until eclosion also did not differ between KD and the controls. In all strains hypoxia prolonged developmental time from embryo until pupation by about 40%, whereas the time from pupation to eclosion was comparable to that observed under normoxia (324–350 h after egg-deposition).

Long-term hypoxia (5% O_2 for up to 40 days) resulted in a reduction of lifespan by ~50% in both female and male flies compared to those reared under normoxia (Fig. 3, Supplementary Fig. S2), confirming previously observed reduced survival rates of adult flies under hypoxic conditions (Rascon and Harrison, 2010; Van Voorhies, 2009; Vigne and Frelin, 2006). Again, no effect of *glob1* overexpression was observed (Supplementary Fig. S3). The CDR strain survived longer than the other strains for unknown

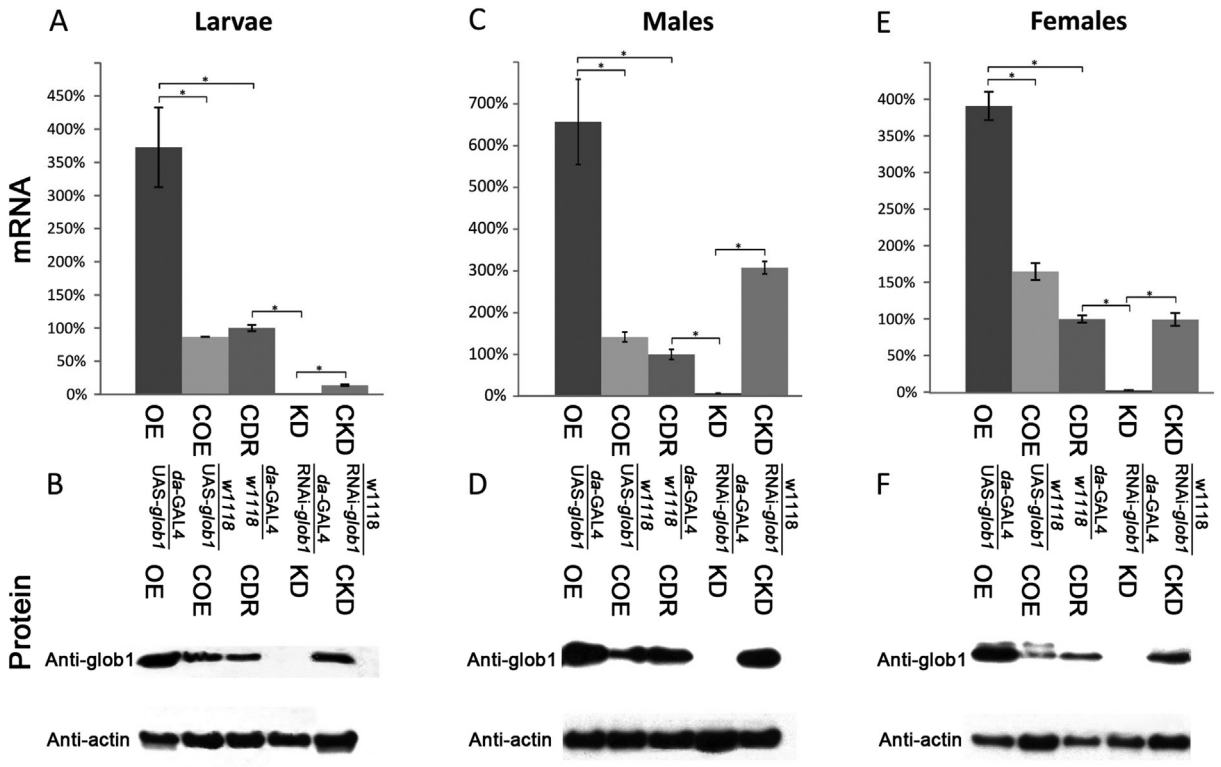

Fig. 1. Modulated *glob1* levels in *D. melanogaster* larvae and flies. Quantification of *glob1* mRNA and protein levels of 3rd instar larvae (A and B), male (C and D) and female flies (E and F) in *glob1* overexpressing (OE) and knockdown (KD) strains compared to corresponding controls (COE, CDR and CKD). mRNA levels (bars) are shown relative to *glob1* expression in the driver control (CDR). For Western blot analysis, actin was visualized by an anti-actin antibody as loading control. Error bars represent standard deviations. * = $p < 0.05$.

reasons. Notably, however, both male and female KD flies showed a significantly reduced overall survival rate and a reduced median survival time at 5% O_2 (females 20 days vs. 28 days in control; males 9.5 days vs. 17.5 days in control) (Fig. 3).

To test the effect of severe, short-term hypoxic challenge, flies were exposed to 1.5% O_2 for 6 h. Shortly after O_2 concentration dropped below 2%, all flies became motionless and remained in this stupor state until re-oxygenation after 6 h (also cf. Haddad et al., 1997a). Under these conditions, female KD flies had a significantly decreased survival rate, with only 5% surviving (compared to 33.3% and 20.8% survival in the controls) (Fig. 4A). Male KD flies also showed a decreased survival rate (4.4% vs. 10.4% and 9.6% in the controls), but the difference was not statistically significant (Fig. 4B). OE flies did not show significant differences in survival of severe hypoxia compared to the controls.

3.4. Effect of glob1 modulation on the onset of and recovery from anoxic stupor in adult flies

Anoxic stupor is a quick response of adult *Drosophila* to almost complete O_2 deprivation that starts within seconds (Csik, 1939; Haddad et al., 1997b). We measured the time of onset of stupor after exposure of the flies to near anoxia ($O_2 < 1\%$). No significant differences in the reaction time to anoxia were observed between KD, OE and control strains. All flies passed into the stereotypic anoxic stupor equally quickly after exposure to near anoxia (120–180 s) (Supplementary Fig. S4A-D). By contrast, the recovery time after 2 h of stupor was significantly shorter in female KD flies (median 120 s) compared to the controls (330 s and 360 s, respectively) (Fig. 5). Recovery times of female OE were in the same range (240 s–400 s) (Supplementary Fig. S5A). Male flies did not show *glob1*-dependent changes in recovery times (Supplementary Fig. S5B, C).

3.5. Effect of glob1 modulation under experimental hyperoxia

Elevated O_2 partial pressures increase O_2 supply, but may also lead to the generation of detrimental ROS. We, therefore, analyzed the lifespan and the development of KD and OE flies under hyperoxia (95% O_2). No effect on developmental times (starting from 3rd instar larvae) was observed. However, irrespective of the genotype, the survival rates of larvae decreased (76–90%). Adult flies were not influenced by hyperoxia during the first 96 h. After the 5th day, however, fly survival rates started to drop dramatically to 50% and resulted in the death of all flies within ~8 days, as previously reported in other studies (Gruenewald et al., 2009; Walker and Benzer, 2004). No differences in the survival rate of *glob1*-modulated and control flies were observed (Fig. 6A,B).

3.6. Phenotypic effects of paraquat exposure in glob1 knockdown and over-expressing flies

The herbicide paraquat is known to act as a chemical ROS generator *in vivo*, which facilitates the accumulation of superoxide anion radicals (O_2^-), resulting in a cascade of production of other, even more potent ROS such as hydrogen peroxide or hydroxyl radicals (Sittipunt, 2005). We tested the ability of 3rd instar larvae and adult flies to withstand paraquat-induced oxidative stress. Most of 3rd instar larvae fed with 20 mM paraquat still developed into adult flies, but died within a few hours after eclosion. Paradoxically, we observed a significantly higher rate of KD larvae that developed into adults (91.7%) compared to the corresponding

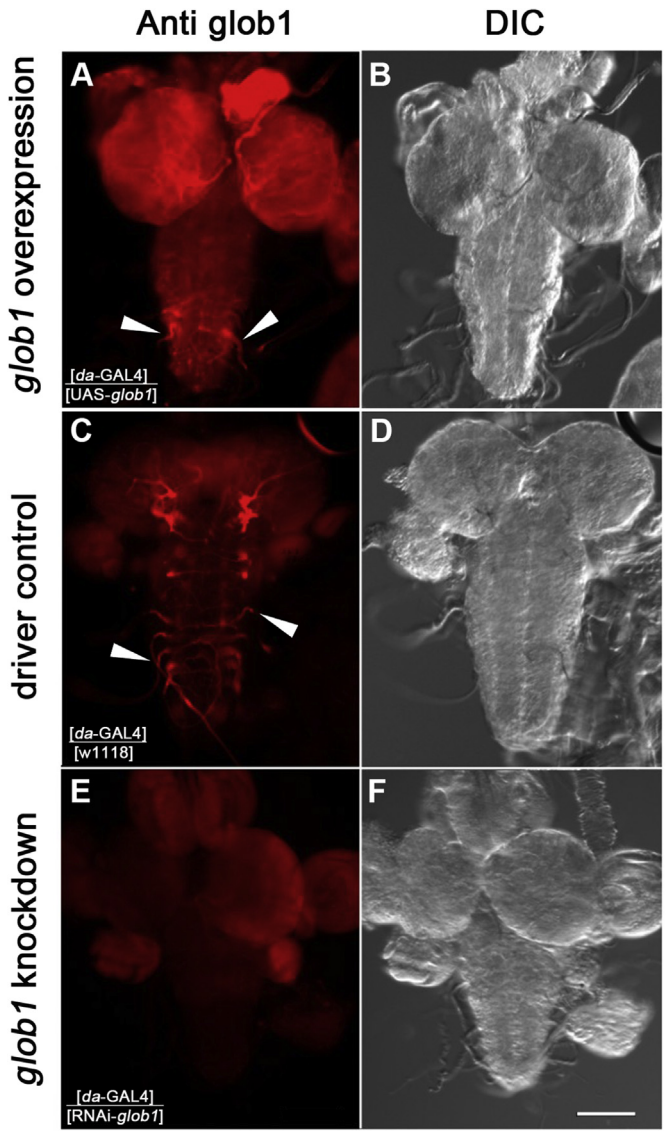

Fig. 2. Evaluation of modulated glob1 protein expression in the larval central nervous system (CNS). Indirect immunofluorescence analyses employing anti-glob1 antibodies on 3rd instar larval CNS with *glob1* over-expression (A), control flies (C) and knock-down (E). B, D, F show the respective differential interference contrast (DIC) images. The arrowheads indicate glob1-specific signals in tracheoles.

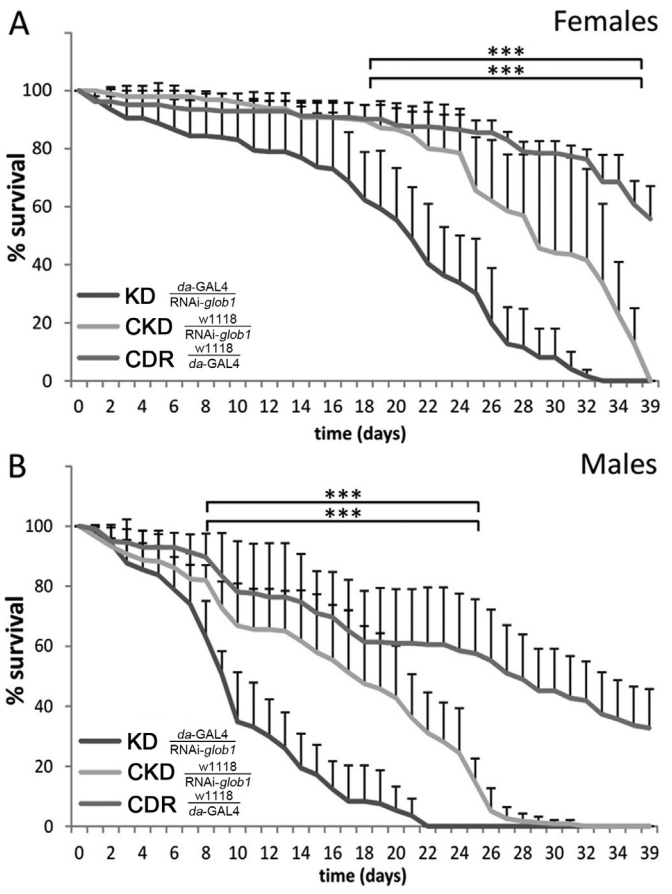

Fig. 3. Survival of adult flies with modulated *glob1* expression under moderate hypoxia (5% O_2). *Glob1* knockdown (KD) leads to decreased overall survival and median survival (dotted line) in female (A) and male (B) flies during hypoxic exposure compared to respective controls (CKD, CDR). Female and male flies with glob1 over-expression (OE) did not show differences in survival of hypoxic treatment compared to respective controls. Error bars represent standard deviations. *** = $p < 0.001$, ANOVA.

controls (75.7% and 53.5%). Survival rate in 3rd instar OE larvae was the same as in controls (Fig. 7A).

Paraquat treatment of adult flies resulted in the death of control and OE flies within 96 h. No statistically significant differences in survival were observed in OE female or male flies (Supplementary Fig. S6A, C). However, a significantly higher survival rate of female KD flies within the first 55 h was observed. Later, the survival rate was similar to that of the control strains (Fig. 7B). We did not observe the same effect in male KD flies (Supplementary Fig. S6B).

We further quantified the damage in lipids and DNA caused by ROS. The amount of lipid peroxidation in adult flies was measured after exposure to 20 mM paraquat or hyperoxia (95% O_2). Lipid peroxidation (LPO) in flies increased to ~17 μM LPO/mg tissue after paraquat treatment and to 22 μM LPO/mg tissue after hyperoxic exposure; however, we found no differences in the amount of LPO in flies with modulated *glob1* expression. Oxidative damage to DNA was analyzed by visualizing apoptotic cells via TUNEL staining. We observed an increase of positive nuclei in sections of adult flight muscles of flies exposed to paraquat, and even more TUNEL positive nuclei in sections obtained from hyperoxia-treated flies (Supplementary Fig. S7), as expected from the literature (Radyuk et al., 2009; Walker and Benzer, 2004). However, again no significant differences were observed in the level of cell death in flies with modulated *glob1* expression.

4. Discussion

The presence of globins in chironomids, some backswimmers, and the botfly is immediately obvious due to the red color of the globin-containing organs or the hemolymph. The respiratory function of these proteins is well established (cf. Burmester and Hankeln, 2007; Weber and Vinogradov, 2001). The functions of globins that are expressed at lower levels and in insects lacking visible globin staining are less clear. Despite its spatially restricted expression, the concentration of glob1 in adult *Drosophila* flies corresponds to roughly 0.1% of total proteins (Burmester et al., 2006). RNAseq data at modENCODE Expression Data (http://flybase.org/reports/FBgn0027657.html) show RPKM values > 300, which also point to a prominent expression of *glob1* mRNA. This hints at an important physiological function in the fly's metabolism. An impairment of endogenous *glob1* expression should, therefore,

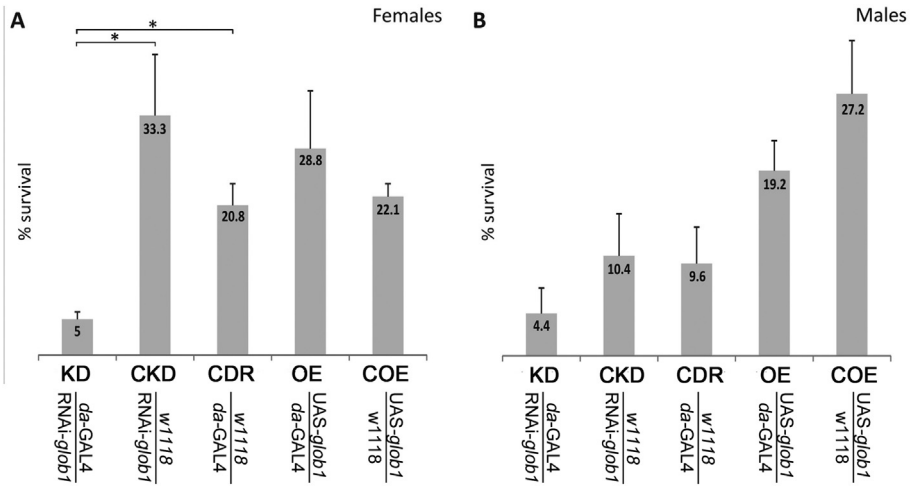

Fig. 4. Survival of adult flies with modulated *glob1* expression under severe hypoxia (1.5% O_2). *Glob1* knockdown (KD) leads to decreased survival of severe hypoxia of female (A) and male (B) flies compared to corresponding controls. Female and male flies with glob1 over-expression (OE) did not show differences in survival of hypoxic treatment compared to respective controls. Error bars represent standard deviations. * = $p < 0.05$, two-tailed t-test.

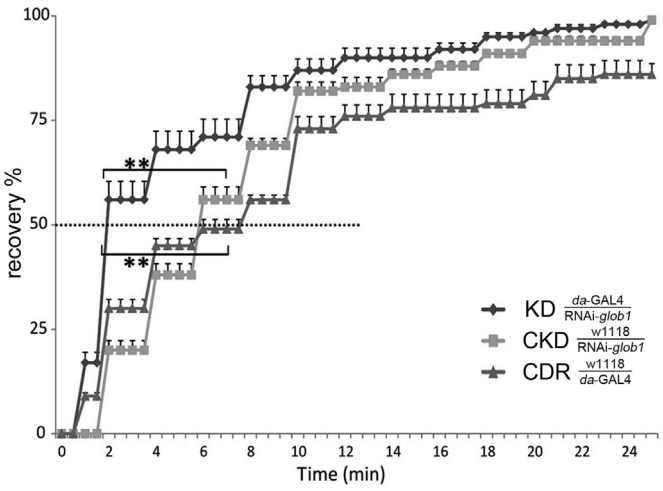

Fig. 5. Recovery time from severe hypoxia of female KD flies. *Glob1* knockdown leads to shortened median recovery time (dotted line) after hypoxic challenge in female flies compared to respective controls. Error bars represent standard deviations. ** = $p < 0.005$, ANOVA.

lead to phenotypic consequences under varying pO_2.

4.1. Glob1-knockdown reduces viability of adult flies under hypoxia, supporting a role in O_2 supply

Neither *OE* nor *KD* flies exhibited obvious phenotypic abnormalities at normoxia. Therefore, we applied experimental hypoxia to challenge the organism and to bypass possible compensatory mechanisms that may take effect under normoxic conditions. Development and reproduction of non-adapted wildtype flies are usually impaired at concentrations lower than 6% O_2, and embryos being exposed to 4% O_2 do not reach adulthood (Zhou et al., 2007). Survival of *Drosophila* embryos is reduced to 60% at 7.5% O_2 and shows a prolonged developmental time until emergence at 10% O_2 (Peck and Maddrell, 2005). Therefore, we applied 5% O_2 to evoke systemic hypoxia. In 3rd instar larvae, experimental and control strains did not display behavioral differences after such hypoxia (data not shown). However, endogenous *glob1* expression is relatively low in late 3rd instar larvae (cf. http://flybase.org/reports/FBgn0027657.html), indicating that glob1 may play only a minor role at that stage.

In adult flies, in contrast, we found that both, moderate (5% O_2) and severe (1.5% O_2) hypoxia caused a significant reduction of the survival rate and median survival time of the *glob1* KD strain. This result clearly shows the importance of *glob1* for an adequate supply of O_2. Although under natural conditions adult flies live in ambient air and are not exposed to systemic hypoxia, their O_2 demands may be high and strongly depend on the animal's activity. Flight movements, for example, result in higher metabolic rates of flies (Lehmann and Dickinson, 1997; Lehmann et al., 2000) and consequently lead to increased O_2 requirements of the flight muscle. Glob1 may play the role of a temporary O_2 store, thus buffering acute O_2 needs and thereby extending ATP homeostasis. In tracheal cells, glob1 may also create an oxygen sink, facilitating a constant flow of O_2 from the tracheal space into the surrounding cells. This hypothesis is in line with the observed O_2 affinity of glob1 (de Sanctis et al., 2005; Hankeln et al., 2002), which resembles that of other high level-expressed insect globins with known O_2 storage or transport functions, such as the hemoglobins of *G. intestinalis* (Keilin and Wang, 1946) or the chironomid midges (Osmulski and Leyko, 1986). A role of glob1 in mediating O_2 homeostasis is further supported by its close evolutionary relationship to the hemoglobin of *G. intestinalis* (Burmester et al., 2006).

4.2. Glob1 does not modulate the onset of anoxic stupor, but its knockdown helps female flies to recover

We further investigated whether altered glob1 levels influence the time it takes for adult flies to fall into, and to recover from, anoxic stupor. Anoxic stupor is a very quick response to complete O_2 deprivation that begins within seconds (Csik, 1939; Haddad et al., 1997b). There were no measurable differences between the OE, KD, and the control flies in the time taken until the onset of anoxic stupor. This observation may be explained by the very fast response to anoxia.

Surprisingly, we observed an *enhanced* recovery from anoxic stupor in female KD flies. A similar tendency was observed in the KD males, although this effect was not significant. Male and female OE flies were not different from the controls. At first sight, the beneficial effect of a glob1 KD appears paradoxical, but may be interpreted in the light of chronic hypoxia that may have been

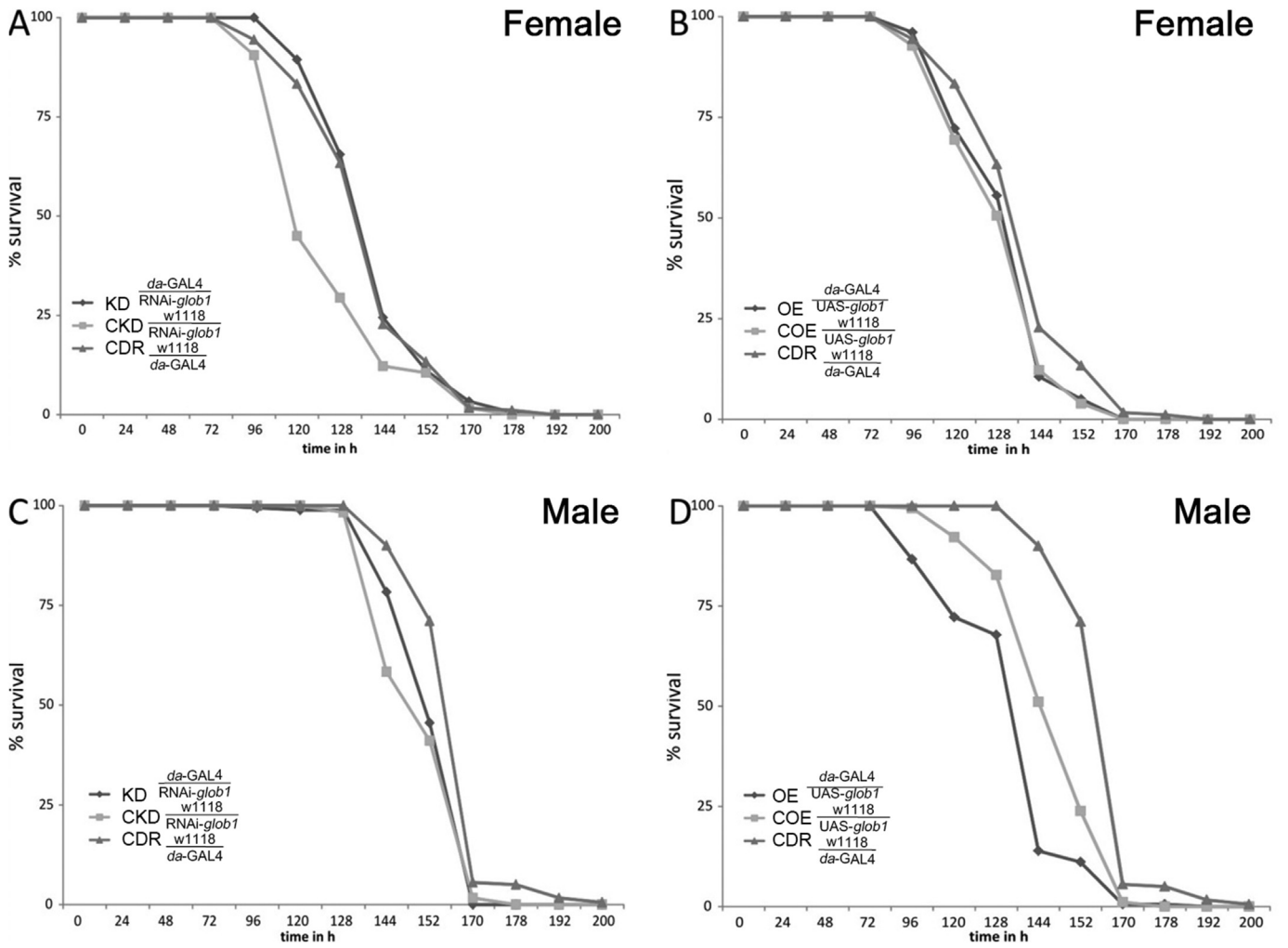

Fig. 6. Survival of adult flies with modulated *glob1* expression under hyperoxia (95% O_2). No significant differences in survival of hyperoxia of female (A and B) and male (C and D) flies with glob1 knockdown (KD), over-expression (OE) and the respective controls.

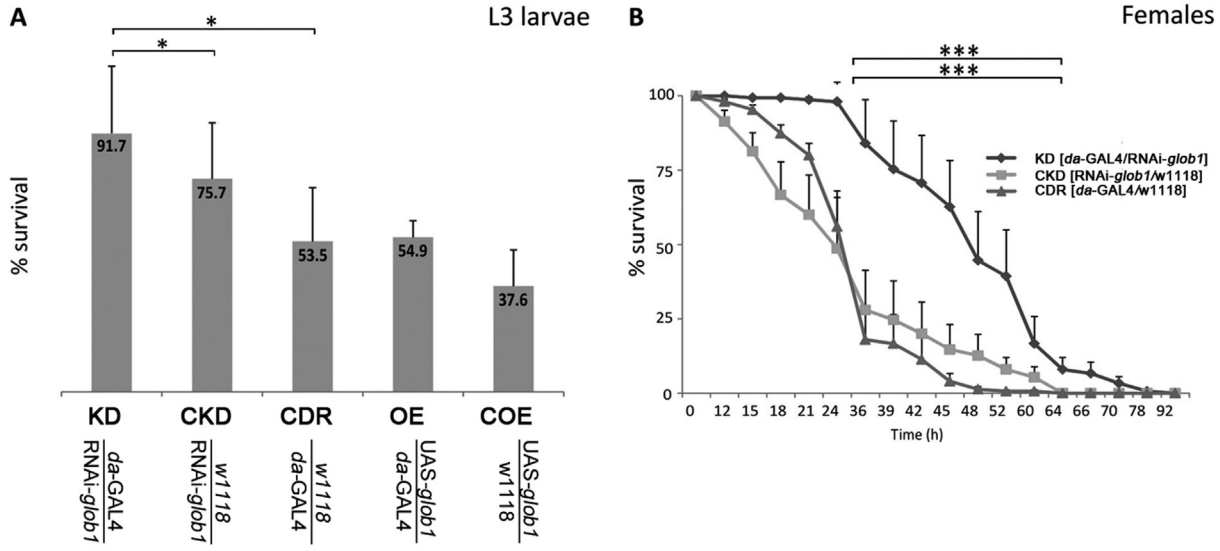

Fig. 7. Survival of larvae and female flies with modulated *glob1* expression under paraquat-induced (20 mM) oxidative stress. (A) *Glob1* knockdown (KD) in 3rd instar larvae increases survival of paraquat treatment compared to respective controls. L3 larvae with *glob1* over-expression (OE) did not show differences in survival of paraquat treatment compared to respective controls. *$p < 0.05$, two-tailed t-test. (B) The survival rate of paraquat-treated female KD flies was increased compared to respective controls. Error bars represent standard deviations. *** = $p < 0.001$, ANOVA.

evoked by a reduced expression of *glob1*, which in turn may have resulted in multiple compensatory adaptations. In fact, (Zhou et al., 2007) found that flies that had been adapted to low O_2 conditions exhibited shorter recovery times from anoxic stupor. Corresponding adaptations to hypoxia may include an increase in the diameter of the trachea (Henry and Harrison, 2004), which then would enhance the O_2 supply after stupor and reduce recovery times. Female flies might particularly benefit from such adaptations because of their larger body mass.

4.3. Glob1 is probably not involved in ROS response

In analogy to vertebrate myoglobin (Flögel et al., 2004), glob1 may also be instrumental in the detoxification of ROS formed during oxidative stress. Therefore, we analyzed the consequences of modulated *glob1* expression in *Drosophila* after experimental hyperoxia and chemical ROS production by the herbicide paraquat (Bus and Gibson, 1984). In *Drosophila*, chronic experimental hyperoxia is deleterious and drastically reduces adult lifespan to about eight days (Gruenewald et al., 2009; Walker and Benzer, 2004). Elevated O_2 levels (O_2 > 80%) result in developmental arrest and death of *Drosophila* embryos (Zhao et al., 2010), showing that a balance in the formation and clearance of ROS is crucial for normal growth and development (Finkel and Holbrook, 2000). The damage resulting from oxidative stress includes peroxidation of membrane lipids (Arking et al., 2000; Gruenewald et al., 2009), protein carbonylation (Gruenewald et al., 2009) and DNA-damage, eventually resulting in apoptosis (Walker and Benzer, 2004). While null-mutations of antioxidant enzymes (e.g., copper-zinc-superoxide dismutase) (Phillips et al., 1989) lead to ROS hypersensitivity, the overexpression of antioxidant proteins can improve resistance to oxidative stress, as shown in flies over-expressing mitochondrial catalase, which exhibited 30–40% increased survival rates when challenged with paraquat (Mockett et al., 2003).

Our data obtained by exposure of *Drosophila* to experimental hyperoxia revealed phenotypic and molecular effects as expected from the literature, but failed to reveal statistically significant differences between KD, OE and control strains. Considering the well-documented susceptibility to oxidative stress of flies with functional impairments in their antioxidant system, the absence of detrimental or beneficial effects on the survival of strains with modulated *glob1* expression argues against a primary role of glob1 as a prominent antioxidant protein. Even if we envisage the possibility that a potential antioxidant role of glob1 must not inevitably lead to different survival rates upon hyperoxic stress, we would at least have expected measurable differences on the molecular level, visible by altered levels of LPO or apoptotic cells.

In addition to hyperoxia, we also tested the ability of *Drosophila* adult flies and 3rd instar larvae with modulated glob1 expression to withstand strong oxidative stress induced by the herbicide paraquat. Again, it has previously been shown that in *Drosophila* increased resistance to paraquat positively correlates with resistance to oxidative stress in general and also prolonged lifespan (Vermeulen et al., 2005), and flies with defects in antioxidant enzymes show increased ROS susceptibility after paraquat exposure (Phillips et al., 1989; Radyuk et al., 2009). In our case, most the of 3rd instar larvae fed with 20 mM paraquat still developed into adult flies, but eventually died a few hours after eclosion. Paradoxically, however, we observed a significantly *higher* rate of KD larvae that developed into adults (91.7%) compared to the corresponding controls (75.7% and 53.5%). This was paralleled by a markedly increased survival of KD female flies (but not males) after paraquat treatment. Both findings suggest an unexpected protective effect towards paraquat in the *absence* of glob1. Our tentative explanation is as follows: provided that glob1 functions as an O_2 store, the O_2 released from glob1 could act as an electron acceptor for paraquat and become reduced to the superoxide anion radical. Therefore, a lower intracellular O_2 concentration in *glob1* knockdowns would hypothetically lead to a *decreased* $O_2^{\bullet-}$ formation and less damage. Unfortunately, the molecular assays could not confirm this postulated ROS decrease in KD animals, possibly due to a lack of sensitivity. Also, ectopic *glob1* overexpression did not deteriorate survival after paraquat exposure as might then have been expected, a fact that could be explained by the only moderate (two-to four-fold) level of over-expression. The sex-specificity of the beneficial effect of glob1 knockdown during paraquat-treatment is best explained by the known differences between the sexes in diverse physiological pathways as a consequence of reproductive activity (Burger and Promislow, 2004). The earlier and far more rapid onset of paraquat-induced mortality, which we and others (Chaudhuri et al., 2007) specifically observed in male flies, could mask possible differences between flies with modulated *glob1* expression and controls.

4.4. Conclusions

The technical ability to genetically modulate intracellular globin expression gave us the opportunity to evaluate phenotypic consequences of *glob1* knockdown and overexpression in *Drosophila*. We showed that a knockdown of *glob1* elicits a phenotypic effect in flies after exposure to hypoxia. In summary, the data suggest a role of glob1 in O_2 homeostasis. Glob1 may provide temporary and/or local O_2 supply, but can also buffer excess O_2 levels, which may transiently occur in both, the tracheal system and fat body tissue. It remains to be shown whether this also applies to the orthologous globins of other insects (Burmester and Hankeln, 2007; Burmester et al., 2007; Hankeln et al., 2006; Kawaoka et al., 2009). From the evolutionary perspective, an O_2 homeostasis role is the most parsimonious assumption, particularly as the globins from backswimmers, chironomids, the horse botfly and *Drosophila* belong to the same globin family (Burmester et al., 2006; Dewilde et al., 1998; Wawrowski et al., 2012). A globin-mediated O_2 homeostasis in insects may also compensate for the loss of the respiratory protein hemocyanin, which occurred in the Eumetabola (Holometabola + Hemiptera) during evolution (Burmester and Hankeln, 2007).

During preparation of the manuscript, a study by Yadav et al. (2015) on the function of glob1 was published. These authors used a P-element insertion mutation inside one of the two candidate promotors of the glob1 gene locus, which reduced *glob1* expression in larvae by ~50%. In this mutant, and without applying additional stress regimes, the authors observed some complex phenotypes (e.g. embryonic semi-lethality, delay in hatching, increased ROS levels in larvae), which they ascribe to the reduced level of *glob1* expression. Due to the vastly different experimental approach employed it is difficult to compare directly and unify those results with our present study, which has focused on revealing effects of *glob1* deficiency in larval and adult flies after stress exposure. Additional experiments will clearly be necessary to define better the role of glob1 in *Drosophila*.

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft (Ha2103/5 and Bu956/6).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2016.03.004.

References

Arking, R., Burde, V., Graves, K., Hari, R., Feldman, E., Zeevi, A., Soliman, S., Saraiya, A., Buck, S., Vettraino, J., Sathrasala, K., Wehr, N., Levine, R.L., 2000. Forward and reverse selection for longevity in *Drosophila* is characterized by alteration of antioxidant gene expression and oxidative damage patterns. Exp. Gerontol. 35, 167–185.

Brand, A.H., Perrimon, N., 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118, 401–415.

Burger, J.M., Promislow, D.E., 2004. Sex-specific effects of interventions that extend fly life span. Sci. Aging Knowl. Environ. 2004 pe30.

Burmester, T., 2005. Physiology: a welcome shortage of breath. Nature 433, 471–472.

Burmester, T., Hankeln, T., 1999. A globin gene of *Drosophila melanogaster*. Mol. Biol. Evol. 16, 1809–1811.

Burmester, T., Hankeln, T., 2007. The respiratory proteins of insects. J. Insect Physiol. 53, 285–294.

Burmester, T., Hankeln, T., 2014. Function and evolution of vertebrate globins. Acta Physiol. Scand 211, 501–514.

Burmester, T., Klawitter, S., Hankeln, T., 2007. Characterization of two globin genes from the malaria mosquito *Anopheles gambiae*: divergent origin of nematoceran haemoglobins. Insect Mol. Biol. 16, 133–142.

Burmester, T., Storf, J., Hasenjager, A., Klawitter, S., Hankeln, T., 2006. The hemoglobin genes of *Drosophila*. FEBS J. 273, 468–480.

Bus, J.S., Gibson, J.E., 1984. Paraquat: model for oxidant-initiated toxicity. Environ. Health Perspect. 55, 37–46.

Bustin, S.A., 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J. Mol. Endocrinol. 29, 23–39.

Centanin, L., Dekanty, A., Romero, N., Irisarri, M., Gorr, T.A., Wappner, P., 2008. Cell autonomy of HIF effects in *Drosophila*: tracheal cells sense hypoxia and induce terminal branch sprouting. Dev. Cell 14, 547–558.

Centanin, L., Gorr, T.A., Wappner, P., 2010. Tracheal remodelling in response to hypoxia. J. Insect Physiol. 56, 447–454.

Chadwick, L., Gilmour, D., 1940. Respiration during flight in *Drosophila repleta* Wollaston: the oxygen consumption considered in relation to the wing-rate. Physiol. Zool. 13, 398–410.

Chaudhuri, A., Bowling, K., Funderburk, C., Lawal, H., Inamdar, A., Wang, Z., O'Donnell, J.M., 2007. Interaction of genetic and environmental factors in a *Drosophila* parkinsonism model. J. Neurosci. 27, 2457–2467.

Cronmiller, C., Cummings, C.A., 1993. The daughterless gene product in Drosophila is a nuclear protein that is broadly expressed throughout the organism during development. Mech. Dev. 42, 159–169.

Csik, L., 1939. The susceptibility to oxygen want in different *Drosophila* species. Z. für Vgl. Physiol. 27, 304–310.

de Sanctis, D., Dewilde, S., Vonrhein, C., Pesce, A., Moens, L., Ascenzi, P., Hankeln, T., Burmester, T., Ponassi, M., Nardini, M., Bolognesi, M., 2005. Bishistidyl heme hexacoordination, a key structural property in *Drosophila melanogaster* hemoglobin. J. Biol. Chem. 280, 27222–27229.

Dewilde, S., Blaxter, M., van Hauwaert, M., van Houte, K., Pesce, A., Griffon, N., Kiger, L., Marden, M.C., Vermeire, S., Vanfleteren, J., Esmans, E., Moens, L., 1998. Structural, functional, and genetic characterization of *Gastrophilus* hemoglobin. J. Biol. Chem. 273, 32467–32474.

Finkel, T., Holbrook, N.J., 2000. Oxidants, oxidative stress and the biology of ageing. Nature 408, 239–247.

Flögel, U., Ding, Z., Hardung, H., Jander, S., Reichmann, G., Jacoby, C., Schubert, R., Schrader, J., 2008. In vivo monitoring of inflammation after cardiac and cerebral ischemia by fluorine magnetic resonance imaging. Circulation 118, 140–148.

Flögel, U., Gödecke, A., Klotz, L.O., Schrader, J., 2004. Role of myoglobin in the antioxidant defense of the heart. FASEB J. 18, 1156–1158.

Foe, V.E., Alberts, B.M., 1985. Reversible chromosome condensation induced in *Drosophila* embryos by anoxia: visualization of interphase nuclear organization. J. Cell Biol. 100, 1623–1636.

Gleixner, E., Abriss, D., Adryan, B., Kraemer, M., Gerlach, F., Schuh, R., Burmester, T., Hankeln, T., 2008. Oxygen-induced changes in hemoglobin expression in *Drosophila*. FEBS J. 275, 5108–5116.

Gleixner, E., Herlyn, H., Zimmerling, S., Burmester, T., Hankeln, T., 2012. Testes-specific hemoglobins in *Drosophila* evolved by a combination of sub- and neofunctionalization after gene duplication. BMC Evol. Biol. 12, 34.

Gorr, T.A., Gassmann, M., Wappner, P., 2006. Sensing and responding to hypoxia via HIF in model invertebrates. J. Insect Physiol. 52, 349–364.

Gorr, T.A., Tomita, T., Wappner, P., Bunn, H.F., 2004. Regulation of *Drosophila* hypoxia-inducible factor (HIF) activity in SL2 cells: identification of a hypoxia-induced variant isoform of the HIFalpha homolog gene similar. J. Biol. Chem. 279, 36048–36058.

Grieshaber, M.K., Hardewig, I., Kreutzer, U., Portner, H.O., 1994. Physiological and metabolic responses to hypoxia in invertebrates. Rev. Physiol. Biochem. Pharmacol. 125, 43–147.

Gruenewald, C., Botella, J.A., Bayersdorfer, F., Navarro, J.A., Schneuwly, S., 2009. Hyperoxia-induced neurodegeneration as a tool to identify neuroprotective genes in *Drosophila melanogaster*. Free Radic. Biol. Med. 46, 1668–1676.

Guillemin, K., Groppe, J., Ducker, K., Treisman, R., Hafen, E., Affolter, M., Krasnow, M.A., 1996. The pruned gene encodes the *Drosophila* serum response factor and regulates cytoplasmic outgrowth during terminal branching of the tracheal system. Development 122, 1353–1362.

Haddad, G.G., 2000. Enhancing our understanding of the molecular responses to hypoxia in mammals using *Drosophila melanogaster*. J. Appl. Physiol. 88, 1481–1487.

Haddad, G.G., 2006. Tolerance to low O_2: lessons from invertebrate genetic models. Exp. Physiol. 91, 277–282.

Haddad, G.G., Sun, Y., Wyman, R.J., Xu, T., 1997a. Genetic basis of tolerance to O_2 deprivation in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. U. S. A. 94, 10809–10812.

Haddad, G.G., Wyman, R.J., Mohsenin, A., Sun, Y., Krishnan, S.N., 1997b. Behavioral and electrophysiologic responses of *Drosophila melanogaster* to prolonged periods of anoxia. J. Insect Physiol. 43, 203–210.

Hankeln, T., Jaenicke, V., Kiger, L., Dewilde, S., Ungerechts, G., Schmidt, M., Urban, J., Marden, M.C., Moens, L., Burmester, T., 2002. Characterization of *Drosophila* hemoglobin. Evidence for hemoglobin-mediated respiration in insects. J. Biol. Chem. 277, 29012–29017.

Hankeln, T., Klawitter, S., Kramer, M., Burmester, T., 2006. Molecular characterization of hemoglobin from the honeybee Apis mellifera. J. Insect Physiol. 52, 701–710.

Hendgen-Cotta, U.B., Flogel, U., Kelm, M., Rassaf, T., 2010. Unmasking the Janus face of myoglobin in health and disease. J. Exp. Biol. 213, 2734–2740.

Henry, J.R., Harrison, J.F., 2004. Plastic and evolved responses of larval tracheae and mass to varying atmospheric oxygen content in *Drosophila melanogaster*. J. Exp. Biol. 207, 3559–3567.

Hetz, S.K., Bradley, T.J., 2005. Insects breathe discontinuously to avoid oxygen toxicity. Nature 433, 516–519.

Heymann, N., Lehmann, F.O., 2006. The significance of spiracle conductance and spatial arrangement for flight muscle function and aerodynamic performance in flying *Drosophila*. J. Exp. Biol. 209, 1662–1677.

Jarecki, J., Johnson, E., Krasnow, M.A., 1999. Oxygen regulation of airway branching in Drosophila is mediated by branchless FGF. Cell 99, 211–220.

Kawaoka, S., Katsuma, S., Meng, Y., Hayashi, N., Mita, K., Shimada, T., 2009. Identification and characterization of globin genes from two lepidopteran insects, *Bombyx mori* and *Samia cynthia ricini*. Gene 431, 33–38.

Keilin, D., Wang, Y.L., 1946. Haemoglobin of *Gastrophilus* larvae. Purification and properties. Biochem. J. 40, 855–866.

Kestler, P., 1985. In: Hoffmann, K.H. (Ed.), Environmental Physiology and Biochemistry of Insects. Springer, Berlin.

Landis, G.N., Abdueva, D., Skvortsov, D., Yang, J., Rabin, B.E., Carrick, J., Tavare, S., Tower, J., 2004. Similar gene expression patterns characterize aging and oxidative stress in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. U. S. A. 101, 7663–7668.

Lavista-Llanos, S., Centanin, L., Irisarri, M., Russo, D.M., Gleadle, J.M., Bocca, S.N., Muzzopappa, M., Ratcliffe, P.J., Wappner, P., 2002. Control of the hypoxic response in *Drosophila melanogaster* by the basic helix-loop-helix PAS protein similar. Mol. Cell. Biol. 22, 6842–6853.

Law, J.H., Wells, M.A., 1989. Insects as biochemical models. J. Biol. Chem. 264, 16335–16338.

Lehmann, F.O., 2001. Matching spiracle opening to metabolic need during flight in *Drosophila*. Science 294, 1926–1929.

Lehmann, F.O., Dickinson, M.H., 1997. The changes in power requirements and muscle efficiency during elevated force production in the fruit fly *Drosophila melanogaster*. J. Exp. Biol. 200, 1133–1143.

Lehmann, F.O., Dickinson, M.H., Staunton, J., 2000. The scaling of carbon dioxide release and respiratory water loss in flying fruit flies (*Drosophila* spp.). J. Exp. Biol. 203, 1613–1624.

Liu, G., Roy, J., Johnson, E.A., 2006. Identification and function of hypoxia-response genes in *Drosophila melanogaster*. Physiol. Genomics 25, 134–141.

Locke, M., 1997. Caterpillars have evolved lungs for hemocyte gas exchange. J. Insect Physiol. 44, 1–20.

Mockett, R.J., Bayne, A.C., Kwong, L.K., Orr, W.C., Sohal, R.S., 2003. Ectopic expression of catalase in *Drosophila* mitochondria increases stress resistance but not longevity. Free Radic. Biol. Med. 34, 207–217.

Osmulski, P.A., Leyko, W., 1986. Structure, function and physiological role of *Chironomus* haemoglobin. Comp. Biochem. Physiol. B 85, 701–722.

Peck, L.S., Maddrell, S.H., 2005. Limitation of size by hypoxia in the fruit fly *Drosophila melanogaster*. J. Exp. Zool. A Comp. Exp. Biol. 303, 968–975.

Phillips, J.P., Campbell, S.D., Michaud, D., Charbonneau, M., Hilliker, A.J., 1989. Null mutation of copper/zinc superoxide dismutase in *Drosophila* confers hypersensitivity to paraquat and reduced longevity. Proc. Natl. Acad. Sci. U. S. A. 86, 2761–2765.

Radyuk, S.N., Michalak, K., Klichko, V.I., Benes, J., Rebrin, I., Sohal, R.S., Orr, W.C., 2009. Peroxiredoxin 5 confers protection against oxidative stress and apoptosis and also promotes longevity in *Drosophila*. Biochem. J. 419, 437–445.

Rascon, B., Harrison, J.F., 2010. Lifespan and oxidative stress show a non-linear response to atmospheric oxygen in *Drosophila*. J. Exp. Biol. 213, 3441–3448.

Sittipunt, C., 2005. Paraquat poisoning. Respir. Care 50, 383–385.

Van Voorhies, W.A., 2009. Metabolic function in *Drosophila melanogaster* in response to hypoxia and pure oxygen. J. Exp. Biol. 212, 3132–3141.

Vermeulen, C.J., Van De Zande, L., Bijlsma, R., 2005. Resistance to oxidative stress induced by paraquat correlates well with both decreased and increased lifespan in *Drosophila melanogaster*. Biogerontology 6, 387–395.

Vigne, P., Frelin, C., 2006. A low protein diet increases the hypoxic tolerance in *Drosophila*. PLoS ONE 1, e56.

Walker, D.W., Benzer, S., 2004. Mitochondrial "swirls" induced by oxygen stress and in the *Drosophila* mutant hyperswirl. Proc. Natl. Acad. Sci. U. S. A. 101,

10290–10295.
Wawrowski, A., Matthews, P.G., Gleixner, E., Kiger, L., Marden, M.C., Hankeln, T., Burmester, T., 2012. Characterization of the hemoglobin of the backswimmer *Anisops deanei* (Hemiptera). Insect Biochem. Mol. Biol. 42, 603–609.
Weber, R.E., Vinogradov, S.N., 2001. Nonvertebrate hemoglobins: functions and molecular adaptations. Physiol. Rev. 81, 569–628.
Wegener, G., 1993. Hypoxia and Post Hypoxic Recovery in Insects: Physiological and Metabolical Aspects, Surviving Hypoxia: Mechanism of Control and Adaptation. CRC-Press, London, Tokyo.
Willmer, P., Stone, G., Johnston, I.A., 2000. Environmental Physiology of Animals. Blackwell Science, Malden, MA.
Wingrove, J.A., O'Farrell, P.H., 1999. Nitric oxide contributes to behavioral, cellular, and developmental responses to low oxygen in *Drosophila*. Cell 98, 105–114.
Wittenberg, J.B., Wittenberg, B.A., 2003. Myoglobin function reassessed. J. Exp. Biol. 206, 2011–2020.
Yadav, R., Kundu, S., Sarkar, S., 2015. Drosophila glob1 expresses dynamically and is required for development and oxidative stress response. Genesis 53, 719–737.
Zhao, H.W., Zhou, D., Nizet, V., Haddad, G.G., 2010. Experimental selection for *Drosophila* survival in extremely high O_2 environments. PLoS ONE 5, e11701.
Zhou, D., Udpa, N., Gersten, M., Visk, D.W., Bashir, A., Xue, J., Frazer, K.A., Posakony, J.W., Subramaniam, S., Bafna, V., Haddad, G.G., 2011. Experimental selection of hypoxia-tolerant *Drosophila melanogaster*. Proc. Natl. Acad. Sci. U. S. A. 108, 2349–2354.
Zhou, D., Xue, J., Chen, J., Morcillo, P., Lambert, J.D., White, K.P., Haddad, G.G., 2007. Experimental selection for *Drosophila* survival in extremely low O_2 environment. PLoS ONE 2, e490.
Zhou, D., Xue, J., Lai, J.C., Schork, N.J., White, K.P., Haddad, G.G., 2008. Mechanisms underlying hypoxia tolerance in *Drosophila melanogaster*: hairy as a metabolic switch. PLoS Genet. 4, e1000221.