Funktionsaufklärung von Atmungsproteinen in Insekten

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INHALT

1 EINLEITUNG

1.1 Globine

Die Globin-Superfamilie umfasst evolutionär alte Atmungsproteine, die in nahezu allen aeroben Organsimen – angefangen von Bakterien über Pflanzen und Pilzen bis hin zu Insekten und Vertebraten – präsent sind. Trotz enormer Unterschiede der Aminosäure-Sequenz, den biochemischen Eigenschaften und physiologischen Funktionen weist die Tertiärstruktur der Globine einen hohen Grad an Konservierung auf. Den Globinen gemein ist die typische Globin-Faltung, bestehend aus sechs bis acht α-Helices (Helix A-H), die eine 3/3-α-helikale Sandwichstruktur einnehmen. Die Helices formen eine hydrophobe ‚Tasche' für die nicht-kovalent gebundene prosthetische Häm-Gruppe (Fe^{2+}-Protoporphyrin IX), die die Globine zur Bindung gasförmiger Liganden wie O_2, NO oder CO befähigt (Kendrew et al., 1958; Schroeder, 1963; Lecomte et al., 2005).

Prominente und sehr gut untersuchte Vertreter der Superfamilie sind die Globine der Vertebraten, kleine, globuläre Proteine mit einer durchschnittlichen Länge von 150 Aminosäuren. Die inzwischen recht lange Liste der Vertebraten-Globine wird angeführt von den wohl bekanntesten Vertretern, dem Hämoglobin und Myoglobin:

- Beim Hämoglobin (Hb) handelt es sich um ein Heterotetramer, bestehend aus zwei α- und zwei β-Proteinketten. Hb ist in Erythrozyten der Vertebraten lokalisiert und übt eine O_2/CO_2-Transportfunktion zur Aufrechterhaltung der Zellatmung aus (Millikan, 1933; Schroeder, 1963; Perutz, 1979).
- Myoglobin (Mb) liegt als Monomer in den Zellen der Herz- und Skelettmuskulatur und in glatter Muskulatur verschiedener Organe vor (Kendrew et al., 1958; Qiu et al., 1998). Dort dient es der O_2-Speicherung zur Pufferung muskulärer O_2-Schwankungen (Millikan, 1939; Wittenberg, 1970; Wittenberg und Wittenberg, 2003) sowie dem intrazellulären O_2-Transport von der Zellmembran zu den Mitochondrien (Wittenberg, 1970; Wittenberg und Wittenberg, 2003; Endeward et al., 2010).

Für beide Globine konnte zusätzlich zu den ‚klassischen' Funktionen als O_2-Speicher- und Transporter eine Nitrit-Oxidase-Aktivität und eine Dioxygenase-Aktivität zur Detoxifizierung schädlichen Nitrits (NO_2^-) und Stickstoffmonoxids (NO) nachgewiesen werden (Flogel et al., 2001; Grange et al., 2001; Pietraforte et al., 2004; Flogel et al., 2008; Hendgen-Cotta et al., 2008). Zusätzlich sind sowohl Hb als auch Mb in der Lage, speziell unter hypoxischen Bedingungen in deoxygeniertem Zustand NO_2^- zu NO zu reduzieren und damit Ischämie-

bedingten Schädigungen der Organe vorzubeugen (Gladwin et al., 2004; Cossins und Berenbrink, 2008; Hendgen-Cotta et al., 2010b).

Bei Cytoglobin (Kawada et al., 2001; Burmester et al., 2002; Trent und Hargrove, 2002) und Neuroglobin (Burmester et al., 2000) handelt es sich um vor etwa zehn Jahren entdeckte Mitglieder der Globinfamilie:

- Cytoglobin (Cygb) wird als Monomer in Fibroblasten und verwandten Zelltypen (Schmidt et al., 2004) sowie in der Retina exprimiert (Schmidt et al., 2005; Ostojic et al., 2006; Ostojic et al., 2008). Welche Rolle es dort übernimmt, ist bisher unklar, jedoch gibt es eine Vielzahl an Hinweisen auf mögliche Funktionen, angefangen bei einer Beteiligung des Cygbs bei der Entgiftung reaktiver Sauerstoff- oder Stickstoffspezies (Hankeln et al., 2005; Hundahl et al., 2010) über eine Myoglobin-ähnliche Funktion als O_2-Speicher (Sawai et al., 2003) und im NO-Metabolismus (Burmester et al., 2007a; Petersen et al., 2008; Halligan et al., 2009), eine Rolle als O_2-Sensor (Sawai et al., 2005) bis hin zu einer Beteiligung als O_2-Lieferant für Enzyme bei der Kollagen-Synthese (Schmidt et al., 2003).
- Neuroglobin (Ngb) ist als Monomer in Neuronen (allerdings nicht in Gliazellen) des zentralen und peripheren Nervensystems, in Zellen endokriner Gewebe (Burmester et al., 2000; Reuss et al., 2002; Wystub et al., 2003; Hankeln et al., 2004), in Photorezeptorzellen und der inneren und äußeren plexiformen Schicht der Retina (Schmidt et al., 2003; Bentmann et al., 2005) lokalisiert. Trotz umfassender Untersuchungen kann auch über die Funktion von Neuroglobin nur spekuliert werden. So gibt es für Ngb Hinweise auf eine respiratorische Funktion ähnlich der des Myoglobins (Reuss et al., 2002; Schmidt et al., 2003; Hankeln et al., 2004; Burmester und Hankeln, 2009), auf eine Beteiligung bei der Entgiftung reaktiver Sauerstoff- und Stickstoffspezies (Herold et al., 2004; Ridnour et al., 2004; Milton et al., 2006; Moncada und Bolanos, 2006; Greenberg et al., 2008; Wang et al., 2008) und auf eine Rolle im NO-Metabolismus (Herold et al., 2004; Brunori et al., 2005; Petersen et al., 2008). Eine Beteiligung in einer G-Protein-abhängigen Signaltransduktionskaskade (Wakasugi et al., 2003; Wakasugi und Morishima, 2005; Watanabe und Wakasugi, 2008) und eine Rolle in der Verhinderung Cytochrom-c-vermittelter Apoptose wurden ebenfalls diskutiert (Fago et al., 2006).
- Androglobin (Adgb) (Hoogewijs et al., submitted) ist das neueste Mitglied der Globinfamilie. Es konnte bisher in über 30 Metazoen-Genomen identifiziert werden. Bei Adgb handelt sich um ein chimäres Protein, bestehend aus einer Globin-Domäne und einer N-terminal gelegenen Cystein-Protease-ähnlichen Domäne, die eine hohe Verwandtschaft zum humanen Calpain-7 aufweist. Die Expression von Adgb findet präferentiell in Testes der

Säuger statt. Aufgrund dessen scheint eine Funktion während der Spermatogenese wahrscheinlich (Hoogewijs et al., submitted).

Die aktuelle Liste der Vertebraten-Globine wird durch weitere Globin-Varianten vervollständigt, die aber ein beschränktes Vorkommen in einigen Taxa aufweisen.

- Globin X (Gb X) ist das monomere Globin der Fische und Amphibien. Im Krallenfrosch *Xenopus* und dem Zebrafisch *Danio rerio* wurde Globin X im Gehirn und den Augen nachgewiesen (Fuchs et al., 2006), im Goldfisch *Carassius gibelio* hingegen konnte Globin X auch in nicht-neuronalem Gewebe detektiert werden (Roesner et al., 2005). Globin X liegt membrangebunden vor, als mögliche Funktionen werden eine Beteiligung bei der Entgiftung reaktiver Sauerstoff- und Stickstoffspezies oder eine Rolle als O_2-Sensormolekül diskutiert (Blank et al., submitted).
- Auch bei Globin Y (Gb Y) handelt es sich um ein monomeres Globin, das zuerst in mehreren Geweben adulter *Xenopus* nachgewiesen wurde (Fuchs et al., 2006). Die Anwesenheit von Gb Y konnte zudem in Platypus *Ornithorhynchus anatinus* (Patel et al., 2008), in Anolis *Anolis carolinensis* (Hoffmann et al., 2010b) und der Bartagame *Pogona vitticeps* (Patel et al., 2010) nachgewiesen werden. Über seine Funktion ist bisher nichts bekannt.
- Globin E ist als Monomer in Photorezeptorzellen von Vögeln lokalisiert (Kugelstadt et al., 2004; Blank et al., 2011). Es wird angenommen, dass es dort eine Myoglobin-ähnliche Funktion in der O_2-Versorgung der Photorezeptorzellen der Vogelretina übernimmt (Blank et al., 2011).

Der Stammbaum der Vertebraten-Globine zeigt eine Verteilung der Globine auf zwei Äste, deren Entstehung vermutlich noch vor der Spaltung der Tiere in Proto- und Deuterostomier vor etwa 600 Millionen Jahren zu datieren ist. Einem der beiden Äste können Neuroglobin und Globin X zugeordnet werden, die aus unabhängigen, anzestralen Duplikationsereignissen hervorgegangen sind (Roesner et al., 2005; Fuchs et al., 2006; Hoffmann et al., 2010a). Der zweite Ast des Vertebraten-Globin-Stammbaums umfasst Myoglobin, die Hämoglobine, Cytoglobin sowie die Globine Y und E, die ebenfalls alle das Produkt Vertebraten-spezifischer Duplikationsereignisse sind (Hoffmann et al., 2010a). Die Vertebraten-Globine können in vier Hauptgruppen unterteilt werden: a) Cytoglobin und die Hämoglobine der Cyclostomata, b) Myoglobin und Globin E, c) α- und β-Hämoglobin-Ketten der Gnathostomata und d) Globin Y (siehe Abb. 1.1). Diese Verwandtschaftsverhältnisse deuten auf die Anwesenheit aller vier Vertebraten-spezifischen Globin-Linien in gemeinsamen Vorfahren der Vertebraten hin (Storz et al., 2011).

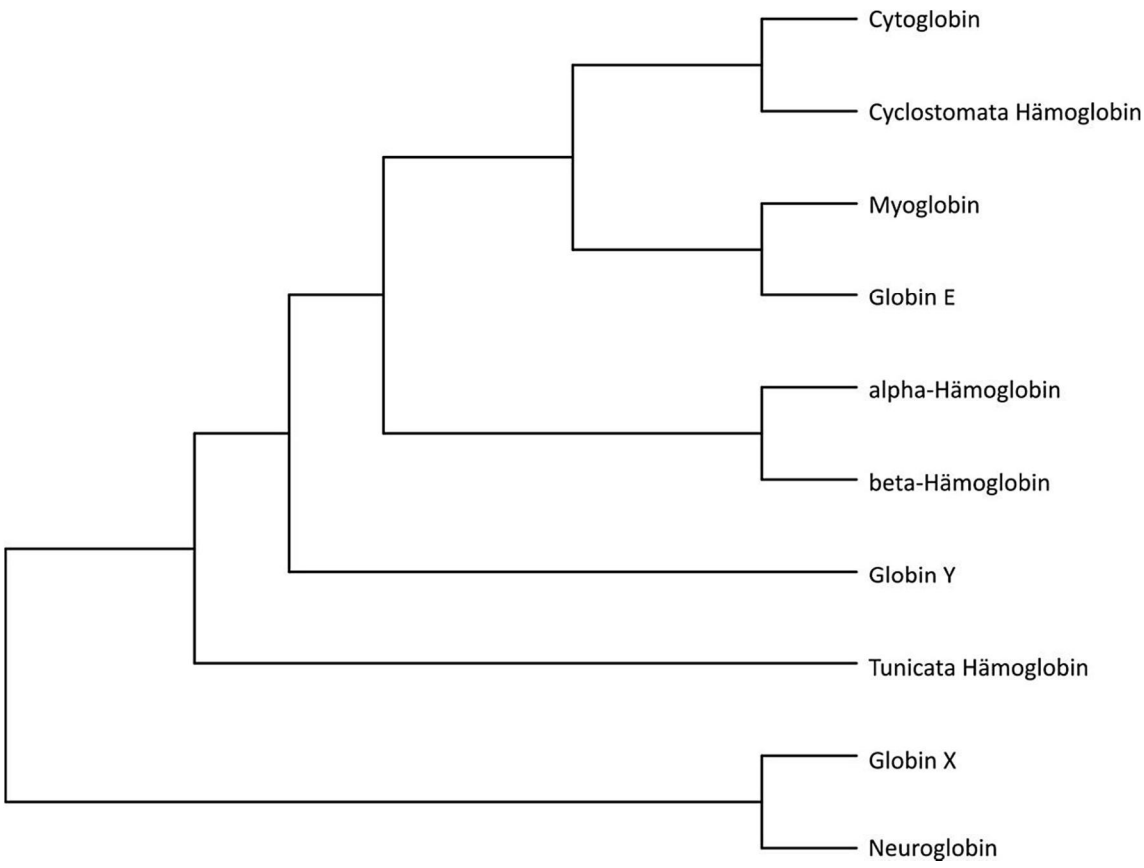

Abb. 1.1: Phylogenie der Globin-Gene ausgewählter Chordaten (verändert nach Storz et al., 2011).

Der Ursprung der Globine in der Evolution geht zurück bis zu Bakterien und Archaea bereits vor der Entstehung eukaryotischer Lebewesen (Hardison, 1996). Bei den heutigen Protoglobinen der Bakterien und Archaea handelt es sich möglicherweise um den Globin-Prototypen, der für die Globin-Evolution Modell stand (Freitas et al., 2004; Vinogradov et al., 2005). Die im Laufe der Globin-Evolution entstandene Funktionsvielfalt kann insbesondere bei den Pflanzen und Bakterien beobachtet werden. So finden sich zum Beispiel Flavohämoglobine (Hämoglobine mit einer N-terminalen FADReduktase-Domäne) in Bakterien (Frey und Kallio, 2003), nichtsymbiontische Hämoglobine in Pflanzen (Bogusz et al., 1988; Garrocho-Villegas et al., 2007), Hämoglobine mit verkürzter Primärstruktur und einer 2/2 α-helikalen Sandwichstruktur (truncated hemoglobins) in Bakterien, Protozoen und Pflanzen (Wittenberg et al., 2002; Lecomte et al., 2005; Vinogradov et al., 2005) sowie Globine mit Sensorfunktion (globin-coupled sensors) (Hou et al., 2000; Hou et al., 2001; Freitas et al., 2003) in Bakterien und Archaea. Alle Bakterienglobine besitzen die Fähigkeit, O_2 zu binden, aber auch zur enzymatischen Entgiftung reaktiver Stickstoff- und Sauerstoffspezies beizutragen (Wu et al.,

2003; Poole, 2005; Vinogradov et al., 2005; Gardner et al., 2006; Bonamore und Boffi, 2008; Vinogradov und Moens, 2008). Zudem können sie, insbesondere im Falle der Globine mit Sensordomänen, den GCS (‚globin-coupled sensors'), aerotaktische und genregulatorische Funktionen in den Zellen übernehmen (Freitas et al., 2005; Gilles-Gonzalez, 2005; Saito et al., 2008).

Ebenso vielfältig wie die physiologischen Funktionen der Globine sind die quartären Strukturen, die von Monomeren mit Einzeldomänen über Polymere mit Einzeldomänen bis hin zu Polymeren mit bis zu 18 kovalent verknüpften Globin-Domänen reichen (Vinogradov, 1985; Vinogradov et al., 1993; Terwilliger, 1998; Weber und Vinogradov, 2001). Insbesondere bei den Globinen der Invertebraten kann man die große Bandbreite der Quartärstruktur beobachten. In Ringelwürmern (Annelida) und Riesenröhrenwürmern (Vestimentifera) finden sich z. B. riesige Globin-Aggregate mit einer Masse von 3600kDa, zusammengesetzt aus vielen hexagonal in zwei Schichten angeordneten Untereinheiten (Lamy et al., 1996). Diese werden noch übertroffen von den Globin-Polymeren der Muscheln, die mehrere Domänen vereinen und bis zu 12000kDa groß sein können (Terwilliger und Terwilliger, 1978).

Diese Extrembeispiele der Globin-Quartärstruktur repräsentieren jedoch lediglich einen kleinen Teil der Invertebraten-Globine. Der größte Teil der Invertebraten-Globine liegt als monomere, Myoglobin-ähnliche Globine mit ca. 150 AS Länge vor, wie sie z. B. in Insekten sowohl intra- als auch extrazellulär auftreten.

1.2 Globine in Insekten

In Insekten findet der Gastransport von und zu den Organen mithilfe des Tracheensystems statt. Dabei handelt es sich um ein System aus tubulären Verzweigungen, die den Körper durchziehen und am Ende durch feine Verästelungen, den Tracheolen, direkt mit den Zellen in Verbindung stehen. Die Verteilung des über die Spirakel aufgenommenen Sauerstoffs erfolgt per Diffusion, dadurch wird eine optimale Versorgung der Organe gewährleistet. Aufgrund der hohen Effizienz des Tracheensystems wurde bis vor einigen Jahren angenommen, dass Insekten auf respiratorische Proteine verzichten können (Willmer et al., 2000). Als Ausnahmen galten Insekten mit offensichtlich sauerstoffarmem Habitat, wie etwa die Larven der Zuckmücke *Chironomus* (Diptera), die einen hohen Gehalt an extrazellulärem Hämoglobin in ihrer Hämolymphe aufweisen, das dem O_2-Transport dient (Ewer, 1942; Osmulski und Leyko, 1985). Auch die Pferdemagenbremse *Gasterophilus intestinalis* (Diptera), deren Larven im Pferdeinneren leben und die Rückenschwimmer *Buenoa confusa*, *Anisops confusa* und *Anisops assimilis* (Hemiptera) greifen auf Globine zur Gewährleistung der O_2-Versorgung zurück. Bei den Hämoglobinen von *G. intestinalis* und den Rückenschwimmern handelt es sich um

intrazelluläre Proteine, die hauptsächlich im Fettkörper und Tracheensystem lokalisiert sind und vermutlich eine dem Myoglobin ähnliche O_2-Speicherfunktion ausüben (Hungerford, 1922; Keilin und Wang, 1946; Bergtrom, 1977; Wells, 1981; Dewilde et al., 1998; Vinogradov et al., 2005; Matthews und Seymour, 2006).

Ausgehend von der Entdeckung eines Globins in *Drosophila melanogaster* (glob1) (Burmester und Hankeln, 1999) wurden jedoch mehr und mehr Globine auch in solchen Insekten gefunden, die in normoxischer Umgebung leben (Abb. 1.2). Globine wurden unter anderem identifiziert in den Blattläusen *Acyrthosiphon pisum*, *Aphis gossypii* (Hemiptera), den Käfern *Tribolium castaneum* und *Dascillus cervinus* (Coleoptera), in der Honigbiene *Apis mellifera* (Hankeln et al., 2006) und dem Seidenspinner *Bombyx mori* (Hymenoptera) (Kawaoka et al., 2009), in den Moskitos *Aedes aegypti* und *Anopheles gambiae* (Burmester et al., 2007b) sowie in der Tsetsefliege *Glossina morsitans* (Diptera) (Hankeln et al., 2006). Die Sequenzanalyse der Insekten-Globine ergab, dass die für die Häm- und O_2-Bindung wichtigen Aminosäuren zumeist konserviert vorliegen. Die Insekten-Globine sind somit höchstwahrscheinlich funktionell, wenngleich ihre exakte physiologische Rolle unklar ist (Burmester und Hankeln, 2007).

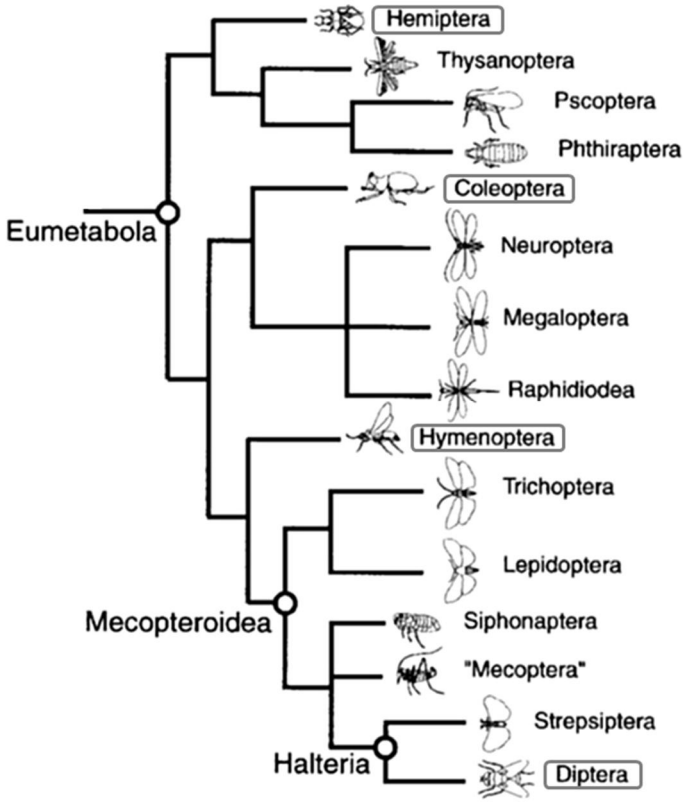

Abb. 1.2: Auftreten von Globinen innerhalb des Stammbaums der Eumetabola (Wheeler, 2001). Die Ordnungen der Spezies mit Globinen sind markiert.

1.3 Globine in *Drosophila*

Drosophila melanogaster besitzt drei Globine, glob1, glob2 und glob3 (Burmester et al., 2006). Alle drei Globine weisen die typische Globinfaltung sowie eine Konservierung der für die Häm- und O_2-Bindung funktionell wichtigen Aminosäuren auf (Hankeln et al., 2002; Burmester et al., 2006).

- Glob1 hat mit 154 AS die für Globine typische Länge und eine mit anderen intrazellulären Insekten-Globinen vergleichbare O_2-Affinität (P_{50} = 0,12 Torr). Im deoxygenierten Zustand liegt das Häm-Eisen des glob1 hexakoordiniert vor. Dies kommt durch die zusätzliche Bindung des proximalen Histidinrests an Position 7 der E-Helix zustande (de Sanctis et al., 2005; de Sanctis et al., 2006). *Glob1* ist ein stark exprimiertes Gen, das in allen Entwicklungsstadien aktiv ist, wobei die stärkste Expression im Fettkörper und Tracheensystem nachgewiesen wurde (Hankeln et al., 2002).
- Bei glob2 und glob3 handelt es sich um Duplikate. Beide Proteine weisen N- und C-terminale Extensionen auf, sie liegen deshalb mit 196 AS und 222 AS deutlich über der durchschnittlichen Globin-Länge. Beide Gen-Paralogen werden exprimiert, was durch das Vorhandensein von ESTs (='expressed sequence tags') gezeigt werden konnte. Über das zelluläre und temporale Expressionsmuster von *glob2* und *3* ist hingegen bisher nichts bekannt. Dies wurde daher im Rahmen der vorliegenden Arbeit analysiert.

1.4 Reaktionen von *Drosophila* auf unterschiedliche Sauerstoffbedingungen

Die steigende Zahl an in Insekten identifizierten Globinen sowie das breite Auftreten in einer Vielzahl von Spezies lässt mittlerweile die Vermutung zu, dass Globine zum Standardrepertoire der Insekten gehören. Diese Annahme wirft die Frage nach dem Nutzen O_2-bindender Proteine in Insekten mit hochentwickeltem Tracheensystem auf, das ja bekanntermaßen eine ausreichende O_2-Versorgung der Organe und Zellen selbst während äußerst aktiver Phasen wie dem Flug gewährleistet.

Betrachtet man jedoch beispielsweise den Lebensraum von *Drosophila,* wird deutlich, dass insbesondere Larven, die in fermentierendem Obst leben, oftmals hypoxischen Bedingungen ausgesetzt sind (Wingrove und O'Farrell, 1999). Es wurde experimentell gezeigt, dass alle Entwicklungsstadien von *Drosophila*, die adulten Tiere eingeschlossen, eine hohe Toleranz gegenüber hypoxischen und sogar anoxischen Bedingungen zeigen (Haddad et al., 1997; Wingrove und O'Farrell, 1999; Haddad, 2006). Diese Hypoxie- und Anoxietoleranz beruht auf Anpassungs- sowie Vermeidungsmechanismen, die von einer hypoxieabhängigen Genregulation gesteuert werden. Die Reaktionen reichen von anoxischem Stupor (Csik, 1939; Haddad et al., 1997), begleitet von einer Drosselung des O_2-Verbrauchs und des gesamten

Metabolismus (Haddad et al., 1997; Haddad, 2000) bis hin zu einer Arretierung des Zellzyklus in Embryonen (Wingrove und O'Farrell, 1999; Lavista-Llanos et al., 2002). Änderungen der Genexpression unter Hypoxie in *Drosophila* werden wie bei Säugern oft durch den Master-Transkriptionsfaktor HIF (='hypoxia inducible factor') induziert und beinhalten beispielsweise die Induktion neuer trachealer Verzweigungen in stark hypoxischen Arealen, ein Mechanismus, der mit der Angiogenese in Mammaliern vergleichbar ist (Lavista-Llanos et al., 2002). HIF ist ein Dimer bestehend aus einer α- und β-Untereinheit. Der Komplex wird unter Hypoxie stabilisiert und bindet an sogenannte HREs ('hypoxia response elements') in der Umgebung der HIF-Zielgene (Wenger et al., 1996; Okino et al., 1998; Ebert und Bunn, 1999; Wenger, 2000; Semenza, 2007), während unter Normoxie eine O_2-abhängige proteasomale Degradation der α-Untereinheit erfolgt und somit kein aktives HIF vorliegt (Maxwell und Ratcliffe, 1998).

In adulten *Drosophila*, die in der Regel zumindest keinem offensichtlichen O_2-Mangel in ihrer Umgebung ausgesetzt sind, scheint weniger die Sauerstoffverfügbarkeit als vielmehr ein O_2-Überschuss Gefahren für den Zellerhalt zu bergen. Die hohe Effektivität des Tracheensystems gewährleistet einerseits eine ausreichende O_2-Versorgung während aktiver Phasen, birgt aber andererseits die Gefahr eines O_2-Überschusses während Phasen geringerer Aktivität (Hetz und Bradley, 2005). Durch die direkte Verbindung der Tracheolen des Tracheensystems mit den Zellen (Guillemin et al., 1996; Jarecki et al., 1999), entsteht somit die Gefahr eines O_2-Überschusses, der über die Bildung von reaktiven Sauerstoffspezies zu oxidativen Zellschäden führen kann.

Viele Insekten schließen oder verengen in Ruhephasen ihre Spirakel und öffnen diese lediglich, wenn der CO_2-Partialdruck zu hoch und der O_2-Partialdruck in den Tracheen zu niedrig wird. Der sogenannte ‚discontinuous gas exchange cycle' (DGC) beschreibt ein azyklisches Öffnen und Schließen der Spirakel (Kestler, 1985; Lighton, 1996), von dem angenommen wird, dass es während Ruhephasen einem O_2-Überschuss im Tracheensystem vorbeugt (Hetz und Bradley, 2005). Für diese Hypothese spricht zudem, dass DGC hauptsächlich in Insekten mit niedriger metabolischer Rate und ausschließlich in Ruhephasen beobachtet wurde (Hetz und Bradley, 2005). Ein hoher O_2-Verbrauch, bedingt durch die stark erhöhte metabolische Rate während aktiver (Flug)Phasen, birgt ebenfalls die Gefahr oxidativer Zellschäden. Der erhöhte O_2-Bedarf fliegender *Drosophila* beispielsweise setzt ein permanentes Öffnen der Spirakel voraus. Zwar sind adulte *Drosophila* in der Lage, die Weite ihrer geöffneten Spirakel in Abhängigkeit des O_2-Bedarfs zu regulieren (Lehmann, 2001; Heymann und Lehmann, 2006), trotzdem birgt die hohe O_2-Konzentration (19kPa) in Tracheolen (Lehmann, 2001) selbst bei hohem O_2-Verbrauch die Gefahr oxidativer Schäden der umliegenden Zellen (Hetz und Bradley, 2005).

Die Entgiftung reaktiver Sauerstoffspezies erfolgt in *Drosophila* wie in Säugern auch mittels eines sehr effektiven, enzymatischen Antioxidations-Systems, dessen Wichtigkeit für den gesamten Organismus deutlich wird, wenn man die Auswirkungen von ‚Loss-of-function'-Mutationen auf den gesamten Organismus betrachtet. Durch Nullmutationen inhibierte Enzyme des Antioxidations-Systems haben gravierenden Einfluss auf die Fertilität und führen zu einer um ein Viertel verkürzten Lebensdauer (Phillips et al., 1989; Parkes et al., 1998; Rogina und Helfand, 2000). Die bei den Untersuchungen zur Hypoxie- und Hyperoxietoleranz von *Drosophila* gewonnenen Erkenntnisse machen deutlich, wie wichtig die Bewahrung des Gleichgewichts zwischen O_2-Verfügbarkeit und -Verbrauch ist, um die normalen metabolischen Prozesse und somit die Gesundheit des gesamten Organismus aufrecht zu erhalten. Zudem offenbaren sie die vielfältigen Mechanismen, denen sich ein Organismus bedient, um dieses Gleichgewicht zu wahren.

Bei den Globinen von *Drosophila* handelt es sich mit großer Wahrscheinlichkeit um funktionelle Atmungsproteine, die aufgrund ihrer typischen Globin-Eigenschaften zur Bindung gasförmiger Liganden befähigt sind. Aufgrund dessen wäre eine Myoglobin-ähnliche O_2-Speicherfunktion denkbar, die das Tracheensystems bei der O_2-Versorgung der Zellen unterstützt. Ebenso denkbar wäre jedoch in Analogie zu Globinen bei Vertebraten eine Beteiligung bei der Entgiftung reaktiver Sauerstoffspezies, die in Insekten durch den Überschuss an O_2 im Tracheensystem in besonderem Maße entstehen sollten.

1.5 Zielsetzung der vorliegenden Arbeit

Für aerobe Organismen ist die Verfügbarkeit von O_2 für die Energieproduktion überlebenswichtig. Der über die Atmungsorgane aufgenommene O_2 wird über das Kreislaufsystem im Körper verteilt, ein Prozess, bei dem oftmals respiratorische Proteine – Globine – beteiligt sind. Die weite phylogenetische Verbreitung von Globinen in Insekten, die dank ihres effizienten Tracheensystems eigentlich bereits eine optimale O_2-Versorgung haben und somit nicht auf die Hilfe von Globinen zur O_2-Versorgung angewiesen sein dürften, wirft die Frage nach der Funktion der Atmungsproteine in Insekten auf. Um dieser Frage nachzugehen, wurde im Rahmen dieser Arbeit auf den Modellorganismus *Drosophila melanogaster* zurückgegriffen, der sich sowohl durch die Anwesenheit dreier Globine (glob1, glob2 und glob3) als auch durch die hervorragenden Möglichkeiten zur genetischen Manipulation auszeichnet.

Ziel des ersten Teils dieser Arbeit war es, mittels Analyse der Genstruktur, der Gen- und Proteinexpression unter verschiedenen Bedingungen sowie durch Manipulation der

endogenen *glob1* Expression Hinweise auf die Funktion des *glob1* Gens von *Drosophila* zu erhalten. Die zu adressierenden Fragen lauteten:

- Ändert sich die endogene *glob1*-Expression in *D. melanogaster* in Abhängigkeit des Sauerstoffgehalts der Umgebung? (Vergleiche Kapitel 2.1: Oxygen-induced changes of hemoglobin expression in *Drosophila*)
  Wildtypische Embryonen, L3 Larven und adulte Fliegen von *D. melanogaster* sollten experimentell sowohl hypoxischen als auch hyperoxischen Bedingungen ausgesetzt und anschließend die *glob1* Genexpression mittels quantitativer Realtime-PCR quantifiziert werden. Aus dem Modus der Genregulation sollten Schlussfolgerungen auf die funktionelle Rolle von glob1 gezogen werden.

- Gibt es entwicklungsabhängige und sauerstoffabhängige Unterschiede im Expressionsmuster der *glob1* Transkriptvarianten? (Vergleiche Kapitel 2.2: Entwicklungs- und gewebeabhängige Regulation der *D. melanogaster glob1* Transkription)
  Hierzu sollte eine *in silico* Sequenzanalyse des *glob1* Genbereichs durchgeführt und etwaige Besonderheiten, wie z. B. mögliche duplizierte Bereiche in den Promotorregionen des *glob1* Gens experimentell charakterisiert werden. Zusätzlich sollte das Expressionsmuster der *glob1* Transkriptvarianten unter normoxischen und hypoxischen Bedingungen mittels quantitativer Realtime-PCR charakterisiert werden. Die Analyse des Expressionsmusters der Transkriptionsvarianten sollte Hinweise auf entwicklungs- oder O_2-spezifische Änderungen der Promotor-Aktivitäten liefern, aus welchen Schlussfolgerungen auf die funktionelle Rolle von glob1 gezogen werde sollten.

- Welche phänotypischen Auswirkungen hat die Manipulation der endogenen *Drosophila glob1* Expression? (Vergleiche Kapitel 2.3: Knockdown of *Drosophila* glob1 evokes a mild phenotype but argues for a role in O_2 supply)
  Durch Verwendung des UAS/Gal4-Systems sollte die endogene *glob1*-Expression in *D. melanogaster* sowohl herauf- als auch RNAi-vermittelt herunterreguliert werden. Eventuelle phänotypische Auswirkungen dieser Manipulation sollten in Larven und in Adulten unter normalen Bedingungen sowie unter experimentellem Stress identifiziert und gegebenenfalls auf zellulärer Ebene eingehender charakterisiert werden.
  Es wurde erwarte, dass Änderungen der glob1-Expression Abweichungen vom normalen Phänotyp, insbesondere unter induzierten Stressbedingungen, hervorruft, die Rückschlüsse auf die Funktion(en) von glob1 zulassen.

Ziel des zweiten Teils dieser Arbeit war die Abgrenzung der Funktion von glob1 relativ zu den anderen beiden Globinen in *D. melanogaster*, glob2 und glob3. Hierzu sollten *D. melanogaster glob2* und *glob3* mittels phylogenetischer Rekonstruktionen, Analysen zur molekularen Evolution sowie durch Analysen des Expressionsmusters beider Globine unter Normoxie und Hypoxie charakterisiert werden. Die Detail-Fragen für das Kapitel 2.4 ‚Testes-specific hemoglobins in *Drosophila* evolved by a combination of sub- and neofunctionalization after gene duplication' lauteten daher:

- Wie stark und wo werden *Drosophila glob2* und *glob3* exprimiert?
  Die *Drosophila glob2*- und *glob3*-Expression sollte in männlichen und weiblichen L3 Larven, Puppen und Adulten mittels quantitativer Realtime-PCR vorgenommen werden. Nach Eingrenzung der Expression auf bestimmte Organe sollte die Lokalisierung der *glob2* und *glob3* Transkripte mithilfe von antisense mRNA *in situ*-Hybridisierung erfolgen.
  Es sollte untersucht werden, ob das Expressionsmuster der *glob2/3* Duplikate mit dem des *glob1* übereinstimmt oder ob unterschiedliche Expressionsmuster auf Unterschiede in der Funktion von glob2/3 und glob1 hindeuten.

- Welche evolutionären Vorgänge können die Herkunft von *glob2* und *glob3* in *Drosophila* erklären?
  Um die Entstehung der Duplikate *glob2* und *glob3* nachvollziehen zu können, sollten alle in den Datenbanken verfügbare Genome auf die Anwesenheit der Duplikate überprüft werden. Anschließend sollte eine Rekonstruktion der evolutionären Vorgänge, die die Verteilung der Duplikate in den untersuchten *Drosophila* Spezies sinnvoll erklären kann, vorgenommen werden.
  Anhand der phylogenetischen Verbreitung der Gene sollten Schlussfolgerungen auf ihre Funktion gezogen werden.

- Gab es im Laufe der Evolution Änderungen auf Sequenzebene von *Drosophila* glob2 und glob3, die Hinweise auf funktionelle Änderungen zulassen?
  Um die Entstehung der Duplikate *glob2* und *glob3* nachvollziehen zu können, sollten alle in den Datenbanken verfügbaren Genome auf die Anwesenheit der Duplikate überprüft werden. Anschließend sollte eine Rekonstruktion der evolutionären Vorgänge, die die Verteilung der Duplikate in den untersuchten *Drosophila* Spezies sinnvoll erklären können, vorgenommen werden. Zusätzlich sollten Sequenzvergleiche der Globine über die Spezies hinweg durchgeführt werden, um die Anzahl synonymer und nicht-synonymer

Aminosäureaustausche zu berechnen. Dies sollte dazu dienen, Aussagen über die Evolution der Globine nach dem Duplikationsereignis treffen zu können, um dadurch eventuell Hinweise auf Funktionsanpassungen eines Duplikats oder beider Duplikate zu erhalten.

2. ERGEBNISSE

2.1 Gleixner, E., Abriss, D., Adryan, B., Kraemer, M., Gerlach, F., Schuh, R., Burmester, T. and Hankeln, T. (2008) Oxygen-induced changes in hemoglobin expression in *Drosophila*. FEBS J. 2008 275:5108-16.

2.2 Entwicklungs- und gewebeabhängige Regulation der *D. melanogaster glob1* Transkription

2.3 Gleixner, E., Schuh, R., Wolf, C., Burmester, T. and Hankeln, T. (2011) Knockdown of *Drosophila* glob1 evokes a mild phenotype but argues for a role in O_2 supply.

2.4 Gleixner, E., Herlyn, H., Zimmerling, S., Burmester, T. and Hankeln, T. (2011) Testes-specific hemoglobins in *Drosophila* evolved by a combination of sub- and neofunctionalization after gene duplication.

2.1 Gleixner, E., Abriss, D., Adryan, B., Kraemer, M., Gerlach, F., Schuh, R., Burmester, T. and Hankeln, T. (2008) Oxygen-induced changes in hemoglobin expression in *Drosophila*. FEBS J. 2008 275:5108-16.

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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

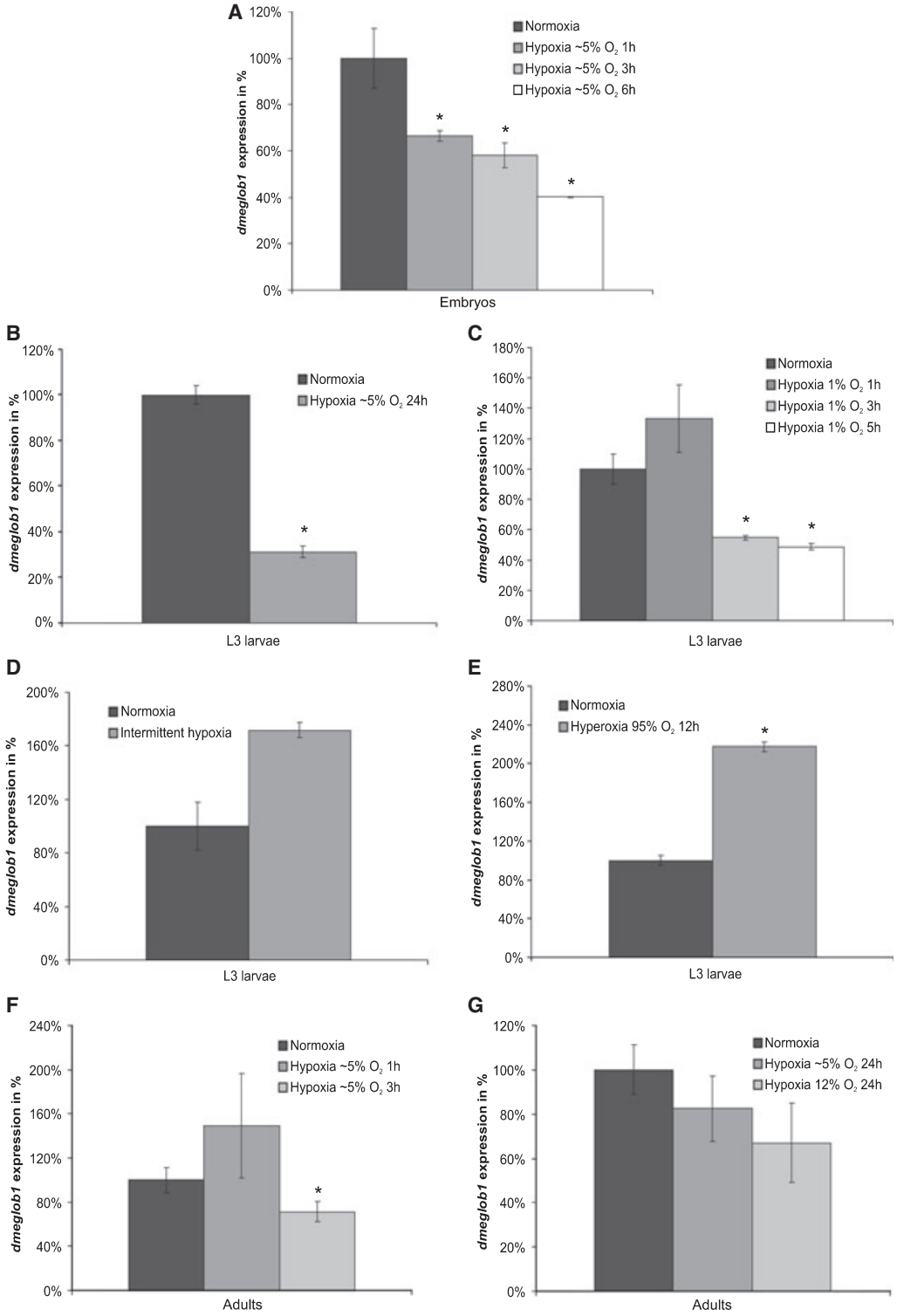

Fig. 1. Regulation of *dmeglob1* mRNA in *Drosophila melanogaster* developmental stages after hypoxia and hyperoxia stress. mRNA levels (bars) are shown relative to gene expression at normoxia (21%). The applied O_2 concentrations, exposure times and developmental stages are indicated. (A) Embryos, pooled 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 ~ 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 ~ 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% 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 (~ 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 ~ 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

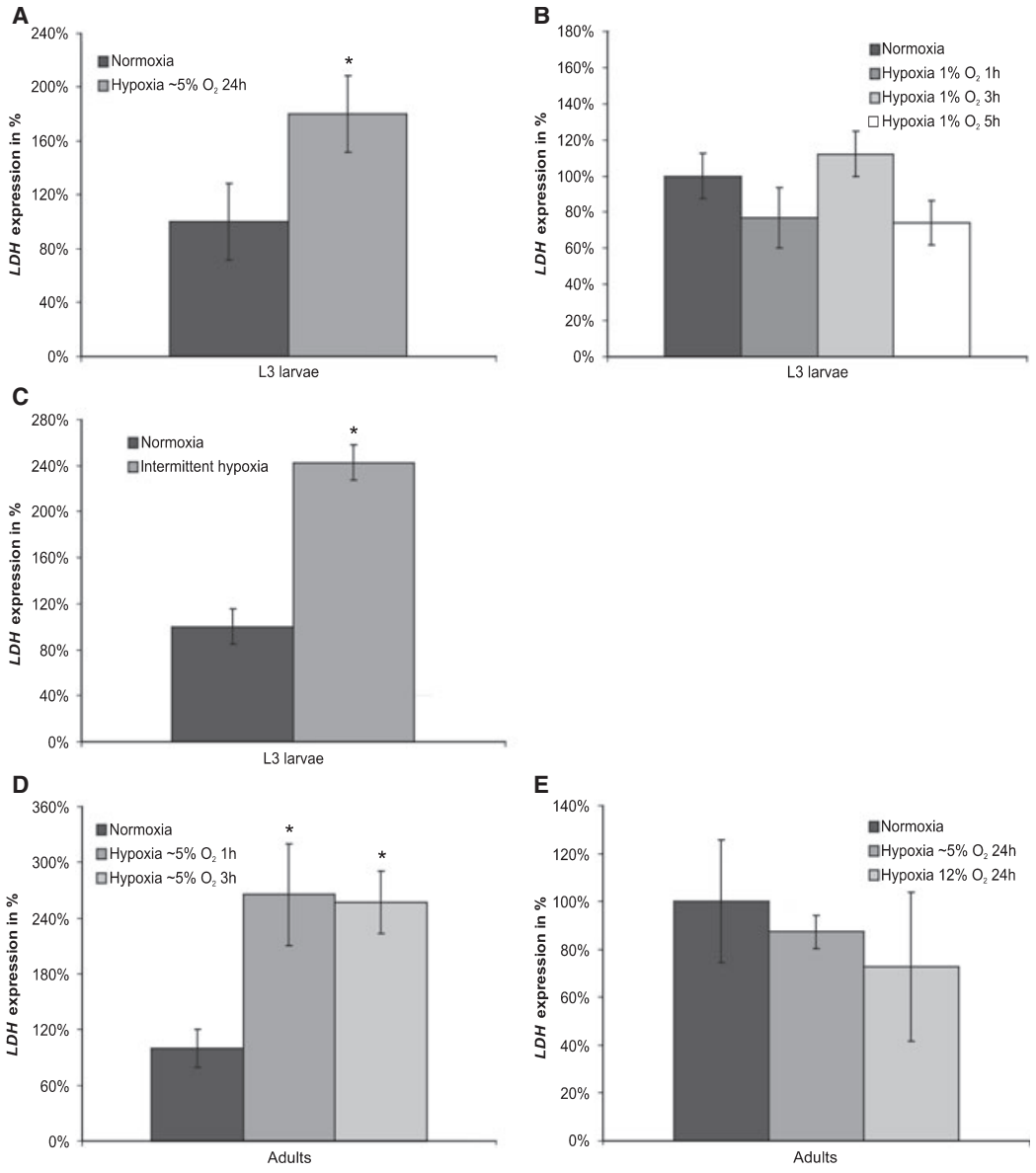

Fig. 2. Regulation of *LDH* mRNA in *Drosophila melanogaster* developmental stages after hypoxia stress. mRNA levels (bars) are shown relative to the gene expression at normoxia (21%). The applied O_2 concentrations, exposure times and developmental stages are indicated. (A) Third instar larvae, ~ 5% 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 $\sim$ 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.

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2.2 Entwicklungs- und gewebeabhängige Regulation der *D. melanogaster glob1* Transkription

Entwicklungs- und gewebeabhängige Regulation der D. melanogaster glob1 Transkription

Einleitung

Das *Drosophila glob1* Gen befindet sich auf Chromosom 3R und überspannt eine 8719bp lange Region. Es enthält insgesamt 6 Exons, wobei lediglich die Exons 4-6 proteinkodierend sind. Die kodierende Region hat eine Länge von 462bp und resultiert in einem Protein mit 17kDa Masse (Burmester und Hankeln, 1999). Durch die Analyse von EST-Datenbanken konnte festgestellt werden, dass insgesamt vier verschiedene *glob1* Transkriptvarianten (Isoform A-D) existieren. Alle diese Isoformen beinhalten die kodierenden Exons 4-6, weisen allerdings unterschiedliche 5´-Enden auf, die sich durch die Prä- bzw. Absenz, sowie die Längen der nicht-kodierenden 5´-Exons unterscheiden (Hankeln et al., 2002). Die Expression von drei der vier Isoformen konnte bereits per Northern Blot und quantitativer Realtime-PCR in unterschiedlichen Entwicklungsstadien experimentell bestätigt werden (Hankeln et al., 2002). Die Überprüfung der Region des *glob1* Gens mithilfe von bioinformatischen Programmen auf mögliche Promotoren ergab die Anwesenheit eines TATA-Box beinhaltenden, putativen Promotors (Promotor I, Abb. 1) 16bp vor Exon1 gelegen. Ein weiterer Promotor mit einer TATA-Box-ähnlichen Sequenz (Promotor II, Abb. 1) wurde 30bp vor Exon3 identifiziert (Hankeln et al., 2002).

Drosophila glob1 ist ein stark exprimiertes Gen. Transkripte finden sich in allen Entwicklungsstadien und in vielen Geweben, wobei die Hauptexpressionsorte der Fettkörper sowie das Tracheensystem sind. Dies konnte mithilfe von mRNA *in situ* Hybridisierung in Embryonen und Larven nachgewiesen werden. In adulten Fliegen konnten die gleichen Hauptexpressionsort zudem mittels Immunhistochemie nachgewiesen werden konnte (Hankeln et al., 2002).

Im Rahmen der vorliegenden Studie wurde mittels Sequenzanalyse eine genaue Untersuchung des *glob1* Genbereichs, insbesondere der Promotorregionen durchgeführt.

Es erfolgte eine genaue Analyse des Expressionsmusters der vier *glob1* Isoformen in unterschiedlichen Entwicklungsstadien von *D. melanogaster*, um dadurch Hinweise auf eine funktionelle Relevanz der alternativ gespleißten Transkripte zu erhalten. Zusätzlich wurde die Expression der Isoformen auf Änderungen in Reaktion auf Hypoxie überprüft.

Um das bisher vorhandene Wissen um die *glob1* mRNA Expressionsorte in Embryonen von *D. melanogaster* auf Proteinebene zu vervollständigen, wurden Immunfluoreszenz-Färbungen unter Verwendung eines glob1-Antikörpers an späten Embryonen durchgeführt.

Material und Methoden

Untersuchung der glob1 Genregion in Drosophila

Für die Untersuchung der *glob1* Genregion in den *Drosophila* Spezies der *melanogaster* Subgruppe, wurden die *glob1* Genregionen inklusive ihrer proximal und distal flankierenden Bereiche (je 2kb) heruntergeladen. Alignments und Sequenzanalysen wurden mithilfe des Programms SeqMan (DNAStar, Lasergene) durchgeführt. DotPlots zweier Sequenzen zur Identifizierung duplizierter Bereiche wurden mithilfe des Programms MegAlign (DNAStar, Lasergene) durchgeführt.

Präparation genomischer DNA und genomische PCR

Die Isolierung genomischer DNA aus 25 L3 Larven der *D. melanogaster* Wildtyp-Stämme Oregon R, Canton S, Berlin und Acharren erfolgte gemäß des Protokolls von Huang et al. (2009). Die experimentelle Bestätigung der durch Sequenzanalyse identifizierten Duplikation in der Genregion des *D. melanogaster glob1* in den Wildtyp-Stämmen erfolgte mittels PCR unter Verwendung der Taq-Polymerase TrueStart (Fermentas, St. Leon Roth) gemäß den Anweisungen der Herstellers. Es wurde folgende Primer verwendet: *Dmeglob1* Dupli for 5´-GTTGAACTACGCCTCTCTCATC-3´ und *Dmeglob1* Dupli rev 5´-GCCGGCAGAGAGTGCGAA-3´.

Hypoxie

Wildtypische *D. melanogaster* Embryonen, L3 Larven, Puppen und adulte Weibchen und Männchen der Linie Oregon R wurden Hypoxie mit 5% O_2 für 24h ausgesetzt. Die Hypoxie-Behandlung erfolgte in einer Hypoxie-Kammer (PRO-OX, BioSpherix, Ltd., New York, USA) mit O_2-Sensor, sowie einem Steuerungselement (E-702, BioSpherix, Ltd., New York, USA) zur Kontrolle der gewünschten O_2-konzentration. Hypoxische Bedingungen wurden durch Begasung mit technischem Stickstoff (Westfalen AG Münster) erzeugt.

RNA-Präparation, cDNA-Synthese und quantitative Realtime-PCR

Gesamt-RNA aus normoxischen und hypoxischen Embryonen, L3 Larven, Puppen und adulten Weibchen und Männchen der wildtypischen *D. melanogaster* Linie Oregon R wurde mithilfe des RNeasy mini-Kits (Qiagen, Hilden) gemäß den Anweisungen des Herstellers isoliert. Für die cDNA Synthese wurde die reverse Transkriptase SuperScriptIII (Invitrogen, Darmstadt) verwendet. Es wurden jeweils 1µg Gesamt-RNA für die reverse Transkription eingesetzt, der Ansatz erfolgte gemäß den Anweisungen des Herstellers.

Die mRNA-Quantifizierung per Realtime-PCR wurde mit dem Gerät 7500 fast Real-time PCR-System (Applied Biosystems, Darmstadt) durchgeführt. Die PCR-Ansätze hatten ein Endvolumen von 10µl

und enthielten SYBR Green (Power SYBR Green PCR Master Mix, Applied Biosystem, Darmstadt), sowie beide Primer in einer Endkonzentration von je 0.33µM. Die pro PCR-Reaktion eingesetzte cDNA-Menge entsprach 25ng Gesamt-RNA. Folgende Primer-Kombinationen wurden verwendet: Dmeglob1 IsoA for 5´-TATCTTCCCCATCGACGGCG-3´; Dmeglob1 IsoB for 5´-TAATACGTTGCCTAAAAGCCA-3´; Dmeglob1 IsoC for 5´-CGTCGTGTGCAGTTGCCTG-3´; Dmeglob1 IsoD for 5´-CGTTGCTTACAATTCCCGTTC-3; Dmeglob1 IsoA-D rev ´5´-CTGCCGGCATGTGCTCGG-3´.
Nach der Hitzeaktivierung der DNA Polymerase (AmpliTaq Gold, Invitrogen) bei 95°C für 15min wurde die Amplifikation nach folgendem Protokoll durchgeführt: 95°C für 15sek, 60°C für 30sek, 72°C für 30sek, wobei die Messung der Fluoreszenz im letzten Schritt eines jeden Zyklus erfolgte. Alle Realtime-PCR-Läufe wurden mithilfe einer Dissoziationskurve am Ende des Laufs auf ihre Spezifität überprüft. Die mRNA Expressionslevel wurden mittels absoluter Quantifizierung durch Messung der Ct-Werte über Standardgeraden ermittelt. Jeder Realtime-PCR Ansatz wurde in Triplikaten gemessen und jedes Experiment wurde einmal repliziert. Die statistische Signifikanz der Daten wurde in Excel mithilfe des t-Tests überprüft.

Immunfluoreszenz-Färbung an D. melanogaster Embryonen

Um Embryonen mit tracheenspezifischer GFP-Expression, aber ansonsten unveränderter Genexpression zu generieren, wurden homozygote, jungfräuliche Weibchen der *Drosophila* Responder-Linie UAS CD8-GFP (Stammsammlung AG Technau) mit Männchen mit dem tracheenspezifischen Treiber breathless Gal4 (btl Gal4) verkreuzt und für 18h auf Gläser mit Apfelsaftagar gesetzt. Nach erfolgter Eiablage wurden die Embryonen zur Dechorionisierung mit 7,5%iger Chlorbleiche überschichtet, durch ein Sieb gespült und bei Raumtemperatur (RT) für 23min auf einem Schüttler in Fixativ (PBS/Formaldehyd/Heptan, im Verhältnis 9/1/10) fixiert. Nach Devitellinisierung der Embryonen (Zugabe von Methanol und 1min Vortexen, einmal wiederholen) wurden die Embryonen viermal für 10min in Methanol gewaschen. Anschließend erfolgten 5 Waschschritte mit PBS mit 0,3% Triton X-100 (PBST) für jeweils 10min. Die primäre Antikörper-Inkubation erfolgte über Nacht bei 4°C auf dem Schüttler mit glob1-spezifischen Antikörpern (1:10 verdünnt in PBST). Nach erfolgter ÜN-Inkubation wurde erneut 5mal 10min mit PBST gewaschen, gefolgt von der Inkubation im sekundären Alexa 466 konjugierten, anti-rabbit-Antikörper (Molecular Probes, 1:400 verdünnt in PBST) für 2Stunden bei RT. Anschließend wurde 5mal für je 10min in PBST gewaschen, zweimal mit PBS gespült und 70% Glycerin/PBS zugegeben. Die Auswertung der Immunfluoreszenz-Färbungen wurde mit dem Lichtmikroskop Leica DM 6000 B (Leica microsystems, Bensheim) durchgeführt.

Ergebnisse

Untersuchung der Drosophila glob1 Genregion

Die bioinformatische Sequenzanalyse der *glob1* Genregion der *Drosophila* Spezies der *melanogaster* Subgruppe ergab das Vorhandensein einer Duplikation im *glob1* Genbereich von *D. melanogaster*. Der duplizierte Bereich hat eine Länge von 2059bp, die Duplikate grenzen unmittelbar aneinander und weisen eine Sequenzidentität von 99,8% auf. Das näher zur codierenden Sequenz von *glob1* gelegene Duplikat (Duplikat2) endet 64bp vor Exon3A, dem nicht-kodierenden Startexon der Transkriptvarianten A und B. Von der Duplikation betroffen sind Exon1 und Exon2, sowie der TATA-Box beinhaltende Promotor vor Exon1 (Promotor I), der Transkriptvarianten C und D. Beide Duplikate zeigen eine exakte Übereinstimmung in der Länge und Sequenz der Exons und Introns, sowie der Lage und Sequenz des putativen Promotors (Abb. 1). Potentielle Bindestellen des Transkriptionsfaktors HIF1 (Semenza und Wang, 1992; Semenza et al., 1996; Camenisch et al., 2001) sind nicht von der Duplikation betroffen.

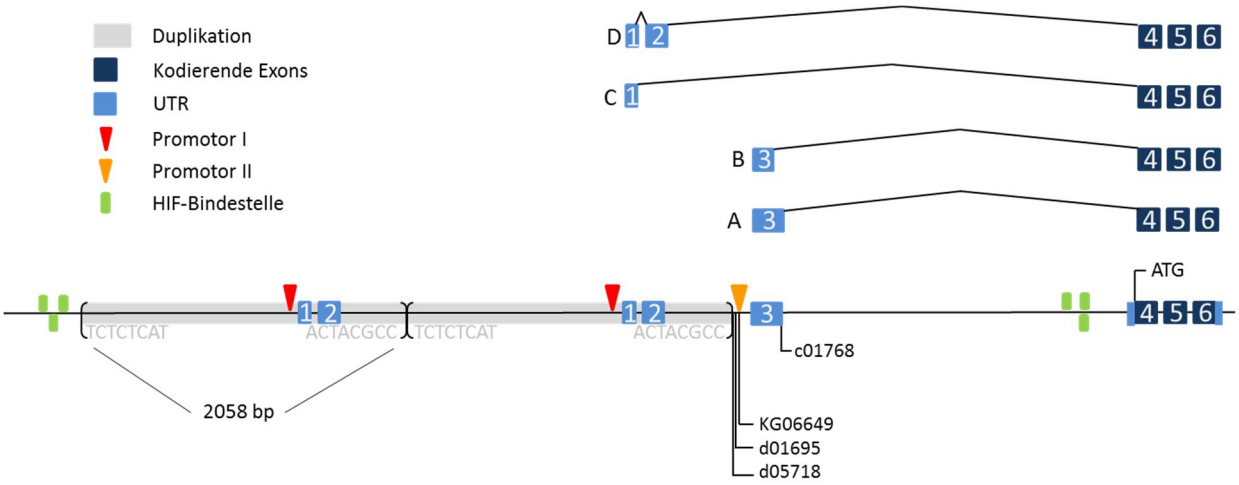

Abb. 1: Organisation des *D. melanogaster glob1* Gens. Dargestellt sind die vier Transkripte (Isoformen A-D) mit unterschiedlichen nicht-kodierenden Exons (hellblau) in den 5´-Bereichen und der kodierenden Region (dunkelblau). Im unteren Teil der Abbildung ist der *glob1* Genbereich mit den Duplikaten (hellgrau), nicht-kodierenden und kodierenden Exons, sowie dem duplizierten Promotor I (rot) dargestellt. Zusätzlich eingezeichnet sind der (TATA-Box-) Promoter II der Transkripte A und B (orange), potentielle HIF-Bindestellen (grün) und P-Element-Insertionsstellen kommerziell erhältlicher, transgener glob1-Stämme.

In den *glob1* Genregionen der übrigen vier *melanogaster* Spezies fanden sich hingegen keine Hinweise auf eine derartige Duplikation.

Um den durch Sequenzanalyse erhaltenen Befund der Duplikation innerhalb der *glob1* Genregion in *D. melanogaster* experimentell zu bestätigen, wurden genomische PCRs mit Duplikat2-spezifischen Primern mit DNA aus vier wildtypischen *D. melanogaster* Linien durchgeführt. Die PCR auf DNA aus

Fliegen der Linien Oregon R, Berlin und Acharren lieferte eine eindeutige Bande der erwarteten Länge von 2,1kb und bestätigte somit das tatsächliche Vorhandensein eines duplizierten Bereichs im *glob1*-Gen. Die PCR mit DNA aus Fliegen der Linie Canton S als Template lieferte in keinem der durchgeführten Experimente ein Amplifikationsprodukt.

Quantifizierung der D. melanogaster glob1 Isoformen in unterschiedlichen Entwicklungsstadien

Mittels Realtime-PCR wurden die Expression sowie die Expressionsstärke aller vier vorhandenen *glob1* Isoformen in Embryonen, L3 Larven, Puppen und adulten Weibchen und Männchen der *D. melanogaster* Wildtyp-Linie Oregon R sowohl unter normoxischen, als auch unter hypoxischen Bedingungen gemessen. In allen fünf untersuchten Entwicklungsstadien konnte die Expression der vier Isoformen sowohl unter normoxischen als auch unter hypoxischen Bedingungen nachgewiesen werden. Das Expressionsmuster der Isoformen war in allen fünf Stadien ähnlich (die absoluten mRNA Kopienzahlen der einzelnen Isoformen können Tabelle 1 entnommen werden): Isoform C zeigte sich unter normoxischen Bedingungen als am stärksten exprimiert, wies jedoch unter Hypoxie eine Abnahme der Expression in Embryonen (Abb. 2A), Larven (Abb. 2B), Weibchen (Abb. 2D) und Männchen (Abb. 2E) auf. Die gemessene Menge an Isoform A mRNA war sowohl unter Normoxie als auch unter Hypoxie in allen Stadien deutlich geringer im Vergleich zu Isoform C. Eine Ausnahme bildete hierbei die Isoform A-Expression unter Hypoxie in Embryonen (Abb. 2A) und in Weibchen (Abb. 2D). In Embryonen wurde eine um das 4,5fache erhöhte Isoform A-Expression unter hypoxischen im Vergleich zu normoxischen Bedingungen gemessen - nur geringfügig schwächer als die Isoform C-Expression unter Normoxie. In Weibchen wich die Isoform A-Expression unter Normoxie und Hypoxie nur leicht voneinander ab und war nur geringfügig schwächer als die hypoxische Isoform C-Expression.

Sowohl Isoform B als auch Isoform D konnten zwar in allen Entwicklungsstadien nachgewiesen werden, die Expression war aber in allen Fällen sehr gering und betrug sowohl unter Normoxie als auch unter Hypoxie lediglich etwa 1-4% der Isoform C-Expression.

Zusammenfassend kann gesagt werden, dass unter Normoxie in allen untersuchten Entwicklungsstadien Isoform C die vorherrschende *glob1* Transkriptvariante darstellt, wobei die stärkste Expression in Embryonen gemessen wurde. Lediglich in Weibchen zeigte Isoform A eine vergleichbar starke Expression (Abb. 3A). Diese Gewichtung verändert sich unter Hypoxie – in L3 Larven, Puppen und Männchen wurde Isoform C nach wie vor als prominenteste *glob1* Transkriptvariante gemessen, wohingegen in Embryonen unter Hypoxie eine deutliche Zunahme der Isoform A-Expression gefunden wurde (Abb. 3B). In Weibchen wurde unter Hypoxie eine vergleichbare Kopienzahl für Isoform A und C gemessen, dieses im Vergleich zu normoxischen

Bedingungen verschobene Expressionsmuster ist auf eine auch unter Hypoxie hohe Isoform A-Expression zusammen mit einer Abnahme der Isoform C-Expression unter Hypoxie zurückzuführen.

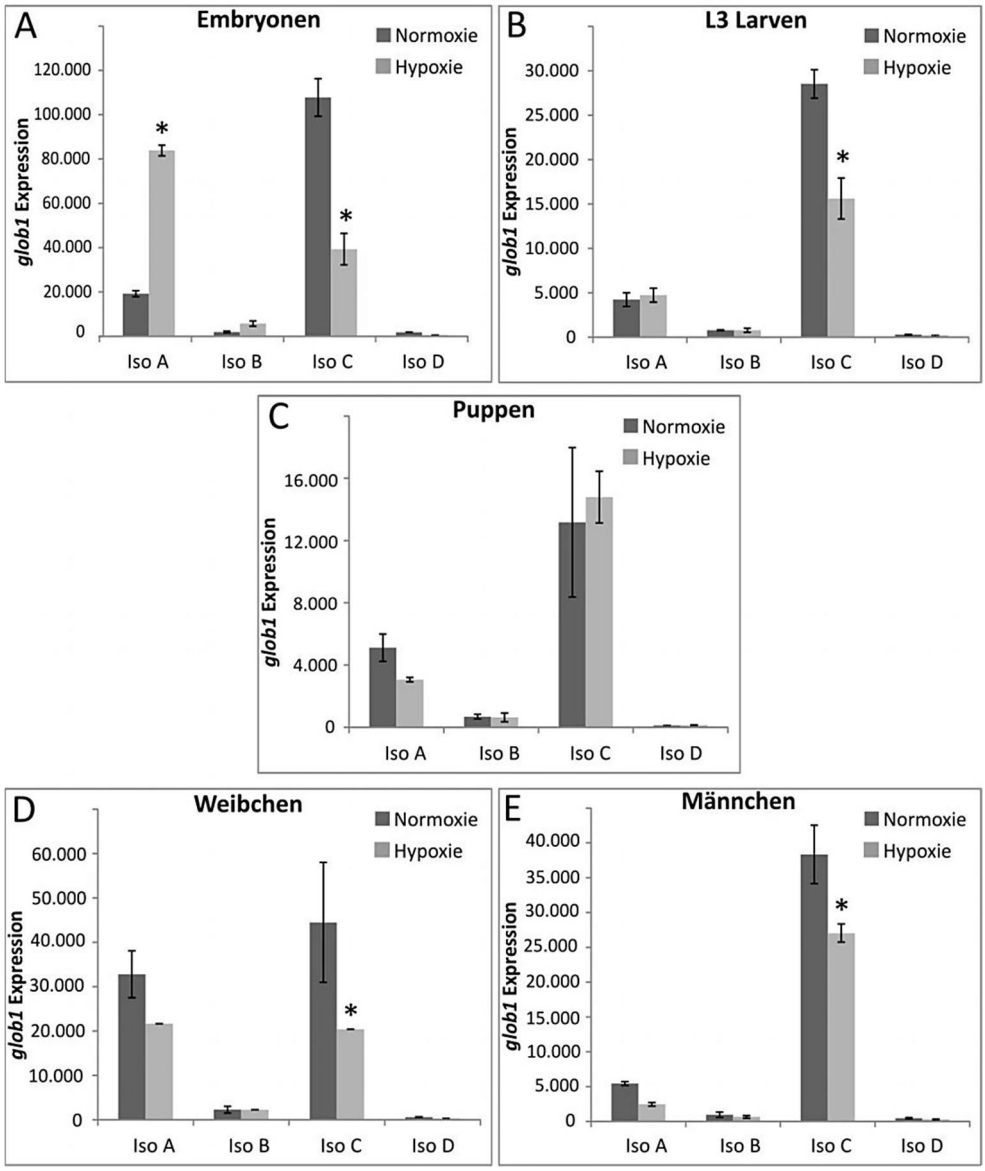

Abb. 2: mRNA Expressionsmuster der *D. melanogaster* (Oregon R) *glob1*-Isoformen A-D in unterschiedlichen Entwicklungsstadien unter verschiedenen O_2-Bedingungen. Bei den dargestellten Balken der Diagramme handelt es sich um absolute mRNA Kopienzahlen der Isoformen A-D gemessen unter Normoxie (dunkelgrau) im Vergleich zu Hypoxie (hellgrau) in Embryonen (A), L3 Larven (B), Puppen (C), Weibchen (D) und Männchen (E). Die Fehlerbalken wurden berechnet aus der Standardabweichung der einzelnen Triplikate zum Mittelwert. *$p < 0,05$, t-Test.

Addiert man die Expression aller Isoformen, so findet man die höchste *glob1* Gesamtexpression in Embryonen, die etwa 2-3mal höher ist als in allen anderen untersuchten Entwicklungsstadien. Eine weitere Besonderheit der *glob1* Gesamtexpression in Embryonen ist die gleichbleibende Expressionsstärke unter hypoxischen Bedingungen. In allen anderen untersuchten Stadien, mit Ausnahme des Puppenstadiums, wurde eine etwa 40%ige Abnahme der *glob1* Gesamtexpression

gemessen (Tabelle 2.1.1). Dies bestätigt die Beobachtung, dass die *glob1*-Expression in Larven unter niedrigen O_2-Bedingungen abnimmt (Gleixner et al., 2008).

		Isoform A	Isoform B	Isoform C	Isoform D	Gesamt
Embryo	Normoxie	18337	1568	100639	1843	122387
	Hypoxie	83971	4776	40090	527	129364
L3 Larve	Normoxie	4879	741	27279	303	33202
	Hypoxie	5044	584	13761	172	19561
Puppe	Normoxie	4384	555	10256	112	15307
	Hypoxie	2957	394	13445	115	16911
Weibchen	Normoxie	28571	1597	36129	626	66923
	Hypoxie	21061	1800	20554	271	43686
Männchen	Normoxie	5207	635	38539	537	44918
	Hypoxie	2479	536	26066	336	29417

Tabelle 1: mRNA Expression der *D. melanogaster glob1* Isoformen A-D in unterschiedlichen Entwicklungsstadien unter Normoxie und Hypoxie. Bei den dargestellten Werten handelt es sich um die absolute Kopienzahl der jeweiligen Isoform, sowie um die Gesamt-mRNA Kopienzahl errechnet aus den mRNA Kopienzahlen der einzelnen Isoformen.

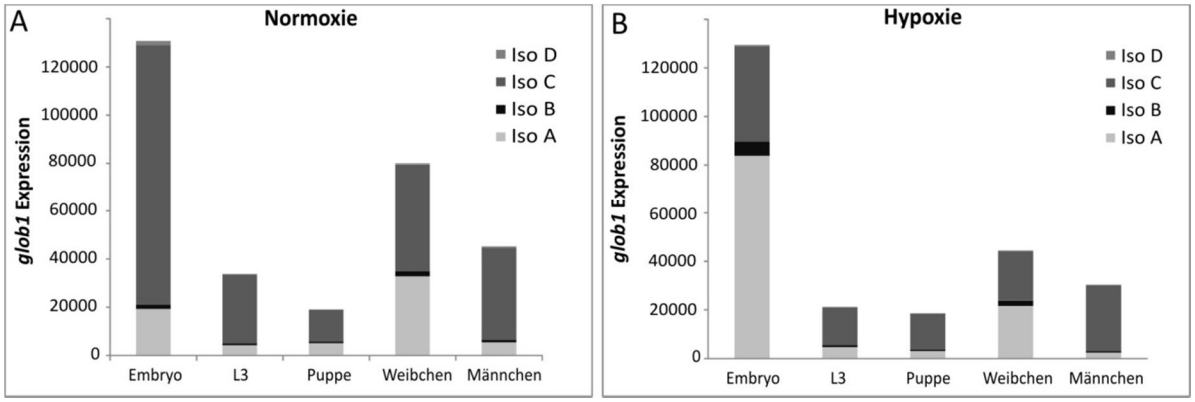

Abb. 3: Expressionsmuster der vier *glob1* Isoformen in unterschiedlichen Entwicklungsstadien im Vergleich. (A) Glob1 Isoform A-D-Expression unter Normoxie in Embryonen, L3 Larven, Puppen , Weibchen und Männchen. (B) *Glob1* Isoform A-D-Expression unter Hypoxie in Embryonen , L3 Larven, Puppen, Weibchen und Männchen. Aufgetragen sind die absoluten mRNA Kopienzahlen der Isoformen A-D.

Immunfluoreszenzfärbung mit Anti-glob1-Antikörpern an Embryonen von D. melanogaster

Das bisherige Wissen um die räumlichen Muster der *glob1*-Expression in Embryonen von *D. melanogaster* beruht vor allem auf mRNA Expressions-Analysen durchgeführt mittels *in situ* Hybridisierung. Der Nachweis von *glob1* Transkripten durch Verwendung markierter antisense RNA-Sonden lieferte Hinweise auf die Anwesenheit von *glob1* Transkripten im Fettkörper und dem Tracheensystem später Embryonalstadien von *D. melanogaster* (Hankeln et al., 2002). Sowohl in L3 Larven als auch in adulten Tieren konnte die glob1-Expression im Fettkörper und Tracheensystem auf Proteinebene bereits bestätigt werden (Hankeln et al., 2002). Die Untersuchung der glob1 Proteinexpression in späten Embryonalstadien von *D. melanogaster* zur Vervollständigung des glob1 Expressionsmusters mithilfe glob1-spezifischer Antikörper wurde im Rahmen dieses Experiments durchgeführt.

Um das Tracheensystem deutlich sichtbar zu machen und somit die glob1 Proteinexpression eindeutig zuordnen zu können, wurden Embryonen mit tracheenspezifischer GFP-Expression für die glob1 Antikörperfärbung verwendet (Abb. 4: UAS GFP x btl). Die lichtmikroskopische Auswertung der Antikörperfärbung in Embryonen des Stadiums 16 ergab ein deutliches glob1-spezifisches Signal im embryonalen Fettkörper, der sich lateral vom anterioren zum posterioren Ende, parallel zu den primären Tracheenästen zieht. Das schwächere, diffuse Signal zentral im Embryo ist mit großer Wahrscheinlichkeit auf eine unspezifische Färbung des Mitteldarms zurückzuführen (Abb. 4: Anti-glob1). Die Überlagerung der Aufnahmen mit glob1-spezifischen Signalen mit Aufnahmen der GFP Expression im Tracheensystem zeigten in Embryonen jedoch kein glob1-spezifisches Signal in den Zellen der Tracheen (Abb. 4: GFP + glob1).

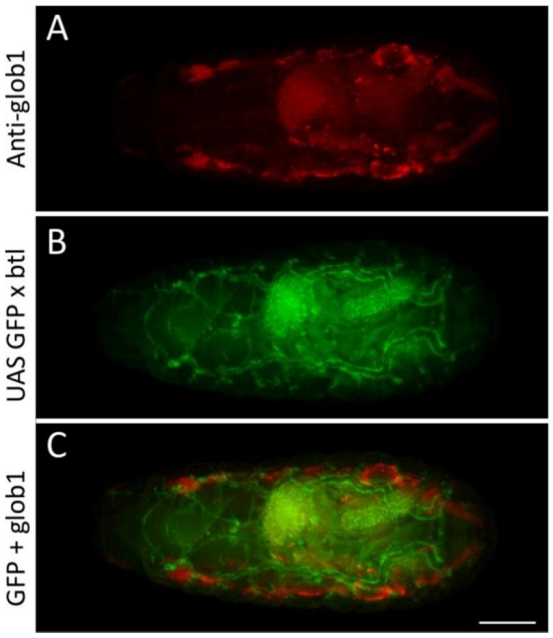

Abb. 4: Immunfluoreszenzfärbung mit Anti-glob1-Antikörpern an *D. melanogaster* Embryo, Stadium 16 mit tracheenspezifischer GFP-Expression. (A) Glob1-spezifisches Signal im Fettkörper. (B) GPF Expression im Tracheensystem (UAS GFP:CD8 x btl Gal4). (C) Kombination aus (A) und (B).

Diskussion

Duplikation im 5´UTR des D. melanogaster glob1 Gens

Die detaillierte Sequenzanalyse des *glob1* Genbereichs der Spezies der *melanogaster* Subgruppe ergab eine Duplikation im Bereich der Exons 1 und 2 des *glob1* Gens in *D. melanogaster*, die experimentell in drei von vier untersuchten *D. melanogaster* Wildtyp-Linien bestätigt wurde. Das Fehlen der Duplikation im *glob1* Genbereich der anderen untersuchten Spezies der *melanogaster* Subgruppe spricht für ein exklusives Duplikationsereignis im Verlauf der *D. melanogaster* Evolution. In der wildtypischen Linie Canton S konnten mithilfe der durchgeführten PCR keine Anzeichen auf ein Duplikationsereignis gefunden werden. Da es sich bei den verwendeten Wildtypen um inzestuöse Laborlinien handelt, folglich keine Vermischung der Linien untereinander stattfindet, kann man aus dem Fehlen der Duplikation in Canton S schließen, dass diese Linie ihren Ursprung vor dem Duplikationsereignis im Vorläufer der anderen *D. melanogaster*-Linien hatte. Ein altenativer, technischer Grund für das Fehlen einer Duplikations-spezifischen Bande in der Canton S-PCR könnten jedoch Linien-spezifische Unterschiede in der Sequenz der Primer-Bindestellen sein. Dies kann nicht ausgeschlossen werden, da der betroffene Bereich nicht sequenziert wurde. Eine Verunreinigung der genomischen DNA mit PCR-inhibierenden Stoffen hingegen ist unwahrscheinlich, dies wurde durch das Mitführen geeigneter Positivkontrollen überprüft.

Der duplizierte Bereich umfasst sowohl Exon1, Exon2 als auch den Promotor I proximal zu Exon1 (Abb. 1). Die Sequenzanalyse zeigte, dass der duplizierte Bereich eine 99,8%ige Übereinstimmung aufweist, und dass Promotor I, sowie Exon1 und Exon2 in beiden Duplikaten unverändert vorliegen. Das Fehlen homologer Sequenzbereiche an den Enden und der Grenze der Duplikate spricht gegen ungleiches Crossing-over als Duplikationsmechanismus. Eine mögliche Erklärung zur Entstehung der Duplikation im *glob1* Gen wäre ‚non-homologous endjoining', ein Mechanismus zur Reparatur von Doppelstrangbrüchen, der auf Sequenzhomologien verzichtet (Moore und Haber, 1996).

Die durchgeführte Realtime-PCR zur Quantifizierung der Expressionsstärke der *glob1* Transkriptvarianten zeigte, dass die Varianten mit Exon1 und Exon2 (Isoform C: Exon1 und Exon4-6; Isoform D: Exon1+2 und Exon4-6, Abb. 1) exprimiert werden, es sich bei Isoform C sogar um die vorherrschende Transkriptvariante handelt. Welcher der beiden duplizierten Transkriptionsstartpunkte tatsächlich verwendet wird und ob oder in welcher Form die Duplikation die Transkription der betroffenen Isoformen beeinflusst, kann durch diese hier durchgeführten Experimente nicht gezeigt werden.

Regulation der glob1 Transkription erfolgt entwicklungsabhängig

Das Auftreten der vier *glob1* Transkriptvarianten ist auf zwei alternative Transkriptionsstartpunkte (Promotor I und Promotor II) zurückzuführen, gefolgt von alternativem Splicing der Transkripte, welches in je zwei Varianten pro Transkriptionsstart resultiert. Die so entstanden *glob1* Isoformen unterscheiden sich in ihrem 5´-untranslatierten Bereich, beinhalten aber alle dieselbe kodierende Region (Abb. 1). Die Verwendung mehrerer Transkriptionsstartpunkte, teilweise mit zeitlich oder räumlich versetzter Transkriptionsinitiation, in Verbindung mit alternativem Splicing ist ein häufig vorzufindender Mechanismus zur Generierung verschiedener Transkriptvarianten eines Gens in *D. melanogaster*. Das Gen *slowpoke* (kodiert für einen BK-Typ Calcium-aktivierten Natrium-Kanal) zum Beispiel wird sowohl ausgehend von mehreren Promotoren gewebespezifisch exprimiert, als auch alternativ gespliced (Butler et al., 1993; Brenner und Atkinson, 1996; Schreiber und Salkoff, 1997). Die Expression dieses Gens resultiert in Transkripten mit unterschiedlichen 5´-Enden, die je nach Promotorspezifität im Nervensystem oder in den Muskeln und Tracheen lokalisiert sind (Brenner und Atkinson, 1996; Bohm et al., 2000). Zusätzlich zur Transkriptionsregulation über gewebespezifische Promotoren durchlaufen die *slowpoke* Transkripte alternatives Splicing, das die C-terminale Aminosäuresequenz des Proteins beeinflusst und zur individuellen Feinregulation des Endprodukts dient (Lagrutta et al., 1994; Schreiber et al., 1999).

Eine ähnliche gewebespezifische Regulation der Transkription ausgehend von den beiden identifizierten Promotoren wäre auch für das *glob1* Gen denkbar. *Glob1* wird hauptsächlich im Fettkörper und den Tracheen exprimiert. Neben dem schwächeren Auftreten von *glob1* Transkripten in anderen Geweben zeigt *glob1* eine deutliche Expression in Ovarien, insbesondere in späten Stadien (Hankeln et al., 2002). Die hier durchgeführte Quantifizierung der *glob1* Transkription zeigte eine deutlich höhere Expression der Isoform A in Weibchen und insbesondere in Embryonen. Isoform C dagegen wurde in allen untersuchten Stadien, mit Ausnahme der hypoxischen Embryonen, als vorherrschende Transkriptvariante identifiziert. Es wäre durchaus denkbar, dass der Promotor II proximal zu Exon 3 die Ovarien-spezifische Expression in Weibchen steuert, womit die im Vergleich zu Männchen erhöhte Isoform A-Expression zu erklären wäre. Der Promotor I proximal zu Exon1 dagegen könnte die Expression der ‚Haupt'-Isoform C im Fettkörper und den Tracheen initiieren.

In Embryonen der frühen Stadien 0-5 konnte die Anwesenheit von *glob1* Transkripten per mRNA *in situ* Hybridisierung nachgewiesen werden. Es wird vermutet, dass diese Transkripte maternalen Ursprungs sind (Hankeln et al., 2002). Die Hypothese des maternalen Ursprungs der frühembryonalen *glob1* Transkripte ließe sich durchaus auf die Anwesenheit der Isoform A in Embryonen übertragen. Somit wäre der per Realtime-PCR quantifizierte hohe Wert an Isoform A Kopien in Embryonen nicht auf aktive Transkription in Embryonen, sondern auf Weibchen- und Ovarien-spezifische Transkription, (ausgehend vom Promotor II) gefolgt von maternaler Vererbung

zurück zu führen. Gegen eine maternale Vererbung der *glob1* Isoform A spricht allerdings der Befund, dass in Embryonen jedoch nicht in Weibchen eine starke Zunahme der Isoform A-Expression unter Hypoxie gemessen wurde. Um eine maternale Vererbung der Isoform A zu überprüfen, müsste die Isoform A Anwesenheit in Embryonen bis Stadium 5 mit Embryonen späterer Stadien verglichen werden. Bei dem hier durchgeführten Experiment wurde jedoch Gesamt-RNA aus Embryonen aller Stadien verwendet. Aufgrund dessen kann hier keine Aussage über die Isoform A-Verteilung in Embryonen früher und später Stadien getroffen werden.

Die Anwesenheit der durch alternatives Splicing entstandenen Isoformen B und D, die in allen untersuchten Stadien eine sehr schwache Expression zeigten kann, zum jetzigen Zeitpunkt nicht funktionell erklärt werden.

Glob1 Proteinexpression im embryonalen Fettkörper

Die hier gezeigte Untersuchung der glob1 Proteinexpression in Embryonen von *D. melanogaster* wurde durchgeführt, um das bisher lediglich auf mRNA *in situ* Hybridisierung basierende Wissen um die embryonale glob1 Proteinexpression zu vervollständigen. Die Immunfluoreszenzfärbung an Embryonen ab Stadium 13 zeigte eine deutliche glob1-spezifische Färbung im Fettkörper, im Tracheensystem konnte jedoch kein glob1 Protein detektiert werden. Es kann nicht ausgeschlossen werden, dass das Ausbleiben eines glob1-spezifischen Signals im Tracheensystem technische Ursachen hat, hervorgerufen etwa durch schlechte Antikörpereigenschaften oder durch zu geringe Gangbarkeit des Gewebes. Ebenso möglich wäre, dass es sich bei den durch *in situ* Hybridisierung nachgewiesenen *glob1* Transkripten in Tracheen um technische Artefakte handelt und in Embryonen keine glob1 Proteinexpression im Tracheensystem stattfindet. Eine zwar unwahrscheinliche aber trotzdem nicht völlig auszuschließende Erklärung die tatsächliche Abwesenheit von glob1 Protein im embryonalen Tracheensystem trotz Anwesenheit von *glob1* mRNA.

Die Entwicklung des Tracheensystems dauert etwa 10 Stunden, beginnt in der Mitte der Embryogenese (Stadium 11), zieht sich bis zu deren Ende und wird teilweise im Larvenstadium fortgesetzt. Während der Embryogenese sind die Tracheen mit Flüssigkeit gefüllt, die kurz vor dem Übergang zum Larvenstadium entfernt wird, so dass das Tracheensystem erst dann seine respiratorische Funktion übernehmen kann (Samakovlis et al., 1996). Vom entwicklungsbiologischen Standpunkt betrachtet wäre es durchaus plausibel, dass glob1 Protein erst zum Beginn des Larvenstadiums, also mit Verwendung des Tracheensystem als Atmungsorgan benötigt wird. Die Anwesenheit von *glob1* Transkripten im embryonalen Tracheensystem in Stadium 15 könnte (Hankeln et al., 2002) durch eine vorzeitige Initiation der Transkription erklärt werden, die das ausreichende Vorhandensein benötigter *glob1* Transkripte bei Inbetriebnahme des Tracheensystems in Larven gewährleistet.

Die hier vorgestellten Untersuchungen zur Expression verschiedener *glob1* Transkriptvarianten in Zusammenhang mit dem Nachweis von glob1 Protein in Embryonen könnten erste Hinweise auf eine entwicklungsabhängige Regulation der *glob1* Transkription sowie mögliche Unterschiede in der Funktion des glob1 Proteins in Embryonen und späteren Entwicklungsstadien liefern.

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2.3 Gleixner, E., Schuh, R., Wolf, C., Burmester, T. and Hankeln, T. (2011) Knockdown of *Drosophila* glob1 evokes a mild phenotype but argues for a role in O_2 supply.

Knockdown of *Drosophila* glob1 evokes a mild phenotype but argues for a role in O_2 supply

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Globin, oxygen, hypoxia, trachea, respiration

Summary

Insects are equipped with an efficient tracheal system developed for sufficient O_2 supply in phases of high metabolic activity. The presence of hemoglobins in insects living in normoxic environments is therefore surprising and raises the question as to the function of those respiratory proteins. We have modulated the expression of *Drosophila melanogaster* globin 1 (glob1), the predominant globin expressed in the tracheal system and the fat body, by obtaining a transcriptional RNAi-mediated knockdown and an over-expression of the protein in larvae and adult flies. There were no phenotypic consequences of glob1 knockdown or over-expression on fly development and longevity. Under stress conditions, however, the strong knockdown of glob1 protein leads to disadvantages of adult flies in survival of both, moderate and severe hypoxic conditions and paradoxically to increased resistance of Paraquat-induced ROS-stress. Those survival parameters after exposure to hypoxia as well as reactive oxygen species (produced by hyperoxia or Paraquat) point towards a role of glob1 in O_2 supply.

Introduction

O_2 supply in insects is maintained by the tracheal system and occurs very efficiently via passive diffusion (Kestler, 1985). In highly active tissues like the flight muscle, terminal tracheal branches are connected with nearly every cell (Guillemin et al., 1996; Jarecki et al., 1999) and can therefore provide for sufficient O_2 supply even in phases of high activity.

On the one hand, this direct connection between cells and the tracheal system is crucial for sufficient O_2 supply, on the other hand it holds the risk of oxidative damage to the cells, caused by an excess of O_2 (Hetz and Bradley, 2005). Adult *Drosophila* have to keep up their spiracles during active phases, to provide for sufficient O_2, although they are able to adapt the width of the opened spiracles to their O_2 need, the partial O_2 pressure within the tracheal system reaches a range (~19kPa) (Lehmann, 2001; Heymann and Lehmann, 2006) – a high and potentially toxic O_2 concentration for tissues and cells for being exposed to directly (Hetz and Bradley, 2005). During phases of low activity and therefore decreased O_2 consumption, the risk of oxidative damage to cells directly connected to tracheal protuberances is in fact even more present (Hetz and Bradley, 2005).

Interestingly, many terrestrial insects, including *Drosophila,* exhibit a striking tolerance towards low O_2 conditions (Wegener, 1993). *Drosophila* embryos, larvae and even adults can survive anoxic periods for hours without suffering tissue injury (Haddad et al., 1997b; Wingrove and O'Farrell, 1999; Haddad, 2006) and can reproduce and develop in 8% O_2 or even lower O_2 conditions (Zhou et al., 2007). The adaptive response of *Drosophila* embryos and larvae to low O_2 conditions can be explained by their life on fermenting fruits, where they have to compete for O_2 with microorganisms. The ability of *Drosophila* to withstand stringent hypoxic or even anoxic conditions is mainly due to a switch to a hypometabolic condition, ranging from motionlessness (Csik, 1939; Haddad et al., 1997b), reduction of O_2 consumption (Haddad et al., 1997b; Haddad, 2000), anaerobiosis (Wegener, 1993; Grieshaber et al., 1994) to cell cycle arrest (Wingrove and O'Farrell, 1999; Lavista-Llanos et al., 2002). Less harsh hypoxic conditions mainly induce hypoxia inducible factor (HIF)-mediated changes of gene expression in the tracheal system comparable to mammalian angiogenesis (Jarecki et al., 1999; Metzger and Krasnow, 1999; Lavista-Llanos et al., 2002) via the receptor breathless and its ligand branchless, the *Drosophila* homologs to the mammalian FGF (=fibroblast growth factor) receptor and FGF (Glazer and Shilo, 1991; Sutherland et al., 1996). This signal results in the secreting of increased doses of branchless in O_2-starved areas and as response, a sprouting of tracheal cells towards those tissue regions (Jarecki et al., 1999; Metzger and Krasnow, 1999; Centanin et al., 2008).

Hypoxia-mediated gene regulation in *Drosophila* is performed by the evolutionary conserved hypoxia-inducible transcription factor (HIF), which binds to hypoxia response elements in the regulatory region of downstream genes (Nagao et al., 1996; Nambu et al., 1996; Sonnenfeld et al., 1997; Jarecki et al., 1999; Metzger and Krasnow, 1999; Lavista-Llanos et al., 2002; Gorr et al., 2004; Gorr et al., 2006). HIF activity increases when O_2 levels drop and peaks at O_2 concentrations between 3 and 5% in *Drosophila* embryos, indicating a strong concentration dependence of HIF activity (Lavista-Llanos et al., 2002). Under more severe hypoxic conditions however, HIF activity was found to decrease, probably caused by general down-regulation of metabolic activity towards cell-cycle arrest as a response to severe O_2 deprivation (Wingrove and O'Farrell, 1999; Haddad, 2000; DiGregorio et al., 2001; Lavista-Llanos et al., 2002; Douglas et al., 2005).

The genetic basis of the striking hypoxia tolerance of *Drosophila* has not been unraveled yet. Several approaches have been made to characterize hypoxia-dependent gene expression. Liu et al (2006) analyzed gene expression of adult flies after exposure to severe (0.5 % O_2) and moderate (5% O_2) hypoxic conditions. After identification of hypoxia-dependent up-regulated genes (n=79 after 0.5% O_2 for 6h), they tested their importance in resistance and survival to hypoxia and found that the candidate hypoxia response genes *hairy*, *ade5*, *astray* and *CG18135* are needed for recovery from hypoxia as well as survival during hypoxic stress (Liu et al., 2006). Transcription studies performed by Zhou et al. (2008) based on adapted flies reared in chronic hypoxic conditions revealed that larvae and flies exhibited up-regulation of several genes from signal transduction pathways, like EGF, insulin, Notch and Toll/Imd pathways and interestingly a dosage reduction of genes involved in metabolic processes (Zhou et al., 2008; Zhou et al., 2011). When comparing hypoxia-dependent gene regulation of larvae and adults, hypoxic larvae showed 20 times more regulated genes, of which nearly half displayed a dosage reduction. Significant down-regulation was especially found in genes regulating cell respiration and energy metabolism, e.g. glycolytic enzymes, the TCA-cycle, lipid β-oxidation and the respiratory chain complex. This down-regulation of metabolic genes was restricted to the larval stage (Zhou et al., 2008). Thus, hypoxia-tolerance of *Drosophila* is based on highly complex genetic (and metabolic) interactions. However, it was shown that changes in gene expression of a single gene, *hairy*, as well as manipulation of genes involved in the Notch pathway (see e.g. Andersson et al. 2011) can influence the hypoxic survival of *Drosophila* (Liu et al., 2006; Zhou et al., 2008; Zhou et al., 2011).

In reverse to their hypoxia-tolerance, *Drosophila* are not able to withstand prolonged exposure to severe hyperoxic conditions for long. When exposed to 95 – 100% O_2, no behavioral changes but a rapid decrease in the survival rate of adult flies, leading to death of all flies after about 8 days, can be observed (Walker and Benzer, 2004; Gruenewald et al., 2009). 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, which can be seen for example by the accumulation of apoptotic cells in the indirect flight muscle deriving from flies reared in 100% O_2 (Walker and Benzer, 2004). Gene-expression studies on flies exposed to 100% O_2 revealed the up-regulation of genes encoding for heat-shock proteins, antioxidant enzymes and proteins involved in immune response (Landis et al. 2004). Furthermore, analyses of the gene-expression pattern of hyperoxia-adapted flies in combination with experimental studies indicated that the up-regulation of two genes from the AMP family (*Diptericin* and *Attacin*) as well as the down-regulation of *Tm1*, *Gpdh*, *UGP* and *CG33129* contribute to survival in high O_2 conditions (Zhao et al. 2010).

In vertebrates, O_2 supply of tissues is maintained by respiratory proteins like hemoglobin and myoglobin (Millikan, 1933; Millikan, 1939; Schroeder, 1963; Wittenberg, 1970; Perutz, 1979; Wittenberg and Wittenberg, 2003). Furthermore, globins can also be involved in the detoxification of reactive oxygen species (ROS) (see e.g. Flogel et al., 2008; Greenberg et al., 2008; Hendgen-Cotta et al., 2010; Hundahl et al., 2010). In insects, the occurrence of respiratory proteins was thought to be limited to insects living in aqueous or hypoxic environment like the Chironomid midges (Rollett, 1861; Lankester, 1872) the horse botfly *Gasterophilus intestinalis* (Keilin and Wang, 1946) or backswimmer (Miller, 1964; Bergtrom, 1977;

Wells, 1981). Starting with the discovery of a globin gene (glob1) in *D. melanogaster* however (Burmester and Hankeln, 1999), more and more globins in insects living in normoxic environment like the honeybee *Apis mellifera* (Hankeln et al., 2006) the silkworm *Bombyx mori* (Kawaoka et al., 2009) or the mosquitoe *Anopheles gambae* (Burmester et al., 2007) were identified, leading to the assumption that globins belong to the standard repertoire of insects. Given this knowledge, it is tempting to speculate that respiratory proteins such as globins contribute to the striking hypoxic and even anoxic tolerance of *Drosophila*.

Drosophila melanogaster harbours three globins, named glob1, glob2 and glob3. Whereas *glob2* and *glob3* seem to be expressed on a low level, *glob1* expression was confirmed in all developmental stages, mainly in the tracheal system and fat body. Little is known about the proteins encoded by *glob2* and *glob3*, however, the high degree of conservation of the amino acids crucial for heme- and O_2 binding found in both proteins indicate their functionality (Burmester et al., 2006). Glob1 protein displays a characteristic 3-over-3 a-helical sandwich structure, exhibits a hexacoordinate binding scheme in its deoxy state and binds O_2 with high affinity (Hankeln et al., 2002; de Sanctis et al., 2005). The close evolutionary relationship of glob1 to other intracellular insects globins with known O_2 storage functions, as well as its high O_2 affinity and expression pattern, both reminding of known insect hemoglobins, point towards a role of glob1 in O_2 supply. However, studies of *glob1* mRNA expression in hypoxic S2 cells, derived from late-stage embryos, as well as *in vivo* studies in embryos, larvae and adult flies in response to hypoxia, revealed a probably HIF-mediated substantial down-regulation of *glob1* mRNA (Gorr et al., 2004; Gleixner et al., 2008) and therefore speak against an O_2 supply or storage function of glob1. Furthermore, several mRNA expression studies revealed an increase of *glob1* transcripts in whole flies (2.3 fold) (Landis et al., 2004) and in adult brains (2.3 fold) (Gruenewald et al., 2009) in response to hyperoxia. This was confirmed *in vivo* by measuring an increase of *glob1* mRNA expression levels in larvae after hyperoxic treatment (Gleixner et al., 2008).

Those first studies therefore rather indicate an involvement of glob1 in the detoxification of ROS, as it was proposed for some vertebrate globins (see e.g. Flogel et al., 2008; Greenberg et al., 2008; Hendgen-Cotta et al., 2010; Hundahl et al., 2010), than a classical O_2 supply function comparable to other known intracellular insect globins like the hemoglobins of *G. intestinalis* (Keilin and Wang, 1946).

Yet, we cannot rule out that altered glob1 expression could influence the response to hypoxia or the ability to withstand phases of hypoxia. Glob1 could be involved in O_2 transport or storage and therefore being necessary in O_2 supply of tissues during O_2 deprivation. The protection-against-oxidative-stress-hypothesis as well as the O_2-supply-hypothesis were tested in this study, by analyzing lifespan, development and fertility in flies with modulated glob1 expression under normal and second as well as under stressful conditions

Material and Methods

Fly strains

For the modulation of glob1 expression, the UAS/Gal4-system (Brand and Perrimon, 1993) was applied. The responder strain for RNAi-mediated glob1 knockdown was obtained from Vienna *Drosophila* RNAi Center (tranformant ID 101830). To obtain transgenic flies for glob1 over-expression, the coding sequence of the *glob1* gene was cloned into the pUAST vector. Microinjection of the sequence-verified pUAST-constructs into the *Drosophila* germline was performed at the

Max Planck Institute for Biophysical Chemistry (Göttingen). For glob1 over-expression in larvae, the pUAST-responder line 'UAS29 Gb1' and in adults the responder-line 'UAS36 Gb1' was used. As a driver line for both RNAi mediated knockdown and glob1 over-expression, daughterless Gal4 [w[1118]; P{da-GAL4.w[-]}3] obtained from Bloomington was used. In all experiments, fly lines with the same genetic background as flies with glob1 knockdown ('control down') or overexpression ('control up') were used as control. These flies had unaltered glob1 expression. Additionally, flies from the da Gal4 driver strain were used as control for both glob1 knockdown and over-expression ('control both').
Flies were reared on standard cornmeal agar in plastic vials at 25°C.

To verify glob1 knockdown and over-expression on both, mRNA and protein level, quantitative realtime RT-PCR (QPCR), Western Blot technique and immunofluorescence were applied to different developmental stages.
For QPCR experiments, total RNA was isolated from 3rd instar larvae and sexed, adult flies of double transgenic flies with either glob1 knockdown or over-expression and the corresponding control strains. Generally, 25 L3 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), including a DNase digestion step according to the manufacturer´s instructions. RNA concentration was measured photometrically with the NanoDrop ND-1000 spectrophotometer. RNA integrity was checked by denaturing formaldehyde gel-electrophoresis. For cDNA by SuperScript III reverse transcriptase (Invitrogen), generally 1µg total RNA was used. 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, Darmstadt, Germany). 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* CAG CGA TGA GGT GCA ACT GAT; *Dmegb1_down_rev* GAC CAT GTC TAC GGA ATC ATC. QPCR primers used for verification of glob1 over-expression were *Dmeglob1_up_for* GGA GCT AAG TGG AAA TGC TCG and *Dmegb1_up_rev* TGC CGT TAG TCA CAT TCC GC. 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 sec, 60°C for 30 sec, 72°C for 30 sec, 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. 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 run in triplicate. The significance of the data was assessed by a two-tailed Student's *t*-test employing the Microsoft Excel spreadsheet program.
For Western Blot analyses, total protein extracts were obtained by homogenizing ~25 3rd instar larvae or adult, sexed flies in heated RIPA lysis buffer. Protein extracts were centrifuge and protein concentration in cleared supernatants were determined by a Bradford assay. Generally 30µg total protein extracts were diluted in 2x Laemmli buffer, separated by SDS-PAGE and transferred to a PVDF membrane for 1h at 100V using the Mini Trans-Blot Electrophoretic Transfer Cell (BioRad, Munich, Germany). PVDF membranes

were blocked in 5% non-fat dry milk and incubated with anti-glob1 antibodies (Hankeln et al., 2002), diluted 1:100 in PBS-T, overnight at 4°C. Membranes were subsequently incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibodies (GE Healthcare), diluted 1:15 000 in PBS-T, for 1h at room temperature. For visualization of immunostained proteins, ECL-Western blotting reagent was used and signals were reported by exposure of the membrane to an X-ray film (Kodak BioMax). To confirm equal loading, PVDF membranes were incubated in mild stripping buffer containing 0.1 % SDS at room temperature. Membranes were subsequently blocked and incubated with monoclonal anti-actin antibodies (Sigma), diluted 1:2000 in PBS-T overnight at 4°C. Secondary antibody incubation and signal detection was performed as described above. Quantification of Western Blot signals was performed with the program ImageJ (Abramoff et al. 2004) by measuring the area under curve (AUC) of the specific signals and the loading control. The value for the 'Control both' samples was set 1, other strains were recalculated. Glob1 signals were normalized to the loading control.

For indirect immunofluorescence analyses of glob1, 3rd instar larvae were dissected in cold PBS, brains were isolated and fixed in 4 % paraformaldehyde/PBS for 30 min. Fixed brains were washed in PBS + 0.1 % Triton X, blocked in blocking solution containing 0.5% cold-water fish gelatin (Sigma) plus 0.1% ovalbumin (Sigma) in PBS. Brains were incubated with anti-glob1 antibodies, diluted 1:10 in PBS-T, overnight at 4°C. Larval brains were subsequently washed with PBS-T and incubated with secondary antibodies conjugated to Alexa® 568 (Molecular Probes) diluted 1:400 in blocking solution, for 2h 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).

Lifespan determination and analysis of developmental times

A total of 180 adult flies per sex and genotype (glob1 knockdown, over-expression and control lines) were used for lifespan determination. Crosses were set up as described above. Flies were sexed and kept in batches of 40-50 flies per vial. Flies were moved to vials containing fresh food every 3-4 days. Dead flies were counted over a period of 18 weeks.

For analysis of developmental times, crosses for glob1 knockdown, over-expression and control lines were set up in two replicates, with 15 virgin females and 5 males. 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.

O_2 conditions

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 was obtained from Westfalen AG (Münster, Germany). Gas concentrations were measured and kept constant by an O_2 sensor (E-702, BioSpherix, Ltd., New York, USA). Constancy of temperature (22°C) was checked regularly.

Phenotypic analyses after experimental hypoxia

For lifespan determination during hypoxia, newly eclosed flies with either glob1 knockdown or over-expression and the corresponding control lines were collected, sexed and kept in batches of 50 flies per vial.

In total, 200 adult flies per sex and genotype were analyzed. Flies were kept under hypoxia with 5% O_2 at 22°C and were moved to vials containing fresh food every 3 days. Dead flies were scored every 24 hours.

For recording developmental times during hypoxic conditions, crosses were set up as above. Adult flies were allowed to lay eggs for three days, than they were moved to vials containing fresh food. Vials with embryos and newly hatched larvae were kept at 5% 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. Time spans of developmental stages were documented regularly, starting with wandering L3 larvae until hatching of adults.

For testing the survival 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 or flies aged for 21 days were collected, transferred to empty vials in batches of 50 flies and exposed to 1.5% O_2 for 6h. After hypoxic treatment, flies were exposed to normoxia. Recovery was recorded until one hour after hypoxic treatment. Flies were counted as viable when they started climbing.

To test hypoxic tolerance of 3rd instar larvae, a total of 250 L3 larvae from each genotype were tested. Larvae were raised under non-crowded conditions and transferred to vials containing fresh food in batches of 50. Larvae were exposed to 1.5% O_2 for 7h and were kept subsequently at normoxia until hatching. Emerging adult flies were counted.

For determination of recovery time from severe hypoxia a total of 100 adult flies per genotype and sex were tested Flies were sexed and kept in batches of 20 in empty plastic vials. They were exposed to 1% O_2 for 2h, following normoxia for recovery. Flies were scored as 'recovered' as soon as they started climbing.

To analyze the reaction time of 3rd instar larvae to hypoxic conditions, a total of 20 larvae were tested. Larvae were kept in batches of 5 in vials containing standard cornmeal agar and a pile of yeast in the center. Hypoxia applied (6 % O_2) caused larvae to clear the yeast. Larvae were scored as 'outside' when they clearly had left and moved away from the yeast.

For testing the ability to withstand complete O_2 deprivation (anoxia), a total of 100 adult flies per sex and genotype were used. Flies were sexed and kept in batches of 20 in empty vials. Vials were capped with gauze and put into a sealed plastic box. Pure nitrogen, bubbled through water previously, was flushed into the plastic box. The time it took flies to fall down was recorded. Flies were scored as "dropped" when they completely stopped moving.

Phenotypic analyses after experimental hyperoxia

A total of 180 adult flies per sex and genotype were used for lifespan determination under constant hyperoxic conditions (95% O_2). Flies were sexed and kept in batches of 40-50. Flies were moved to vials containing fresh food every 3 days and dead flies were counted.

For measuring developmental times during hyperoxia, adult flies were allowed to lay eggs for three days and were then moved to vials containing fresh food. Vials with embryos and newly hatched larvae were kept at 95% O_2 at 22°C. Developmental stages were recorded regularly, starting with wandering L3 larvae until hatching of adults.

ROS exposure by Paraquat treatment of adult flies and 3rd instar larvae

A total of 150 adult flies per sex and genotype were exposed to Paraquat (Sigma). Flies were kept in batches of 50 in plastic vials containing filter paper soaked with 20mM Paraquat dissolved in 5% sucrose. Flies were checked regularly and dead flies were counted over a period of four days.

A total of 135 L3 larvae were used to test survival under Paraquat. Larvae were kept in batches of 20-30 in plastic vials containing Formula 4-24 instant *Drosophila* Medium (Carolina Biological Supply, USA) soaked with 20mM Paraquat dissolved in 5% sucrose. Larvae were placed directly in the mush and were checked regularly. Pupae and hatching flies were counted. The percentage of surviving flies was calculated by comparing the number of larvae to hatched adult flies.

Anaylses 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.

The flies and 3rd instar larvae used for measurement were maintained under normoxia, 20mM Paraquat for 24h or hyperoxia with 95 % O_2 for 48h. For each measurement about 60 larvae or sexed flies per genotype were used. 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 500nm was measured using a µ Quant 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.

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 12µm thick cryo-sections deriving from thoraces of female flies either reared under normoxia or treated with 20mM Paraquat for 24h or 95% O_2 for 96h. 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 (Adobe Systems, San Jose, CA, USA).

Statistics

All experiments were repeated at least three times and diagrams represent pooled data with error bars indicated. Two-sided ANOVA with Bonferroni post-hoc test was performed to compare survival of flies during hypoxia with 5% O_2, recovery times of flies after exposure to 1% hypoxia for 2h and flies treated with Paraquat. For statistical analyses of all other experiments, two-tailed Student´s t-test was applied.

Results and Discussion

Characterization of modulated glob1 expression

By applying the UAS/Gal4-system (Brand and Perrimon, 1993) we were able to successfully modulate the endogenous *glob1* mRNA as well as protein expression in *Drosophila*. Employing a daughterless (da) Gal4 driver line, we obtained ubiquitous glob1 knockdown and over-expression in all developmental stages, as expected from the daughterless promoter (Cronmiller et al., 1988; Cui and DiMario, 2007; Munoz-Soriano and Paricio, 2007; Wicks et al., 2009).

Glob1 knockdown and over-expression in 3rd instar larvae and male and female flies was confirmed on mRNA level by quantitative Real-time RT-PCR and on protein level by Western Blot analysis. Glob1 knockdown in embryos was confirmed on mRNA level, and in pupae on protein level (Suppl. Fig. 1A+B). For

our phenotypic experiments we settled on larvae and adult flies, since those developmental stages are motile and therefore are able to respond visibly to various external stimuli.

Double transgenic larvae and sexed flies with *glob1* knockdown and over-expression were compared to control strains of the same genotype having unaltered *glob1* expression. Measurements of transcript copy numbers showed an RNAi-mediated decrease of *glob1* mRNA down to 2% of the corresponding control in larvae (Fig. 1A) and female flies (Fig. 1E) and a reduction to 6% of the corresponding control in male flies (Fig. 1C).

Western Blot analyses revealed a decrease of glob1 protein below the detection level in larvae, male and female flies (Fig. 1B, 1D and 1F).

Ubiquitous over-expression in larvae resulted in a 3.7fold increase of *glob1* mRNA (Fig. 1A) and a doubling of glob1 protein (Fig. 1B) compared to the corresponding controls. In male flies, glob1 over-expression resulted in a 6.6fold increase of mRNA (Fig. 1C) and a 2fold increase of protein levels (Fig. 1D) compared to the corresponding controls, whereas ubiquitous over-expression in females increased *glob1* mRNA levels by 3.9fold (Fig. 1E) and glob1 protein by 4fold (Fig. 1F).

To further confirm the ubiquitous expression pattern of the applied da Gal4 driver line in combination with the used UAS-glob1 constructs, we performed immunofluorescence staining with anti-glob1 antibodies on brains of glob1 knockdown and over-expression larvae. Larval brains with ectopic glob1 over-expression exhibited a strong fluorescent staining in both central brain hemispheres as well as the ventral ganglion (Fig. 2, upper panel). In addition to the prominent glob1 signals in the larval brains, staining in the tracheoles surrounding the ventral ganglion could be detected (Fig. 2 upper panel, arrowheads), which possibly reflect endogenous glob1 expression. In larval brains derived from the control strain, the endogenous glob1 staining in tracheoles is visible (Fig. 2, middle panel). Dissected and stained brains from larvae with glob1 knockdown did not show specific signals (Fig. 2, lower panel), indicating the successful RNAi-mediated down-regulation of glob1. Altogether, all three methods applied reveal a robustly working system for glob1 knockdown and over-expression of both, mRNA and protein.

Drosophila glob1 is a highly conserved functional respiratory protein with strong expression in all developmental stages (Hankeln et al., 2002; Burmester et al., 2006). It has a functional globin fold (de Sanctis et al., 2005; de Sanctis et al., 2006) and binds O_2 with relative high affinity (Hankeln et al., 2002). Consequently, its presence in *Drosophila* should be accompanied by an important physiological function in the fly´s oxidative metabolism. An impairment of endogenous glob1 expression should therefore most probably lead to phenotypic consequences.

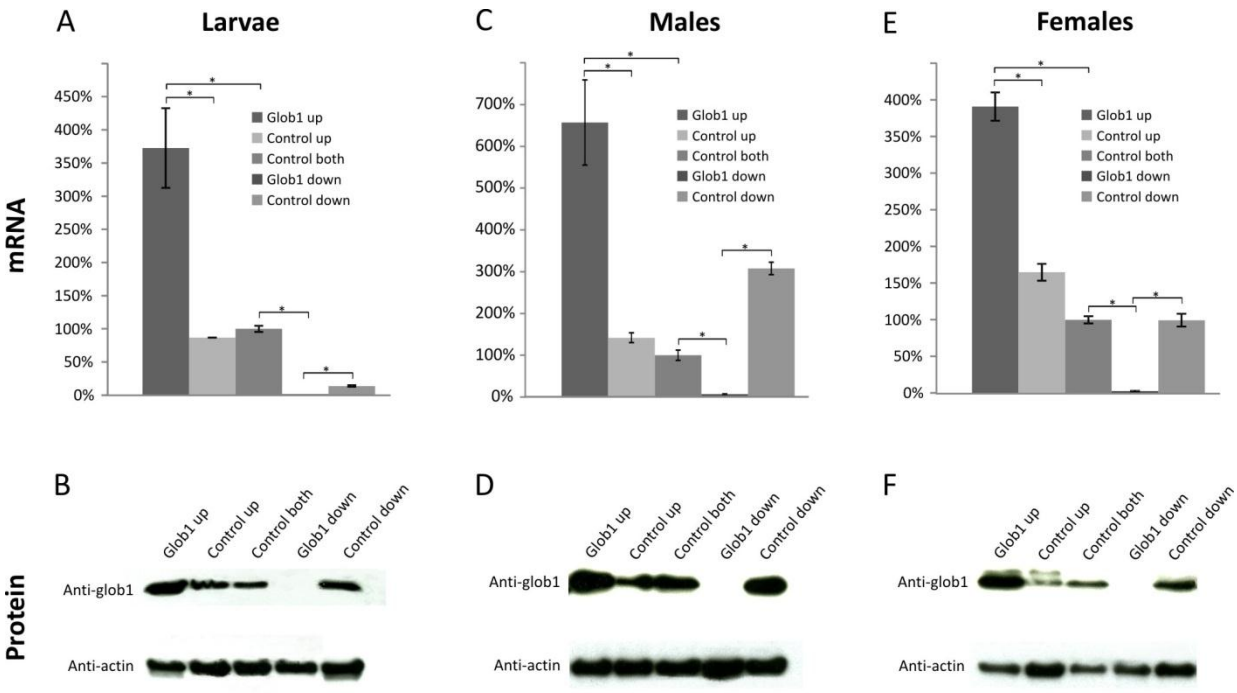

Figure 1: Verification of modulated glob1 expression in whole larvae and flies. Quantification of *glob1* mRNA and protein expression of 3rd instar larvae (A+B), male (C+D) and female flies (E+F) with *glob1* over-expression ('Glob1 up') and knockdown ('Glob1 down') compared to corresponding controls ('Control up', 'Control down', 'Control both', see Material & Methods section for further explanation). mRNA levels (bars) are shown relative to *glob1* expression of the control strain for both, over-expression and knockdown ('control both'). For Western Blot analysis, anti-actin antibodies were used as loading control. Error bars represent standard deviations. *p<0.05.

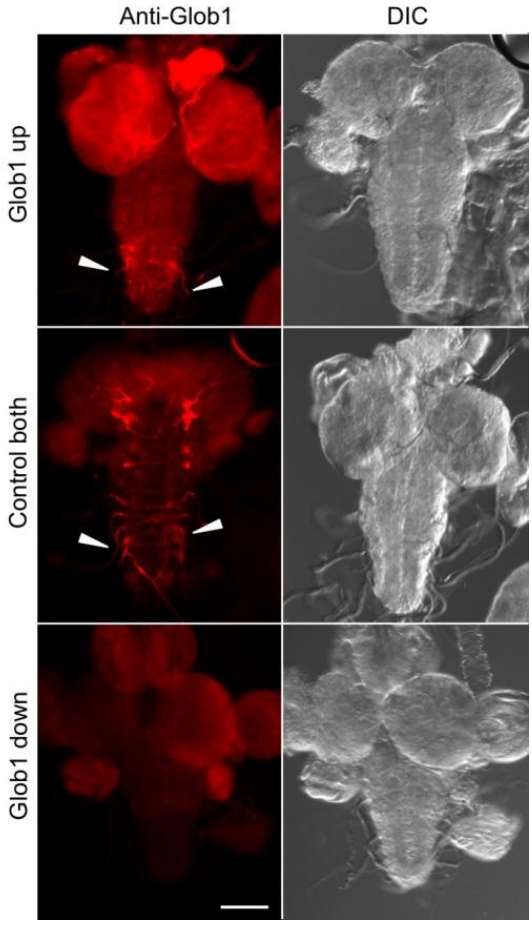

Figure 2: Verification of modulated glob1 expression in larval brains. Indirect immunofluorescence analyses with anti-glob1 of larval brains (3rd instar) with glob1 over-expression ('Glob1 up', upper panel) and knockdown ('Glob1 down', lower panel) compared to control brains ('Control both', middle panel) (left column) and differential interference contrast (DIC) images of brains (right column). Arrowheads indicate glob1-specific signals in tracheoles.

No effect of glob1 knockdown and over-expression on development and lifespan under normoxia

We initially analyzed the influence of the strong knockdown and a moderate 2-4fold over-expression of glob1 on longevity and development of flies kept under normal conditions, i.e. without experimental stress. Lifespan of adult, sexed flies with either glob1 knockdown or over-expression was monitored at constant temperature and otherwise constant food and breeding conditions. The median lifespan of glob1 knockdown flies

(females 93 ± 2 days, males 68 ± 2 days) did not show significant differences compared to the controls (females 86 ± 2 days and 79 ± 5days, males 69 ± 2 days and 67 ± 2 days). Median lifespan of glob1 over-expressing flies (females 85 ± 4 days, males 65 ± 3 days) also did not deviate from median lifespan of the corresponding control strains (females 80 ± 4 days and 79 ± 5days, males 61 ± 4 days and 67 ± 2 days) (Suppl. Fig. 2A-D). Female flies generally outlived their male counterparts by about two to three weeks. Developmental times, starting from late-stage embryos, of strains with glob1 knockdown or over-expression did not differ from developmental times of the corresponding control strains (not shown).

The deleterious effects of impaired functions of antioxidant enzymes like Cu/Zn-SOD or Catalase and chaperones like Hsp22 on lifespan and oxidative stress resistance of *Drosophila* have been convincingly demonstrated (Phillips et al., 1989; Griswold et al., 1993; Rogina and Helfand, 2000; Morrow et al., 2004a; Morrow et al., 2004b). If glob1 would be involved in detoxification of an excess of O_2 or ROS, as suggested by the increase of *glob1* mRNA expression under hyperoxic conditions (Gleixner et al., 2008), one may have expected a negative effect on lifespan or development in flies with glob1 knockdown. The unaltered lifespan and developmental times however, argue against a primary function of *Drosophila* glob1 as a classical antioxidant.

An involvement of a vertebrate myoglobin (Mb), in development was recently shown in zebrafish, where the Mb knockdown caused morphological and anatomical impairment in developing embryos and increased embryonic mortality over time (Vlecken et al., 2009). The mechanisms responsible for these developmental defects as a consequence of Mb-knockdown are not unraveled yet. In light of the absence of a developmental phenotype in the fly, a direct involvement of glob1 in embryonic development comparable to Mb in zebrafish appears unlikely.

Hypoxic stress: no effect of glob1 on development and larval behavior

Since flies with altered glob1 expression did not exhibit obvious phenotypic abnormalities under normoxia, we applied experimental hypoxic stress in order to challenge the organism and to bypass possible compensatory mechanisms that may take effect under normoxic conditions.

We first analyzed the influence of constant, moderate hypoxia on the development of embryos with altered glob1 expression. Development and reproduction of non-adapted flies in hypoxic conditions start to be impaired at concentrations of 6% O_2, and embryos being exposed to 4% O_2 do not reach adulthood (Zhou et al 2007). Another study found survival of *Drosophila* embryos to be reduced to 60% at 7.5%.O_2 and observed an increase in the time until emergence at 10% hypoxia compared to normoxic flies (Peck and Maddrell, 2005). We therefore monitored the development of *Drosophila* with either glob1 knockdown or over-expression at 5% O_2 at 22°C, starting from embryos collected overnight (0-18h). The number of embryos that reached the adult state did not differ between the strains analyzed. However, we observed a decrease of the overall survival rate of all embryos kept under hypoxic conditions to about 80% as expected under this stress regime (Zhou et al., 2007). Developmental times from embryonic to pupal stage and until eclosion also did not differ in *Drosophila* with glob1 knockdonw or over-expression compared to the corresponding controls. An overall delay in time (about 40%) from embryo until pupariation (about 40%) was noticed in all strains, whereas the time from pupariation to eclosion was comparable to normoxic conditions (about 324 – 350h after egg-deposition).

Next we were interested if altered glob1 expression in 3rd instar larvae would influence the time larvae can withstand hypoxia before clearing the food. *Drosophila* larvae are buried in the food with their posterior ends with the spiracles protruding for respiratory purpose. When hypoxia is applied, they respond within seconds and stop feeding, start leaving the food and finally wander onto the surface in order to escape hypoxic areas (Wingrove and O'Farrell, 1999). Our experimental setup was chosen according to Wingrove and O´Farrell (1999), but with hypoxic conditions of 5%, since we wanted to avoid that larvae became motionless under more severe hypoxia.

The measurement of escape time of larvae under hypoxic conditions again did not reveal any statistically significant differences between glob1 knockdown (111 sec), over-expression (142 sec) and control animals (119 sec, 101 sec and 105 sec) (Suppl. Fig. 3).

The larval stage is a challenging stage regarding sufficient O_2 supply, when larvae compete with bacteria and fungi living in the same environment. In case of an involvement of glob1 in O_2 supply one would expect it to be instrumental during hypoxic phases in larval life. However, the unaltered, stereotypic reaction to hypoxia of larvae with modulated glob1 expression at least indicates that larvae living in their natural habitat do not rely on globins as *primary* O_2 supply proteins. Furthermore, the endogenous glob1 expression, quantified by QPCR, is lowest in 3rd instar larvae, possibly indicating a secondary role of glob1 in the larval stage. Yet, we could clearly replicate the known detrimental effects of hypoxia on *Drosophila* as shown by overall delay of developmental times and decreased survival of embryos.

Glob1 causes improved recovery from, but does not influence the onset of anoxic stupor

We then investigated if altered glob1 levels would influence the time it takes for adult flies to fall into, and to recover from, anoxic stupor. The anoxic stupor is a very quick response to complete O_2 deprivation that begins within seconds (Csik, 1939; Haddad et al., 1997b). Zhou et al. (2007) observed a significant decrease of recovery time after anoxic stupor in flies adapted to low O_2 conditions. Ma et al. (2001) could show that the over-expression of an anoxia-regulated gene (*fau*), whose function is unknown, resulted in shortened recovery time after anoxic treatment. It has also been shown that recovery time increases with prolonged anoxic treatment (Haddad et al., 1997b; Ma et al., 2001).

We first exposed flies to severe hypoxia (O_2 < 1%) and measured the time of onset of anoxic stupor, but could not observe pronounced, significant differences in the reaction to anoxia between flies with glob1 knockdown or over-expression compared to the corresponding controls. All flies passed into the stereotypic anoxic stupor equally shortly after exposure to severe hypoxia (120-180s) (Suppl. Fig. 4A-D). The measurements of the recovery time after two hours of anoxic stupor, in contrast, revealed a significantly shorter (median) time it took 50% of *female* flies with glob1 knockdown (120 sec) to recover from anoxic stupor, compared to that of control flies (330 sec and 360 sec). Median recovery times of female flies with glob1 over-expression were at the same level as control strains, ranging from 240sec to 400sec (Suppl. Fig. 5A). Male flies did not show glob1-dependent changes in median recovery times (ranging from 330sec to 420 sec of; Suppl. Fig. 5B+C). The absence of measurable differences in the onset of anoxic stupor of flies with modulated glob1 expression may be explained by the very quick response, occurring within seconds, of flies exposed to severe hypoxia or anoxia. Under such stringent stress conditions, flies inevitably fall into stupor that possible glob1-specific benefits or disadvantages possibly cannot take effect.

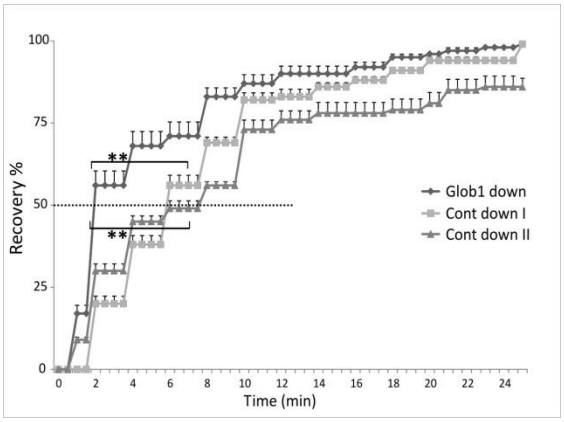

Figure 3: Recovery time from severe hypoxia (O_2 < 1%) of female flies with modulated glob1 expression. Glob1 knockdown ('Glob1 down') leads to shortened median recovery time (dotted line) after severe hypoxic challenge in female flies compared to respective controls. Error bars represent standard deviations. **p < 0.005, ANOVA.

At first sight, the *improved* recovery time from anoxic stupor of female flies with glob1 knockdown appears to be a paradoxical effect. We interpret these data in light of the observation made by Zhou et al. (2007) that flies adapted to low O_2 conditions over generations exhibit shorter recovery times from anoxic stupor. Further adaptations of those chronically hypoxic flies included differential gene expression in signal transduction and metabolic pathways, a decreased size and body weight and increased O_2 consumption (Zhou et al., 2008; Zhou et al., 2011), possibly due to hypoxia-dependent increase in the diameter of the dorsal trunks of the tracheal system (Henry and Harrison, 2004). Possibly, the glob1 knockdown situation has created similar adaptive compensatory effects. Such physiological compensations for a loss of glob1 function would also explain the lack of observed phenotypes in lifetime, development and larval behavior. Preliminary microarray expression studies of female glob1 knockdown flies in comparison with control flies (not shown) indeed revealed differential expression of a variety of genes, indicating compensatory effects on the gene regulatory level which have to be confirmed and further analyzed. The expression level of the two additional *Drosophila* globins *glob2* and *glob3*, however, were not affected by a glob1 knockdown.

Glob1 knockdown causes increased sensibility to prolonged, moderate hypoxic stress of adult flies

The phenotypic results so far indicate that we did not fully succeed to identify and apply the correct stress conditions under which glob1 function turns essential in *Drosophila* embryos, larvae and adults. Also, some of our experiment read-outs may have been too coarse to detect fine-grained differences between the glob1 mutants and wildtype flies. We therefore applied additional regimes of hypoxic stress, long-term moderate and short-term severe to the adult stage, where the reaction of flies has been documented best (see e.g. Csik, 1939; Haddad et al., 1997a; Haddad et al., 1997b; Zhou et al., 2007) and where we were able to apply hypoxia for days to weeks without taking developmental changes into account.

A long-term hypoxic treatment (5% O_2 for up to 40 days) resulted in a 50% lifespan reduction of female and male flies compared to those reared under normoxia, confirming previously observed reduced survival rates of adult flies under hypoxic conditions (Vigne and Frelin, 2006; Van Voorhies, 2009; Rascon and Harrison, 2010). Female and male flies with glob1 knockdown as well as over-expression were dead after 39 and 30 days, respectively (Fig. 4 A+B). The same was true for flies of the control strain I, which reflect the genetic background of glob1 knockdown flies, butwith unaltered glob1 expression. Flies of the control strain II, in contrast, still exhibited 50% survival after 39 days of hypoxia. This higher tolerance of control II flies cannot be explained sufficiently, but might be due to differences in their specific 'white$^-$ /daughterless' genetic background. Flies with

glob1 over-expression did not show differences in survival of hypoxic treatment compared to control strains (Suppl. Fig. 6A+B). Most interestingly, however, female flies with glob1 knockdown (Fig. 4A) showed a significantly reduced overall survival and a higher vulnerability to hypoxic treatment as well as a significantly reduced median survival time (18.5 days) compared to control flies (Control I: 25.5 days). The same was observed for male flies with glob1 knockdown (Fig. 4B), which also exhibited significantly decreased overall survival, and higher vulnerability to hypoxic treatment, indicated by a significantly decreased median survival rate (9.5 days) compared to control flies (Control I: 17.5 days).

To test the consequences of more severe, short-term hypoxic challenge, flies were exposed to 1.5% O_2 for 6h. Shortly after O_2 concentration dropped below 2%, all flies became motionless and remained in this state until re-oxygenation after 6h, as it was expected according to several previous studies (Csik, 1939; Haddad et al., 1997b). Under these conditions, female flies with glob1 knockdown had a significantly decreased survival rate compared to control strains. Only 5% of female flies with glob1 knockdown survived the severe hypoxic treatment, compared to 33.3% and 20.8% survival rates of control strains (Fig. 5A). Male flies with glob1 knockdown also showed a decreased survival rate (4.4%) compared to the control strains (10.4% and 9.6%) however this difference was not statistically significant (Fig 5B). Flies with glob1 over-expression did not show significant differences in survival of severe hypoxia.

Altogether, these data indicate for the first time a clear beneficial phenotypic effect of the presence of glob1 in *Drosophila* adults. These phenotypic consequences are only seen in the transcriptional *glob1* knockdown. Possibly, the tissue-specific levels of glob1 over-expression mediated by the da driver are not sufficient to reveal additional beneficial effects, suggesting that wildtype levels of glob1 are already at optimal levels.

Although adult flies live in ambient air and therefore are not directly exposed to systemic hypoxic conditions, their O_2 demand strongly differs depending on activity. Flight movements clearly increase the metabolic activity of flies (Lehmann and Dickinson, 1997; Lehmann et al., 2000) and consequently lead to higher O_2 demand. Resting phases, in contrast, bear low O_2 consumption and should increase the risk of toxic O_2 levels (Lehmann and Schutzner, 2010). Adult *Drosophila* therefore are able to adjust the opening width of their spiracles to the O_2 demand. They thus keep a balance between O_2 need, the potential risk of an excess of O_2 and the risk of desiccation caused by water loss through opened spiracles (Lehmann, 2001; Heymann and Lehmann, 2006; Lehmann and Schutzner, 2010). It was suggested that this balance provides enough respiratory reserves for sudden increases in oxygen consumption (Lehmann and Schutzner, 2010). The decreased survival of moderate and severe hypoxia of flies with glob1 knockdown may indicate a possible involvement of glob1 in O_2 supply of adult flies. An additional support in O_2 supply by respiratory proteins in phases of high O_2 consumption appears well conceivable. The globin could fulfill the role of a temporary O_2 store, thus buffering acute O_2 needs and extending ATP homeostasis. This hypothesis is further supported by the finding that the oxygen affinity of glob1 (Hankeln et al., 2002; de Sanctis et al., 2005) is comparable to other insect globins with known O_2 storage or transport functions, like the hemoglobins of *G. intestinalis* (Keilin and Wang, 1946) or the Chironomid midges (Osmulski and Leyko, 1985). Moreover, the close evolutionary relationship of *Drosophila* glob1 to insect globins known to fulfill O_2 delivery or storage roles (Burmester et al., 2006), provides

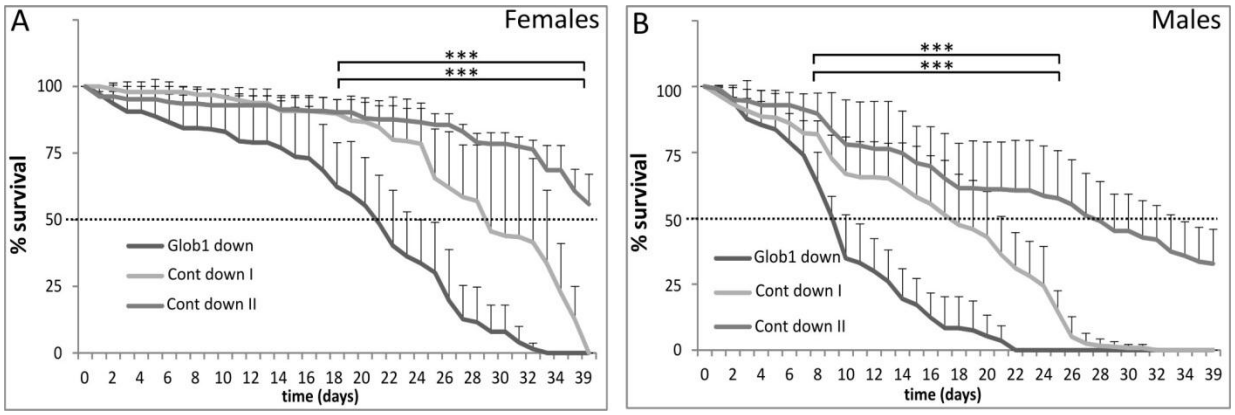

Figure 4: Survival of moderate hypoxia (5% O_2) of adult flies with modulated glob1 expression. Glob1 knockdown ('Glob1 down') leads to decreased overall survival and median survival (dotted line) in female (A) and male (B) flies during hypoxic exposure compared to respective controls. Female and male flies with glob1 over-expression ('Glob1 up') did not show differences in survival of hypoxic treatment compared to respective controls. Error bars represent standard deviations. ***p < 0.001, ANOVA.

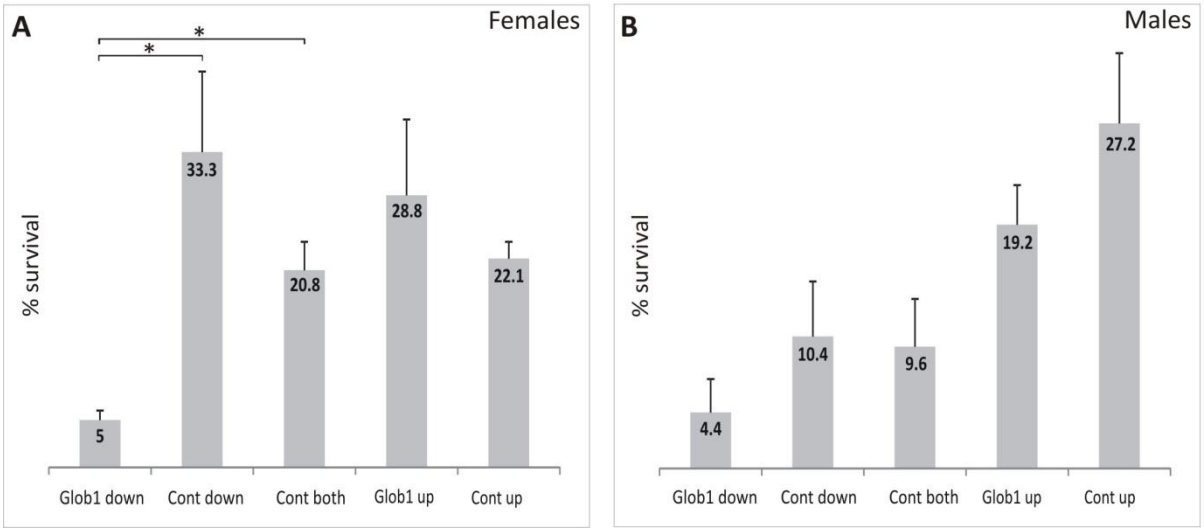

Figure 5: Survival of severe hypoxia (1.5% O_2) of adult flies with modulated glob1 expression. Glob1 knockdown ('Glob1 down') leads to decreased survival of severe hypoxia of female (A) and male (B) flies compared to corresponding controls. Female and male flies with glob1 over-expression ('Glob1 up') 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.

indirect evidence for a function of glob1 in O_2 supply of *Drosophila*.

A role of glob1 in alleviating oxidative stress?

The results obtained from the hypoxic experiments so far thus indicate a possible involvement of glob1 in O_2 supply. Considering previous results obtained from *glob1* mRNA expression studies, the observed increase of *glob1* mRNA after hyperoxic treatment can also be interpreted in the context of other possible globin functions like the detoxification of an excess of O_2 or ROS (Gleixner et al., 2008). Thus, glob1 could be instrumental in the detoxification of ROS formed during hyperoxic conditions, in analogy to vertebrate myoglobin (Flogel et al., 2004). We therefore analyzed the phenotypic consequences of modulated glob1 expression in *Drosophila* after experimental hyperoxia and chemical ROS production by the herbicide Paraquat (Bus and Gibson, 1984).

No effect of glob1 knockdown on development and lifespan under hyperoxia

Drosophila, exposed to high O_2 concentrations, do not show behavioral responses like those observed under hypoxic conditions. However, the effect of chronic hyperoxia on survival is deleterious since adult lifespan is reduced drastically to about 8 days (Walker and Benzer, 2004; Gruenewald et al., 2009). Modulations in expression of antioxidant enzymes, however, can influence resistance to oxidative stress positively, as in flies over-expressing mitochondrial Catalase, which exhibited increased survival rates (30-40%) when treated with Paraquat (Mockett et al., 2003). Null-mutations of antioxidant enzymes like e.g. Cu/Zn-SOD, on the other hand, can lead to hypersensitivity to Paraquat-induced oxidative stress (Phillips et al., 1989). Elevated as well as decreased ROS levels influence the development of organisms. It has been shown that a fine balance in the formation and clearance of ROS is crucial for normal growth and metabolism under normoxia (Finkel and Holbrook, 2000) and that elevated O_2 levels (O_2 > 80%) result in developmental arrest and death of *Drosophila* embryos (Zhao et al., 2010).

We therefore analyzed lifespan and development of flies with either glob1 knockdown or over-expression relative to appropriate control flies under hyperoxic conditions with 95% O_2. Developmental times of *Drosophila*, starting from 3rd instar larval stage, were not influenced by hyperoxia. However, as expected, survival rates of larvae irrespective of their genotype were decreased, resulting in development rates from larvae to adults of 76-90%. When exposing adult *Drosophila* to hyperoxia, the flies were not visibly influenced during the first 96 hours. After the 5th day, however, fly survival rates started to drop dramatically to 50% and resulted in the death of all flies after about 8 days, as expected from the literature (Walker and Benzer, 2004; Gruenewald et al., 2009). Again, we did not observe any differences in the survival rate of flies with glob1 knockdown or over-expression relative to controls (Suppl. Fig. 7A+B). These data so far indicate that a role of glob1 in antioxidant defense, in the sense of classical antioxidant proteins like Cu/Zn-SOD or Catalase (Lee et al., 1981; Mackay and Bewley, 1989), is rather improbable.

Glob1 knockdown leads to increased resistance to oxidative stress induced by Paraquat

The herbicide Paraquat is known to act as a potent chemical ROS generator *in vivo*. It strongly facilitates the accumulation of reactive superoxide anions upon ingestion, which results in a cascade of production of other, even more reactive O_2 species like hydrogen peroxide or hydroxyl radicals (Sittipunt, 2005). In numerous studies, Paraquat concentrations of 10-20mM were used to test the ability of flies to withstand oxidative insult (Phillips et al., 1989; Reveillaud et al., 1991), and it has been shown that increased resistance to Paraquat positively correlates with increased resistance to oxidative stress in general and also prolonged lifespan (Vermeulen et al., 2005). Furthermore it has been shown that flies with defects in enzymes of the antioxidant system show increased susceptibility to oxidative stress caused by Paraquat (Phillips et al., 1989; Radyuk et al., 2009). We therefore tested the ability of adult flies and 3rd instar larvae with modulated glob1 expression to withstand Paraquat-induced oxidative stress. Most of 3rd instar larvae fed with 20mM Paraquat were still able to develop into adult flies, but eventually died a few hours after eclosion. Paradoxically, we observed a significantly *higher* rate of larvae with glob1 knockdown that developed into adults (91.7%) compared to the corresponding controls (75.7%; 53%).

Survival rate of L3 larvae with glob1 over-expression was the same as controls (Fig. 6A).

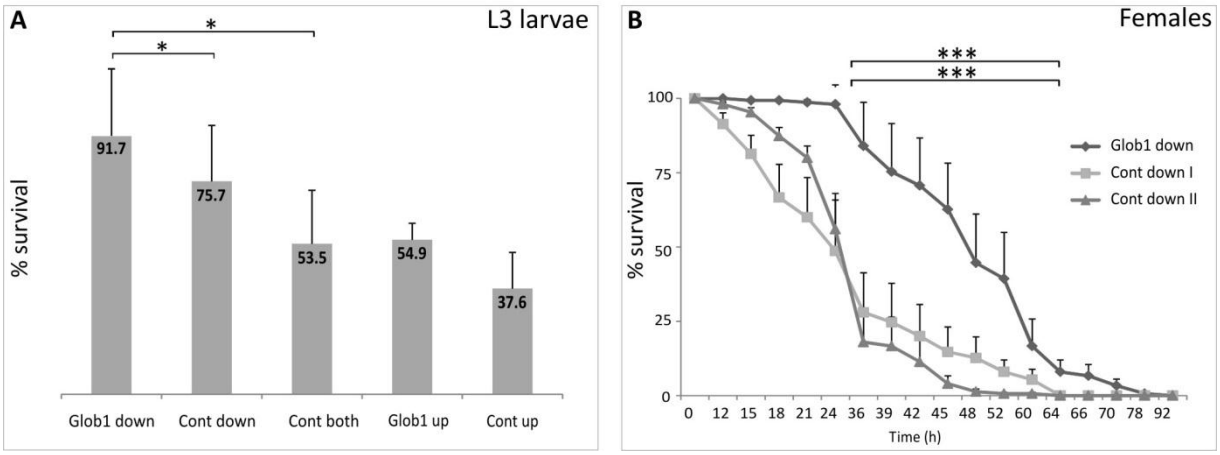

Figure 6: Survival of Paraquat-induced (20mM) oxidative stress of larvae and female flies with modulated glob1 expression. (A) Glob1 knockdown (´Glob1 down') in 3rd instar larvae increases survival of Paraquat treatment compared to respective controls. L3 larvae with glob1 over-expression ('Glob1 up') did not show differences in survival of Paraquat treatment compared to respective controls. * $p < 0.05$, two-tailed t-test. (B) Glob1 knockdown in female flies ('Glob1 down') increases survival of Paraquat treatment compared to respective controls. Error bars represent standard deviations. ***$p < 0.001$, ANOVA.

For adult flies, Paraquat treatment resulted in death of all individuals within 96h. No differences in survival of Paraquat treatment was observed in female or male flies with glob1 over-expression compared to respective controls (Suppl. Fig. 8A+C). However, we observed a significantly *higher* survival rate specifically of female flies with glob1 knockdown during the first 55 hours. After this point, the survival rate adjusted to those of the control strains (Fig. 6B). We could not observe the same effect in male flies with glob1 knockdown (Suppl. Fig. 8B). This could be due to the earlier and far more rapid onset of Paraquat-induced mortality, which we and others (Chaudhuri et al., 2007) specifically observed in male flies, and that could mask possible differences between flies with modulated glob1 expression and controls.

The observed increased resistance to Paraquat in female glob1 knockdown flies led us to quantify the amount of damage to lipids and DNA caused by ROS. A level of reactive O_2 species that exceeds the capacity of an organism´s antioxidant system leads to increased oxidative damage and ultimately to an impairment of cellular functions up to death. In *Drosophila* the damage resulting from oxidative stress to cells is diverse, ranging from peroxidation of membrane lipids (Arking et al., 2000; Gruenewald et al., 2009) to protein carbonylation (Gruenewald et al., 2009) and DNA-damage, eventually resulting in apoptosis (Walker and Benzer, 2004).

We measured the amount of lipid peroxidation in male and female adult flies after exposure to 20mM Paraquat or hyperoxia with 95% O_2. We observed the expected increase in the amount of lipid peroxidation (LPO) in flies treated with Paraquat (~17µM LPO/mg tissue), as well as after hyperoxic exposure (~22µM LPO/mg tissue), but did not detect differences in the amount of LPO in flies with modulated glob1 expression. We also tried to quantify the amount of oxidative damage to DNA by visualizing apoptotic cells via TUNEL staining revealed an increase in 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 (Suppl. Fig 9), as expected from the literature (Walker and Benzer, 2004; Radyuk et al., 2009). However, again no significant differences were observed

in the amount of cell death in flies with modulated glob1 expression.

Considering the well-documented susceptibility to oxidative stress of flies with functional impairments in their antioxidant system (see e.g. Phillips et al., 1989; Mockett et al., 2003; Walker and Benzer, 2004), the absence of detrimental or beneficial effects to the survival of strains with modulated glob1 expression argues against a primary role of glob1 in ROS detoxification. Even if we envisage the possibility that a potential antioxidant role of glob1 must not inevitably lead to different survival rates upon oxidative stress, we would at least have expected measurable differences on the molecular level, visible by altered levels of LPO or apoptotic cell. Yet, we indeed observed an increase in cell damage caused by oxidative stress, but to the same extent in all strains analyzed. Although there is still a possibility that glob1 fulfills a 'background' antioxidant role, whose effect might be masked under the stringent conditions which we applied in our study, its dominant function should rather be considered elsewhere.

The surprising resistance to Paraquat of female flies and L3 larvae with glob1 knockdown is difficult to explain. However, if glob1 really functions as an O_2 store, the O_2 released from glob1 could possibly act as an electron acceptor for Paraquat and get reduced to superoxide anion. Therefore, a lower intracellular O_2 concentration in glob1 knockdowns would hypothetically lead to decreased O_2^- formation and less damage. Unfortunately, our molecular assays could not confirm the postulated decreased ROS formation in the glob1 knockdown, possibly due to a lack of sensitivity. Also, ectopic glob1 over-expression did not deteriorate survival of Paraquat exposure as might then have been expected, a fact that could possibly be explained by the moderate (2-4fold) 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 reproduction activity (Burger and Promislow, 2004). Notwithstanding these confining aspects, our interpretations of the phenotypic effects of ROS-stress, in particular the paradoxical amelioration of survival rates in Paraquat-treated glob1 knockdown females, support the idea that glob1 acts as an O_2-binding storage and supply protein.

To confirm our hypothesis, the intracellular oxygenation status in tissues of flies with modulated glob1 expression should be addressed. This could for example be done indirectly by analyzing the cellular HIF-status via electrophoretic mobility shift assay (EMSA) of flies with glob1 knockdown and over-expression, to quantify the amount of stabilized HIF-1α and thereby indirectly determining the intracellular O_2 status. Another approach could be the mRNA quantification of known HIF-regulated genes in flies with modulated glob1 expression, whose increased expression would indicate low intracellular O_2 concentrations.

Conclusion

The ability to robustly modulate the endogenous globin expression gave us the opportunity to screen for phenotypic abnormalities as a consequence of glob1 knockdown and over-expression. We show for the first time here that a transcriptional knockdown of the *glob1* gene produces a phenotypic effect in *Drosophila* flies after exposure to experimental hypoxia. These data, in conjunction with indirect evidence obtained by exposure to ROS stress, suggest a role of glob1 as an O_2 supply protein. Glob1, predominantly expressed in tracheal cells and fat body, possibly acts as a short-term O_2 buffer, which is able (1) to bind O_2 in case of excessive presence in the tracheal system an

(2) deliver O_2 in case of acute need, e.g. during abrupt onset of flight.

The observation that under normoxia, neither the lack of glob1, nor it over-expression was apt to produce overt phenotypic abnormalities was unexpected and points to the necessity to apply suitable stress conditions while testing for gene function. The situation is reminiscent of the myoglobin-deficient knockout mice (Garry et al., 1998; Godecke et al., 1999) which appeared to be completely normal, but only at second sight revealed morphological compensatory effects like increased capillary density and elevated coronary flow (Godecke et al., 1999). In *Drosophila*, the tracheal system responds to low intracellular O_2 conditions by ramifying towards oxygen starved areas (Jarecki et al., 1999; Metzger and Krasnow, 1999; Lavista-Llanos et al., 2002), a reaction comparable to angiogenesis in vertebrates (Jarecki et al., 1999; Metzger and Krasnow, 1999; Lavista-Llanos et al., 2002). It is thoroughly possible that glob1 knockdown influences intracellular O_2 concentrations and thereby causes yet to be defined morphological and metabolic adaptations in the tracheal system and the fat body.

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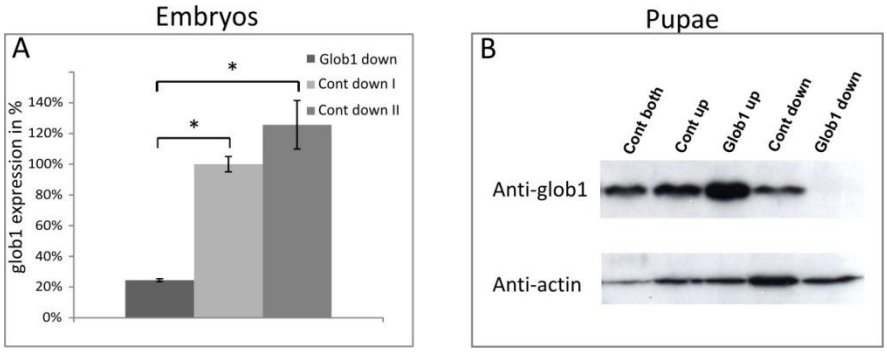
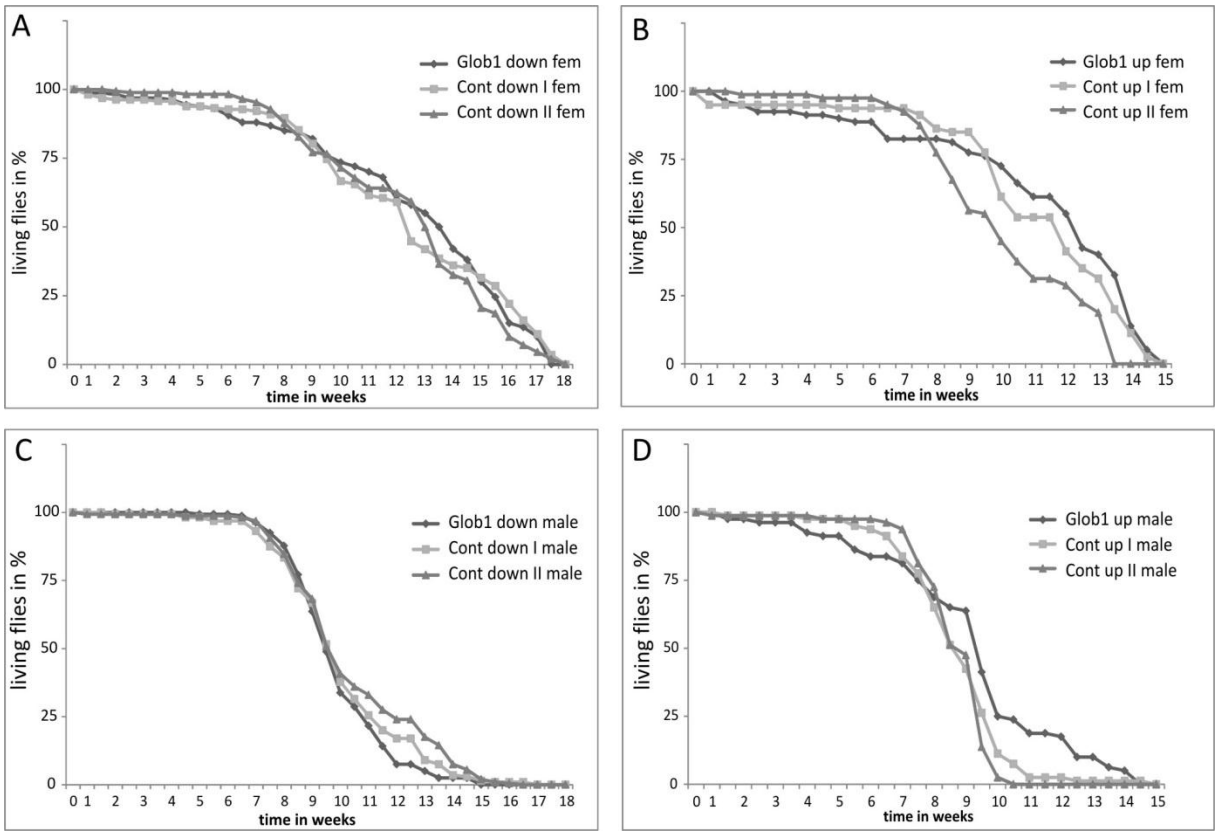
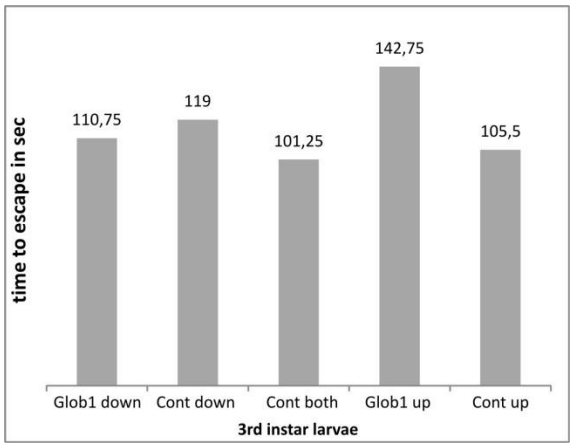
Supplementary Material



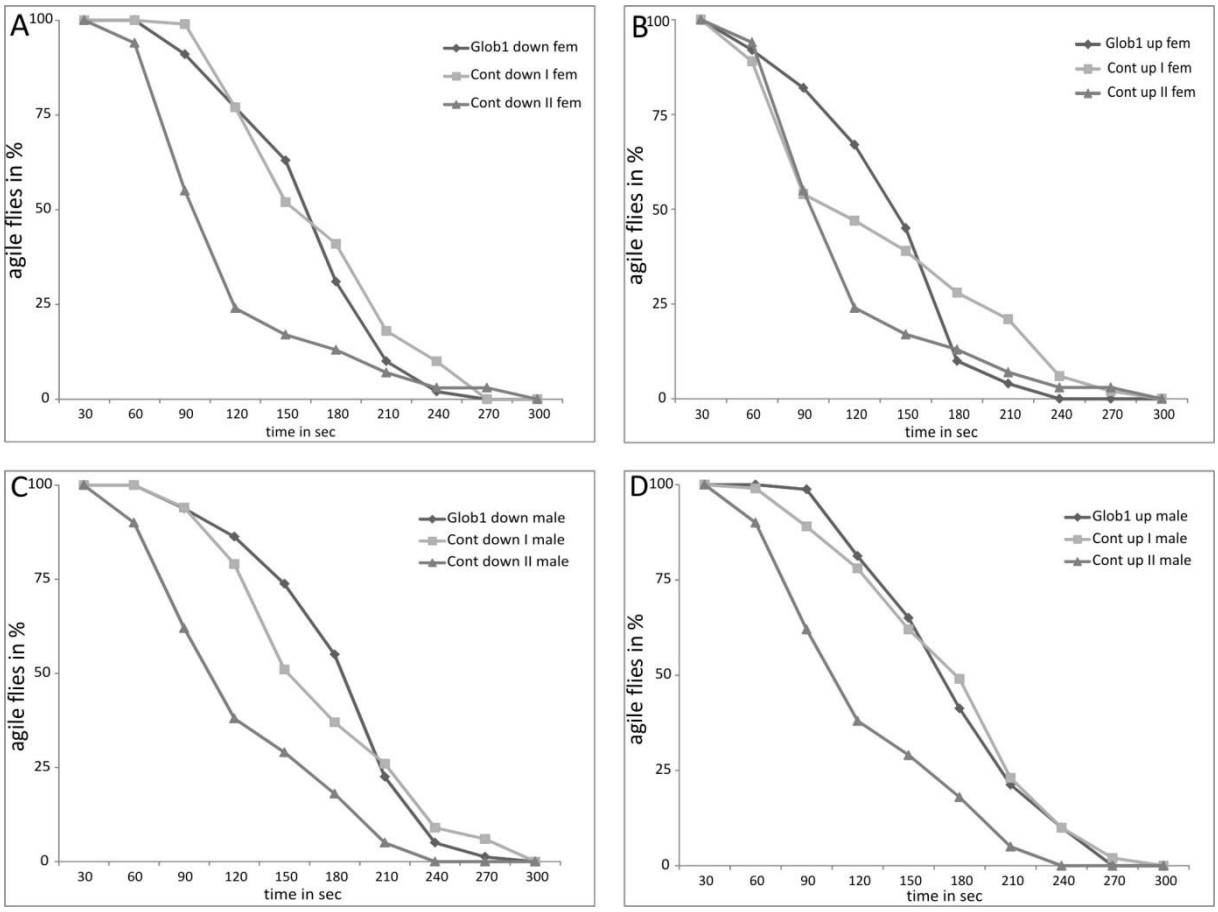
Supplementary Figure 1: Modulated glob1 expression in embryos and pupae confirmed either on mRNA or on protein level. (A) Quantification of *glob1* mRNA expression in embryos: *glob1* knockdown ('Glob1 down') compared to respective controls. mRNA levels (bars) are shown relative to *glob1* expression of control I. Error bars represent standard deviations *p < 0.05. (B) Modification of glob1 expression in pupae confirmed on protein level by Western Blot: glob1 over-expression ('Glob1 up') and knockdown ('Glob1 down') compared to respective controls stained with anti-glob1 antibodies (upper panels) and anti-actin antibodies (lower panels) as loading control.



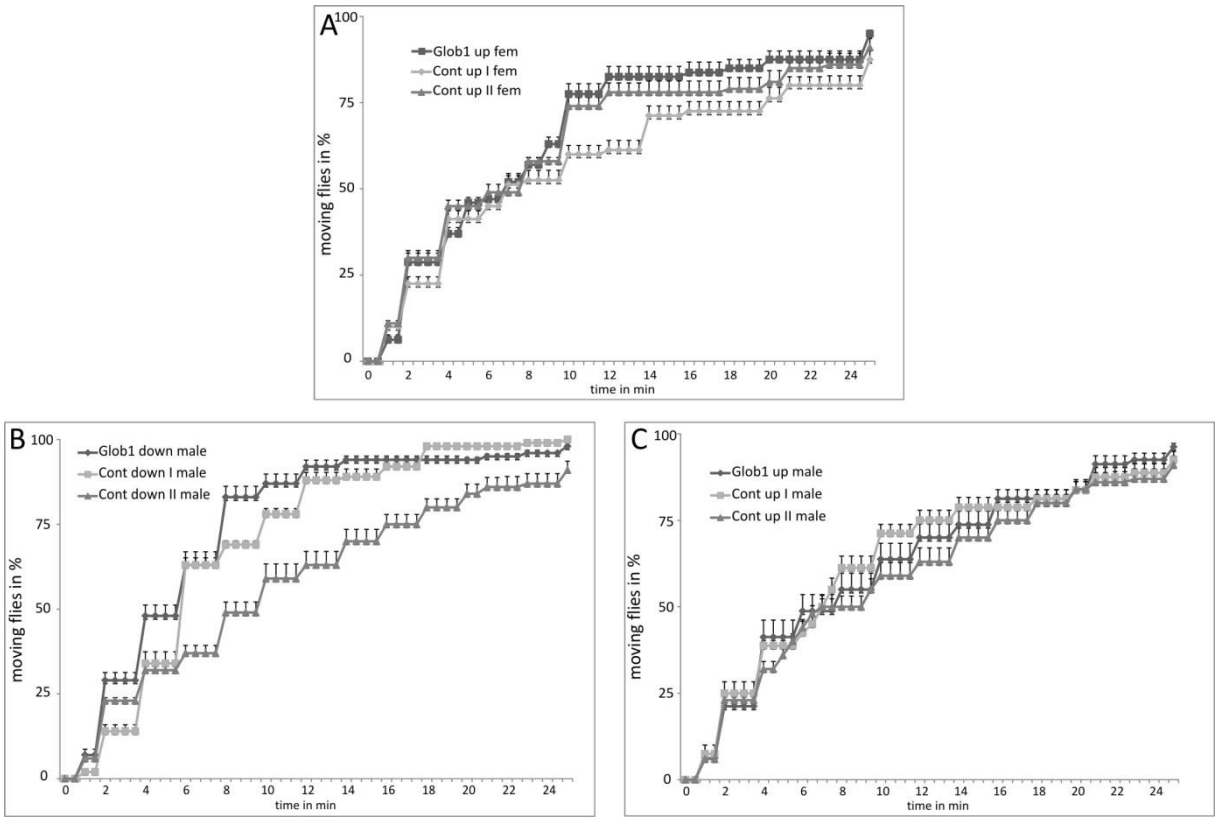
Supplementary Figure 2: Lifespan of adult flies with modulated glob1 expression under normoxia.
No differences in lifespan of female flies with glob1 knockdown ('Gob1 down') (A) and over-expression ('Glob1 up') (B) compared to corresponding controls. No differences in lifespan of male flies with glob1 knockdown (C) and over-expression (D) and respective controls.



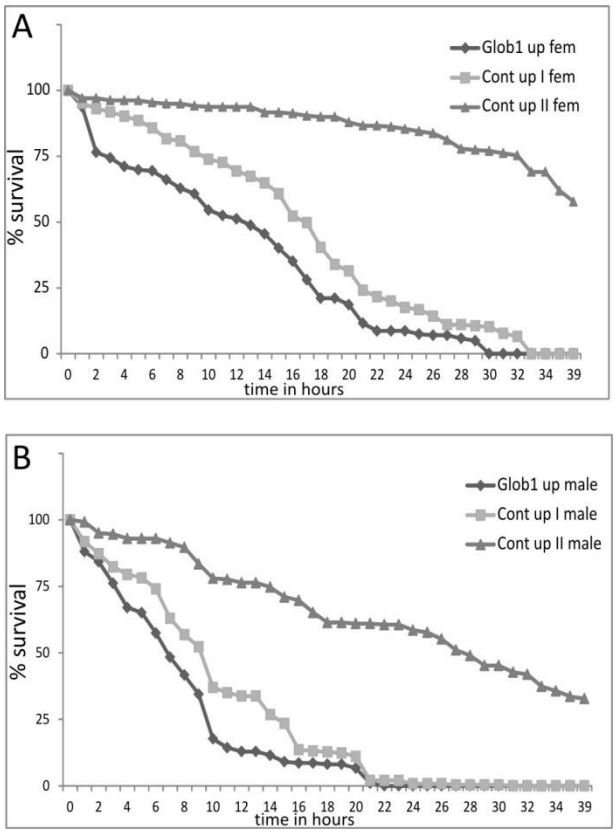
Supplementary Figure 3: Response of 3rd instar larvae with modulated glob1 expression to moderate hypoxia (5% O_2). No differences in response to hypoxia of larvae with glob1 knockdown ('Glob1 down'), over-expression ('Glob1 up') and respective controls.



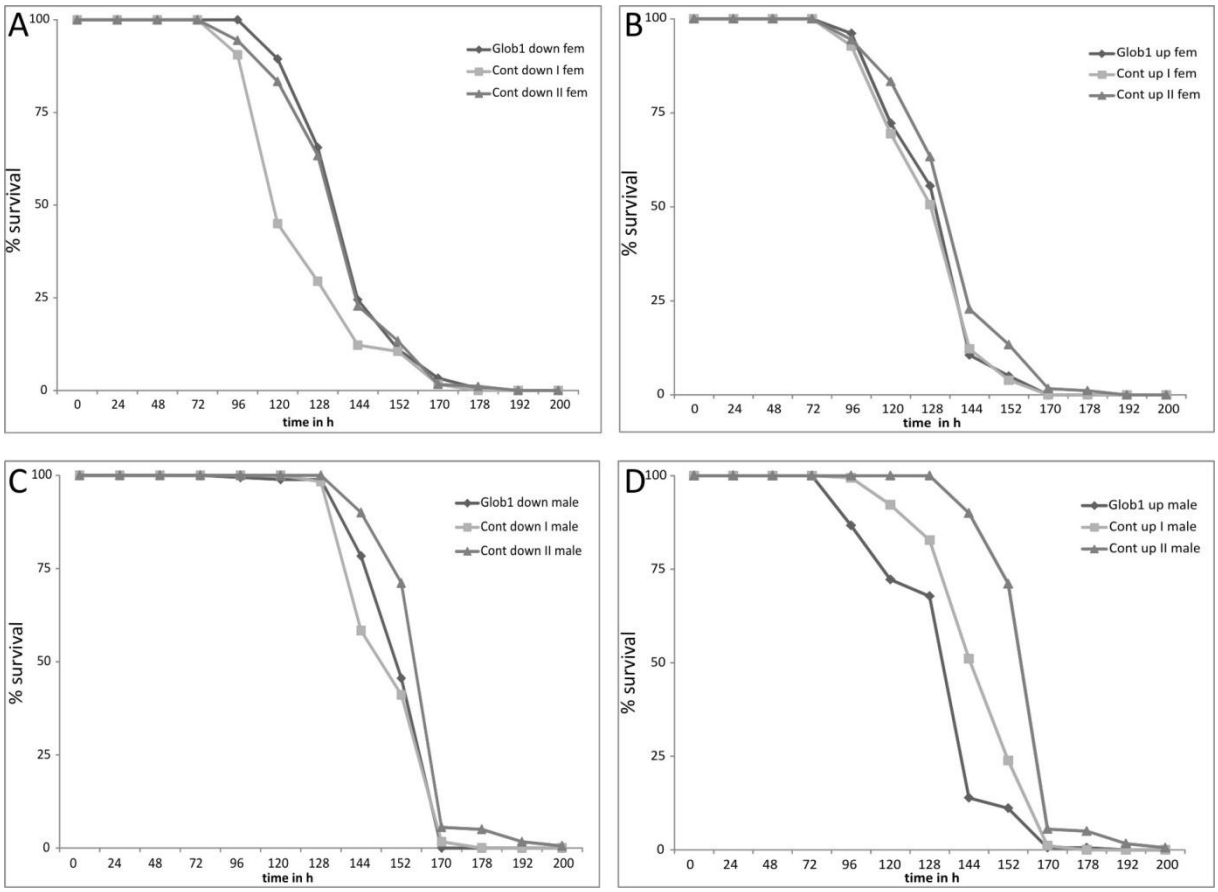
Supplementary Figure 4: Reaction to anoxia of adult flies with modulated glob1 expression.
No differences in reaction to anoxia of female (A+B) and male (C+D) flies with glob1 knockdown ('Glob1 down'), over-expression ('Glob1 up') and respective controls.



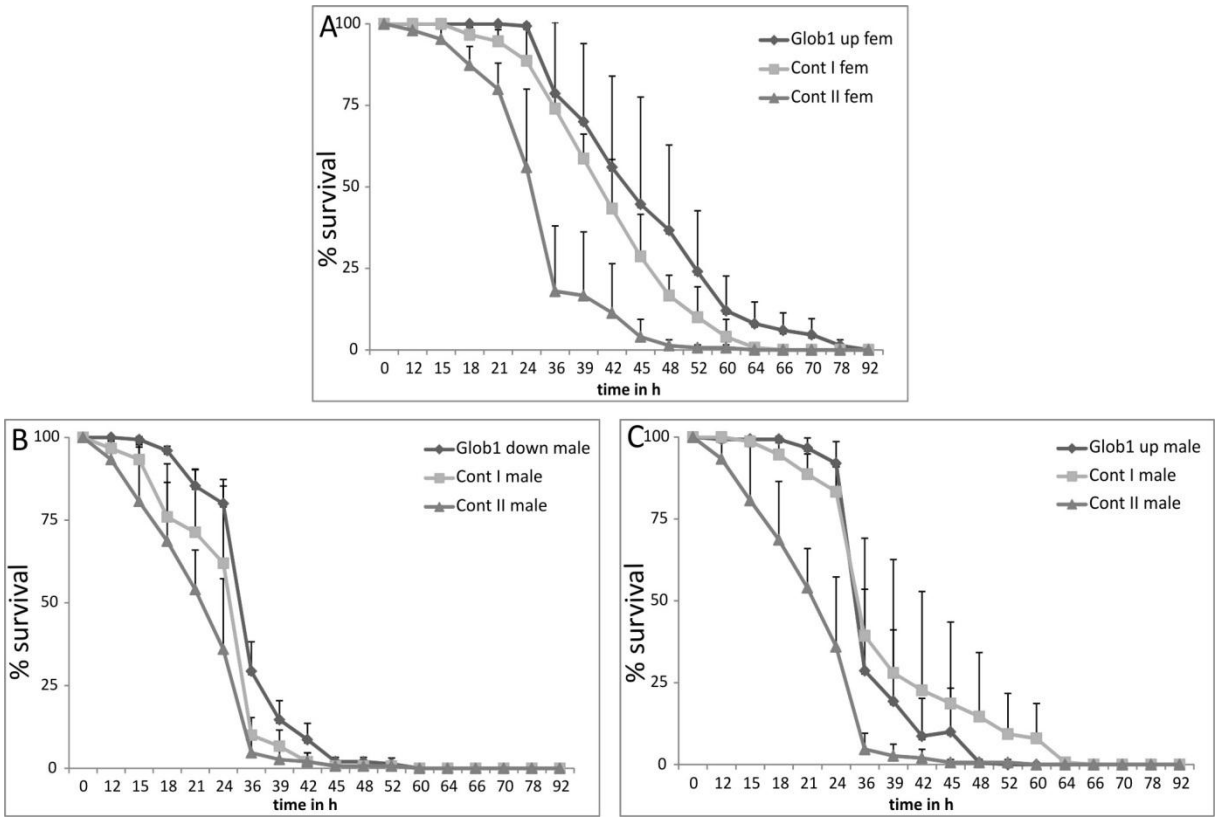
Supplementary Figure 5: Recovery from severe hypoxia (O_2 < 1%) of adult flies with modulated glob1 expression.
No differences in recovery time of female flies with glob1 over-expression ('Glob1 up') (A) and male flies with glob1-knockdown ('Glob1 down') (B) and over-expression (C) and respecitve controls. Error bars respresent standard deviations.



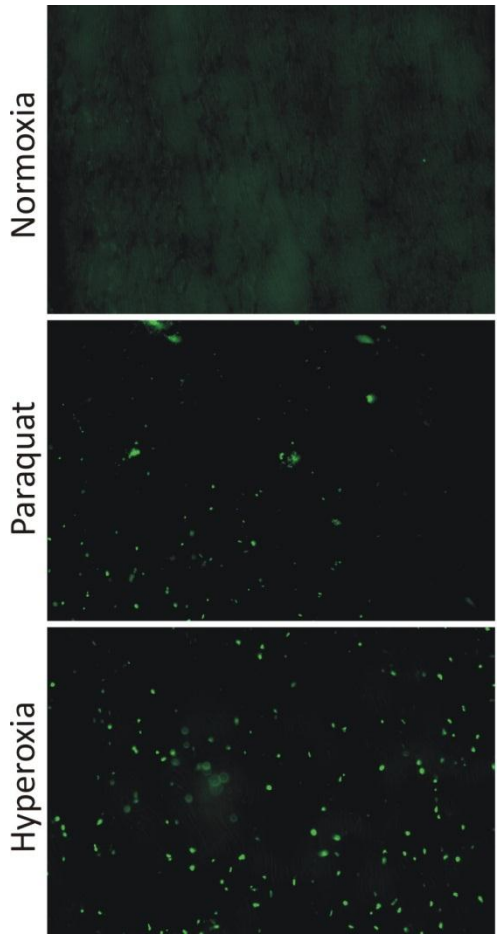
Supplementary Figure 6: Survival of long-term hypoxia (5% O_2) of adult flies wit glob1 over-expression.
No differences in survival rates of female (A) and male (B) flies with glob1 over-expression ('Glob1 up') and to respective controls.



Supplementary Figure 7: Survival of hyperoxia (95% O_2) of adult flies with modulated glob1 expression.
No differences in survival of hyperoxia of female (A+B) and male (C+D) flies with glob1 knockdown ('Glob1 down'), over-expression ('Glob1 up') and respective controls.



Supplementary Figure 8: Survival of Paraquat (20mM) of adult flies with modulated glob1 expression.
No differences in survival of Paraquat-induced ROS-stress of female (A) and male (B) flies with glob1 over-expression ('Glob1 up') and male flies with glob1 knockdown ('Glob1 down') (C) and respective controls.



Supplementary Figure 9: TUNEL staining of apoptotic nuclei in cryo-sections of adult flight muscles.
TUNEL staining of flight muscles obtained from flies kept in normoxia (upper panel), after Paraquat-treatment (middle panel) and after hyperoxic treatment (lower panel). An increase in TUNEL-positive nuclei after oxidative stress induced by Paraquat and hyperoxia was observed.

Abbreviations

Cu/Zn-SOD, copper/zinc-superoxide dismutase; da, daughterless; DIC, differential interference contrast; EMSA, electrophoretic mobility shift assay; HIF, hypoxia-inducible factor; Hsp, heat shock protein;. LPO, lipid peroxidation; Mb, myoglobin; ROS, reactive oxygen species; TUNEL, TdT-mediated dUTP-biotin nick end labeling.

2.4 Gleixner, E., Herlyn, H., Zimmerling, S., Burmester, T. and Hankeln, T. (2011) Testes-specific hemoglobins in *Drosophila* evolved by a combination of sub- and neofunctionalization after gene duplication.

Testes-specific *Drosophila* hemoglobins evolved by a combination of sub- and neofunctionalization after gene duplication

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Keywords
Globin, gene duplication, oxygen, hypoxia

Summary

Since the discovery of the first globin gene, *glob1*, in *Drosophila melanogaster*, it is now well established that globins belong to the standard repertoire of insect genomes. Insect globins usually share a conserved expression pattern in tracheae, the fat body and Malpighian tubules. Here we show that the more recently discovered *D. melanogaster* globin variants *glob2* and *glob3* both display a male-specific expression in the reproductive tract during spermatogenesis. Both paralogs are transcribed at equivalent mRNA levels and largely overlap in their cellular pattern of expression during spermatogenesis. Phylogenetic analyses showed that *glob2* and *glob3* derive from a gene duplication event which occurred in the ancestor of the *Sophophora* subgenus at least 40 million years ago, so that flies of the *Drosophila* subgenus harbor only one *glob3*-like gene. Molecular evolutionary sequence analyses indicate an evolution of *glob2/3* duplicates by a combination of sub- and neofunctionalization. Considering their testes-specific restricted expression, an involvement of glob2/3 in the oxidative metabolism of spermatogenesis is conceivable.

Introduction

O_2 supply in insects is mainly accomplished by the highly specialized and effective tracheal system. Respiratory proteins such as the hemo- and myoglobins (Hb, Mb) of vertebrates were long considered dispensible in insects (Brusca and Brusca, 1990; Willmer et al., 2000), except for a few taxa that are specifically adapted to an hypoxic environment (Vinogradov et al., 2005). Among these, larvae of the horse botfly *Gasterophilus intestinalis* and backswimmer of the genus *Anisops* possess intracellular Hbs, which probably carry out Mb-like O_2-storage functions (Keilin and Wang, 1946; Dewilde et al., 1998; Vinogradov et al., 2005; Matthews and Seymour, 2006). Extracellular Hbs for oxygen transport and storage are present in the hemolymph of the hypoxia-tolerant aquatic larvae of chironomid midges (Ewer, 1942; Osmulski and Leyko, 1985).

In recent years, genomic sequence data have provided evidence that *Hb* genes are indeed a standard component of the insect genome (Burmester and Hankeln, 2007). Recent discoveries of *Hb*s in insects include the honeybee *Apis mellifera* (Hankeln et al., 2006), the mosquitoes *Anopheles gambiae* and *Aedes aegypti* (Burmester et al., 2007), and other dipteran, lepidopteran, coleopteran and hymenopteran species (Burmester and Hankeln, 2007; Kawaoka et al., 2009). The insect model organism *Drosophila melanogaster* was initially shown to possess at least one *Hb* gene named *glob1* (Burmester and Hankeln, 1999). *Glob1* is predominantly expressed at substantial amounts in the fat body and the tracheal system of *Drosophila* embryos, larvae and adults. These expression sites, which appear to be conserved features of Hbs in other insect species as well, suggest that glob1 function is associated with O_2 supply and oxidative metabolism (Hankeln et al., 2002; Burmester and Hankeln, 2007). The intracellular glob1 protein binds O_2 at high affinities ($P_{50}(O_2)$ = 0.12-0.15 Torr) and forms a typical globin fold, in which the heme iron atom is hexacoordinated (Hankeln et al., 2002; de Sanctis et al., 2005). The *glob1* gene is downregulated upon experimental hypoxia *in vitro* and *in vivo* (Gorr et al., 2004b; Gleixner et al., 2008), while hyperoxia and intermittent hypoxic regimes trigger a slight transcriptional upregulation. These data indirectly suggest that glob1 might also be instrumental in binding excess O_2 or noxious reactive oxygen species (ROS) in the tracheal system (Gleixner et al., 2008)

In addition to *glob1*, two other globin genes named *glob2* and *glob3* were identified in the *Drosophila* genome (Burmester et al., 2006). These two genes represent related paralogous copies, which in *D. melanogaster* both reside on chromosome 3R about 800 kb apart in head-to-tail orientation. Phylogenetic analyses showed that *glob2/3* are only distantly related to *glob1* and most other insect Hb genes (Burmester et al., 2006). The basal position of *glob2/3* in the insect Hb phylogenetic tree and the monophyly of the two duplicates were further substantiated by their exon-intron pattern (Burmester et al., 2006). Conceptual translation of *D. melanogaster glob2* and g*lob3* produces proteins of 222 and 195 amino acids, respectively, thus exceeding the typical globin length of about 140-150 amino acids due to N- and C-terminal extensions. In the globin fold, however, amino acid residues functionally important for heme and ligand binding (e.g. the PheCD1 and the proximal and distal histidines E7 and F8) are well conserved in glob2/3 proteins. In our initial analysis (Burmester et al., 2006), *glob2* appeared to be expressed at a much lower level than *glob1* as evidenced by only a few corresponding expression sequence tag (EST) entries in databases, while *glob3* lacked EST transcriptional evidence. Towards studying the functional roles of glob2 and 3 we now

conducted a more detailed expression analysis of both genes across *Drosophila* developmental stages, gender and tissues. Additionally, the availability of completely sequenced *Drosophila* genomes representing nine species of the *Sophophora* subgenus and three species of the *Drosophila* subgenus (Clark et al., 2007) allowed us to gain novel insight into the molecular evolution and phylogeny of the *glob2* and *glob3* paralogs, which is relevant for functional interpretations.

Materials and methods

Sequence retrieval, gene phylogeny and molecular evolution

Coding sequences of *Drosophila glob2* and *3* genes were extracted from flybase (http://flybase.org/). Genomic loci were identified by BLASTN search (Altschul et al., 1990), using coding sequences as query. The dataset contained 33 *glob1*, *glob2*, and *glob3* sequences of 9 representatives of the *Sophophora* subgenus and 3 representatives of the *Drosophila* subgenus plus *glob1* of *Gasterophilus intestinalis* and hemoglobin *CTTIII* of *Chironomus thummi thummi* as outgroup representatives. Globin nucleotide sequences were aligned in the amino acid mode using the ClustalX algorithm implemented in BioEdit (Hall 1999). After removal of highly divergent 5' and 3' ends the alignment had a length of 447 bp, comprising the globin domain.

We tested for potential gene conversion between paralogs at the nucleotide level using Geneconv version 1.81 (Sawyer, 1989; Sawyer, 1999), which tests for imbalances in the distribution of segments among homologous DNA sequences. To estimate the rates of amino acid sequence evolution of *Drosophila* glob1, glob2 and glob3, pairwise protein distances were calculated using the program MatGat (http://bitincka.com/ledion/matgat/) and applying the PAM matrix. We followed the accepted divergence estimates of drosophilid taxa as proposed by Tamura et al. (2004).

In order to infer the phylogenetic relations among *glob1*, *glob2* and *glob3* tree reconstructions were carried out at the nucleotide level using a maximum likelihood (ML) approach and Bayesian phylogenetic inference. To minimize the possible influence of saturation on tree reconstruction, only first and second codon positions were used. Applying the Akaike Information Criterion and assuming 4 rate categories, Treefinder (Jobb et al., 2004) identified a special case of the GTR model, called J1[Optimum,Empirical]:G[Optimum]:4 in the Treefinder terminology, as the model of best fit. The J1 model assumes the same rates for i) TA and TG substitutions, and ii) CA and CG substitutions. Except for this constraint, base frequencies, substitution rates and gamma shape parameter were freely estimated from the data. Branch support was estimated by Local Rearrangements of tree support-Expected Likelihood Weights (LR-ELWs) (Strimmer and Rambaut, 2002) on the basis of 10.000 replicates. The Bayesian analysis was performed using MrBayes v3.1.2 (Huelsenbeck et al., 2001; Ronquist and Huelsenbeck, 2003). As the J1 model cannot be specified in MrBayes, we assumed the original GTR model instead. We used 2 independent runs, each with four chains and 1.000.000 generations, discarding the first 200.000 generations as "burnin". Trees were further edited using TreeView (Page, 1996). We further elucidated the phylogeny within a clade uniting all *glob2* and *glob3* sequences. Therefore, parametric bootstrap testing was performed specifying a topology reflecting the '*glob2/3* duplication-in-*Sophophora* subgenus' scenario as alternative hypothesis H1 and a topology reflecting the '*glob2* deletion-in-*Drosophila* subgenus' as null hypothesis (H0). At the ortholog level, both trees followed the

generally accepted phylogeny within the *Drosophila* genus (Clark et al., 2007).

We employed the ML approach implemented in CODEML (PAML v4.4 package) (Nielsen and Yang, 1998; Yang et al., 2000; Yang, 2007) for the detection of possible signatures of positive selection among sites and lineages given an unrooted phylogeny ("intree") using the ratio of non-synonymous to synonymous nucleotide substitution rates ($\omega = d_N/d_S$) as a measure. The 'complete' dataset contained 33 *glob1*, *glob2*, and *glob3* sequences of 9 representatives of the *Sophophora* subgenus and 3 representatives of the *Drosophila* subgenus plus *glob1* of *Gasterophilus intestinalis* and hemoglobin *CTTIII* of *Chironomus thummi thummi* as outgroup representatives. The 'reduced' dataset contained the same sequences as the 'complete' dataset, but without the 12 glob1 sequences. Codon frequencies were estimated from the data using a F3x4 matrix. The intree reflected the '*glob2/3* duplication-in-*Sophophora* subgenus' scenario. To test for the presence of candidate sites of positive selection, we compared the fit of models M7 (beta) and M8 (beta plus ω) per likelihood ratio test (LRTI). M7 and M8 describe the codon distribution in the ω interval (0, 1) as a beta function. However, while M7 confines ω to (0, 1), M8 allows for a positively selected extra site class. To avoid local optima, we ran M8 twice with different initial ω values (0.6 and 1.6). In order to test for lineage specificity of sequence evolution, we additionally carried out LRTII, comparing the fit of the free-ratio model, which allows for independent ω-values among branches, and the one-ratio model, which assumes a constant ω-value for the entire phylogeny. The free-ratio model was run twice in order to assess the stability of the results in the light of the rather short sequence data set (447 bp). For LRTI, twice the log likelihood difference ($2\Delta l$) between the nested models was compared to critical values from a chi-square distribution with degrees of freedom (df) equal to the difference in the number of free parameters between the models, which is 4 – 2 = 2. For LRTII, twice the log likelihood difference ($2\Delta l$) between the nested models was compared to critical values from a chi-square distribution with df 66 (= number of branches minus 1).

Fly stocks

Hypoxia experiments were carried out using *D. melanogaster* wild type strain Oregon R. *In situ* hybridization and developmental stage-specific *glob2* and *glob3* mRNA expression analyses were carried out using *D. melanogaster* wild type strain Oregon R and *D. virilis*. Flies were kept on standard cornmeal agar in cylindrical plastic vials sealed with a foam stopper at 25°C

Hypoxia and hyperoxia experiments

Generally, 25 adult *D. melanogaster* were experimentally exposed to moderate hypoxia (5 % O_2 for 24h), severe hypoxia (1% O_2 for 1h, 3h and 4h) and hyperoxia (95% O_2 for 12h), using a translucent PRO-OX chamber (BioSpherix Ltd., New York, USA). During hypoxia treatments, animals were checked for vitality and the occurrence of phenotypic reactions, known to be caused by the applied O_2 concentrations (see e.g. (Wingrove and O'Farrell, 1999). Flies were kept at 25°C at the pre-adjusted O_2 concentration obtained by mixing nitrogen or oxygen with ambient air. Technical nitrogen and oxygen were obtained from Westfalen AG (Münster, Germany). Gas concentrations were measured and kept constant by an oxygen sensor (E-702, BioSpherix, Ltd., New York, USA). After the desired time, animals were immediately collected and shock-frozen in liquid N_2. Samples were stored at -80°C until use.

RNA preparation and quantitative real-time reverse transcription-PCR (QPCR)

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany), quantified and

checked on integrity. Reverse transcription was carried out with 1 µg total RNA employing the Superscript II RNase H^- reverse transcriptase (Invitrogen, Karlsruhe, Germany). QPCR experiments were carried out on the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Darmstadt, Germany). (for primer sequences see supplemental material). mRNA expression levels were calculated by the standard-curve approach, measuring Ct-values. Factors of differential gene expression in developmental stages were calculated relative to the gene expression in adult males. *D. melanogaster glob2*, *glob3* and *LDH* expression data under different oxygen conditions were normalized relative to expression of the ribosomal protein gene *L17A*, which is unregulated by hypoxia according to microarray experiments (B. Adryan and R. Schuh, Göttingen, personal communication). Factors of differential gene regulation were calculated relative to the normoxic condition (21% O_2). Statistical evaluation was performed by calculating the mean values of the factors of regulation and their standard deviation. Two independent experiments (biological replicates) were performed for each condition, and each QPCR 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.

Preparation of genomic DNA and genomic PCR

Genomic DNA was isolated from 20 L3 larvae according to the protocol from Huang et al. (2009). Genomic PCR was performed using Taq polymerase (Sigma-Aldrich Chemie GmbH, München) according to the manufacturer`s recommendations (for primer sequences see supplemental material).

mRNA in situ hybridization

Probes for mRNA *in situ* hybridization experiments were prepared by *in vitro* transcription from the coding sequence of *D. melanogaster glob2*, *glob3* and *D. virilis glob3* using the DIG RNA Labelling Kit (Roche Applied Science, Mannheim, Germany). As a positive control for testes-specific mRNA expression, *D. melanogaster* β-tubulin 85D was used (Bialojan et al., 1984). It revealed the expected staining of cells in meiosis II (not shown). Negative control did not show stained regions. *In situ* hybridization to whole testes was carried out according to the protocol from Tautz and Pfeifle (1989) with the following changes: testes were dissected in testes buffer (Hennig, 1967; Pisano et al., 1993; Cenci et al., 1994), fixed with 4% formaldehyde/PBS for 30min and washed briefly in PBS + 0.1% (v/v) Tween-20. Tissues were pre-incubated with hybridization solution (50% (v/v) 10x Diethylpyrocarbonate (DEPC)-SSC, 50% (v/v) formamide, 0.1% (v/v) Tween-20) containing 0.1mg/ml salmon sperm carrier DNA at 55°C. Hybridization was carried out at 55°C overnight in hybridization solution containing 0.1mg/ml salmon sperm DNA and 0.5 µg/mL antisense probe. Washing was performed in PBS + 0.1% (v/v) Tween-20 at 65°C. Detection and staining were carried out using an anti-DIG alkaline phosphatase-conjugated antibody and NBT/BCIP solution (Roche Applied Science, Mannheim, Germany) according to the manufacturer`s recommendations.

Bioinformatical promotor prediction

The identification of possible binding-sites of RNA polymerase II was performed with the program Neural Network Promoter Prediction (NNPP) on the Berkeley *Drosophila* Genome Project homepage (http://www.fruitfly.org/seq_tools/promoter.html). The NNPP program recognizes RNA polymerase II promoter via a TATA box and the Initiator transcription start site (Reese et al., 1997; Reese, 2001). Additionally, we used the hidden Markov model-based program McPromoter (http://tools.igsp.duke.edu/generegulation/McPromoter/) (Ohler et al., 2002; Ohler, 2006)

with the genomic sequences of *D. melanogaster glob2*, *glob3,* and *D. simulans* and *D. sechellia glob3* as input.

Results

Genomic organization of glob2 and glob3 paralogs in Drosophila

While *glob3* orthologs are present in all twelve fly genomes studied, we could detect *glob2* orthologs exclusively in the nine *Drosophila* species belonging to the *Sophophora* subgenus (Fig. 1). The 5' genetic neighbourhood of *glob3* varies across the investigated *Drosophila* species. In contrast, the 3' environment of *glob3* is conserved throughout the taxon sample. In all twelve fly genomes studied, however, is located on the Muller element E, which is equivalent to chromosome 3R in *D. melanogaster*. Chromosomal location and synteny relationships are more complex in *glob2*: *D. ananassae* and *D. pseudoobscura/persimilis* harbor *glob2* and *glob3* as tandem duplicates in head-to-tail orientation with less than 400 bp space between the gene copies. This *glob2-glob3* tandem resides on a chromosome arm, which is equivalent to arm 3R in *D. melanogaster* (Fig. 1). In *D. willistoni*, which branches off even closer to the base of the *Sophophora* subgenus, the *glob2* copy is located on arm 2R corresponding to 2L in *D. melanogaster* (Fig. 1). Finally, *glob2* and *glob3* are linked on arm 3R, but separated by about 800 kb in the representatives of the *D. melanogaster* subgroup.

Reconstruction of a globin phylogenetic tree

Pairwise sequence comparisons of nucleotide sequences using Geneconv did not reveal hints for complete or partial gene conversion between paralogs. Subsequent analyses should thus not be impaired by concerted sequence evolution of paralogs within species. ML based tree reconstruction revealed a monophyletic origin of *glob2* and *glob3* sequences of the genus *Drosophila* (LR-ELW 99.17) under exclusion of *glob1* sequences (Suppl. Fig. 1A). Within this clade, *Sophophora glob2* and *glob3* each constitute a monophlyum with moderate support (LR-ELW 82.55) under exclusion of *glob3* sequences from the *Drosophila* subgenus which appear paraphyletic. Among the representatives of the *Sophophora* subgenus, the observed phylogeny widely corresponds to the accepted species phylogeny introduced by Clark et al. (2007). Bayesian inference essentially confirmed the ML based phylogeny, though support for a monophyletic *Sophophora glob2/3* clade was low and *Drosophila* (subgenus) *glob2* appeared as monophyletic (Suppl. Fig. 1B).

Branch lengths estimated by ML and Bayesian inference consistently illustrate that *glob1* accumulated less substitutions than did *glob2/3*, following gene duplication. Within the glob2/3 clade, *Sophophora glob2* appears to be more derived than *Sophophora glob3*, compared to the hypothetical ancestor of both gene copies. An acceleration of sequence evolution of glob2/3 versus glob1 and, slightly so of glob3 versus glob2 is also seen when rates of amino acid sequence change are calculated using a PAM substitution matrix assuming the *Drosophila* divergence times suggested by Tamura et al (2004). The evolutionary rates measured in replacements per site per year for glob2 and glob3 are $2.8*10^{-9}$ and $3.0*10^{-9}$. Glob1 exhibits a clearly slower evolution rate with about $9.5*10^{-10}$ replacements per site per year.

Parametric bootstrap testing as implemented in Treefinder identified the '*glob2/3* duplication-in-*Spohophora* subgenus' topology as the significantly better fit ($p = 0.96$) of the data, compared to a tree topology reflecting the '*glob2* deletion-in-*Drosophila* subgenus' scenario. Due to consistent results from unconstraint tree reconstruction and hypotheses testing, we took the topology reflecting the '*glob2/3* duplication - in -

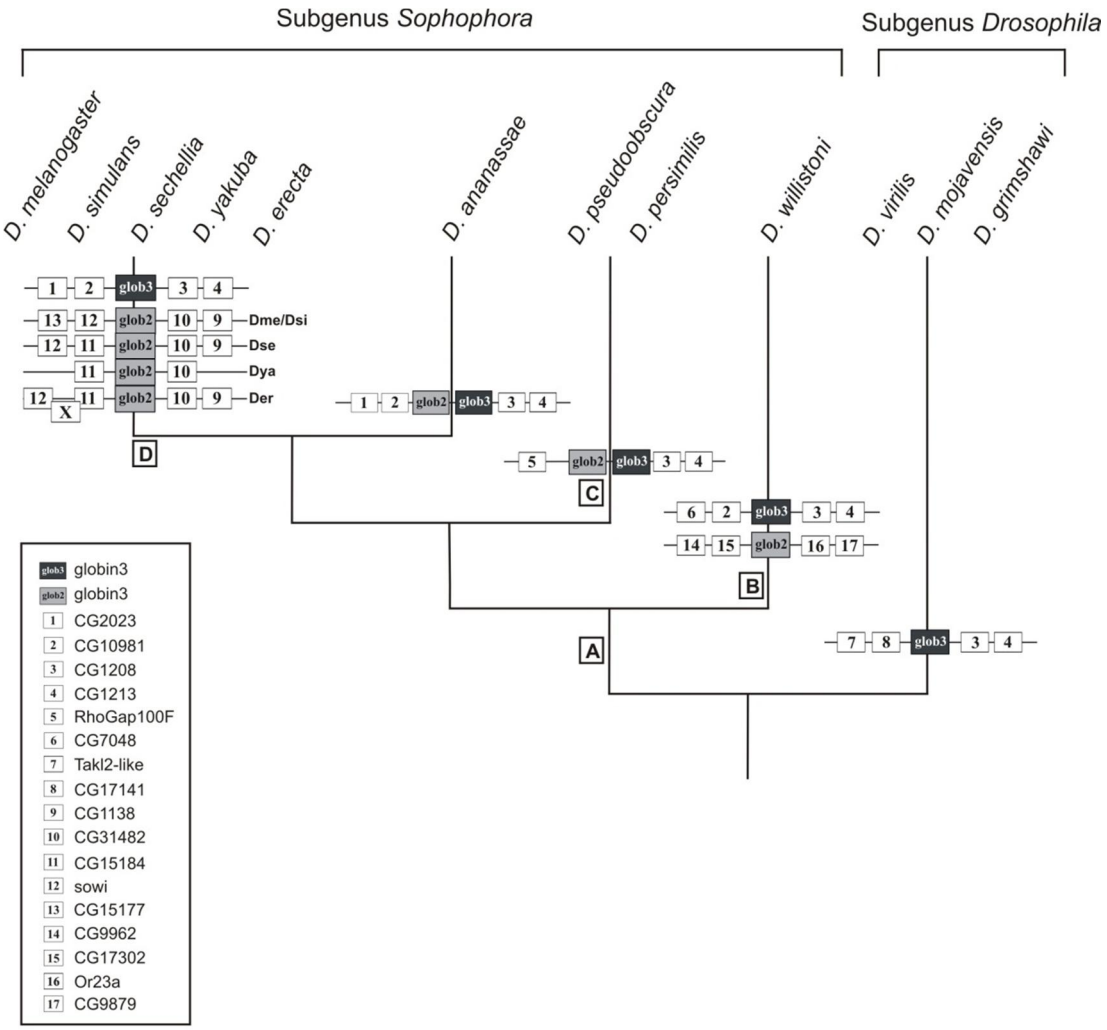

Figure 1: Hypothetical scenario of evolutionary events leading to globin 2 and globin 3 genes in drosophilids. The chromosomal localization and organisation of *glob2* and *glob3* genes and flanking regions in different subgroups of *Drosophila* phylogeny are displayed. [A] tandem duplication of *glob3* ancestor; [B] separation of glob2 to Chr 2R (= *Dme* Chr 2L); [C] tandem *glob2/3*, but chromosome breakage distal to *glob2/3*; [D] transposition of *glob2* to 3R 83F4, 800kb apart from *glob*3.

Spohophora subgenus' scenario as an intree for subsequent analyses of sequence evolution.

Molecular evolution

The LRTI analysis did not support the presence of positively selected sites across the analyzed *glob1/2/3* dataset, neither for the complete 35 species sample nor for the reduced 23 species sample ($p > 0.05$). The prevalence of negative purifying selection was reflected by average d_N/d_S estimates of 0.123 (beta null M7) and 0.111 (one ratio M0) for the entire phylogeny. LRTII (log likelihood values: one-ratio model = - 8067.330 / free-ratio model = - 8004.725) however provided highly significant support for branch specificity of sequence evolution ($p = 0.000$; df = 66, $2\Delta l = 125.21$). It is important to note that the free-ratio model produced stable results in two runs starting with different initial omega values (0.6 and 1.6). Differences were confined to decimal places. The free-ratio model suggests elevated omega values indicating positive selection ($d_N/d_S > 1$) for a total of ten out of 66 branches (Suppl. Fig. 2). The two most basal branches with evidence of positive selection within the *glob2/3* clade were the stem lineages of the *Sophophora glob2/3* (36 non-synonymous / zero synonymous substitutions) and *Sophophora glob3* (24 non-synonymous / zero synonymous substitutions) clades. The high

significance of LRTII might partly be due to saturation artefacts. However, all ten branches with signatures of positive selection were characterized by M0 branch length estimates < 1 (Suppl. Fig. 3), making it unlikely that these branches were strongly affected by multiple substitutions leading to saturation. Rather, saturation has underestimated the number of exchanges along a total of four branches with M0 branch length estimates > 3, i.e. the branches to outgroup representatives *G. intestinalis glob1* and *C. thummi thummi CTTIII* and the stem lineages of the *Drosophila* (genus) *glob2/3* and Sophophora *glob2* clade. Branch-specificity of sequence evolution is thus a likely characteristic of *Drosophila* globin evolution, as is positive selection at both, the ortholog and paralog level.

Quantification of Drosophila glob2 and glob3 mRNA expression

In our earlier study, six expressed sequence tag (EST) entries in the database of *D. melanogaster* revealed that *glob2* is actually expressed, albeit at a rather low level (Burmester et al., 2006). Seven additional *D. melanogaster* ESTs can now be reported (EC252960, EL878213, EL878330, EL878331, EL878455, EC067562, EC061683), most of which derive from adult flies. ESTs corresponding to *glob2* are also present for *D. simulans*, *D. sechellia* and *D. yakuba* adults, and eight *D. simulans* ESTs derive from 3rd instar larvae.

Because of the absence of *glob3* EST entries for *D. melanogaster* and due to our initial failure to recover cDNA from larval or adult RNA by RT-PCR we (erroneously) assumed that *glob3* is not expressed (Burmester et al., 2006). Since 2007, however, there are three *glob3* ESTs reported for adult *D. melanogaster* (EL876979, EL877168, EL877169). In addition, we could identify three ESTs from *D. simulans* (2 adult, one 3rd instar larvae), three ESTs from adult *D. sechellia* and one EST from adult *D. yakuba*. Based on these findings, we systematically re-analyzed *glob2* and *glob3* mRNA expression by quantitative real-time RT-PCR in embryos, male and female larvae, pupae and adult males/females of *D. melanogaster* and the distantly related *D. virilis*.

In embryos, the mRNA expression of *D. melanogaster glob2* was minimal (not shown). *Dmeglob2* turned out to be maximally expressed in male adult flies (Fig. 2A). Expression levels in male larvae and pupae were at 43 % and 78 % relative to male adults. In female larvae, pupae and adults *dmeglob2* expression was always beyond the detection limit. The developmental expression pattern of *dmeglob3* showed a high similarity to *dmeglob2*. *Dmeglob3* mRNA expression in embryos (not shown) as well as in female larvae, pupae and adult flies was not detectable. The amounts of *dmeglob3* mRNA in male larvae and pupae were about 16 % and 50 % of the maximal mRNA expression level obtained in male adult flies (Fig. 2B). The developmental expression pattern of the single-copy *glob3* gene in *D. virilis* was generally matching those of the *D. melanogaster* paralogs. In embryos, no expression of *dviglob3* expression could be measured (not shown), while adult males showed maximum expression (Fig. 2C). Male and female larvae expressed 5 %, male pupae 38 %, and female pupae and adults 2 % and 6 % of the male adult level (Fig. 2C).

We also compared absolute mRNA copy numbers and observed that *D. melanogaster glob2* and *glob3* expression in male adults was about the same level, with 181,675 copies and 150,026 copies of mRNA, respectively. The amount of *D. virilis glob3* mRNA (311,673 mRNA copies) in male adult flies was approximately equivalent to the sum of the *D. melanogaster glob2* and *glob3* mRNA copy numbers (Suppl. Fig. 4A-C).

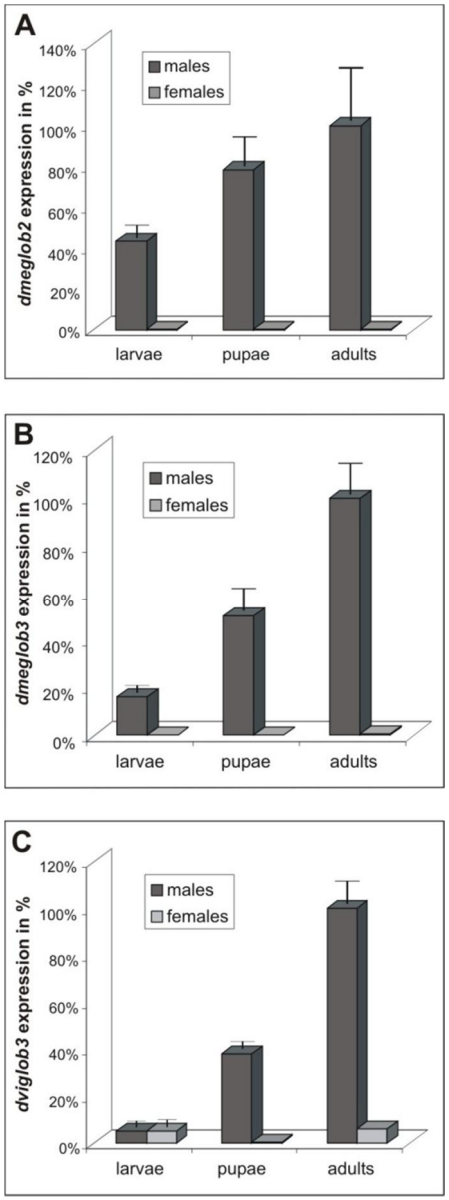

Figure 2: Quantification of mRNA expression of *glob2* in *D. melanogaster* and *glob3* in *D. melanogaster* and *D. virilis* in sexed 3rd instar larvae, pupae and adults. mRNA levels (bars) are shown relative to glob-expression in adult males. (A) *dmeglob2* expression. (B) *dmeglob3* expression. (C) *dviglob3* expression.

During the analyses of the genomic region of *D. melanogaster glob3*, we identified a non-LTR-retrotransposable element of the Jockey family 234 bp upstream of the coding sequence of *dmeglob3*. The TE has a length of about 3.1 kb, its 5´end is truncated, it is located on the minus-strand, its 3´end exhibits an A-rich stretch and it is specific for *D. melanogaster*. By applying genomic PCR on different wild type strains of *D. melanogaster* (Oregon R, Canton S, Berlin and Acharren), we could confirm the fixation of the transposon in these wild-type strains. By comparison with genomic data from *D. sechellia*, we found that the retrotransposon ends 142 bp upstream of the 5´UTR of *dmeglob3* (Suppl. Fig. 5). To find out, if the retrotransposon interferes with the promoter of *dmeglob3*, we predicted possible transcription start sites applying the hidden Markov model-based program McPromoter (Ohler et al., 2002; Ohler, 2006) and the time-delayed neuron network based program NNPP (Reese et al., 1997; Reese, 2001). Only the latter identified a candidate Pol II promoter for *D. simulans glob3* and *D. sechellia glob3* (but not for *D. melanogaster* g*lob2* and *glob3)*, which is located 216 bp upstream of the translation start-site and 124 bp upstream of the 5´UTR (of *dmeglob3*) spanning the corresponding integration site of the TE in *D. melanogaster* (Suppl. Fig. 5).

Tissue expression patterns of glob2 and glob3

The expression patterns of *glob2* and *glob3* were examined in head, thorax and abdomen of *D. melanogaster* adult flies by mRNA *in situ* hybridization. In head and thorax, no staining could be observed for both globin genes (data not shown). In the abdomen, the only tissues stained were the testes from male flies, as already expected from the male expression levels in QPCR. The hybridization experiments were repeated, using dissected testes of *D. melanogaster* and *D. virilis*. Staining signals indicated a *glob2* expression in several regions of the *D. melanogaster* adult testes, corresponding to several stages of spermatogenesis. Hybridization signals could be observed for primary spermatocytes, meiotic spermatocytes, round spermatids and early differentiating spermatids (Fig. 3A). No staining was obtained in stem cells, mitotic cells, late elongating spermatids and mature sperm. *Glob3* in *D. melanogaster* exhibited a very similar expression pattern, although with a slightly weaker intensity of staining (Fig. 3B).

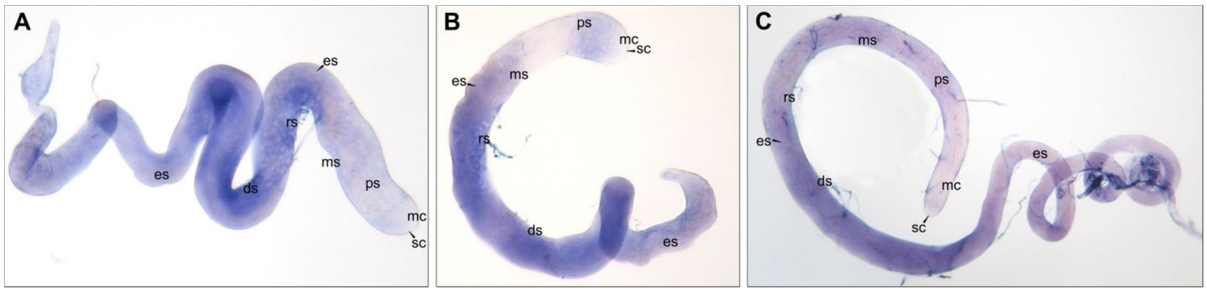

Figure 3: mRNA *in situ* hybridization of *Drosophila* adult testes with *glob2* and *glob3* antisense RNA-probes. Arrows indicate stained regions. (A) *dmeglob2* expression. (B) *dmeglob3* expression. (C) *dviglob3* expression. All three globins analysed show expression in primary spermatocytes, meiotic spermatocytes, round spermatids and early differentiating spermatids. (sc) stem cells; (mc) mitotic cells; (ps) primary spermatocytes; (ms) meiotic spermatocytes; (rs) round spermatids; (ds) early differentiating spermatids; (es) late elongating spermatids.

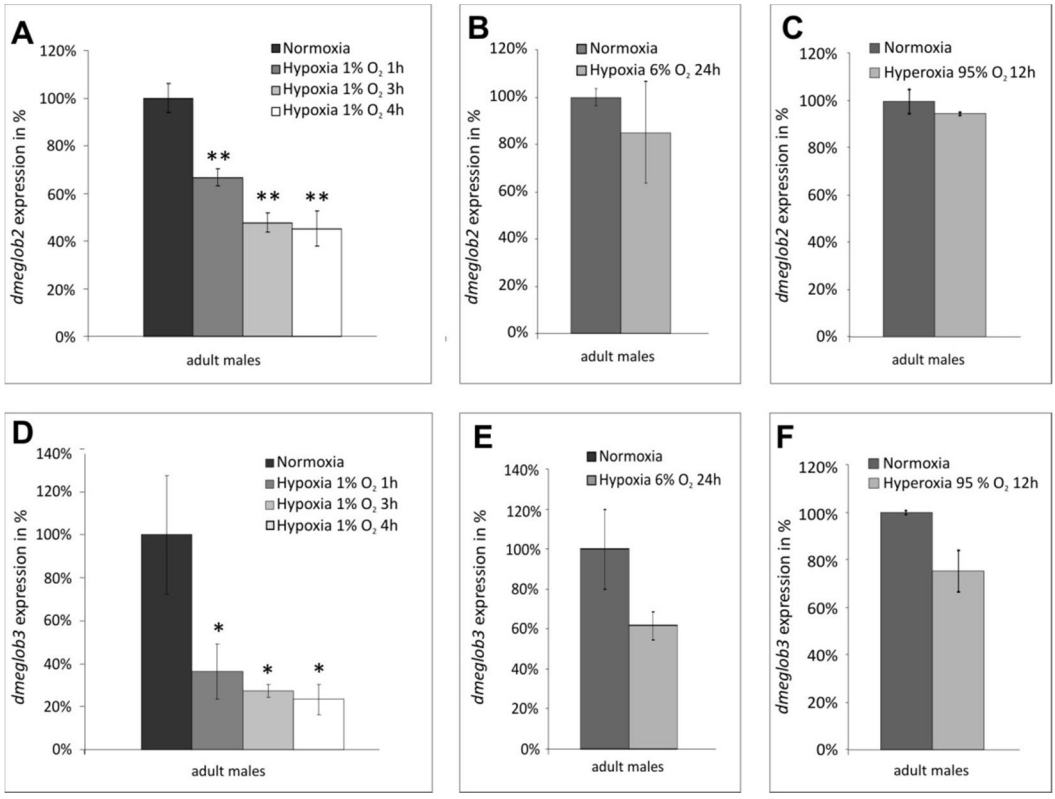

Figure 4: Regulation of *glob2* and *glob3* mRNA in *D. melanogaster* male adults after hypoxic and hyperoxic stress. mRNA levels (bars) are shown relative to the gene expression at normoxia (21%). (A) *dmeglob2* expression after 1 % O_2 for 1h, 3h and 4h. (B) *dmeglob2* expression after 6 % O_2 for 24h. (C) *dmeglob3* expression after 95% O_2 for 12h. (D) *Dmeglob3* expression after 1 % O_2 for 1h , 3h and 4h. (E) *dmeglob3* expression after 6 % O_2 for 24h. (F) *dmeglob3* expression after 95% O_2 for 12h (*p < 0.05; **p < 0.01).

In *D. virilis*, in situ hybridization with a *glob3* antisense probe also resulted in very similar signals in primary spermatocytes, meiotic spermatocytes, round spermatids and early differentiating spermatids (Fig. 3C).

Regulation of dmeglob2 and dmeglob3 expression under hypoxia and hyperoxia

Glob2 and *glob3* mRNA levels were measured by QPCR in normoxic (21% O_2) and experimentally hypoxic as well as hyperoxic male adult *D. melanogaster*. *Lactate-dehydrogenase* (*Ldh*), analysed as a positive control for hypoxia up-regulation via the HIF-1

pathway (Douglas et al., 2005), showed the expected increase in mRNA expression (Suppl. Fig. 6 A+B).

A short-term severe hypoxia regime (1% O_2 for 1 h, 3 h and 4 h) resulted in a significant, gradual decrease of *dmeglob2* mRNA levels to about 45% of the normoxic control (Fig. 4A). Long-term moderate hypoxia (6% O_2 for 24 h) triggered only a slight down-regulation of *dmeglob2* mRNA expression (Fig. 4B). *Dmeglob3* mRNA expression was even more reduced (to 23 % after 1% O_2 for 3 h; Fig 4D). After long-term hypoxia (6% O_2 for 24h), *dmeglob3* mRNA level decreased to about 60% compared to the normoxic control (Fig. 4E). After hyperoxic treatment, now significant changes mRNA levels of both, *dmeglob2* and *3* were detected (Fig. 4C+F). A bioinformatical search for hypoxia-responsive elements (Camenisch et al., 2001) in the twelve genomes, conducted by the rVISTA program (Loots et al., 2002); http://rvista.dcode.org/), did not yield evidence for interspecifically conserved HRE motifs.

Discussion

Globin expression in Drosophila testes: a novel or traditional function?

Since the identification of a globin gene (*glob1*) in *D. melanogaster* (Burmester and Hankeln, 1999) it has become clear that globins belong to the standard repertoire of most, if not all insects, including those living in normoxic environment (Burmester and Hankeln, 2007). A conserved common feature of the insect globins studied so far in more detail was their predominant expression in cells of the tracheal system and in the fat body (Hankeln et al., 2002; Burmester et al., 2006; Burmester and Hankeln, 2007). Here we convincingly show that the two additional globin genes in the *D. melanogaster* genome, *glob2* and *glob3* (Burmester et al., 2006), and also their single orthologous counterpart *glob3* in *D. virilis* display an exceptional expression pattern, being almost exclusively transcribed in the adult male reproductive tract during several phases of spermatogenesis. Our QPCR and mRNA *in situ* hybridization results are in agreement with a recent microarray study by Vibranovski et al. (2009) confirming the male-specific expression of *dmeglob2/3*. In fact, this study suggests that *dmeglob3* expression is stronger in post-meiotic compared to meiotic cell populations, whereas *dmeglob2* exhibits a signal distribution characteristic for genes transcribed during meiosis.

Although globins are traditionally known as O_2 supply proteins of the respiratory system, the expression of globins in the male reproductive system is not an entirely new fact. The vertebrate neuroglobin, for example, which is well recognized for its cell-protective role in nerve cells, is also expressed for yet unknown reasons in spermatogonia and primary spermatocytes of mouse testes (Reuss et al., 2002). Very recently, we reported the identification of a highly specialized chimeric protein of yet unknown function, in which a globin domain is fused to a protease-like domain (Hoogewijs et al., submitted). This chimeric globin was named androglobin due to its predominant expression in testes tissue. While this new member of the globin family is evolutionary ancient and extremely conserved, being present in vertebrates, other chordates, lophotrochozoa, ecdysozoa, more basal animal phyla and even choanoflagellates, the available genomes of *Drosophila* do not contain an androglobin ortholog (Hoogewijs et al., submitted). It is therefore tempting to speculate that flies have evolved the *glob2/3* genes to compensate somehow for a loss of their androglobin lineage. Since glob2/3 are single-domain globins without a fused protease part, they would under this scenario have to interact via their N- and C-terminal extensions (Burmester

et al., 2006) with other partner proteins to mimic the role of androglobin.

After all, with the findings for neuroglobin, androglobin and *Drosophila* glob2/3, the expression of globins in the male reproductive system and, more specifically, in the process of spermatogenesis appears to reflect an important aspect of globin function. Over the last decade, the traditional globin functions in O_2 supply and storage (Wittenberg, 1970; Dickerson and Geis, 1983) have been complemented by other, equally important physiological roles, e.g. in the detoxification of harmful reactive oxygen species (ROS; Merx et al., 2001; Flogel et al., 2004; Weber and Fago, 2004), in the scavenging and/or production of the bio-active gas nitric oxide (NO; Flogel et al., 2001; Gladwin et al., 2004), in redox-mediated cell signaling and apoptosis regulation (Fago et al., 2006) and in lipid metabolism (Sriram et al., 2008; Reeder et al., 2011). At current, we have only indirect data to distinguish between these possibilities for glob2/3. Thus, we consider a conventional role of glob2/3 in O_2 supply in testes rather unlikely in light of the observed transcriptional down-regulation of both genes after experimental hypoxia, while traditional hemoglobins like those in the midge *Chironomus* or the crustacean *Daphnia magna* increase expression under hypoxic conditions (Osmulski and Leyko, 1985; Zeis et al., 2003; Gorr et al., 2004a). Alternatively, *Drosophila* glob2/3 may be instrumental in alleviating oxidative stress in the male reproductive tract, which is routinely exposed to reactive oxygen species formed as by-products during oxidative metabolism. Spermatozoa are sensitive to oxygen-induced damage mediated by lipid peroxidation (Jones et al., 1979; Sanocka and Kurpisz, 2004). This sensitivity is enforced by the shortage of antioxidants in the spermatozoa (Aitken et al., 1996; Aitken and Baker, 2004). The importance of an effective antioxidants system for correct spermatogenesis in *Drosophila* has been shown e.g. in male flies with a null mutation in the gene for Cu-Zn superoxide dismutase, which leads to a reduced fertility among other phenotypic abnormalities (Phillips et al., 1989; Parkes et al., 1998). While exposure of flies to oxidative stress was accompanied by an upregulation of antioxidant genes (Landis et al., 2004), we could not detect such a response for *dmeglob2/3* after experimental hyperoxia. Thus, currently no correlative evidence for an involvement of glob2/3 in ROS detoxification exists. Also, biophysical data on the ligand binding characteristics of the two proteins are lacking, because recombinant expression has not successfully been performed up to now. The primary sequences of both globins, however, have retained during evolution all key residues necessary for heme- and gas ligand-binding (Burmester et al., 2006). In light of the delicate oxygen homeostasis, which is necessary in the testes to produce undamaged sperm at 'high-throughput', we therefore envisage glob2/3's role to lie in some pathway of oxygen-dependent sensing and signalling.

Molecular evolution of the Drosophila globin gene family: a complex scenario of neo- and subfunctionalization?

The highly specialized expression pattern of glob2/3 has to be interpreted on the background of the evolutionary history of the *Drosophila* globin gene family. With the notable exception of the very large globin gene family of chironomids, which provides the midge larvae with huge amounts of extracellular globins for survival in hypoxic aquatic habitats, all other insect taxa studied so far on the genome sequence level have only one (*Bombyx mori*), two (*Apis mellifera, Aedes aegypti*, *Anopheles gambiae*) or three (*Drosophila* spec.) globin genes (Burmester and Hankeln, 2007; Kawaoka et al., 2009). Molecular phylogenetic reconstructions based on globin amino acid sequence data have

shown that *Drosophila* glob1 forms a clade with the globins of chironomids and the horse botfly *G intestinalis* (Burmester et al., 2006). Glob1 is thus representative for class of presumably O_2-supplying globins from brachyceran and nematoceran dipterans. In contrast, glob2/3 appeared at a basal position in the globin phylogenetic tree, with no clear affiliations to other insect globins (Burmester et al., 2006). Evolutionary rate calculations (Burmester et al., 2006; this paper) show that glob2/3 have evolved about 3fold faster than glob1, so that the ancestral tree position might result from a long-branch attraction artefact. Instead, the absence of *glob2/3* orthologs in other insect genome sequences (our unpublished observations) strongly suggests that this testes-specific globin-type has evolved secondarily in the ancestor of the *Drosophila* genus. This derived evolutionary origin of glob2/3 would be in agreement with the idea that it may have evolved to compensate for the taxon-specific loss of the testes-expressed androglobin gene lineage.

Gene synteny data enabled us to reconstruct the history of *glob2* and *3* within the genus. We identified *glob3* orthologs in all 12 sequenced *Drosophila* genomes. *Glob2* orthologs were found exclusively in the nine species, which belong to the *Sophophora* subgenus. Given the widely accepted phylogenetic relations among the twelve species (Clark et al., 2007), this distribution suggests the duplication of a *glob2/3* ancestor in the stem lineage of the *Sophophora* subgenus and thus before the radiation of this clade at least 40 million years ago (Tamura et al., 2004). This *'glob2/3* duplication-in-*Sophophora* subgenus' scenario is more parsimonious than the alternative '*glob2* deletion-in-*Drosophila* subgenus' hypothesis, which implies the duplication of *glob2/3* in the ancestor of the *Drosophila* genus and the subsequent loss of the *glob2* paralog in the stem lineage of the *Drosophila* subgenus. Our tree reconstructions and hypotheses testing are fully in line with the more parsimonious interpretation of the *glob2/3* history in the sampled species. The finding of introns in all gene copies suggests that the duplication of the *glob2/3* ancestor resulted from unequal crossing, rather than retroposition. Probably, the duplication event in the *Sophophora* ancestor initially resulted in a head-to-tail orientation of *glob2* and *glob3* on a chromosome arm, which is equivalent to arm 3R in *D. melanogaster* (event A, Fig. 1). This situation is today conserved in *D. ananassae*, *D. pseudoobscura* and *D. persimilis*. In *D. willistoni* the *glob2* ortholog has most probably been transposed onto chromosomal arm 2R, equivalent to 2L in *D. melanogaster* (event B, Fig.1). This corresponds to an increased activity of transposable elements in the *D. willistoni* (Clark et al., 2007). Likewise, the separation of *glob2* and *glob3* by about 800 kb in the representatives of the *D. melanogaster* subgroup probably has been caused by a subsequent transposition of the *glob2* paralog (event D, Fig. 1).

The evolutionary fate of gene duplicates and the consequences of gene duplication for the evolution of novel adaptive traits are matters of intense research and debate (Lynch and Conery, 2000). Early studies (e.g. Force et al., 1999) have contrasted the alternative models of neofunctionalization (i.e. one gene copy stays conserved, the other evolves a novel function) and subfunctionalization (i.e. both gene copies loose part of their functions and have to complement each other). While the real evolutionary scenarios might be more complex than anticipated before (He and Zhang, 2005), recent sequence data from *Drosophila* suggest that unexpectedly large fractions of the fly genome are under purifying or even adaptive, positive selection (Sella et al., 2009). Regarding the *glob2/3* gene pair as a possible model case for connecting molecular evolution to the phenotype, we

applied codon-based maximum likelihood analyses of sequence evolution, calculating ratios of non-synonymous to synonymous nucleotide substitutions (d_N/d_S), at the codon- and branch-level to infer selective regimes acting on the globin gene duplicates. The *'glob2/3* duplication-in-*Sophophora* subgenus' hypothesis, supported by both, parsimony criteria and hypothesis testing, served as the basis for these analyses. While support for site-specificity of positive selection was not significant, the alternative free-ratio model turned out to be a significantly better fit of the sequence data than the one-ratio model. Branch-specific estimates of d_N/d_S >1, suggestive of episodes of positive selection and hence adaptive evolution, were estimated for 5 of the altogether 66 branches of the combined ortholog/paralog tree (Suppl. Fig. 3). From a theoretical point of view it is interesting that positive selection was indicated for the branch uniting *Sophophora glob3* orthologs, while the algorithm inferred negative selection for the stem lineage of the *Sophophora glob2* clade. Such a pattern is not in line with the duplication > degeneration > complementation or subfunctionalization models (Jensen, 1976; Orgel, 1977; Hughes, 1994; Force et al., 1999), nor with the recently introduced model of subneofunctionalization (He and Zhang, 2005), which both assume similar evolutionary fates of the two gene copies, either as a consequence of relaxed functional constraint (Force et al., 1999) or by a succession of relaxed functional constraint and positive selection along both lineages (He and Zhang, 2005). The observation of negative and positive selection along the stem lineages of *Sophophora glob2* and *Sophophora glob3*, respectively, reminds more of the classical concept of neofunctionalization *sensu* (Ohta, 1988), assuming the conservation of one gene copy by negative selection, while the paralog evolves a new function under the influence of positive selection. However, taking branch length estimates under assumption of the one-ratio model (M0) into account, the present situation deviates as well from the simple expectations under neofunctionalization. Thus, negative selection is indicated for the longer *Sophophora glob2* stem lineage, while positive selection is estimated for the comparatively short *Sophophora glob3* stem lineage. Positive selection in the *Sophophora glob3* stem lineage is thus not accompanied by a general acceleration of sequence evolution, compared to its paralog's stem lineage, as might have been expected. The data rather reflect that the ancestral *Sophophora glob3* was more conserved than its paralog following gene duplication. At the same time, however, the non-synonymous exchanges in the *Sophophora glob3* ancestral sequence occurred under the influence of positive selection, while (most of) the more abundant non-synonymous exchanges in the faster evolving *Sophophora glob2* ancestor did not. Such a scenario could also explain why *Drosophila glob3* sequences are more similar to *Sophophora glob3* than to *Sophophora glob2* sequences, and why unconstraint tree reconstructions sometimes group *Drosophila glob3* and *Sophophora glob3* sequences monophyletically under exclusion of *Sophophora glob2* sequences.

It is well-known that genes encoding proteins involved in sex and reproduction are often subjected to a wide array of selective forces like sexual conflict and sperm competition (Herlyn and Zischler, 2007; Nadeau et al., 2007) and actually show signatures of positive selection and adaptive evolution (Civetta and Singh, 1995; Singh and Kulathinal, 2000; Civetta, 2003; Clark et al., 2006; Clark et al., 2007; Zhang et al., 2007). For *glob2* and *3*, negative selection apparently prevails across the investigated paralog and ortholog phylogeny. However, branch-specific analyses reveal that the investigated globins might as well have undergone phases of adaptive

evolution as indicated by d_N/d_S values >1, although, currently, the potential adaptive values of amino acid exchanges along the ten branches with signatures of positive selection must remain unknown. On the other hand, the *glob2* and *3* genes in the *Sophophora* species appear to complement each other in terms of quantitative and regional RNA expression patterns, which is consistent with a sub-functionalization regime. A complex mixture of changing selective constraints over evolutionary time may therefore be a realistic alternative to simple models of gene duplicate evolution.

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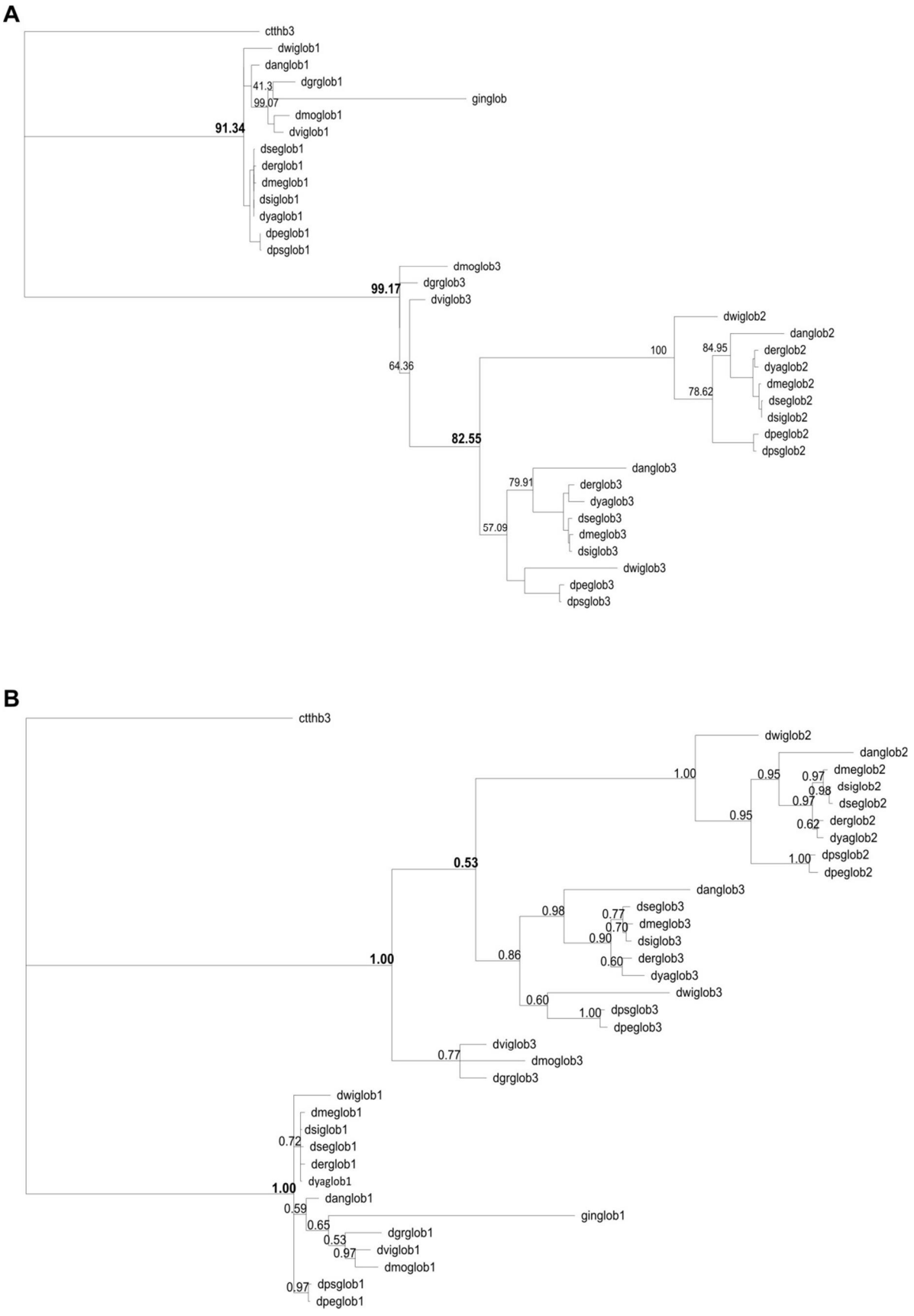
Supplementary Material

Oligonucleotides and PCR conditions

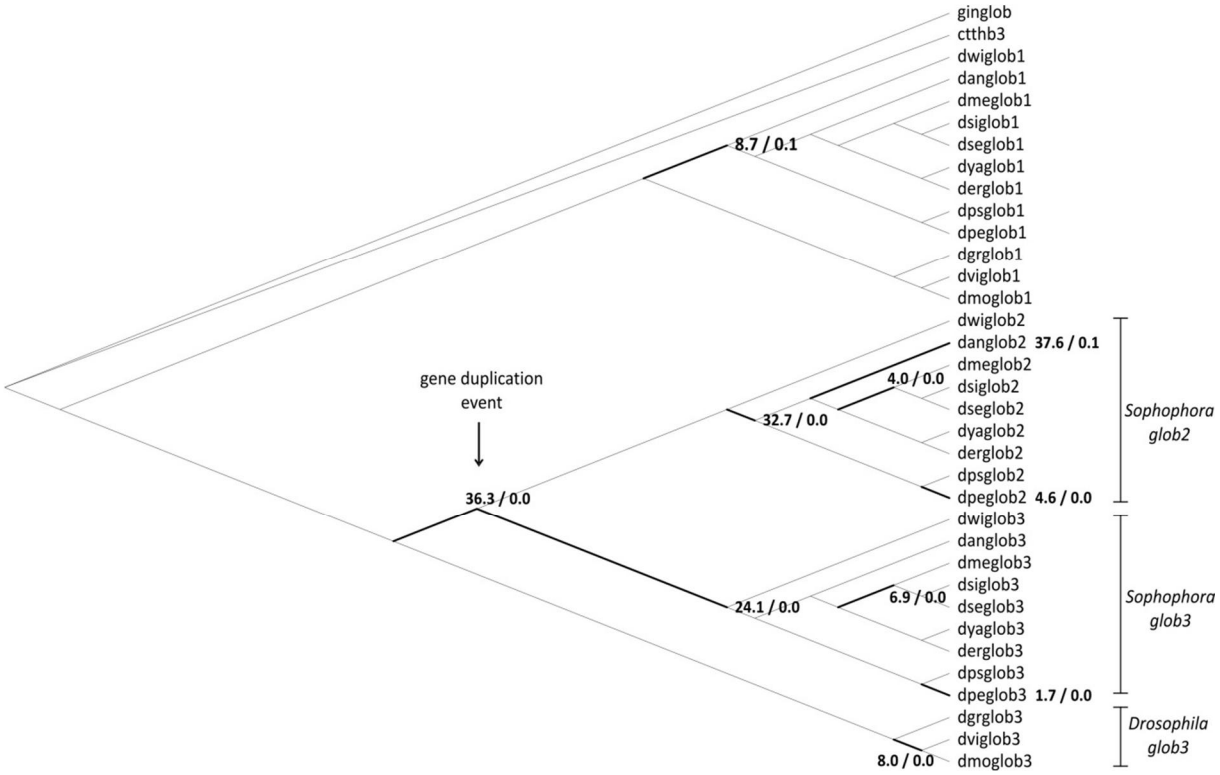
For QPCR experiments we used the following oligonucleotide primer combinations: *dmeglob2* 5´- ata tga gga tgc tgg cc acc -3´ and 5´- cgt caa cag tcg tcc taa gg -3´; *dmeglob3* 5´- gtt aat att ggg cat ctg gcc -3´ and 5´- gta ttc gac tgc tgg tcc tg -3´; *dviglob3* 5´- gag cat atg agg atg ttg atg aac -3´ and 5´- gcg aag tct gag caa aga tag c -3´; *dmeL17A* 5´- taa cca gtc cgc gag cag c -3´ and 5´- gtc atg cct gcc gtg gtt att -3´; *dmeLDH*, 5'- cta aca gat cca ttc gca aca cc -3' and 5'- act tga tgc tac gat tcg tgg -3'. The gene for ribosomal protein *dmeL17A* was used as a reference for normalization, the gene for lactate dehydrogenase *dmeLDH* served as a positive control for hypoxia-induced changes in gene expression. After activation of the polymerase at 95°C for 15 min, amplification was performed in a three-step protocol: 94°C for 15 sec, 60°C for 30 sec, 72°C for 30 sec, measuring the fluorescence during the last step of each cycle. All PCR experiments were followed by dissociation curves at a temperature range from 60°C to 95°C to analyse the specificity of the amplification reactions. No unspecific products or primer dimers were detected by melting curve analysis and gel electrophoresis of PCR amplificates. For detection of the Jockey transposable element the following oligonucleotide primer were used: *dmeglob3* Jockey 5´-ttg acc ttc cgt tgg tgt ttg-3´ and 5´-ttg ggt agg taa gac ttg aca ag-3´.

Quantification of lactate dehydrogenase expression as positive control for hypoxia

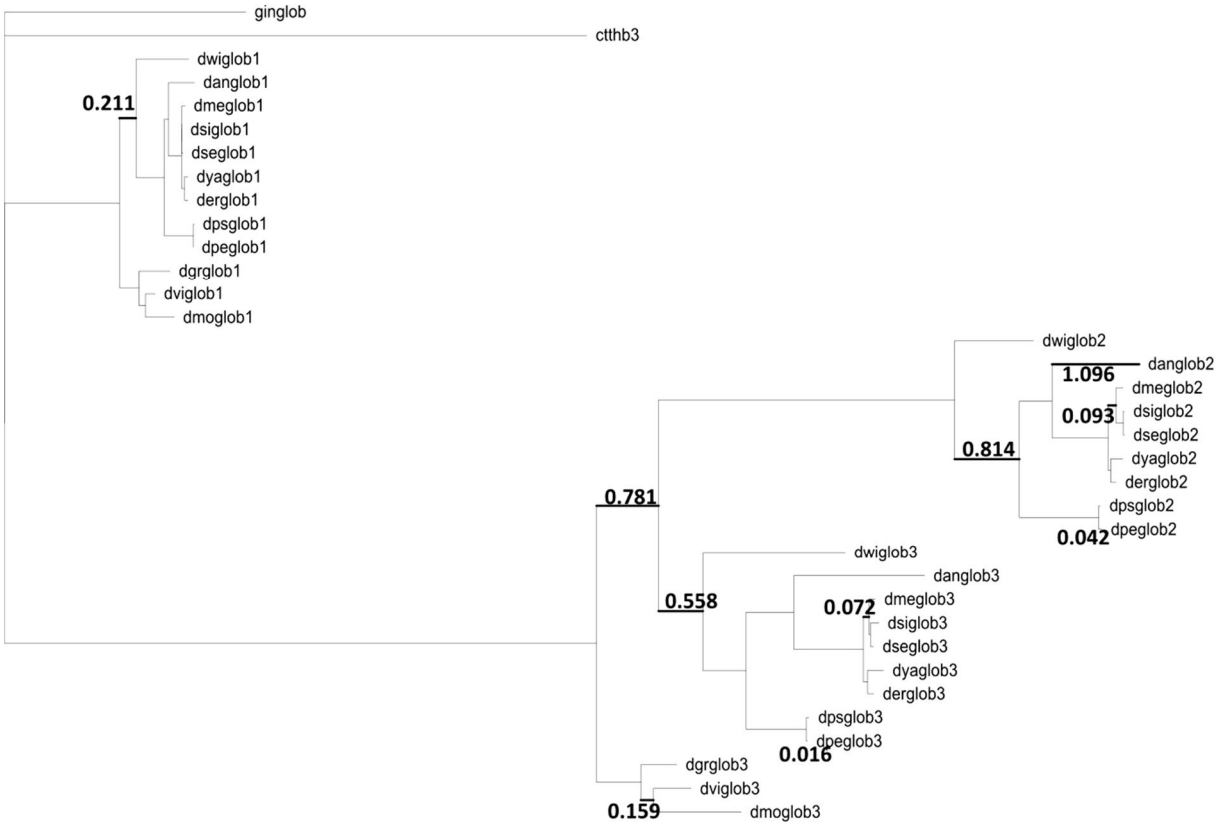
To confirm the observed changes in *dmeglob2 and dmeglob3* expression levels under hypoxia, we used *LDH* as a positive control for hypoxia-induced changes in gene expression. *LDH* expression in *Drosophila* cell culture is up-regulated eightfold under O_2 deprivation (1% O_2) via the hypoxia-inducible factor 1 (HIF-1) pathway (ZITAT). Severe, short-term hypoxia (1% O_2 for 1 h, 3 h and 4 h) was applied to adult male flies. After 1 h of hypoxia, no alteration in *LDH* expression could be detected. 3 h of hypoxia caused the *LDH* mRNA levels to increase about 2,2 fold and 4 h of hypoxia to about 3,4 fold compared to the normoxic control (Fig 3E). After applying long-term moderate hypoxia with 6 % O_2 for 24 h, an increase in *LDH* mRNA expression to about 1,5 fold could be detected (Fig 3F).



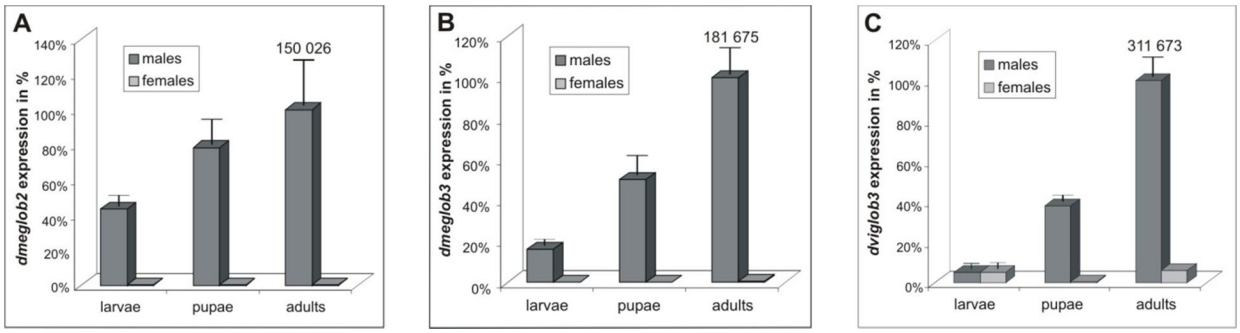
Supplementary Figure 1: Phylogenetic reconstruction of *Drosophila glob1*, *glob2* and *glob3* including *G. intestinalis glob1* and *C. thummi thummi hblII* at the nucleotide level (using only first and second codon positions) (A) by applying a Maximum likelihood approach implemented in Treefinder and (B) a Bayesian analysis using MrBayes.



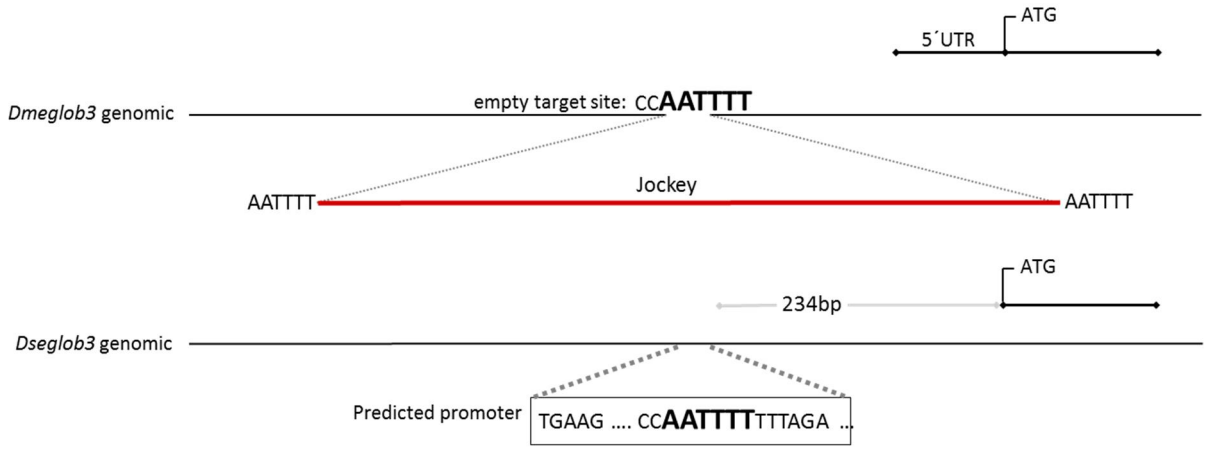
Supplementary Figure 2: Maximum likelihood approach applying CODEML (PAML v4.2 package) for the detection of signs of positive selection along lineages, assuming '*glob2/3* duplication-in-*Sophophor* subgenus' topology. Elevated omega ratios, indicated by d_N/d_S rates, were found along ten out of 66 branches (in bold).



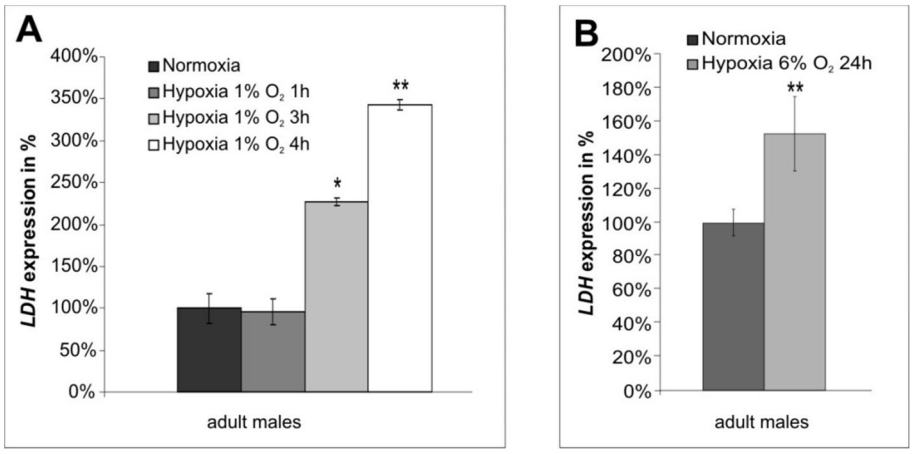
Supplementary Figure 3: Characterization of branches by M0 revealed branch length estimates < 1 of the ten branches (in bold) with signatures of positive selection.



Supplementary Figure 4: *Glob2* and *glob3* absolute mRNA copy number in sexed 3rd instar larvae, pupae and adult flies of *D. melanogaster* and *D. virilis*, measured via QPCR. *Glob2* (181675 copies) (A) and *glob3* (150026 copies) (B) copy number in *D. melanogaster* summed up is equal to *glob3* copy number in *D. virilis* (311673 copies) (C).



Supplementary Figure 5: Schematic diagram of the genomic region of *D. melanogaster glob3* including the transposable element inserted downstream of the *glob3* gene in comparison to the corresponding genomic region of *D. sechellia* glob3. In *D. melanogaster*, the insertion and the putative duplicated sequences are indicated. In *D. secheliia*, the predicted promoter sequence spanning the transposon insertion sequence in *D. melanogaster* are highlighted. Exons and 5´UTR and distances between 3´end of transposable element and Exon1 in both *D. melanogaster* and *D. sechellia* are plotted.



Supplementary Figure 6: Regulation of *LDH* mRNA in *D. melanogaster* male adults after hypoxic stress. mRNA levels (bars) are shown relative to the gene expression at normoxia (21%). (A) *LDH* expression after 1% O_2 for 1 h , 3 h and 4 h. (B) *LDH* expression after 6 % $O_2$for 24h. (*p < 0.05; **p < 0.01).

Abbreviations

aa, amino acids; DEPC, Diethylpyrocarbonate; d_N/d_S, non-synonymous to synonymous nucleotide substitution rates; EST, expressed sequence tags; Hb, hemoglobin; LR-ELW, Local Rearrangements of tree support-Expected Likelihood weight; LRT, likelihood ratio test; Mb, myoglobin; ML, maximum likelihood; non-LTR, non-long terminal repeat; ROS, reactive oxygen species; TE, transposable element.

3. DISKUSSION UND AUSBLICK

3.1 Evolution der Globine in *Drosophila*

Drosophila melanogaster besitzt drei Globine, glob1, glob2 und glob3, deren Funktion bislang unklar ist. Glob1 weist die typische Globinfaltung auf, die für eine Häm- und O_2-Bindung funktionell wichtigen Aminosäuren sind konserviert und die Hauptexpressionsorte sind der Fettkörper und das Tracheensystem (Burmester und Hankeln, 1999; Hankeln et al., 2002). Die Varianten *glob2* und *glob3* sind durch N- und C-terminale Verlängerungen der Globin-Domäne gekennzeichnet und weisen ebenfalls eine Konservierung der für die Häm- und O_2-Bindung wichtigen Aminosäuren auf (Burmester et al., 2006; Kapitel 2.4). Bei *glob2* und *glob3* handelt es sich um duplizierte Gene, deren Expressionsmuster streng auf die männliche Keimbahn beschränkt ist (Kapitel 2.4). Betrachtet man die Lage der *Drosophila*-Globine im Stammbaum der Insekten-Globine (Burmester und Hankeln, 2007), so wird deutlich, dass glob1 eine nahe Verwandtschaft mit den O_2-speichernden und -transportierenden Hämoglobinen der Chironomiden und der Pferdemagenbremse *G. intestinalis* aufweist, die sich auf Sequenzebene zudem durch die gemeinsame Lage der Introns an den Positionen D7.0 und G7.0 widerspiegelt (Hankeln et al., 2002; Burmester et al., 2006). Die Exon-Intron-Struktur der *glob2/3*-Duplikate hingegen zeigt mit den Introns an den Positionen B12.2 und G7.0 eine anzestrale Organisation, die in derselben Form auch z. B. bei Vertebraten-Globinen konserviert ist. Dies führte zu der Annahme, dass glob2/3 eine basale Position im Globin-Stammbaum innehaben (Burmester et al., 2006). Im Rahmen der vorliegenden Arbeit wurden nun erweiterte phylogenetische Rekonstruktionen unter Berücksichtigung aller momentan zugänglichen orthologen Globin-Sequenzen von Drosophiliden sowie dem *glob1* von *G. intestinalis* und dem *hbIII* von *C. thummi thummi* durchgeführt, um Rückschlüsse auf die Evolution der Globine innerhalb des Genus *Drosophila* ziehen zu können. Die phylogenetischen Rekonstruktionen wiesen auf einen monophyletischen Ursprung von *glob1* einerseits und *glob2* sowie *3* andererseits hin und bestätigten die basale Anordnung der Duplikate *glob2* und *3* relativ zum *glob1*. Diese basale Lokalisation ist jedoch mit großer Wahrscheinlichkeit auf die schnelle Evolutionsrate der Duplikate zurück zu führen, die deutlich höher als die des glob1 ist (Kapitel 2.4). *Drosophila* glob1 weist eine nahe Verwandtschaft mit den Hämoglobinen der Chironomiden und von *G. intestinalis* auf (Burmester et al., 2006), deren Beteiligung an der O_2-Versorgung unumstritten ist (Ewer, 1942; Keilin und Wang, 1946; Bergtrom et al., 1976; Osmulski und Leyko, 1985; Weber und Vinogradov, 2001) auf. Dies deutet zusammen mit dem Auftreten des *Drosophila* glob1 im Fettkörper und Tracheensystem, das ebenfalls deutliche Parallelen zu den intrazellulären Hämoglobinen von *G. intestinalis* zeigt, auf eine grundständige Funktion des

Drosophila glob1 innerhalb des O_2-Metabolismus hin (beispielsweise als O_2-Speicher oder –Transportprotein). Im Gegensatz dazu konnten bisher keine *glob2/3* Orthologen in anderen Dipteren identifiziert werden, was darauf hindeutet, dass dieser Typ von Testes-spezifischen Globinen auf die Drosophiliden beschränkt zu sein scheint. Tatsächlich zeigte die kürzliche Entdeckung des Androglobins, eines chimären Globins, das ausschließlich in Testes exprimiert wird und bisher in über 30 Metazoen identifiziert wurde, dass Testes-spezifischen Globine weit verbreitet sind (Hoogewijs et al., submitted). Interessanterweise konnten Hoogewijs et al. bei der im Rahmen ihrer Studie durchgeführten *in silico* Suche keine zum Androglobin orthologen Gene in den Genomen der Drosophiliden identifizieren. Dieser Befund spricht dafür, dass die Entstehung der Testes-spezifischen Globine glob2 und glob3 in *Drosophila* als Kompensation für den Verlust des Androglobins in den Drosophiliden diskutiert werden kann.

3.2 *Glob1*-Expression in Abhängigkeit vom Sauerstoffgehalt der Umgebung

Um weitere Hinweise auf die physiologische Funktion des *Drosophila* glob1 zu erhalten, wurden Versuche zur Regulation der *glob1*-Expression in Abhängigkeit unterschiedlicher O_2-Bedingungen durchgeführt. Zwar konnte ein direkter Einfluss der O_2-Konzentration der Umgebung auf die *glob1*-Expression gemessen werden, jedoch resultierte dieser erstaunlicherweise in einer Abnahme der *glob1*-Expression unter Hypoxie und in einer Zunahme unter Hyperoxie (Gleixner et al., 2008). Diese Antwort der *glob1*-Expression auf Hypoxie und Hyperoxie entspricht nicht dem erwarteten Muster einer Zunahme der Expression unter Hypoxie, das bei klassischen O_2-Speicherproteinen wie z. B. den Hämoglobinen der *Chironomiden* Larven und den Hämoglobinen des Wasserflohs *Daphnia magna* zu beobachten ist (Weber und Vinogradov, 2001; Gorr et al., 2004a). Das beobachtete Expressionsmuster stellt somit die naheliegende Funktion des *Drosophila* glob1 bei der O_2-Versorgung in Frage und verlangt danach, weitere physiologische Aufgaben der Globine als mögliche Funktion des *Drosophila* glob1 zu berücksichtigen.

Beinahe alle Globine der Vertebraten besitzen neben der Eigenschaft der Bindung von Liganden wie O_2 oder CO die Fähigkeit zur enzymatischen Entgiftung reaktiver Stickstoff- und Sauerstoffspezies (siehe z.B. Flogel et al., 2008; Greenberg et al., 2008; Hendgen-Cotta et al., 2010b; Hundahl et al., 2010). Im Hinblick auf die Zunahme der *glob1*-Expression unter Hyperoxie in *D. melanogaster* wäre daher eine Funktion des glob1 bei der Entgiftung reaktiver Sauerstoffspezies, die unter hyperoxischen Bedingungen drastisch zunehmen (siehe z.B. Jamieson et al., 1986), durchaus denkbar. Dies schließt eine zusätzliche, konstitutive, d. h. nicht stressabhängig induzierbare O_2-Speicherfunktion des *Drosophila* glob1, insbesondere über kurze Zeiträume hinweg, nicht aus.

Eine Regulation der Genexpression unter hypoxischen Bedingungen verläuft bei *Drosophila*, wie auch bei Vertebraten, oftmals HIF-vermittelt. Bei HIF (=‚hypoxia inducible factor') handelt es sich um einen Master-Transkriptionsfaktor, der durch Hypoxie induziert wird und über die Bindung an sogenannte HREs (=‚hypoxia response elements') in der Umgebung der Zielgene deren Expression unter Hypoxie beeinflusst (Wenger et al., 1996; Okino et al., 1998; Ebert und Bunn, 1999; Semenza, 2007). Bereits im Jahr 2004 konnten Gorr und Kollegen *in vitro* eine Beteiligung des Transkriptionsfaktors HIF bei der Abnahme der *glob1*-Expression unter Hypoxie nachweisen (Gorr et al., 2004b), die durch die 2008 durchgeführten *in vivo* Expressionsstudien des *glob1* unter Hypoxie bestätigt wurde. Die daraufhin durchgeführte *in silico* Suche nach HREs im Bereich des *glob1* Gens ergab die Anwesenheit von potenziellen HREs direkt proximal zur codierenden Sequenz und etwa 900bp proximal zum Promotor I (siehe Kapitel 2.2, Abb. 1) (Burmester et al., 2006). Die Anwesenheit mehrerer solch potenzieller HREs in der *glob1* Genregion spricht dafür, dass die beobachtete Abnahme der *glob1*-Expression unter Hypoxie tatsächlich auf den verminderten O_2-Gehalt in der Umgebung zurückzuführen ist.

Eine ebenfalls Hypoxie-induzierte Abnahme der Globin-Expression, vergleichbar mit der des *glob1* Gens in *D. melanogaster*, wurde in den unterirdisch lebenden Blindmäusen *Spalax judaei* und *Spalax galili* beobachtet (Avivi et al., 2010). Die Blindmäuse zeigen eine hohe Toleranz gegenüber hypoxischen Bedingungen und weisen im Vergleich zur Ratte einen konstitutiv stark erhöhten Gehalt an Myoglobin, Neuroglobin sowie Cytoglobin auf, was für eine Beteiligung dieser Globine bei der Hypoxie-Toleranz der untersuchten Blindmäuse spricht (Burmester et al., 2007a; Avivi et al., 2010). Umso überraschender war die Beobachtung, dass sowohl Neuroglobin als auch Myoglobin unter Langzeit-Hypoxie eine Abnahme der mRNA Expression aufwiesen (Avivi et al., 2010). Eine mögliche Erklärung für die unerwartete, Hypoxie-induzierte Abnahme der Globin-Expression in *Spalax* und *Drosophila* besteht darin, anzunehmen dass diese Globine als O_2-Speicher fungieren. Es wäre unsinnig eine Zunahme an O_2-Speicherprotein zu fordern, wenn der zu speichernde Sauerstoff unter Hypoxie kaum mehr vorhanden ist. Für diese Interpretation spricht, dass die Menge an Globin Protein sowohl in *S. judaei*, in *S. galili* (Avivi et al., 2010) als auch in *D. melanogaster* (Gleixner, persönliche Beobachtung, unpubliziert) unter Hypoxie unverändert bleibt und wohl nur die Transkription herunterreguliert wird.

3.3 Entwicklungsabhängige Regulation der *glob1* Transkription

Glob1 wird in *D. melanogaster* ausgehend von zwei alternativen Startpunkten transkribiert (Promotor I und II, siehe Kapitel 2.2, Abb. 1). Dadurch entstehen zwei alternative Transkripte (Isoform A und C), die nach alternativem Splicing in zwei weiteren Transkripten (Isoform B und

D) resultieren (Hankeln et al., 2002; Kapitel 2.2, Abb.1). Die Transkriptvarianten unterscheiden sich an ihren 5´-Enden, besitzen jedoch alle dieselbe kodierende Sequenz, so dass davon ausgegangen werden kann, dass die Translation aller in demselben Protein resultiert. Auf mRNA *in situ* Hybridisierung basierende Analysen zur Lokalisation der *glob1* Transkripte zeigten die Anwesenheit von *glob1* mRNA in frühen Embryonalstadien (Stadium 0-5) sowie dann erst wieder in späten Embryonalstadien (ab Stadium 12) (Hankeln et al., 2002). Das Fehlen von *glob1* Transkripten in Embryonen ab Stadium 6 zusammen mit der Anwesenheit von *glob1* mRNA in frühen Embryonalstadien deutet auf eine maternale Herkunft der *glob1* Transkripte in den Embryonalstadien 0-5 hin (Hankeln et al., 2002), während die *glob1* Transkripte ab Stadium 12 höchstwahrscheinlich durch aktive Transkription im Embryo entstanden sind. Die mittels quantitativer Realtime-PCR durchgeführte Analyse des Expressionsmusters der Isoformen lieferte erste Hinweise darauf, dass Promotor II eine stärkere Aktivität in Weibchen aufweist (Kapitel 2.2, Abb. 2). Es ist folglich durchaus denkbar, dass es sich bei Promotor II um einen Weibchen-spezifischen Promotor handelt, der für die Transkription der maternal vererbten *glob1* Transkripte zuständig ist. Die Expression von Isoform C würde dann entsprechend entwicklungs- und geschlechtsunabhängig ausgehend von Promotor I initiiert. Gegen eine solche maternale Vererbung von Isoform A spricht allerdings, dass in Embryonen (gepoolt, Stadien 0-17) eine starke Zunahme der Isoform A-Expression gemessen wurde, die in adulten Weibchen hingegen nicht vorhanden war. Um zu klären, ob Isoform A tatsächlich primär maternal exprimiert wird und es sich bei Promotor II um einen Weibchen-spezifischen Promotor handelt, müsste die Isoform A-Expression in frühen Embryonalstadien (bis Stadium 5) mit der in späten Embryonalstadien (ab Stadium 12) von *D. melanogaster* mittels quantitativer Realtime-PCR verglichen werden. Eine deutlich höhere Anzahl von Isoform A Transkripten in frühen Embryonen würde dann für die maternale Herkunft der Transkripte sprechen.

Isoform C wird (ausgehend von Promotor I) in allen untersuchten Entwicklungsstadien am stärksten exprimiert, jedoch konnte in allen untersuchten Stadien ebenfalls die Expression der Isoformen A, B und D detektiert werden. Da die drei Isoformen im Vergleich zu Isoform C jedoch nur in sehr geringer Kopienzahl nachgewiesen wurden, besteht die Möglichkeit, dass die Expression von Isoform A, B und D gegenüber der starken Präsenz von Isoform C zu vernachlässigen ist (siehe Kapitel 2.2 Abb. 2). Allerdings wäre es auch durchaus denkbar, dass die Isoformen Gewebespezifität aufweisen und die geringe Kopienzahl der Isoformen B und D durch eine starke lokale Spezialisierung der Genexpression zustande kommt. Um dies zu klären wäre es sinnvoll, die Lokalisation der vier Isoformen in den verschiedenen Entwicklungsstadien zu überprüfen, so dass ausgeschlossen werden kann, dass es sich um gewebespezifische

Transkriptvarianten handelt. Die Lokalisation könnte mittels mRNA *in situ* Hybridisierung unter Verwendung von antisense-Sonden, die spezifisch für die jeweiligen 5´-Enden der Transkriptvarianten sind, bestimmt werden.

Wie bereits erwähnt, handelt es sich bei Isoform C um die prominenteste *glob1* Transkriptvariante in allen untersuchten Entwicklungsstadien (siehe Kapitel 2.2, Abb. 2). Die Quantifizierung der Transkriptvarianten unter Hypoxie zeigte eine deutliche Abnahme der Isoform C Expression (siehe Kapitel 2.2, Abb. 2). Dieser Befund spricht dafür, dass es sich bei Promotor I um den Hypoxie-regulierten, unter HIF-Einfluss stehenden Promotor handelt, der auch für die von Gleixner et al. (2008) beobachtete Abnahme der *glob1* Transkription unter Hypoxie verantwortlich ist.

Die genaue Charakterisierung der spezifischen Verwendung der beiden alternativen Promotoren des *glob1* Gens sowie der alternativen Spliceprodukte könnte neben der Klärung der glob1 Funktion zudem als Modell für die funktionelle Relevanz alternativer Promotoren (und auch des alternativen Splicings) allgemein herangezogen werden. Das humane Myoglobin-Gen beispielsweise wird überraschenderweise ausgehend von mehreren alternativen Promotoren transkribiert, was in einer Vielzahl an 5´-unterschiedlichen Transkripten resultiert, die jedoch ähnlich wie *D. melanogaster glob1* dieselbe kodierende Region beinhalten (Diplomarbeit Anne Bicker, 2010). Die funktionelle Relevanz dieser alternativen Transkripte konnte auch hier bisher nicht geklärt werden. *D. melanogaster* glob1 und dessen alternative Transkription wäre ein attraktives leichter experimentell zu manipulierendes Modell für die funktionelle Relevanz alternativer Promotoren und Transkripte.

3.4 Glob1: O_2-Lieferant oder O_2-Entsorger?

Um zusätzliche Hinweise auf die Funktion des *Drosophila* glob1 zu erhalten, wurde die endogene glob1 Expression mithilfe des UAS/Gal4-Systems manipuliert. Dies resultierte in einer ubiquitären Überexpression sowie in einem RNAi-vermittelten, fast vollständigen Knockdown des glob1 Proteins in L3 Larven und adulten Tieren (Kapitel 2.3, Abb. 1B,D,F). Da weder der glob1 Knockdown noch die Überexpression zu sofort offensichtlichen phänotypischen Abweichungen unter normalen Bedingungen führten, wurden sowohl Larven als auch Adulte verschiedenen experimentellen Stressbedingungen ausgesetzt. Die phänotypischen Experimente zeigten, dass adulte Tiere mit glob1 Knockdown eine höhere Anfälligkeit gegenüber hypoxischen Bedingungen aufweisen und sowohl milde Langzeit- als auch strenge Kurzzeit-Hypoxie signifikant schlechter überleben (Kapitel 2.3, Abb. 2A+B und 3A+B). Unter Hyperoxie konnte dagegen kein Unterschied in der Überlebensrate der Fliegen

und Larven mit glob1 Überexpression oder Knockdown festgestellt werden. Diese Befunde sprechen für eine Beteiligung von glob1 an der O_2-Versorgung, die insbesondere unter hypoxischen Bedingungen zum Tragen kommt. Das Ausbleiben phänotypischer Unterschiede der glob1 Knockdown Fliegen unter normoxischen Bedingungen könnte damit erklärt werden, dass das Fehlen des glob1 beispielsweise durch eine stärkere Verzweigung der Tracheolen und somit einer effektiveren Verteilung des O_2 über das Tracheensystem kompensiert wird. Ein solch kompensatorischer Mechanismus wäre vergleichbar mit der höheren Kapillardichte in auf den ersten Blick phänotypisch ebenfalls unauffälligen Myoglobin-Knockout Mäusen (Godecke et al., 1999; Flogel et al., 2001; Merx et al., 2001). Um zu überprüfen, ob tatsächlich kompensatorische Mechanismen für das Ausbleiben eines Phänotyps unter normoxischen Bedingungen verantwortlich sind, könnte der Verzweigungsgrad der Tracheen, insbesondere der terminalen Äste, in glob1 Knockdown Larven im Vergleich zu Kontroll- und glob1 Überexpressions-Larven überprüft werden. Das Tracheensystem in *Drosophila* ist insbesondere im Larvenstadium sehr dynamisch und besitzt die Fähigkeit, aktiv auf schwankende O_2-Bedingungen zu reagieren. Hypoxische Gewebe-Areale induzieren über die Ausschüttung eines Wachstumsfaktors (dFGF=‚*Drosophila* fibroblast growth factor', kodiert von dem Gen branchless) die Migration der terminalen Äste (Lee et al., 1996; Sutherland et al., 1996; Jarecki et al., 1999). Es konnte gezeigt werden, dass der Verzweigungsgrad der terminalen Tracheenäste unter chronisch hypoxischen Bedingungen zu- und unter hyperoxischen Bedingungen abnimmt (Jarecki et al., 1999; Centanin et al., 2008). Ginge man von einer Beteiligung des glob1 am O_2-Transport von den Tracheolen zu den umliegenden Geweben aus, könnte ein durch den Knockdown bedingter Mangel zu einer chronischen O_2-Unterversorgung des Gewebes und somit hypoxischen Arealen führen. Dies würde auf längere Sicht zu einer stärkeren Verzweigung der terminalen Tracheenäste führen, die den durch die Abwesenheit von glob1 hervorgerufenen intrazellulären O_2-Mangel unter normoxischen Bedingungen kompensieren könnte.

Die Hypothese der intrazellulären Hypoxie, hervorgerufen durch einen Mangel an glob1, wird zusätzlich durch den Befund unterstützt, dass weibliche Fliegen mit glob1 Knockdown eine verkürzte Erholungszeit nach hypoxischem Stupor zeigen (Kapitel 2.3, Abb. 4). In Langzeit-Hypoxieversuchen mit adulten *Drosophila* konnte gezeigt werden, dass Hypoxie-adaptierte Fliegen eine deutlich verkürzte Erholungszeit sogar nach anoxischem Stupor aufweisen (Zhou et al., 2007). Ginge man von einer Anpassung der glob1 Knockdown-Fliegen an die chronische intrazelluläre Hypoxie aus, so würde dieser Anpassungseffekt die beobachtete verkürzte Erholungszeit nach hypoxischem Stupor erklären.

Die Toleranz von *Drosophila* mit manipulierter glob1-Expression gegenüber oxidativem Stress wurde überprüft, indem Larven und Fliegen zum einen 95% O_2 ausgesetzt und zum anderen mit der ROS-generierenden Chemikalie Paraquat gefüttert wurden (Phillips et al., 1989; Mockett et al., 2003; Walker und Benzer, 2004). Während unter hyperoxischen Bedingungen keinerlei phänotypische Unterschiede zwischen glob1 Knockdown- und Überexpressions-Fliegen zu beobachten war, zeigten sowohl Larven als auch weibliche Fliegen mit glob1 Knockdown überraschenderweise eine höhere Resistenz gegenüber Paraquat-induziertem ROS-Stress, die sich bei Larven durch eine signifikant höhere Überlebensrate und in adulten Weibchen durch signifikant längeres Überleben bemerkbar machte (Kapitel 2.3, Abb. 5A+B). Die Hyperoxie- und Paraquat-Behandlungen zeigten zwar einen quantifizierbaren Anstieg an ROS-induzierten Zellschäden, ein Unterschied zwischen Fliegen mit glob1 Knockdown und Überexpression konnte jedoch nicht festgestellt werden. Bei einer Beteiligung von glob1 an der Entgiftung reaktiver Sauerstoffspezies, wie sie aufgrund der Zunahme der mRNA Expression unter Hyperoxie von uns zunächst postuliert wurde (Gleixner et al., 2008), würde man jedoch erwarten, dass der durch den Knockdown bedingte Mangel an glob1 zu nachteiligen und die glob1 Überexpression zu positiven Effekten während oxidativen Stresses führte. Da unter Paraquat aber sogar der gegenteilige Effekt beobachtet werden konnte, scheidet eine primäre Rolle von glob1 bei der Entgiftung reaktiver Sauerstoffspezies als Funktionshypothese aus. Vielmehr spricht auch die höhere Toleranz gegenüber Paraquat, die in glob1 Knockdown Adulten und Larven beobachtet wurde, für eine Beteiligung des glob1 bei der O_2-Versorgung: Paraquat induziert nämlich hauptsächlich die Entstehung des Superoxid-Anions, indem es ein Elektron auf molekularen O_2 überträgt und diesen damit zu O_2^- oxidiert. Weniger glob1 Protein und somit eine geringere Menge an gespeichertem intrazellulären O_2 würden unter Paraquat-Behandlung somit zu einer geringeren Menge an O_2^- und daraus resultierenden ROS-Schäden führen.

Zusammengefasst sprechen die beschriebenen Ergebnisse der Untersuchungen der *Drosophila* Larven und Fliegen mit manipulierter glob1-Expression mehrheitlich für eine Beteiligung des glob1 an der O_2-Versorgung. Trotzdem ist es nach wie vor verblüffend, dass das hochkonservierte und relativ stark exprimierte glob1 (Burmester und Hankeln, 1999; Hankeln et al., 2002; Burmester et al., 2006), dem wohl eine tragende Rolle im O_2-Metabolismus von *D. melanogaster* zuzusprechen ist, nach einem experimentellen Knockdown der Genexpression keine drastischeren phänotypischen Effekte hervorruft. Der hier erzeugte RNAi-vermittelte Knockdown des *glob1* Gens resultierte zwar in einer deutlichen Reduktion der *glob1* mRNA und des Proteins, trotzdem konnten in der durchgeführten qPCR noch *glob1* Transkripte

detektiert werden. Es kann daher nicht vollkommen ausgeschlossen werden, dass selbst die sehr geringe Anzahl an *glob1* mRNA in Knockdown-Fliegen noch ausreichend ist, um deutliche phänotypische Effekte zu verhindern. Aufgrund dessen wäre es sinnvoll, beispielsweise durch gezieltes Ersetzen des *glob1* Gens mittels homologer Rekombination (Rong und Golic, 2000; Gong und Golic, 2003; Huang et al., 2008) glob1 Knockout-Mutanten zu generieren. Die so erzeugten glob1 Knockout-Fliegen sollten dann zur Verifizierung der im Rahmen der vorliegenden Arbeit durchgeführten Experimente verwendet werden. Zusätzlich sollten die in glob1 Knockout-Fliegen beobachteten phänotypischen Effekte durch Rescue-Experimente bestätigt werden, um sicher zu gehen, dass die beobachteten Effekte tatsächlich auf das Fehlen von glob1 zurück zu führen sind. Dazu müssten die glob1 Knockout-Mutanten mittles Transgenese wieder dazu gebracht werden, funktionelles glob1 zu exprimieren. Dies sollte dann zu einem Aufheben der durch den glob1 Mangel hervorgerufenen Effekte führen.
In den im Rahmen der vorliegenden Arbeit durchgeführten Experimenten zur Identifizierung möglicher Auswirkungen der manipulierten glob1-Expression konnten keinerlei phänotypische Effekte in Larven oder Adulten mit glob1 Überexpression festgestellt werden. Zur Generierung der Überexpression wurde auf die ubiquitär treibende daughterless (da) Gal4-Linie zurückgegriffen, deren Verwendung in einer 2-4fachen glob1 Überexpression resultierte (Kapitel 2.3, Abb. 1B+E und Suppl. Abb. 1B). Möglicherweise war die dadurch erzeugte Zunahme von glob1 Protein in den relevanten Geweben (wie dem Fettkörper und Tracheensystem) nicht ausreichend, um phänotypische Effekte hervorzurufen. Aufgrund dessen wäre es sinnvoll, in zukünftigen Experimenten gewebespezifische Treiber zu verwenden, um insbesondere in den endogenen Expressionsorten des glob1 eine drastische Hochregulation zu erzeugen. Eine starke glob1 Überxpression im Fettkörper unter Verwendung der Responderlinie UAS36 in Kombination mit dem Treiber collagen Gal4 (cg Gal4) und besonders im Tracheensystem unter Verwendung der Responderlinie UAS29 mit dem Treiber breathless Gal4 (btl Gal4) könnte unter Stressbedingungen die physiologische Relevanz von glob1 deutlicher zeigen.

3.5 Globine und NO

Im Rahmen der vorliegenden Arbeit wurde die Beteiligung des *Drosophila* glob1 bei der O_2-Versorgung sowie bei der Entgiftung reaktiver Sauerstoffspezies untersucht. Jedoch ist das Spektrum der möglichen Globin-Funktionen weitaus vielfältiger. Eine Fähigkeit, die vielen Globinen gemein ist, ist das Entgiften schädlicher Stickstoffspezies, wie z. B. Nitrit (NO_2^-) oder Stickstoffmonoxid (NO) unter Normoxie, gleichzeitig aber auch die Reduktion von NO_2^- zu NO unter Hypoxie (siehe z.B. Flogel et al., 2008; Greenberg et al., 2008; Hendgen-Cotta et al.,

2010a; Hundahl et al., 2010). In Säugern spielt NO insbesondere unter hypoxischen Bedingungen eine wichtige Rolle, indem es als Signalmolekül bei der Vasodilatation mitwirkt (Ignarro, 1999; Ungvari und Koller, 2001). In *Drosophila* wurde eine Beteiligung von NO als Signalmolekül bei der Hypoxie-abhängigen Entwicklung des Tracheensystems beschrieben (Wingrove und O'Farrell, 1999; Teodoro und O'Farrell, 2003). Bei zukünftigen Untersuchungen zur Funktion des *Drosophila* glob1 sollte folglich eine mögliche Beteiligung des glob1 am Stickstoffmetabolismus berücksichtigt werden.

Eine Möglichkeit, die Beteiligung von glob1 an der Entgiftung von NO unter Normoxie zu untersuchen, wäre, Larven oder Adulten mit gewebespezifischer Überexpression einen NO-Donor wie z. B. SNAP (S-Nitroso-*N*-acetylpenicillamin) zu verabreichen. Anschließend könnte die Menge an NO unter Verwendung des Fluoresceins DAF2/DA (Kojima et al., 1998) mittels Fluoreszenzmikroskopie lokalisiert und quantifiziert werden. Dadurch könnte beobachtet werden, ob die gewebespezifische glob1 Überexpression zu einer lokalen Abnahme an NO führt.

Um die Beteiligung von glob1 an der Synthese von NO unter Hypoxie zu untersuchen, könnte die NO-Synthase in Larven oder Adulten mit gewebespezifischer glob1 Überexpression mithilfe der Chemikalie L-NAME inhibiert (Wingrove und O'Farrell, 1999) und anschließend die Gewebe mit glob1 Überexpression fluoreszenzmikroskopisch auf die Anwesenheit von NO überprüft werden. Ein deutlicher Anstieg der NO-Menge in glob1 überexprimierenden Geweben spräche für eine Beteiligung des glob1 bei der NO-Synthese unter hypoxischen Bedingungen.

3.6 *Drosophila* glob2 und 3: Duplizierte Globine mit spezialisierter Funktion?

Bei *Drosophila glob2* und *3* handelt es sich um duplizierte Gene, die beide in den neun Spezies des Subgenus *Sophophora* vorhanden sind. Im Genom der drei Spezies des Subgenus *Drosophila* existiert jedoch lediglich die *glob3* Kopie. Diese Verteilung von *glob2* und *3* in den 12 sequenzierten *Drosophila* Spezies kann durch zwei Hypothesen erklärt werden: A) Vor der Radiation des Subgenus *Sophophora* fand eine Duplikation des *glob3* Gens statt (‚*glob2/3* duplication-in-*Sophophora* subgenus'); B) Es gab ein frühes Duplikationsereignis, gefolgt von einer Deletion des *glob2* Gens in den Spezies des Subgenus *Drosophila* (‚*glob2* deletion-in-*Drosophila* subgenus'). Die Überprüfung beider Hypothesen mithilfe phylogenetischer Rekonstruktionen ergab, dass die gegenwärtige Verteilung von *glob2* und *3* am besten durch Hypothese A), also durch ein Duplikationsereignis vor der Entstehung des Subgenus *Sophophora* vor mindestens 40 Millionen Jahren, erklärt werden kann (Kapitel 2.4, Abb.1). Untersuchungen der Genexpression der Globin-Duplikate in *D. melanogaster* sowie des einzelnen *glob3* Gens in *D. virilis* ergaben ein zwischenartlich konserviertes Expressionsmuster

in männlichen Larven, Puppen und Adulten. Auch die Lokalisation der Transkripte in der männlichen Keimbahn adulter *D. melanogaster* bzw. *D. virilis* mittels mRNA *in situ*-Hybridisierung zeigte ein übereinstimmendes Muster (Kapitel 2.4, Abb. 2A-C und 2A-C). Die Untersuchungen zur molekularen Evolution der Globine auf Sequenzebene lieferten Hinweise auf positive Selektion entlang des Astes der *Sophophora glob2* und *3* Orthologen sowie entlang der Stammlinie der *Sophophora glob3* Gene (Kapitel 2.4, Suppl. Abb. 3). Dies könnte darauf hinweisen, dass *glob2* und *3* nach der Duplikation eine Kombination aus Sub- und Neo-Funktionalisierungsprozessen durchlaufen haben (Ohta, 1988; He und Zhang, 2005), bei welchen zumindest einer der aus der Duplikation hervorgegangenen paralogen Äste (*Sophophora* glob3 Orthologe) positiver Selektion unterlag.

Da die Untersuchungen zur mRNA Expression der Testes-Globine jedoch keine Unterschiede im Expressionsmuster und der Lokalisation der Transkripte ergaben, kann vom momentanen Wissensstand aus keine eindeutige Aussage über das evolutionäre Schicksal von *glob2* und *3* nach der Duplikation und damit einhergehende funktionelle Anpassungen oder Spezialisierungen getroffen werden. Eine wichtige Rolle bei der Beantwortung dieser Frage spielt die exakte Lokalisation der glob2 und 3 Proteine in Testes, die mittels Antikörperfärbungen bestimmt werden müsste. Dadurch könnten sowohl wichtige Hinweise auf das evolutionäre Schicksal der Duplikate als auch indirekte Hinweise auf die Funktion der Globine in der männlichen Keimbahn gefunden werden. Aufgrund der ausschließlichen Expression von *glob2* und *3* in Testes scheint eine Funktion der Globine während der Spermatogenese naheliegend. Hierbei könnte die Fähigkeit der Globine, reaktive Sauerstoffspezies zu entgiften (Weber und Fago, 2004; Hankeln et al., 2005; Hundahl et al., 2010), einen Beitrag zum Schutz der Spermatogenese vor oxidativen Schäden leisten. Die durchgeführten Untersuchungen zur O_2-abhängigen Regulation der *glob2*- und *3*-Expression in *D. melanogaster* zeigten jedoch keinen Anstieg der *glob2* und *3* mRNA Kopien unter Hyperoxie, wie man sie von einem akut induzierbaren ROS Entgiftungs-Protein erwarten würde. Eine weitere mögliche Funktion der Testes-Globine 2/3 wäre eine Beteiligung am korrekten Ablauf der Spermatiden-Individualisierung, bei der Apoptose-ähnliche Prozesse zur Entfernung überschüssigen cytoplasmatischen Materials notwendig sind (Arama et al., 2003). Untersuchungen am Neuroglobin lieferten Hinweise auf eine Beteiligung des Ngbs bei der Verhinderung Cytochrom c-vermittelter Apoptose durch Reduktion von Eisen(III)-Cytochrom c zu Eisen(II)-Cytochrom c (Fago et al., 2006). Es wäre denkbar, dass die Testes-Globine in *D. melanogaster* eine Rolle bei der Aufrechterhaltung des optimalen Eisen(II)-Cytochrom c-Levels spielen und somit zum Apoptose-basierten, korrekten Ablauf der Spermatogenese beitragen.

Zukünftige Untersuchungen zur Funktion der Testes-Globine sollten sich in erster Linie auf die zelluläre Lokalisation der glob2 und 3 Proteine konzentrieren. Über die Lage der Proteine könnte geklärt werden, an welchem Punkt der Spermatogenese diese Globine funktionell beteiligt sind.

4. ZUSAMMENFASSUNG

Globine sind kleine globuläre Proteine mit nahezu ubiquitärem Vorkommen in allen Tiergruppen. Sie weisen eine typische Sandwichstruktur auf, die in der Regel aus acht α-Helices mit einer zentralen prosthetischen Häm-Gruppe besteht und die Proteine zur Bindung gasförmiger Liganden befähigt. Die Funktionen der Globine reichen von O_2-Transport und –Speicherung, über eine Beteiligung bei der Entgiftung reaktiver Sauerstoff- und Stickstoffspezies bis hin zu sensorischen physiologischen Aufgaben.

Innerhalb der Klasse der Insekten schien das Vorhandensein von Globinen zunächst auf Insekten mit offensichtlich hypoxischen Habitaten beschränkt zu sein. Die Entdeckung des Globins glob1 in *Drosophila melanogaster* deutete jedoch eine sehr viel weitere Verbreitung der Globine in Insekten an, die sich durch die Identifizierung von Globingenen in einer Vielzahl von normoxisch lebenden Insekten, wie z.B. *Apis mellifera* oder *Aedes aegypti* bestätigte.

D. melanogaster besitzt drei Globine, glob1, glob2 und glob3. Glob1 ist eng mit anderen intrazellulären Insektenglobinen verwandt, was zu der Annahme führte, dass es sich bei glob1 um das ursprüngliche und bei glob2 und glob3 um abgeleitete *D. melanogaster* Globine handelt. Glob1 wird in allen Entwicklungsstadien exprimiert, wobei die Hauptexpressionsorte der Fettkörper und das Tracheensystem sind. Die Transkription des *glob1* startet von zwei alternativen Promotoren (Promotor I und II), wodurch in Kombination mit alternativem Splicing vier Transkriptvarianten (Isoform A-D) entstehen, deren Translation jedoch in einer Proteinvariante (glob1) resultiert. Hypoxische Bedingungen führen zu einer vermutlich HIF (=‚hypoxia-inducible factor') -vermittelten Abnahme der *glob1* Genexpression, wohingegen Hyperoxie eine leichte Zunahme der *glob1* mRNA Menge bewirkt. Der mithilfe des UAS/Gal4-Systems erzeugte, RNAi-vermittelte glob1 Knockdown führt zu einer schlechteren Überlebensrate adulter Fliegen unter hypoxischen Bedingungen, einer verkürzten Erholungszeit nach hypoxischem Stupor in Weibchen sowie zu einer erhöhten Resistenz gegenüber dem ROS (='reactive oxygen species') -generierenden Herbizid Paraquat in Larven und adulten Weibchen. Diese Beobachtungen sprechen für eine Funktion des *Drosophila* glob1 innerhalb der O_2-Versorgung. Unter hyperoxischen Bedingungen hingegen wurde kein Unterschied zwischen Fliegen mit wildtypischer und manipulierter glob1-Expression festgestellt, wodurch eine Beteiligung des glob1 bei der Entgiftung reaktiver Sauerstoffspezies als mögliche Funktion vorerst ausscheidet.

Bei *glob2* und *glob3* handelt es sich um duplizierte Gene. Auf phylogenetischen Rekonstruktionen basierend konnte die Entstehung der Globin-Duplikate auf ein Duplikationsereignis vor der Radiation des Subgenus *Sophophora* vor mindestens 40 Millionen

Jahren zurückgeführt werden. Die durchgeführten Analysen zur molekularen Sequenzevolution der Globin-Duplikate deuten darauf hin, dass *glob2* und *glob3* nach der Duplikation eine Kombination aus Sub- und Neo-Funktionalisierungsprozessen durchlaufen haben. *Glob2* und *glob3* zeigen eine deckungsgleiche mRNA Expression, die auf die männliche Keimbahn beschränkt ist. Aufgrund des hohen Konservierungsgrads der für die Häm- und O_2-Bindung essentiellen Aminosäuren kann von der Funktionalität beider Proteine ausgegangen werden. Die streng auf die männliche Keimbahn begrenzte Expression von *glob2* und *glob3* deutet auf eine Rolle der Globin-Duplikate innerhalb der Spermatogenese hin, die möglicherweise in einem Schutz der Spermatogenese vor oxidativem Stress besteht. Auch eine Beteiligung beim korrekten Ablauf der Spermien-Individualisierung, beispielsweise durch Regulation von Apoptoseprozessen wäre denkbar.

SUMMARY

Globins are small globular proteins with a ubiquitous distribution in nearly all kingdoms of life. They exhibit a typical sandwich structure, consisting of eight α-helices embedding a prosthetic heme group in their center which enables the proteins to bind gaseous ligands. Globins display a broad functional diversity ranging from O_2-binding and –delivery, an involvement in detoxification of reactive oxygen and nitrogen species to sensory physiological functions.

In the class Insecta, the occurrence of globins was initially believed to be restricted to insects living in hypoxic environments. The discovery of the hemoglobin glob1 in *Drosophila melanogaster*, however, indicated a far broader distribution in insects. This assumption in fact was verified by the identification of globin genes in a high number of insects living in normoxic habitats like for example *Apis mellifera, Aedes aegypti* or *Bombyx mori.*

D. melanogaster harbours three globins named glob1, glob2 and glob3. Glob1 is closely related to other intracellular insect globins, leading to the assumption that glob1 might be a basal, conserved globin type, whereas the Drosophila-specific glob2 and glob3 might be more derived. Glob1 is expressed in all developmental stages, the main expression can be found in the tracheal system and the fat body. Transcription of *glob1* starts from two alternative promoters (Promoter I and II), resulting in four alternative transcripts (Isoform A-D), all four being translated into the same protein. Hypoxic conditions induce a probably HIF (=hypoxia-inducible factor) -mediated decrease of *glob1* mRNA expression, whereas hyperoxia causes a slight increase in *glob1* mRNA levels. The RNAi-mediated knockdown of glob1, achieved by applying the UAS/Gal4-system, resulted in diminished survival of adult flies exposed to hypoxic conditions, but also in beneficial effects like a shortened recovery time after hypoxic stupor and an increased resistance of larvae and female flies to the ROS (=reactive oxygen species) –generating herbicide Paraquat. These observations – although in part seemingly paradoxical – can be interpreted in terms of a function of glob1 in O_2 supply. Experimental hyperoxia, however, did not reveal phenotypic differences in flies with manipulated glob1 expression compared to control flies. Thus, an involvement of glob1 in the detoxification of ROS appears unlikely at present.

Glob2 and *glob3* originate from a duplication event which could be dated based on phylogenetic reconstructions before the radiation of the subgenus *Sophophora*, at least 40 million years ago. Molecular evolutionary sequence analyses indicate that *glob2* and *glob3* evolved via a combination of sub- and neo-functionalization. *Glob2* and *glob3* exhibit a similar mRNA expression pattern, restricted to the male germline. This highly specific expression

pattern indicates a function of the glob2/3 duplicates within spermatogenesis, e.g. by a protection of spermatogenesis from oxidative stress.

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6. ANHANG

6.1 Zusätzliche Daten

6.1.1 Glob1 Überexpression in verschiedenen Geweben von *D. melanogaster* L3 Larven

Die gewebespezifische *glob1* Überexpression in *D. melanogaster* L3 Larven wurde mithilfe des UAS/Gal4-Systems generiert (Brand und Perrimon, 1993). Als gewebespezifische Treiber wurden die Linien collagen (cg) Gal4 und breathless (btl) Gal4 in Kombination mit der Responder-Linie UAS36 Gb1 verwendet. Bei cg Gal4 handelt es sich um einen fettkörperspezifischen Treiber (Takata et al., 2004; Hennig et al., 2006), wohingegen btl Gal4 im Tracheensystem treibt (Shiga et al., 1996).

Bei den hier dargestellten Kreuzungen handelt es sich um die Kombinationen aus Treiber-und Responder-Linien, die die stärksten *glob1* Überexpressionen zeigten.

Die Quantifizierung der *glob1* mRNA Expression mithilfe quantitativer Realtime-PCR ergab eine 25fache *glob1* Überexpression in Larven der Kreuzung UAS36 x cg Gal4 verglichen mit den Kontrolllinien (Abb. 7.1B). Die Kreuzung UAS36 x btl Gal4 resultierte in einer 2,5fachen *glob1* Überexpression verglichen mit den Kontrolllinien (Abb. 7.1B). Die Charakterisierung erfolgt lediglich auf mRNA Ebene und ausschließlich im larvalen Stadium.

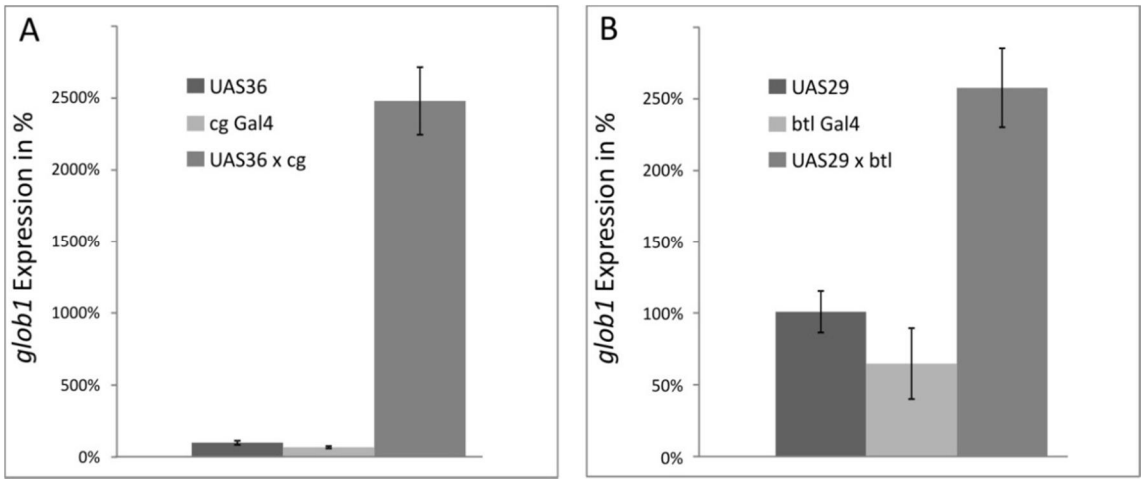

Abb. 6.1: Gewebespezifische *glob1* Überexpression in *D. melanogaster* L3 Larven.

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6.1.2 Microarray-Experiment

Das Microarray-Experiment wurde mit GeneChip® Drosophila Genome 2.0 Arrays (Affymetrix) durchgeführt. Dafür wurde Gesamt-RNA aus adulten *D. melanogaster* Weibchen mit glob1 Knockdown (RNAi Vi Gb1 x da Gal4) und zwei Kotrolllinien (RNAi Vi Gb1 x white$^-$ und da Gal4 x white$^-$) verwendet. Das Experiment wurde einmal durchgeführt, das Transkriptionsmuster in Weibchen mit glob1 Knockdown wurde mit dem Transkriptionsmuster beider Kontrollen verglichen. Gene wurden als ‚reguliert' gewertet, wenn eine mindestens zweifache Regulation in beiden Vergleichen (glob1 Knockdown vs Kontrolle I und glob1 Knockdown vs Kontrolle II) nachgewiesen werden konnte. Insgesamt zeigten 110 Gene eine differentielle Regulation in glob1 Knockdown Weibchen im Vergleich mit beiden Kontrollen. Davon wurden 34 Gene als hochreguliert identifiziert, von denen sechs Gene ausschließlich in glob1 Knockdown-Weibchen detektiert wurden, deren Expression in beiden Kontrollen nicht nachgewiesen werden konnte. Eine Abnahme der Expression in glob1 Knockdown-Weibchen zeigten 76 Gene, von denen 36 unterhalb der Detektionsgrenze lagen. Die Expression dieser 36 Gene konnte in glob1 Knockdown-Weibchen folglich nicht (mehr) nachgewiesen werden. Da 66 der insgesamt 110 in glob1 Knockdown-Weibchen differentiell exprimierten Gene nicht annotiert sind, war eine Aussage über mögliche Gemeinsamkeiten der regulierten Gene nicht möglich.
Eine Liste der in glob1 Knockdown-Weibchen regulierten Gene befindet sich im elektronischen Anhang.

6.2 Geleistete Beiträge der einzelnen Autoren zu den Publikationen

6.2.1 Oxygen-induced changes in hemoglobin expression in *Drosophila*

Die Quantifizierung der *glob1*-Expression in Embryonen (Fig. 1A) sowie die *glob1* und *LDH* Expression in adulten *D. melanogaster* Wildtypen (Fig. 1F+G und Fig. 2D+E) wurde im Rahmen der Diplomarbeiten von Daniela Abriss und Melanie Krämer unter der Betreuung von Frank Gerlach begonnen. Die *glob1* und *LDH* Quantifizierung in L3 Larven sowie die Entwicklung und Etablierung neuer Assays für die Realtime-PCR wurde von Eva Gleixner durchgeführt (Fig. 1B-E und Fig.2 A-C). Zudem war Eva Gleixner mit der Durchsicht sowie der Auswertung der Daten und dem Schreiben des Manuskripts betraut. Boris Adryan und Reinhard Schuh wurden zur Beratung der *Drosophila*-Haltung und –Experimente herangezogen. Die Koordination sowie die Hauptverantwortung der Arbeit lagen bei Prof. Thorsten Burmester und Prof. Thomas Hankeln.

6.2.2 Knockdown of *Drosophila* glob1 evokes a mild phenotype but argues for a role in O_2 supply.

Die transgenen UAS-Responder-Linien (UAS36 und UAS29), die zur Erzeugung der glob1 Überexpression verwendet wurden, wurden von Reinhard Schuh vom Max Planck Institut für Biophysikalische Chemie (Göttingen) bereitgestellt.

Das Ansetzen der *Drosophila*-Kreuzungen zur Manipulation der endogenen *glob1* Expression, sowie die Charakterisierung der manipulierten *glob1*-Expression auf mRNA und Protein-Ebene wurden von Eva Gleixner durchgeführt. Alle Experimente zur Identifizierung von Phänotypen, die auf die manipulierte *glob1*-Expression zurückzuführen sind, wurden ebenfalls von Eva Gleixner durchgeführt. Dasselbe gilt für das TUNEL-Experiment an Flugmuskeln adulter Weibchen mit *glob1* Knockdown, sowie die photospektrometrische Quantifizierung der Lipid-Peroxidation an L3 Larven und adulten *D. melanogaster* mit *glob1* Knockdown und Überexpression.

Die Koordination des Projektes lag bei Prof. Thomas Hankeln, der Kooperationspartner Prof. Thorsten Burmester und Christian Wolf (Intervet Innovation GmbH, Drug Discovery, Schwabenheim) leisteten Beiträge zur Interpretation und Diskussion der Ergebnisse.

6.2.3 Testes-specific hemoglobins in *Drosophila* evolved by a combination of sub- and neofunctionalization after gene duplication

Die Quantifizierung der *glob2*- und *glob3*-Expression in männlichen und weiblichen L3 Larven, Puppen und adulten *D. melanogaster* und *D. virilis* (Abb. 2A-C), sowie die Quantifizierung der *D. melanogaster glob2*- und *glob3*- und *LDH*-Expression in adulten Männchen unter verschiedenen O_2-Bedingungen wurden von Eva Gleixner durchgeführt. Die Entwicklung und Etablierung der Realtime-PCR-Assays, die Hypoxie- und Hyperoxiebehandlung adulter Männchen, die RNA-Isolierung und cDNA-Synthese wurden ebenfalls von Eva Gleixner durchgeführt. Dasselbe gilt für die Durchführung der mRNA *in situ* Hybridisierung an Testes adulter *D. melanogaster* und *D. virilis* (Abb. 3A-C), sowie die Herstellung und Überprüfung der dafür benötigten antisense RNA-Sonden. Stefan Zimmerling war für die Herstellung der Plasmide für die *in vitro* Transkription der *D. melanogaster glob2*-, *glob3*- und der *ß-tubulin*-Sonden, sowie für die methodische Etablierung der mRNA *in situ* Hybridisierung an adulten Testes verantwortlich. Das Durchsuchen der in den Datenbanken vorhandenen Genome verschiedener *Drosophila*-Spezies auf die Anwesenheit von *D. melanogaster glob2* und *glob3* Orthologen, sowie deren Extraktion und Alignierung wurden von Eva Gleixner durchgeführt. Dasselbe gilt für die phylogenetische Rekonstruktion der Verwandtschaftsverhältnisse von *glob1*, *glob2* und *glob3*, durchgeführt mit dem Programm MrBayes v3.1.2. Die auf ‚Maximum

Likelihood‘ basierende phylogenetische Rekonstruktion der Verwandtschaftsverhältnisse von *glob1*, *glob2* und *glob3* durchgeführt mit dem Programm Treefinder, sowie alle notwendigen Vorarbeiten wurden von PD Dr. Holger Herlyn durchgeführt. Ebenfalls von PD Dr. Holger Herlyn durchgeführt wurde die Suche nach positions- und astspezifischen Anzeichen positiver Selektion von *glob1*, *glob2* und *glob3* unter Verwendung des Programms CODEML (PAML v4.4). Die Koordination des Projektes lag bei Prof. Thomas Hankeln, der Kooperationspartner Prof. Thorsten Burmester leistete Beiträge zur Interpretation und Diskussion der Ergebnisse

6.3 Abkürzungsverzeichnis

Adgb	Androglobin
AS	Aminosäure
CO_2	Kohlenstoffdioxid
Cygb	Cytoglobin
dFGF	'*Drosophila* fibroblast growth factor'
DGC	‚discontinous gas exchange cycle'
ESTs	'expressed sequence tags'
GCS	‚globin coupled sensors'
Gb E	Globin E
Gb X	Globin X
Gb Y	Globin Y
Glob1	Globin1
Glob2	Globin2
Glob3	Globin3
Hb	Hämoglobin
HIF	'hypoxia inducible factor'
HRE	'hypoxia response element'
kDa	Kilodalton
kPa	Kilopascal
Mb	Myoglobin
mRNA	'messenger RNA'
Ngb	Neuroglobin
NO	Stickstoffmonoxid
NO_2^-	Nitrit
O_2	Sauerstoff
O_2^-	Dioxid Anion
PCR	'polymerase chain reaction'
RNAi	RNA Interferenz
ROS	'reactive oxygen species'
UAS	'upstream activating sequences'

7 LEBENSLAUF

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Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, dass ich meine Dissertation selbstständig und nur unter Verwendung der angegebenen Hilfsmittel angefertigt habe. Ich habe keinen anderen Promotionsversuch unternommen.

Mainz, den

Eva Maria Gleixner