Insect Molecular Biology

Insect Molecular Biology (2017) **26**(4), 461–468 doi: 10.1111/imb.12310

Aerobic respiration by haemocyanin in the embryo of the migratory locust

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Abstract

It remains unresolved how insect embryos acquire sufficient oxygen to sustain high rates of respiratory metabolism during embryogenesis in the absence of a fully developed tracheal system. Our previous work showed that the two distinct subunits (Hc1 and Hc2) of haemocyanin (Hc), a copper-containing protein, display embryo-specific high expression that is essential for embryonic development and survival in the migratory locust *Locusta migratoria*. Here we investigated the role of haemocyanins in oxygen sensing and supply in the embryo of this locust. Putative binding sites for hypoxia-regulated transcription factors were identified in the promoter region of all of the *Hc1* and *Hc2* genes. Embryonic expression of haemocyanins was highly upregulated by ambient O_2 deprivation, up to 10-fold at 13% O_2 content. The degree of upregulation of haemocyanins increased with increasing levels of hypoxia. Compared with low-altitude locusts, embryonic expression of haemocyanins in high-altitude locusts from Tibetan plateau was constitutively higher and more robust to oxygen deprivation. These findings strongly suggest an active involvement of haemocyanins in oxygen exchange in embryos. We thus propose a mechanistic model for embryo respiration in which haemocyanin plays a key role by complementing the tracheal system for oxygen transport during embryogenesis.

First published online 28 April 2017.

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Keywords: haemocyanin, oxygen transport, embryo respiration, hypoxia, tracheal system, locust.

Introduction

Aerobic metabolism in most animals requires an adequate supply of oxygen (O_2) to cells. Many terrestrial arthropods possess a powerful tracheal system, which is a tubular network of tracheae and tracheoles that enables diffusive gas transport to all parts of the body (Brusca *et al.*, 2003). For example, insects have an elaborate tracheal system, through which O_2 is delivered in gaseous phase mainly by diffusion and convection (Brusca *et al.*, 2003; Socha *et al.*, 2010; Harrison *et al.*, 2013). However, the tracheal system differs in the level of maturation and O_2 carrying capacity in different developmental stages of an individual. For example, insect eggs possess an underdeveloped tracheal system; the system does not fully mature until the nymph and adult stages in hemimetabolous insects (Myat, 2005; Woods, 2010). Furthermore, the tracheal tubes are filled with liquid during early embryogenesis, thus impeding O_2 transport (Forster & Woods, 2013). However, accelerated embryonic development involves a high rate of aerobic metabolism, which causes a high demand for O_2 (Rakshpal, 1962; Ingrisch, 1987). Such an incongruity raises questions as to how embryos survive oxygen shortages in nature and whether an alternative strategy for embryo respiration exists.

The findings of recent research involving haemocyanins specifically expressed in embryos in the migratory locust *Locusta migratoria* (Orthoptera) provide an opportunity to resolve the above questions. Haemocyanins are copper-containing proteins that are composed of multiple subunits, each containing a dinuclear copper site that is able to bind one O_2 molecule (Linzen *et al.*, 1985). Genes encoding haemocyanins have been found in many insect species since 2004, mostly belonging to ametabolous and hemimetabolous insects (Hagner-Holler *et al.*, 2004; Burmester & Hankeln, 2007). Haemocyanins in *L. migratoria* consist of two distinct subunits, Hc1 and Hc2, which are encoded by four *Hc1* genes and three *Hc2* genes, respectively (Chen *et al.*, 2015b).

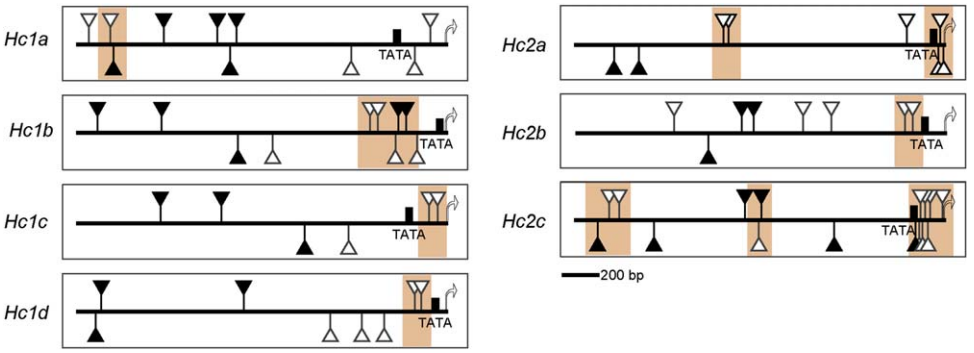

Figure 1. The distribution of putative hypoxia-responsive sequence elements (HREs) in the promoter region of the migratory locust haemocyanin subunit 1 (*Hc1*) and haemocyanin subunit 2 (*Hc2*). The binding sites of HREs were searched for in the 2-kb region upstream of the translation starting codon (ATG). Open triangles represent the hypoxia-inducible factor 1 (HIF-1) binding motif 5'-A/GCGTG-3' in direct (above) and inverted (below) orientation. Filled triangles represent the erythropoietin (EPO) binding sequence 5'-CACAG-3' in forward (above) and reverse (below) orientation. The potentially functional HREs, ie the closely spaced motif combinations of either two HIF binding sites or one HIF plus one EPO site ($<$ 50 bp), are highlighted in brown. The arrows indicate the ATG translation start codon. [Colour figure can be viewed at wileyonlinelibrary.com]

The two subunits are strictly conserved with six histidines that can bind with two Cu^{+} ions for oxygen binding. Haemocyanins are highly abundant in the embryonic stage but are not expressed in late nymphal and adult stages. Functional analysis demonstrated that haemocyanins are crucial for embryonic development and survival in this locust (Chen *et al.*, 2015b). A similar pattern of haemocyanin expression was also observed in the grasshopper *Schistocerca americana* and cockroach *Blaptica dubia*, in which expression of haemocyanin is restricted to embryos (Sanchez *et al.*, 1998; Pick *et al.*, 2010). Taken together, these findings indicate a specialized function of haemocyanins, probably related to gas exchange or sensing in embryos. However, whether the embryo-specific expression of haemocyanins is adaptive and regulated by environmental oxygen conditions remains unknown.

If haemocyanins are essential for embryo respiration, we would expect to see altered expression of haemocyanins evolved in insects living under long-term hypoxic conditions. The locust populations thriving on the Tibetan Plateau appear to have adapted well to the high-altitude hypoxic environment and thus provide a good model for such analysis. The Tibetan Plateau represents one of the most extreme environments for high elevation (with an average elevation of more than 4000 m above sea level) and thus shortage of ambient oxygen. The partial oxygen pressure is about 40% lower in Tibet compared to that in lowland China. Locusts colonized the Tibetan Plateau more than 30 000 years ago (Ma *et al.*, 2012). As an evolutionary consequence, these Tibetan locusts exhibit much higher resistance to oxygen deprivation than lowland locusts (Zhang *et al.*, 2013; Zhao *et al.*, 2013).

In this study, we hypothesized that the embryo-specific expression of haemocyanins is regulated by ambient O_2 availability and has evolved for enhanced oxygen supply in Tibetan locusts, thereby implicating a mechanism for oxygen supply by haemocyanins that complements the tracheal system during embryogenesis. To validate this hypothesis, we examined the expression of *Hc1* and *Hc2* genes in the locusts under different levels of hypoxic conditions. Furthermore, we compared the expression response of the two subunits to hypoxia between lowland and Tibetan locusts. We found that *Hc1* and *Hc2* are highly upregulated under conditions of O_2 deprivation, and that Tibetan locusts have evolved a constitutive high expression of haemocyanin and present robustness to hypoxia exposure. Based on these findings and previous reports, a mechanistic model for embryonic respiration involving the complementary role of haemocyanins and the tracheal system is proposed here. These findings provide new insights into the mechanism for respiration in insect embryos, which is crucial for their adaptation to natural hypoxic environments.

Results

Hypoxia responsive elements (HREs) in the regulatory region of haemocyanin genes

We first examined if there are potentially functional HREs present in the 2 kb promoter region of the four *Hc1* genes, ie *Hc1a*, *Hc1b*, *Hc1c*, *Hc1d*, and the three *Hc2* genes, ie *Hc2a*, *Hc2b*, *Hc2c* (Chen *et al.*, 2015b). HREs are crucial sequence motifs of binding sites by hypoxia-inducible factor 1 (HIF-1) in the regulatory region of many genes and are responsible for transcription activation under hypoxic conditions (Forsythe *et al.*, 1996; Semenza *et al.*, 1996). A functional HRE is defined by having either two potential HIF-1 binding sites or one HIF-1 site plus one erythropoietin (EPO) site that are $<$ 50 bp apart (Semenza *et al.*, 1996; Hankeln *et al.*, 2002). All the *Hc* genes possess at least one HRE

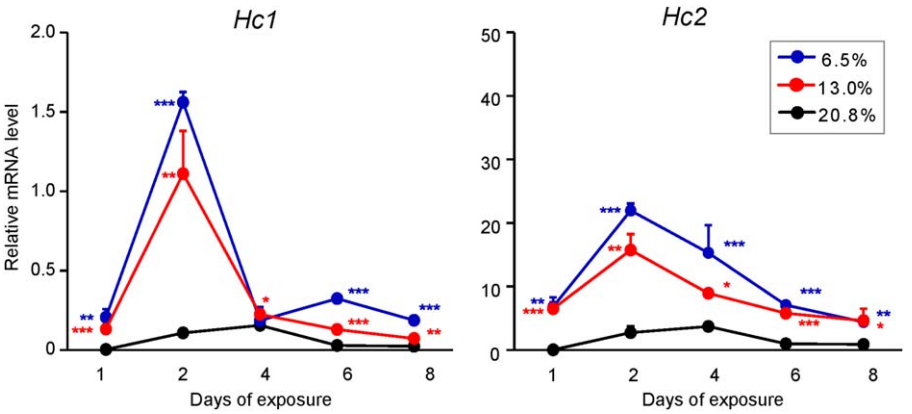

Figure 2. Expression upregulation of the haemocyanin genes *Hc1* and *Hc2* in response to oxygen deprivation in the lowland migratory locust population. Expression of the *Hc1* and *Hc2* genes in locust eggs was measured after the eggs were exposed to three hypoxia levels (6.5, 13.0 and 20.8% O_2) for a range of days (1, 2, 4, 6 and 8 days). The O_2 concentration of 20.8% was used as the normoxic condition (control). *Hc1* and *Hc2* mRNA levels were quantified by real-time quantitative PCR and normalized against that of the internal control (*β-actin*). Three biological replicates of 15 eggs each were used for each treatment group. Error bars indicate +1 SD. Asterisks indicate the significance level as evaluated by a *t*-test: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

binding site in their promoter, but the number of HREs varies amongst gene copies (Fig. 1). *Hc1b* has three HREs and the other three *Hc1* genes have one HRE. *Hc2a* has three HREs and *Hc2b* has one HRE. Remarkably, *Hc2c* harbours 10 HIF-1 binding motifs and six EPO binding motifs, which could potentially constitute more than seven HREs. Most (71%) of the HREs are located in the proximal promoter region (within 500 bp of ATG) (Fig. 1).

We then analysed the natural mutations occurring in the regulatory region, ie within 5 kb upstream of the *Hc1* and *Hc2* genes, in wild locusts. We compared the genomic sequences of 12 locusts collected in the Tibetan Plateau region and 13 locusts in a lowland region in China (Table S1). The results revealed fixed point mutations only in the regulatory region of the *Hc1b* gene (Table S2). There are 10 mutations within this 5 kb region, five of them within a 2 kb region. However, no mutations were associated with the HIF-1 and EPO binding sites.

Embryonic expression of haemocyanins is highly upregulated by oxygen deprivation

We next asked whether haemocyanin expression is associated with ambient O_2 availability. If yes, we would expect to see up- or downregulation of haemocyanin expression in response to O_2 deprivation. To test this, we examined the embryonic expression of *Hc1* and *Hc2* after locust eggs were exposed to different levels of hypoxia in a programmable air chamber. Because the gene copies of *Hc1* or *Hc2* produce identical transcripts (Chen *et al.*, 2015b), we determined the collective transcription level of all *Hc1* or *Hc2* copies. Five-day-old locust eggs were exposed to O_2 concentrations of 20.8 (normoxic control), 13.0 or 6.5% for 1, 2, 4, 6 and 8 days. The results showed that expression of both *Hc1* and *Hc2* was strongly upregulated by O_2 deprivation (Fig. 2). One-day exposure at 13.0 and 6.5% O_2 caused significant upregulation of *Hc1* compared with that under normoxic condition (Students' *t* test, $P<0.001$ at 13.0% O_2 and $P=0.002$ at 6.5% O_2). Under 2-day exposure, *Hc1* expression increased up to 10-fold higher at 13.0% O_2 and 14-fold higher at 6.5% O_2 relative to expression under normoxic conditions. *Hc1* expression decreased at 4 days of exposure, but remained significantly higher than that under normoxic conditions on the sixth day and the eighth day (all $P<0.01$).

Likewise, *Hc2* gene expression was also significantly upregulated after 1 day of exposure to hypoxia ($P<0.001$ at 13.0% O_2 and $P=0.001$ at 6.5% O_2; Fig. 2). *Hc2* expression level peaked on the second day of exposure, reaching fivefold higher at 13.0% O_2 and sevenfold higher at 6.5% O_2 compared to that under normoxia. Then, *Hc2* gene expression gradually decreased from day six to day eight, but still remained significantly higher relative to that under normal air conditions (all $P<0.05$; Fig. 2). Thus, *Hc1* and *Hc2* in eggs exhibit similar patterns of gene expression changes in response to oxygen deprivation, ie expression of both *Hc1* and *Hc2* is readily upregulated to cope with ambient low oxygen supply, and the degree of upregulation of haemocyanins is directly proportional to the severity of hypoxia.

Tibetan locusts exhibit higher constitutive expression of haemocyanins than lowland locusts

We then explored whether the migratory locust has also evolved upregulation of haemocyanins in a naturally hypoxic environment. To test this, we compared the

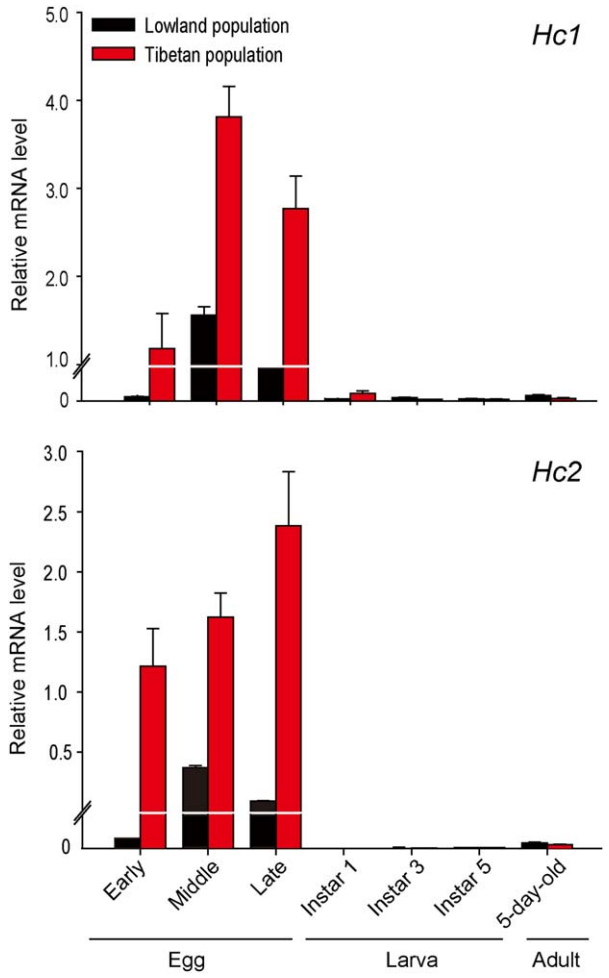

Figure 3. Expression of *haemocyanin genes Hc1* and *Hc2* in migratory locusts from the low-altitude and Tibetan Plateau populations. Early, middle and late stage eggs were those collected when eggs had developed for 3, 7 and 11 days, respectively, under standard conditions (see Experimental procedures). Three to four biological replicates of 15 eggs each were used. The gene expression was normalized against that of the internal control *β-actin* gene. The error bars represent +1 SEM.

expression of haemocyanins in the low-latitude plain population and the Tibetan Plateau population of locusts. The results showed that the two haemocyanin subunits had much higher constitutive expression in embryos in the Tibetan locusts than the plain locusts (Fig. 3). Specifically, *Hc1* expression in the Tibetan locusts was 19-fold, threefold and 15-fold higher than that in the plain locust in the early, middle and late stages, respectively, of embryo development. The differences between the two populations were statistically significant at all three developmental stages ($P = 0.046$, 0.003 and 0.002). Likewise, *Hc2* expression in the Tibetan locusts was significantly higher than that of the plain locusts at all three embryonic stages ($P = 0.018$, 0.003 and 0.007). The difference in *Hc2* expression level between the two populations was 232-fold, threefold and 25-fold in the early, middle and late stages, respectively, of embryo development. As a contrast, *Hc1* and *Hc2* exhibited almost undetectable expression in larval and adult stages in both the lowland and Tibetan populations. Thus, Tibetan locusts appear to have evolved elevated expression of *Hc1* and *Hc2* in embryos to cope with the local hypoxic conditions, which corroborates the role of haemocyanins in oxygen exchange.

Tibetan locusts are less sensitive to oxygen deprivation in haemocyanin expression

If the upregulation of haemocyanins observed in Tibetan locusts is adaptive, we would expect to see well-maintained expression of haemocyanin genes in the embryos of Tibetan locusts exposed to oxygen deprivation. To validate this, we investigated the embryonic expression of haemocyanins in Tibetan locusts exposed to different hypoxia levels. *Hc1* expression at 13.0% O_2 was significantly downregulated compared with that under normoxic conditions at day two ($P = 0.030$) and day four ($P = 0.002$) (Fig. 4). Then, *Hc1* expression increased gradually, reaching a level higher than under normoxia on day eight ($P = 0.033$). *Hc1* expression under 6.5% O_2 was not higher than at normoxia until day six ($P = 0.027$) and remained significantly higher on day eight ($P = 0.034$). Significantly higher *Hc2* expression was elicited by 13.0% O_2 exposure at day six ($P = 0.008$), and by 6.5% O_2 exposure at day two ($P = 0.045$) and day four ($P = 0.002$) (Fig. 4). The results clearly indicate that, compared with the lowland locusts (Fig. 2), the Tibetan locusts exhibit a much lower and delayed upregulation of haemocyanin under environmental hypoxia.

Discussion

This work shows a correlation between haemocyanin expression and oxygen level. Our study also demonstrates that the embryonic expression of haemocyanins is tightly regulated by atmospheric oxygen level, and has evolved to a high level in a high-altitude environment. First, the oxygen responses of haemocyanins in the embryo can be activated by oxygen deprivation possibly through HIF-1, which can bind with HREs present in haemocyanin genes. Different numbers of putative HREs are present in the promoter regions of the *Hc1* and *Hc2* gene copies, implying their potential capacity for transcription activation in response to hypoxia induction. Second, if haemocyanin expression is important for O_2 supply and aerobic metabolism, we would expect upregulation of haemocyanins in response to O_2 deprivation. Indeed, we found that expression of haemocyanins can be readily upregulated by hypoxia. The degree

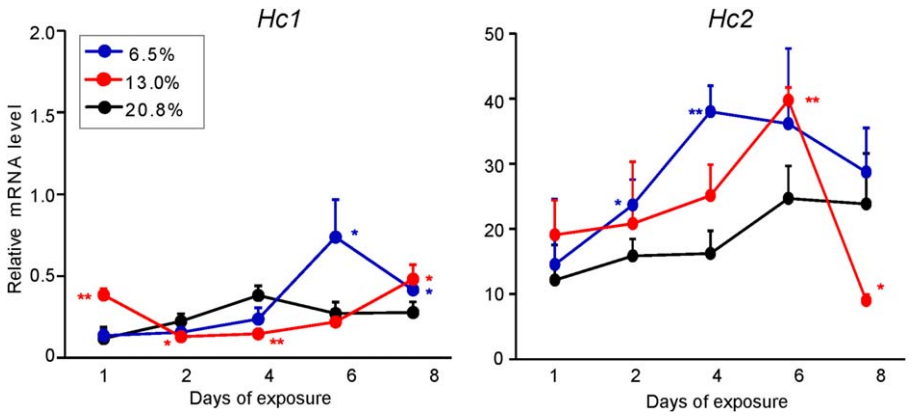

Figure 4. Expression of the haemocyanin genes *Hc1* and *Hc2* in response to oxygen deprivation in the Tibetan Plateau migratory locust population. Expression of the *Hc1* and *Hc2* genes in locust eggs was measured after the eggs were exposed to three hypoxia levels (6.5, 13.0 and 20.8% O_2) for a range of days (1, 2, 4, 6 and 8 days). The O_2 concentration of 20.8% was used as the normoxic condition (control). *Hc1* and *Hc2* mRNA levels were quantified by real-time quantitative PCR and normalized against that of the internal control (*β-actin*). Three biological replicates of 15 eggs each were used for each treatment group. Error bars indicate +1 SD. Asterisks indicate the significance level as evaluated by a *t*-test: *, $P < 0.05$; **, $P < 0.01$.

of upregulation of haemocyanin expression increased with intensity of ambient hypoxia. Third, the haemocyanin expression in Tibetan locusts has evolved to a level several-fold higher than in lowland locusts. The result suggests that the upregulation of haemocyanins is functionally important and thus has been selected for by the hypoxic environment of the Tibetan Plateau. In addition to our findings, a previous oxygen binding assay demonstrated significant oxygen affinity in adult haemolymph containing haemocyanins in stoneflies (Hagner-Holler *et al*., 2004). Given these results, it is unlikely that haemocyanins in eggs act mainly as immune factors or storage proteins that provide energy and amino acids (Coates & Nairn, 2014). Locust haemocyanins are mainly present in the embryo epidermis (Chen *et al*., 2015b). A haemocyanin homologue in the grasshopper *Sc. americana* is expressed in haemocytes attached to the basal membrane of embryos (Sanchez *et al*., 1998). These data suggest the involvement of haemocyanins in embryonic respiration as well as sensing of the environment.

Embryo-specific high expression of haemocyanins seems to be associated with aerobic respiration and development of tracheal systems during embryogenesis. The rate of oxygen consumption increases steadily and rapidly during embryonic development (Rakshpal, 1962; Madhavan, 1975; Ingrisch, 1987). The oxygen consumption by developing eggs of the cricket *Eupholidoptera smyrnensis* increases from 3.5 μl per gram per hour at stage 4 to 37.5 μl at stage 20 of the total 24 stages at 24 °C. The oxygen consumption rises to 56.1 μl at stage 20 at 30 °C (Ingrisch, 1987). In the aquatic hemipteran *Sphaerodema molestum*, the rate of oxygen consumption during embryogenesis shows an increase from 8.5 μl per 100 eggs per hour at the freshly laid stage to 123.8 μl at hatching (Madhavan, 1975). Therefore, embryos have a rapidly increasing, high oxygen demand during development. However, the tracheal systems in embryos are far from functionally mature for gas exchange. The tracheal network starts to form from plates of ectodermal epithelial cells only from stage 11 of the total 17 stages of *Drosophila* embryonic development (Wilk *et al*., 1996; Myat, 2005). Furthermore, the tracheal systems in embryos are initially filled completely with liquid (Forster & Woods, 2013). Thus, there is a deficit of oxygen available for respiration and metabolism owing to the high rate of oxygen consumption for aerobic respiration and low rate of oxygen supply through the tracheal systems during embryogenesis. Here, we propose a mechanistic model for the oxygen supply required for embryo respiration based on our findings (Fig. 5). In this model, the respiratory protein haemocyanin is sufficiently synthesized to meet the high oxygen demands of embryos until the hatching stage, when advanced tracheal systems are fully developed. In other words, haemocyanin serves as an essential functional complement to the tracheal systems for oxygen transport in locust embryos. This complementary pathway featuring haemocyanin as an O_2 carrier serves as a safe and efficient mechanism of respiration during periods of low oxygen supply to the egg or periods of accelerated embryonic growth with high rates of oxygen consumption. Therefore, insects might be amongst the animals that possess both respiratory mechanisms for O_2 exchange.

The mutually supplementary roles of haemocyanin and the tracheal system for tissue O_2 supply are conserved in many arthropod species. Phylogenetic analysis

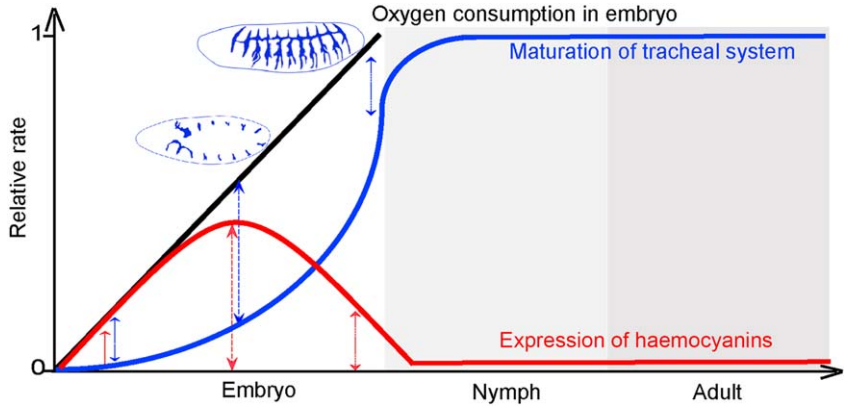

Figure 5. Schematic representation showing complementary mechanisms of oxygen supply for aerobic metabolism in migratory locust embryos. Data from two orthopteran species have shown that, in general, the rate of oxygen consumption increases rapidly during embryogenesis from the freshly laid stage to hatching (Rakshpal, 1962; Ingrisch, 1987) (see black line). Data for relative development rate of the tracheal system were obtained from studies in *Drosophila* (Wilk *et al.*, 1996; Myat, 2005) (see blue line). The tracheal network is formed from plates of ectodermal epithelial cells starting at stage 11 of the 17 total embryonic stages (Myat, 2005). Images at the top are schematic drawings of the tracheal system in middle-term embryos (ie at stage 11 in *Drosophila*) and late-term embryos (ie at stage 14) (Myat, 2005). The expression pattern of haemocyanins is based on results recently reported (Chen *et al.*, 2015b) and in Fig. 3 (see red line). The vertical lines with double arrows in blue indicate oxygen deficit between the rate of oxygen consumption for aerobic respiration and the rate of oxygen supply through the tracheal system at the early, middle and late embryonic stages. The developmental pattern of haemocyanin expression is consistent with that of oxygen deficit, indicating that oxygen shortage is balanced by increased oxygen transport by haemocyanins (indicated by vertical lines with double arrows in red).

indicates that haemocyanins in hemimetabolous insects may be derived from crustacean haemocyanins that possess oxygen-carrying capacity (Hagner-Holler *et al.*, 2004; Chen *et al.*, 2015b). Holometabolous insects lost their haemocyanins, and the majority of them instead evolved haemoglobins, which are not found in eumetabolous insects (mostly non-holometabolous) (Burmester & Hankeln, 2007). The role of supplying O_2 in embryos of the insect species lacking haemocyanin may be carried out by other respiratory proteins, such as haemoglobins (Hankeln *et al.*, 2002), or other physiological mechanisms (Hoback & Stanley, 2001; Harrison *et al.*, 2006). Mutual complementation of these two respiratory systems is not necessarily limited to embryos and may be extended to later developmental stages, such as the adult stage in stoneflies and firebrats (Hagner-Holler *et al.*, 2004; Pick *et al.*, 2008). In addition to hemimetabolous insects, myriapods (millipedes and centipedes) also have both a tracheal system and circulating haemocyanins (Damsgaard *et al.*, 2013). Therefore, evolution of a tracheal system for efficient O_2 supply in insects has not caused loss of respiratory proteins.

The high expression of haemocyanins in embryonic stages probably enhances oxygen transport capacity, which could be essential for embryo development and survival in nature. Insect eggs are often exposed to hypoxic environments, such as high altitude, aquatic conditions, soil or fermenting fruits where eggs are deposited, or subjected to temporary flooding (Hoback & Stanley, 2001). Tibetan locusts have evolved two- to six-fold higher constitutive expression of embryonic haemocyanins compared to the low-altitude locusts studied here. A few fixed point mutations were found in the regulatory region of the *Hc1b* gene in the Tibetan locusts compared with the lowland locusts. The observed *cis*-regulatory mutations may be responsible for the variation in expression level. However, *trans*-regulatory changes, such as those in the transcription factors or protein complexes that bind to the promoters of *Hc* genes, are more likely to be the factors governing the differential expression response to hypoxia. The high expression of haemocyanins may impart enhanced oxygen transport capacity to embryos of Tibetan locusts. Induction of haemocyanin synthesis may be one of the most primary responses of the embryo to lowered O_2 levels, which could influence or even coordinate a cascade of systematic responses to hypoxia. Therefore, upregulation of haemocyanin is of adaptive significance when insect eggs are exposed to natural hypoxic conditions.

Experimental procedures

Wild and laboratory-reared samples of locusts

Sources of the low-altitude plain population and Tibetan Plateau wild population of locusts are the same as reported previously (Zhang *et al.*, 2013). Briefly, more than 500 solitary locust nymphs were field caught in China: the low-altitude plain population was collected in Wudi (64 m altitude, 37°41′N, 117°31′E), Shandong province, whereas the high-altitude plateau population was collected in Maizhokunggar (4000 m, 29°46′N, 91°46′E), Tibet. The collected locusts were reared under standard conditions (see below) in our laboratory in Beijing (< 50 m

in altitude). Locust samples had been maintained for at least five generations prior to use in our experiments. The laboratory lines of locusts, which were used for all experiments unless stated otherwise, were derived from the plain populations. The locust samples subject to genome resequencing were collected from four localities in the Tibetan Plateau and five localities in the lowland region (Table S1).

Locusts were collected and maintained in the laboratory according to a method reported previously (Chen *et al*., 2015b). Briefly, locusts were reared in cages (each 40 × 40 × 40 cm) equipped with sand box at a density of approximately 400 individuals per cage. Nymphs and adults were fed on fresh wheat seedlings and bran and maintained under a 14:10 h (light : dark) photoperiod at 30 ± 2 °C. Egg pods deposited in sand were collected twice a day and kept in a plastic cup (diameter of 6 cm and height of 9 cm) filled with sterilized sand with 8% humidity. The eggs were maintained in a 30 ± 1 °C incubator until use for hypoxia treatment, RNA extraction or double-stranded RNA injection.

Normobaric hypoxic treatments

To study the responses of haemocyanin expression to ambient oxygen availability, eggs were subjected to a series of oxygen partial pressures. Normobaric hypoxic treatments were performed in an enclosed air chamber (FLYDWC-50, Fenglei Oxygen Chamber Co. Ltd, Guizhou, China) in which air and nitrogen supply, oxygen partial pressure and temperature can be controlled precisely. Differential oxygen partial pressure was achieved by inputting different proportions of normoxic air and pure nitrogen. Five-day-old eggs were exposed to composite air containing 6.5, 13 and 20.8% O_2 (control for normoxic air) for 1, 2, 4, 6 and 8 days at 30 ± 1 °C. Fifteen eggs were used in each of the three replicate treatments. Samples were then frozen in liquid nitrogen prior to RNA purification.

Quantification of haemocyanin gene expression

Although the four gene copies of *Hc1* and the three gene copies of *Hc2* differ in gene sequences, the gene copies of *Hc1* or *Hc2* produce identical mRNA transcripts (Chen *et al*., 2015b). Thus, the mRNA level of gene copies cannot be quantified separately using gene copy-specific primers in quantitative PCR but instead must be measured in a collective manner using *Hc1*- or *Hc2*-specific primers. mRNA level was measured by quantitative real-time PCR using a SYBR Green I kit (Roche, Mannheim, Germany). PCR was performed on a LightCycler® 480 Real-Time PCR System (Roche, Rotkreuz, Switzerland). The PCR programme was as follows: 95 °C for 2 min and then 40 cycles of 95 °C for 20 s, 58 °C for 20 s and 68 °C for 20 s. *β-actin* was used as an internal control (Wang *et al*., 2006). The specificity of amplification was confirmed by melting curve analysis. Relative expression was quantified by the comparative cycle threshold method ($2^{-\Delta Ct}$ method) after normalizing against the internal control (Chen *et al*., 2015a). All reactions were performed in triplicate. The primers of *Hc1* and *Hc2* were obtained from a previous report (Chen *et al*., 2015b) and the primers of *β-actin* were from another previous report (Wang *et al*., 2006). Analysis of the genomic sequences indicated that no mutations occurred at the primer sites (Table S2). Thus, the primers apply to both the Tibetan and lowland locusts.

Sequence and data analysis

The 2-kb genomic regions upstream of the translation start codon (ATG) of the four *Hc1* genes and the three *Hc2* genes were obtained from the locust genome sequence and gene annotations (Wang *et al*., 2014; Chen *et al*., 2015b). The promoter regions were screened for putative HIF-1-responsive elements ('core' sequence: 5'-A/GCGTG-3') and the EPO binding sequence (5'-CACAG-3'). We used locusts from the Wudi (low-altitude) population for the HRE analysis. The genomic sequences of *Hc1* and *Hc2* genes of wild locusts were obtained through population genome resequencing (genome data unpublished). The 8× whole genomes of 12 locusts collected from the Tibetan Plateau and 13 locusts from the lowland area were sequenced (Table S1). The insertion/deletion and single nucleotide mutations in the gene region and within 5 kb downstream or upstream of the genes were analysed. An independent *t*-test was performed to compare differences in gene expression. Differences were considered statistically significant if $P < 0.05$, with the significance level indicated as * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Data were analysed using SPSS 16.0 software (SPSS, Chicago, IL, USA).

Acknowledgements

Funding was provided by the National Science Foundation of China (31661143048, 91331106 and 31472048).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Localities of sample collection sites for each locust subject to genome resequencing.

Table S2. The single nucleotide polymorphisms (SNPs) in the *haemocyanin 1* (*Hc1*) and *Hc2* genes in natural populations of the migratory locusts. A total of 12 locusts from four localities in the Tibetan Plateau and 13 locusts from five localities in a lowland region were analysed. Detailed information on the sampling sites is provided in Table S1. Downstream_2K means the genomic region located as far as 2 kb from the 3′ end of the *Hc* genes. Downstream_5K means the region located between 2 and 5 kb from the 3′ end of the *Hc* genes. Scaffold position for each gene: gene *Hc1a* at scaffold3213: 2217826:2344859; gene *Hc1b* at scaffold3213: 2030847:2180556; gene *Hc1d* at scaffold3213: 2146155:2156478; gene *Hc2a* at scaffold8098:543695:566378; gene *Hc2b* at scaffold8098:609791:637215. – represents not covered by sequencing.