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Haemocyanin is essential for embryonic development and survival in the migratory locust

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Abstract

Haemocyanins are commonly known as copper-containing oxygen carriers within the haemolymph of arthropods, and have been found in many orders of insects. However, it remains unresolved why haemocyanins persist in insects that possess elaborate tracheal systems for oxygen diffusion to cells. Here we identified haemocyanins in the migratory locust *Locusta migratoria* that consists of two distinct subunits, Hc1 and Hc2. Genomic sequence analysis indicated that *Hc1* and *Hc2* have four and three gene copies, respectively, which may have evolved via gene duplication followed by divergent evolution of introns. The two subunits exhibit abundant and embryonic-specific expression at the mRNA and protein level; their expression peaks in the mid-term embryo and is not detectable in the late nymphal and adult stages. A larger proportion of the haemocyanins is present in the yolk compared with that in the embryo. Immunostaining shows that haemocyanins in the embryo are mainly expressed in the epidermis. Knockdown of *Hc1* and *Hc2* results in significant embryonic developmental delay and abnormality as well as reduced egg hatchability, ie the proportion of hatched eggs. These results reveal a previously unappreciated and fundamental role for haemocyanins in embryonic development and survival in insects, probably involving the exchange of molecules (eg O_2) between the embryo and its environment.

Keywords: haemocyanin, respiratory protein, embryonic development, locust, developmental delay.

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Introduction

Haemocyanins are copper-containing proteins that transport oxygen in many arthropods and molluscs (Linzen *et al.*, 1985). Arthropod haemocyanins originated more than 550 million years ago from oxygen-consuming phenoloxidases (Burmester, 2004). Haemocyanins are hexamers or oligomers of hexamers, freely dissolved in the haemolymph (Markl & Decker, 1992). A subunit of hexamer can bind one oxygen molecule between two copper atoms, each of which is coordinated by three histidines in two distinct binding sites (Linzen *et al.*, 1985; Decker *et al.*, 2007). Haemocyanins acting as respiratory proteins have been thoroughly investigated in various arthropod subphyla (Burmester, 2001, 2004). Besides being oxygen carriers, haemocyanins exhibit multiple functionalities in homeostatic and physiological processes, eg moulting, hormone transport, innate immunity, osmoregulation and protein storage (Decker *et al.*, 2007; Coates & Nairn, 2014).

In insects, gas exchange is usually facilitated by their elaborate tracheal system, which consists of highly branched air-filled tubes connecting the inner tissues with the atmosphere (Brusca *et al.*, 2003). Oxygen is delivered in gaseous form through this system to every part of the body mainly by diffusion and convection (Socha *et al.*, 2010; Harrison *et al.*, 2013). Therefore, respiratory proteins were thought to be unnecessary for oxygen delivery in insects (Rubin, 2000; Willmer *et al.*, 2004). However, contrary to this assumption, haemocyanin genes have been found in many insect species since 2004, including ametabolous and hemimetabolous insects (Hagner-Holler *et al.*, 2004). These findings of the widespread occurrence of haemocyanin in insects have implications for their potential association with insect respiration (Burmester, 2001; Burmester &

Hankeln, 2007; Pick *et al*., 2008, 2010). For example, studies with the stonefly *Perla marginata* demonstrated oxygen-binding properties of haemolymphs containing haemocyanin (Hagner-Holler *et al*., 2004). However, it remains unresolved why haemocyanins persist as respiratory proteins in insects that possess elaborate tracheal systems for oxygen diffusion to cells.

Unlike most other animals, insects go through distinct developmental stages that involve living in different oxygen conditions. For example, hemimetabolous insects have three developmental stages, ie egg, nymph and adult. These stages differ in oxygen availability and the oxygen carrying capacity of the tracheal system. The tracheal system is very rudimentary at the egg stage and does not become fully mature until the nymph stage (Myat, 2005; Woods, 2010). Furthermore, the tracheal tubes are initially filled with liquid during embryogenesis, thus impeding transport of oxygen (Forster & Woods, 2013). However, the high rate of aerobic metabolism involved in accelerated embryonic development creates a high demand for oxygen (Rakshpal, 1962; Ingrisch, 1987), which cannot be met by simple diffusion. Furthermore, because eggs are small, and almost always isothermal with their local microclimate, they are often challenged by oxygen shortage in fluctuating environments, even at only mildly high temperatures (32–37°C; Woods & Hill, 2004; Woods *et al*., 2005). It is thus critical for embryos to have a reliable mechanism for the storage and transport of oxygen. Pick *et al*. (2010) found that the expression of haemocyanin is restricted to late embryos and first-instar nymph in the cockroach *Blaptica dubia*. Embryonic expression of haemocyanin in *B. dubia* and *Schistocerca americana* implies that haemocyanin may evolve a specialized function related to gas exchange or storage in embryos (Sanchez *et al*., 1998; Pick *et al*., 2010). However, how haemocyanin expression influences embryonic development and to what extent embryonic survival relies on haemocyanin functioning still remain elusive.

In this study, we identified haemocyanins in the migratory locust *Locusta migratoria* (Orthoptera: Acrididae) and found that haemocyanin exhibits high expression at the embryonic stage. We hypothesized that haemocyanins are critical elements of embryonic respiration physiology, and are thus important for embryonic development and survival. To validate this, we cloned and characterized the two subunits of haemocyanin, namely Hc1 and Hc2, in the locust. Genomic sequence analysis suggested that copies of the haemocyanin genes have expanded probably through gene duplication events. Whole-mount immunostaining revealed a high level of expression of haemocyanin in embryonic cuticles and the underlying epidermis. Embryonic RNA interference (RNAi) of the two subunits resulted in significant developmental retardation and abnormality in the embryos. These findings provide new insight into the mechanism for embryonic respiration and regulation of embryogenesis in insects.

Results

Molecular and phylogenetic characterization of locust *Hc1* and *Hc2*

Full-length cDNA sequences of *Hc1* and *Hc2* (GenBank accession number: KJ713391 and KJ713392) obtained through 5′- and 3′-rapid amplification of cDNA ends (RACE) were subjected to BLAST search against the whole-genome sequence of the migratory locust (Wang *et al*., 2014). BLAST results with alignment threshold *E* value = 0 revealed that the *Hc1* gene has four copies in the locust genome, ie *Hc1*a, *Hc1*b, *Hc1*c and *Hc1*d, whereas *Hc2* has three, ie *Hc2*a, *Hc2*b and *Hc2*c (Fig. 1). The four *Hc1* genes are located in tandem in one orientation (Fig. 1A). The *Hc1* genes harbour nine exons that are the same amongst the gene copies, and eight introns that differ greatly in length amongst them. The three *Hc2* genes are also located in tandem in one orientation (Fig. 1B). Each of the *Hc2* genes has 12 exons that are same amongst the gene copies, and 11 introns that differ greatly in length amongst them. The coding sequences of the gene copies of *Hc1* and *Hc2* are thus identical as no alternatively spliced transcripts were found in the embryonic transcriptomes (Chen *et al*., 2010). Sequence alignment showed that the introns of *Hc1* and *Hc2* exhibit high sequence similarity amongst gene copies. For example, the overall amongst-gene copy pairwise distance of intron 2 in four copies of *Hc1* is 0.209 whereas that of intron 3 is 0.208. However, no nucleotide sequences similar to any of these introns were found in the National Center for Biotechnology Information nucleotide database (http://www.ncbi.nlm.nih.gov/). These results suggest that the introns in the four *Hc1* genes as well as the introns in the three *Hc2* genes have a common and locust species-specific origin. Furthermore, these data suggest that locust haemocyanin genes evolved via gene duplication followed by divergent evolution of introns.

The transcript of *Hc1* contains a 2016-bp open reading frame (ORF) encoding 672 amino acids, with a predicted molecular weight of 78.0 kDa. *Hc2* contains a 2046-bp ORF encoding 682 amino acids, with a predicted molecular weight of 78.2 kDa. The two subunits have three functional domains (Supporting Information Fig. S1). The second domain, ie the oxygen binding domain, is highly conserved, and the other two domains are divergent in sequence. The two subunits are strictly conserved with six histidines in the second domain that can bind with two Cu^{+} ions and are thus crucial for oxygen binding (Linzen *et al*., 1985). The conserved residues also include three

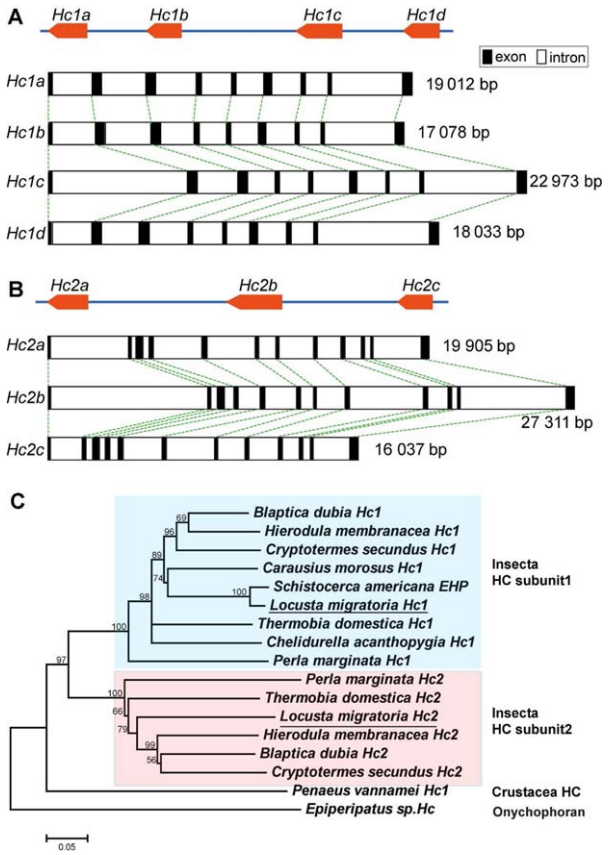

Figure 1. Gene structure and phylogenetic tree of the two haemocyanin subunits Hc1 and Hc2. (A) Genomic organization and gene structure of the four *Hc1* gene copies *Hc1*a, *Hc1*b, *Hc1*c and *Hc1*d (top to bottom). The four gene copies occur in tandem in one orientation on the same scaffold. Each gene copy has nine exons, and each exon is identical amongst the four copies. The number beside each bar denotes the length of the gene. (B) Genomic organization of the three *Hc2* gene copies and their gene structures. The three *Hc2* copies, namely, *Hc2*a, *Hc2*b and *Hc2*c, also occur in tandem in the same orientation. Each copy has 12 exons, which are identical amongst the three copies. (C) Phylogenetic relationships of the two subunits of arthropod haemocyanins. The phylogenetic tree was deduced from amino acid alignment of the two haemocyanin subunits of the locust *Locusta migratoria* and 14 other arthropod species. *Penaeus vannamei* Hc1 and *Epiperipatus* sp. Hc were used as outgroups. The numbers at the nodes represent bootstrap support values obtained by the neighbour-joining method. The scale bar represents 0.05 substitutions per site. The two branches shaded in blue and red indicate clusters of Insecta *Hc1* and *Hc2*, respectively. The locust *Hc1* and *Hc2* genes that are the subjects of this study are underlined. EHP, embryonic haemolymph protein.

phenylalanines that stabilize binding of oxygen in the first and second domains. In addition, *Hc1* and *Hc2* are predicted to contain an 18-bp and a 20-bp typical signal peptide sequence, respectively (Fig. S1).

Amino acid sequences of *Hc1* and *Hc2* in nine insect species, one crustacean species and one onychophoran species were used to construct a phylogenetic tree (Fig. 1C). Insect haemocyanin subunits formed a monophyletic clade; all insect *Hc1* clustered together as a subclade and *Hc2* clustered as another subclade. This suggests that the two haemocyanin subunits occurred in the common ancestor of these insects and evolved independently after the speciation event.

Extensive expression of haemocyanin Hc1 and Hc2 in locust embryo

mRNA levels of *Hc1* and *Hc2* in the locust eggs were examined at three developmental stages, namely, egg, nymph and adult, by real-time quantitative PCR. *Hc1* exhibited low expression in 2-day-old eggs, but increased by 73-fold in 5-day-old embryos, which have identifiable abdominal appendages (Fig. 2A). *Hc1* expression increased further in 8-day-old eggs, reaching 3.3-fold higher than that in 5-day-old eggs. However, *Hc1* expression decreased thereafter, reaching 12% of the level in 8-day-old eggs by the 11th day. The new hatchlings, ie the first instar nymphs, also had very low *Hc1* expression, at only 1% of the level found in 8-day-old eggs. No *Hc1* expression was detected in third and fifth instar nymphs. Likewise, no *Hc1* expression was detected in the whole-body and leg tissue of adults, except in the ovaries, where the expression level of *Hc1* was extremely low, at 1% of that observed in 8-day-old eggs (Fig. 2A).

The expression pattern of *Hc2* throughout development was highly similar to that of *Hc1*. Expression of *Hc2* increased sharply with development and peaked on day eight, reaching 307-fold higher than that on day two. *Hc2* expression then decreased six-fold relative to that on day eight by the 11th day. *Hc2* expression in new hatchlings was also low, at only 0.6% of that on day eight. No *Hc2* expression was detected at later nymphal stages or in adults (Fig. 2A).

Western blot analysis showed a developmental pattern of protein expression of Hc1 and Hc2 consistent with that of the mRNA level determined by real-time quantitative PCR except in first-instar nymphs. Hc1 protein was barely detectable in 2-day-old eggs, but highly abundant in 5-day-old and 11-day-old eggs. Hc1 protein was also detected in first-instar nymphs, but not in later nymphal stages and adults. The Hc2 protein level followed the same pattern as that observed for Hc1 throughout development (Fig. 2B).

In order to determine the abundance of haemocyanin in eggs, we measured the levels of expression of *Hc1* and *Hc2* relative to that of *actin* in 8-day-old eggs. Expression levels of *Hc1* and *Hc2* were 36-fold and 24-fold higher, respectively, than that of *actin* (Fig. S2A). This suggests that haemocyanins constitute a large proportion of the proteins present in locust eggs. The early egg is full of yolk, which is a rich source of nourishment for embryo development and provides a substrate for exchange between the embryo and the environment

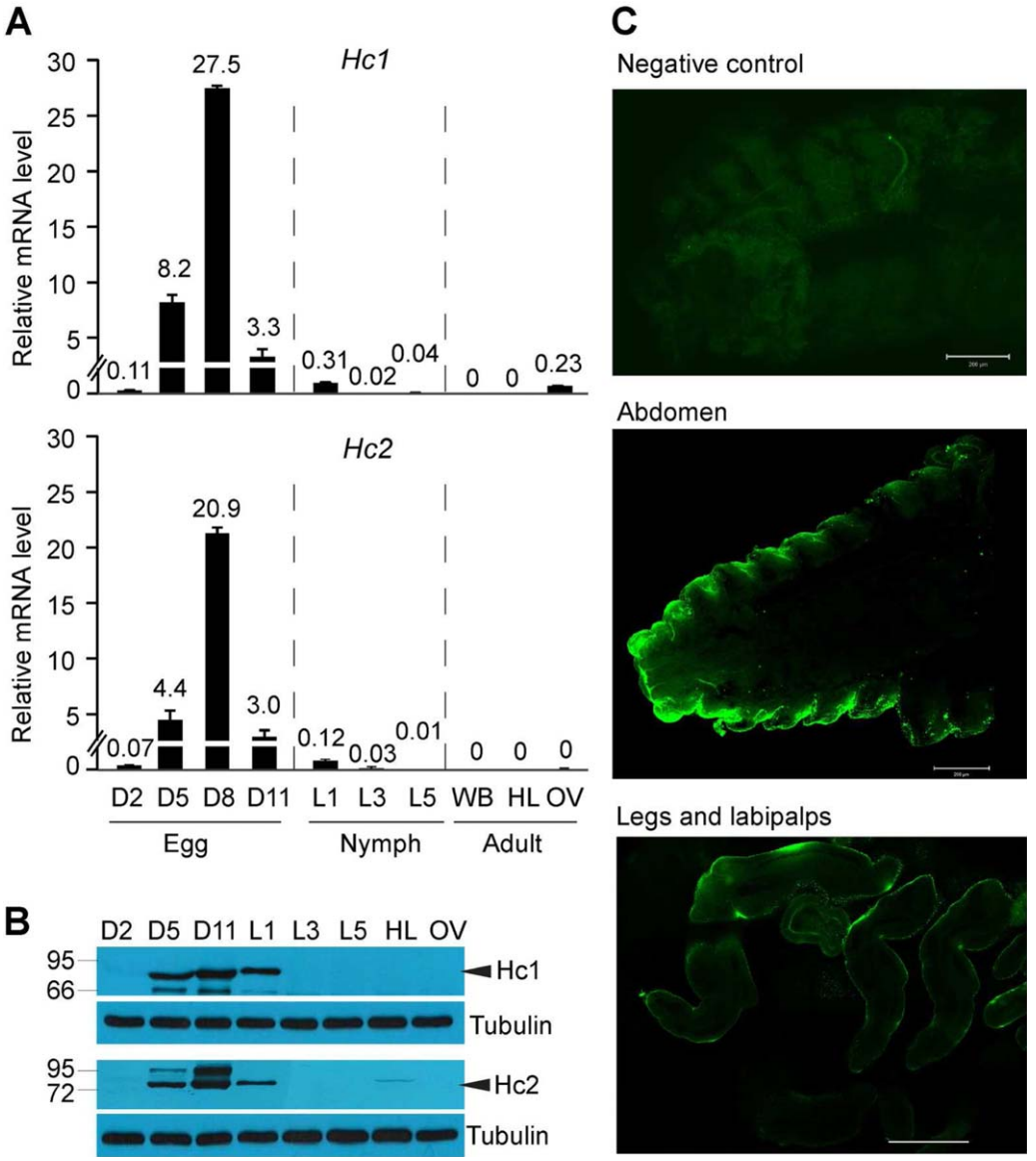

Figure 2. Developmental expression of the *haemocyanin subunit 1* and *2* (*Hc1* and *Hc2*) genes in the locust *Locusta migratoria*. (A) mRNA levels of *Hc1* and *Hc2* at embryo, nymph and adult stages. D2, D5, D8 and D11 denote 2, 5, 8 and 11 days post-egg deposition, respectively. L1, L3 and L5 denote the first, third and fifth instar of the nymph stage, respectively. WB, HL and OV denote the whole-body tissue, haemolymph and ovary of 5-day-old adults, respectively. The exact mRNA level for each stage is labelled on the top of each bar. The mRNA level was quantified by real-time quantitative PCR and normalized against the internal control *ribosomal protein 49 rp49*. Five biological replicates were used. The error bars indicate ±1 SEM. (B) Western blot analysis for expression of Hc1 and Hc2 proteins in the different developmental stages. β-tubulin was used as an internal control for total protein amounts. The protein marker (molecular mass) is shown on the left. (C) Immunostaining for Hc1 protein distribution in the abdomen (middle image), legs and labipalps (bottom image) of 9-day-old embryos. The negative control shows the abdomen in which no primary Hc1 antibody was used (upper image). Scale bars = 200 μm.

(Peel & Akam, 2007). We therefore sought to compare the expression of haemocyanins between yolks and embryos. *Hc1* expression in yolks (including the haemolymph) was nine-fold higher than that in embryos, whereas *Hc2* expression in yolks was seven-fold higher that in embryos (Fig. S2B). These results indicate that a large proportion of the haemocyanins in mid-term locust eggs is present as soluble protein in yolk and in the haemolymph.

To further investigate the distribution of haemocyanin in embryos, we examined the localization of the Hc1 protein in embryos by whole-mount immunostaining. The primary antibody for Hc1, which demonstrated high specificity in Western blot assays (Fig. S3), was selected for the staining. Confocal imaging revealed high levels of expression of Hc1 in embryonic cuticles and the underlying epidermis (Fig. 2C). Specifically, Hc1 expression was found to be extremely high in the epidermis of the

abdomen and between all abdominal segments. Hc1 staining was also rich in the epidermis of appendages such as the legs and labipalps. No signal was detected in the same tissues in non-antibody controls, which confirmed the specificity of our staining.

Inhibition of haemocyanin expression caused developmental retardation and abnormalities in the embryo

Next, developmental changes of the locust embryo were examined after knockdown of *Hc1* expression. Double-stranded RNA (dsRNA) of *Hc1* was injected into 5-day-old eggs, and gene expression was examined on the second and on the fourth days after the injection to test the efficacy of RNAi-mediated knockdown (Fig. 3A). On day two, expression of *Hc1* in eggs with *Hc1* dsRNA injection was reduced by 54%, which was significantly different from that in the control eggs, which received an injection of *green fluorescent protein* (*GFP*) dsRNA ($P = 0.008$). No significant difference was found in the expression of *Hc1* and *Hc2* between *GFP*-injected and non-injected samples. On day four, expression of *Hc1* still remained at a significantly lower level compared with the control ($P = 0.018$). Injection of *Hc1* dsRNA did not affect expression of *Hc2* on days two and four (Fig. 3A). The effect of RNAi on Hc1 expression was validated at the protein level by Western blot (Fig. 3B).

Different doses of *Hc1* dsRNA were applied to 5-day-old eggs in which the embryos had developed to stages 17–18 of the 27 total stages, and the effects on developmental rate of visibly normal embryos were examined. On day two after dsRNA injection, embryos developed significantly slower than the control at four doses: 0.5, 1.0, 2.0 and 5.0 μg/μl (Fig. 3C). Specifically, embryos injected with *Hc1* RNAi at these four doses lagged behind control embryos by 1.6, 1.9, 2.6 and 3.5 stages, respectively (Mann–Whitney *U*-test, $P < 0.001$ for the four treatments). On day four, a significant difference in development was detected at dosages of 0.5, 1.0 and 2.0 μg/μl ($P = 0.012$, <0.001 and <0.001, respectively). On the sixth day, significant difference was observed at 1.0 and 2.0 μg/μl ($P = 0.013$ and 0.014, respectively). Thus, repression of *Hc1* gene expression resulted in delay of embryonic development. Furthermore, the developmental delay was *Hc1* dsRNA dosage-dependent and increased with dosage of *Hc1* dsRNA at least in the range of 0.5 to 2.0 μg/μl. The effects of 5.0 μg/μl dsRNA were weaker than that of lower dosages probably because this dsRNA dosage was oversaturated for target recognition, leading to an inefficient trigger of the RNAi system (Haley & Zamore, 2004).

Hc1 repression also caused apparent developmental arrest and abnormalities. These embryonic abnormalities included involution failure, shrinking embryonic size, transformed or deformed appendages, disorganized thorax or abdomen, and deformed- or black-faceted eyes. Meanwhile, yolk pileup in eggs was observed upon *Hc1* or *Hc2* knockdown by RNAi (Fig. 3D, Fig. S4). Compared with the control, knockdown of *Hc1* expression resulted in a significant increase in the percentage of abnormalities by 11-fold, fourfold and fivefold at 1.0, 2.0 and 5.0 μg/μl dsRNA concentrations, respectively (Fisher's exact test, $P = 0.009$, 0.005 and 0.033; Fig. 3E). The abnormality frequency reached a maximum of 77% at 2.0 μg/μl *Hc1* dsRNA injection. There was no significant difference at 0.5 μg/μl.

Hc1 knockdown also resulted in high mortality at ecdysis, thus reducing the egg hatchability that is defined as the proportion of eggs hatched successfully (Fig. 3F). *Hc1* knockdown caused a 2.5-fold reduction in egg hatchability at 1.0 μg/μl and 7.0-fold reduction at 2.0 μg/μl. Hatchability was not significantly different between embryos injected with ds*Hc1* and ds*GFP* at 0.5 μg/μl.

We also investigated the effects of subunit *Hc2* knockdown on embryonic development. Two days after microinjection of *Hc2* dsRNA, *Hc2* expression was significantly reduced by 47% compared with the control ($P = 0.007$) without any effect on expression of *Hc1* (Fig. S5A). The microinjection of *Hc2* dsRNA also reduced the protein level of Hc2 (Fig. S5B). *Hc2* knockdown at 1.0 μg/μl also caused a delay in the development of embryos. On days four and six after microinjection, embryos injected with *Hc2* dsRNA presented developmental delays of 1.3 and 0.6 stages, respectively, which were both significantly different from the control ($P = 0.001$; Fig. S5C). *Hc2* knockdown also caused prominent developmental abnormalities in embryos. These abnormal traits were similar to those caused by *Hc1* knockdown (Fig. S4). The frequency of abnormal embryos increased significantly by 92% upon *Hc2* knockdown ($P = 0.018$; Fig. S5D). However, no difference in the hatchability of eggs was observed between ds*Hc2* and ds*GFP* (Fig. S5E). These results indicate that knockdown of *Hc2* had a milder effect on embryonic development than knockdown of *Hc1*.

If haemocyanins play a role in oxygen transport in the embryo, the proper functioning of haemocyanin could be affected by oxygen deprivation. To test this, we examined the responses to hypoxia of embryos subjected to *Hc1* knockdown. Compared with normoxia treatment (Fig. 3), 4-day hypoxia exposure to 5% oxygen resulted in slower embryonic development (Fig. S6A), higher percentage of embryonic abnormalities (Fig. S6B) and lower egg hatchability in ds*GFP*-injected eggs (Fig. S6C). *Hc1* knockdown also significantly slowed down embryonic development by 1.2 stages ($P < 0.001$; Fig. S6A), increased the frequency of embryonic

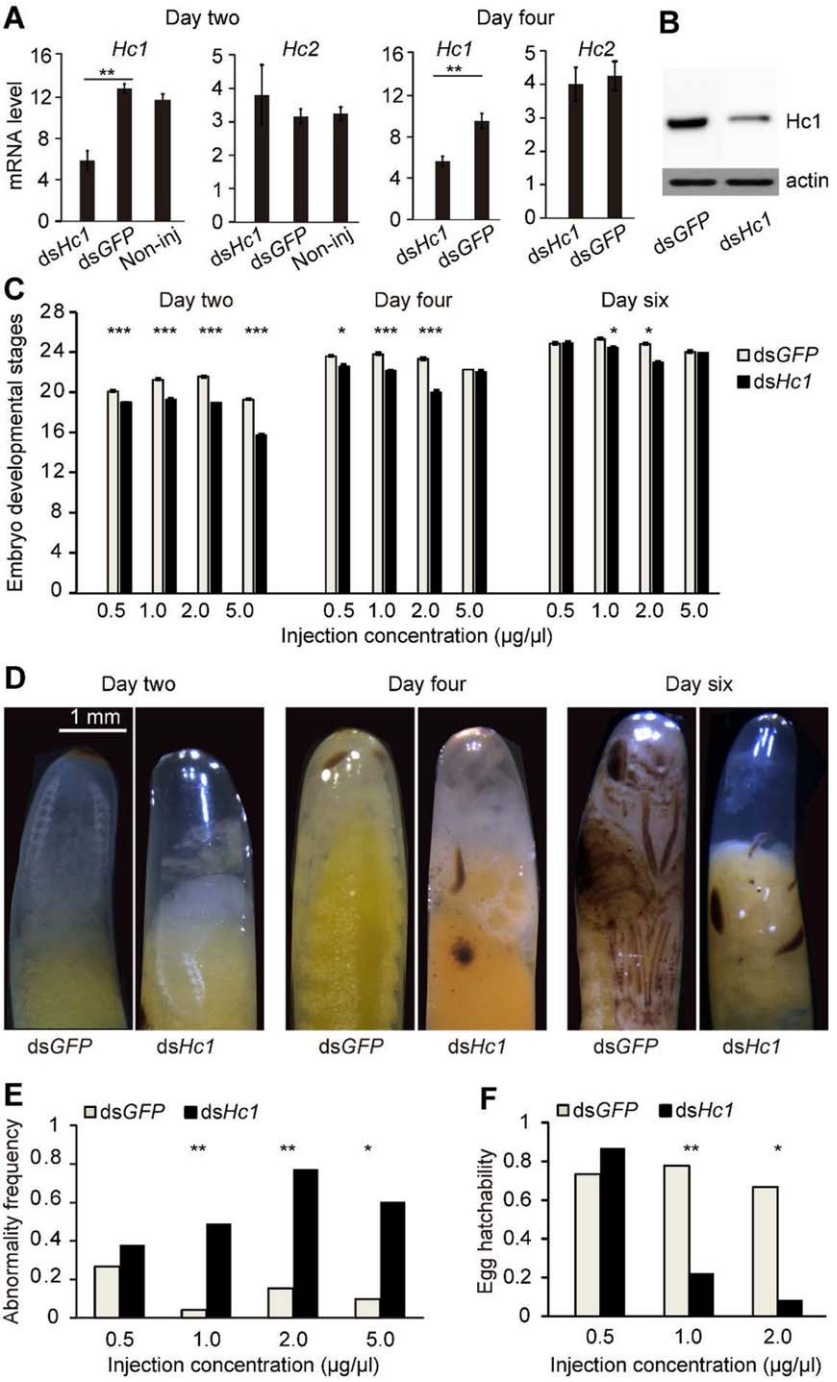

Figure 3. Effects of knocking down *haemocyanin subunit 1* (*Hc1*) gene expression on embryonic development in *Locusta migratoria*. (A) Effect of RNA interference (RNAi) on *Hc1* expression on days two and four after double-stranded RNA (dsRNA) microinjection. A total of 63 nl of 1 μg/μl dsRNA of *Hc1* (ie ds*Hc1*) and *green fluorescent protein* (*GFP*; ie ds*GFP*) was injected into 5-day-old embryos. Non-inj, non-injected sample. Expression levels of *Hc1* (left figure) and *Hc2* (right figure) were measured on days two and four post-dsRNA injection. The mRNA levels of *Hc1* and *Hc2* were quantified by real-time quantitative PCR and normalized against that of the internal control *ribosomal protein 49 rp49*. Five biological replicates of 10 eggs each were prepared for each treatment group. (B) Western blot analysis for the level of Hc1 protein in the samples of ds*Hc1* and ds*GFP* on day two post-dsRNA injection. β-tubulin was used as an internal control for total protein amounts. (C) Retardation of embryonic development caused by *Hc1* RNAi. The developmental stages of 30 embryos were examined on days two, four and six post-dsRNA injection at four different dosages, ie 0.5, 1.0, 2.0 and 5.0 μg/μl. Statistical differences in the frequency distribution of developmental stages between ds*GFP* and ds*Hc1* were evaluated using the Mann–Whitney *U*-test. (D) Developmental abnormality of embryos associated with *Hc1* knockdown. (E) Frequency of affected embryos that exhibited abnormal phenotypes at different doses. Phenotypes of embryos were examined on day six post-injection. (F) Effect of *Hc1* knockdown on egg hatchability. Fisher's exact test was performed to compare the difference in abnormality frequency of embryos and in the hatchability of eggs between the treatment and control groups. Error bars indicate ±1 SEM. Asterisks indicate significance level: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

abnormalities by 34% ($P = 0.001$; Fig. S6B) and reduced egg hatchability by 56% ($P < 0.001$; Fig. S6C).

Discussion

Whether insects require respiratory proteins in addition to their tracheal systems for normal living has been a subject of debate for a long time (Burmester & Hankeln, 2007; Willmer *et al*., 2004). Our study presents several lines of evidence that locust haemocyanins may have the capacity for oxygen transport and that these haemocyanins are essential for both embryonic development and survival. First, sequence analysis of the two haemocyanin subunits revealed that both subunits have amino acid residues required for reversible oxygen binding and subunit cooperativity. Each subunit can transport one O_2 molecule through the reversible binding of two Cu^+ ions, which are coordinated by six histidines in the central second domain of the native polypeptide chain (Linzen *et al*., 1985). Each subunit also harbours three phenylalanines that protect the active core and stabilize oxygen binding in the first and second domains (Hazes *et al*., 1993). Second, knockdown of haemocyanin expression in locust embryos resulted in significant developmental slowdown and arrest of embryos. These results indicate that normal expression of haemocyanins is crucial for proper embryonic development and growth as well as egg survival. Thus, our findings reveal a previously unappreciated and fundamental role for haemocyanins in embryonic development and survival in insects.

In arthropods, haemocyanins are generally free-floating in the haemolymph. For example, haemocyanins account for 90–95% of all crustacean haemolymph proteins (Markl & Decker, 1992). Haemocyanin is also highly abundant in locust haemolymph, which may result from active transcription of multiple haemocyanin gene copies in the genome (Fig. 1). Hypoxic exposure has been shown to increase the expression and concentration of haemocyanin (Brouwer *et al*., 2004; Zhou *et al*., 2014), as well as haemocyanin oxygen affinity in crabs and shrimps (Lallier & Truchot, 1989). This response of haemocyanin to hypoxia could be activated by HIF-1, which can bind with HIF-1 responsive elements probably present in the promoters of *Hc1* and *Hc2* genes (Semenza *et al*., 1996).

Unlike other arthropods (Burmester, 2004; Hagner-Holler *et al*., 2004), haemocyanin is present only at early developmental stages in the locust, mainly in the embryos, and absent in the haemolymph of late nymphal and adult stages. A similar pattern of haemocyanin expression has also been observed in the cockroach *B. dubia*, in which expression of haemocyanin is restricted to embryos (Pick *et al*., 2010). However, a different pattern of *Hc1* expression in developmental stages was reported in *L. migratoria* (Yin *et al*., 2012); the results may be caused by nonspecific amplification of the gene with the degenerate primers, which have been confirmed by our sequence analysis and replication experiments (data not shown). Based on its expression pattern, Sanchez *et al*. (1998) and Pick *et al*. (2010) suggested that insect haemocyanin has acquired a specialized function that may involve oxygen transfer or storage in the embryo. High haemocyanin levels in the yolk and the haemolymph that surround the embryo strongly implies its possible association with local storage of oxygen and timely supply of oxygen in the embryo that possesses only an immature tracheal system. As the embryo develops, the amount of yolk decreases, but the expression of haemocyanin continues to increase (Fig. 2). This indicates that haemocyanin is continuously generated by the embryo, and is not limited to those stored in the yolk. Quantitative expression analysis demonstrated that haemocyanins are abundantly distributed in the epidermis of locust embryos, suggesting that haemocyanin may be involved in the exchange of molecules (eg O_2) between the embryo and its environment, such as the yolk and the haemolymph surrounding the embryo. In the grasshopper *S. americana*, a haemocyanin homologue is expressed in haemocytes attached to the basal membrane of embryos (Sanchez *et al*., 1998). Knockdown of haemocyanin expression caused a delay in embryonic development, which returned to a normal pace as haemocyanin expression recovered (Fig. 3). Hypoxia can enhance the *Hc1* knockdown effects by causing a larger delay in the embryonic development and greater penetrance of embryonic abnormalities based on the knockdown effects. Thus, haemocyanins play a critical role in embryogenesis, probably by mediating oxygen supply to respiring tissues via transfer of oxygen from the surface, as well as local storage of oxygen. Meanwhile, the haemocyanins may have other functions related to oxygen transport, for example, buffering the oxygen supply to embryos, controlling the timing and intensity of oxygen supply, or acting as a hypoxia sensor exporting signals to the eggshell that increase its conductance and influx of oxygen to the embryo. However, because haemocyanins also participate in several physiological processes in addition to oxygen transport (Coates & Nairn 2014), it is not excluded that haemocyanins may affect embryonic development through other regulatory pathways.

Experimental procedures

Laboratory samples of locusts

Locust nymphs were collected in Wudi (64 m in altitude, 37.7°N, 117.6°E), Shandong province, China. The collected locusts were reared using a standard method according to Ma *et al*. (2011) and Guo *et al*. (2011) in our laboratory in Beijing.

Briefly, locusts were cultured in a large cage (40 × 40 × 40 cm) 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. The egg pods collected were kept in a plastic cup (diameter of 6 cm and height of 9 cm) filled with sterilized sand with 8% humidity and maintained in a 30 ± 1°C incubator. Locust samples had been maintained for at least five generations prior to use in our experiments.

cDNA cloning, gene copy validation and molecular phylogenetic analyses

The RNA sample was purified using a RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Two micrograms of purified total RNA were reverse transcribed with an oligo-dT adaptor primer to synthesize the first single-stranded cDNA with Moloney Murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). Full-length cDNA sequences of *Hc1* and *Hc2* were obtained by 5′- and 3′-RACE using a SMART RACE cDNA amplification kit (Clontech, Mountain View, CA, USA) according to the manufacturer's instructions. All primers used for RACE are listed in Table S1.

The genomic sequences of *Hc1* and *Hc2* gene copies were validated by PCR and sequencing. Three primer pairs, located at the 5′ end, in the middle and at the 3′ end of each gene copy, were designed to amplify the three different parts of the gene. One of the two primer sequences is located at the gene-coding region, and another at the gene copy-specific noncoding region [ie 5′ untranslated region (UTR), intronic region or 3′ UTR]. The amplified fragments were sequenced and compared against the locust genome to confirm their identity. All primers are listed in Table S1.

Alignments of nucleic acid sequences and amino acid sequences were constructed using MEGA6 (Tamura *et al.*, 2013). Construction of phylogenetic trees using amino acid sequences was performed by the neighbour-joining and maximum parsimony methods and the p-distance substitution model. Gaps were excluded based on pairwise distance estimation. Reliability of the trees was tested by a bootstrapping procedure with 1000 replications. The data set comprised complete sequences including N- and C-terminal segments. We adopted the tree created by the neighbour-joining method in our results because the trees based on the two methods were consistent except for order within the insect species. *Penaeus vannamei* Hc1 and *Epiperipatus* sp. Hc were used as outgroups. GenBank accession numbers for the sequences are as follows: *B. dubia* Hc1, FM242646; *Hierodula membranacea* Hc1, FM242642; *Cryptotermes secundus* Hc1, FM242644; *S. americana* EHP, AF038569; *L. migratoria* Hc1, KJ713391; *Thermobia domestica* Hc1, FM165288; *Chelidurella acanthopygia* Hc1, FM242641; *Perla marginata* Hc1, AJ555403; *Per. marginata* Hc2, AJ555404; *Thermobia domestica* Hc2, FM165289; *L. migratoria* Hc2, KJ713392; *H. membranacea* Hc2, FM242643; *B. dubia* Hc2, FM242647; *Cr. secundus* Hc2, FM242645; *Pen. vannamei* Hc1, AJ250830; *Epiperipatus* sp. Hc1, CAD12808. Signal peptides were predicted using the online tool SignalP 4.1 (Petersen *et al.*, 2011). The overall pairwise distance between two introns was measured by the proportion of nucleotide sites at which the two sequences differed based on the maximum composite likelihood model (Tamura *et al.*, 2013).

Measurement of mRNA level by real-time quantitative PCR

Gene expression was determined by real-time quantitative PCR using the SYBR Green I kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. PCR was performed on a LightCycler 480 Real-Time PCR system (Roche, Basel, Switzerland). *Ribosomal protein 49* (*Rp49*) was used as internal controls for the locust species (Guo *et al.*, 2013). The specificity of amplification was confirmed by melting curve analysis. Relative expression was quantified by the comparative cycle threshold ($2^{-\Delta Ct}$) method after normalizing against the internal control (Chen & Wagner, 2012; Guo *et al.*, 2013). To eliminate the difference in amplification efficiency amongst the genes *actin*, *Hc1* and *Hc2*, plasmid DNA that contained a fragment of the target gene was constructed. A standard curve was prepared based on the plasmid DNA. All reactions were performed in triplicate. Primers for real-time quantitative PCR are listed in Table S1.

Developmental expression analysis

To study the developmental expression of haemocyanin, tissues from egg, nymphal and adult stages of locusts were collected. Newly deposited eggs were collected every 4 h during daytime and incubated under standard conditions as described above. When eggs developed to the 2-, 5-, 8- and 11-day-old stage, 15 eggs in the middle part of an egg pod were taken out and used as one biological replicate for each stage. The nymphal stage consists of five instars and whole-body tissues were collected from first-, third- and fifth-instar nymphs. Each biological replicate contained 10 female nymphs. Adult whole-body tissues were collected from five females, adult haemolymph was withdrawn from 20 females and adult ovaries were dissected from 10 females for each replicate when adults reached the 5-day-old stage. Five biological replicates were prepared for each of these tissues and immediately frozen in liquid nitrogen for determination of gene expression.

Preparation of Hc1 and Hc2 antibodies

The coding regions of *Hc1* and *Hc2* were cloned separately into the expression vector pET28a using the *Bam*H and *Hin*dIII sites. The recombinant plasmids were transformed into the BL21 *Escherichia coli* strain. Hc1 and Hc2 expression was induced by adding isopropyl-β-D-thiogalactopyranoside (0.4 μM). The bacterial cells were harvested, ruptured by sonication and purified by nickel column chromatography. The target proteins were checked using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue. Polyclonal antibodies were generated by immunizing healthy rabbits with purified fusion proteins as antigens. Protein conjugation, immunization, antiserum purification and specificity tests were all carried out by Beijing Protein Innovation Co. Ltd., China (Beijing).

Immunoblotting assay

Total proteins were extracted with 1 mM phenylmethylsulphonyl fluoride. A total of 150 μg of protein was separated on 12% gel

using denaturing SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). Membranes were blocked in Tris-buffered saline with 5% Tween-20 containing 5% skimmed milk for 2 h at room temperature. The membranes were probed with the primary anti-Hc1 antibody (1:5000 dilution) or anti-Hc2 antibody (1:10 000) at 4°C overnight before washing and incubating with secondary antibodies (Horseradish Peroxidase-conjugated mouse anti-rabbit monoclonal antibody; Sigma A1949, USA) for 1 h at room temperature. Finally, the blot was detected under an enhanced chemiluminescence system (Bio-Rad) and recorded on X-ray films (Kodak, Rochester, NY, USA). β-tubulin (1:5000) was used as the internal control for determining total protein amounts. Images were scanned and the intensity of each band was captured using Quantity One v. 4.4 (Bio-Rad). For each sample, three biological replicates were used for Western blot analysis.

Immunostaining and imaging using confocal microscopy

Nine-day-old eggs were dissected and rinsed with saline. Intact embryos were fixed in 4% formaldehyde overnight at 4°C. After washing in 0.1 M phosphate-buffered saline (PBS; pH 7.4), embryos were incubated in 0.1 M PBS containing 5% normal goat serum (Boster, Beijing, China) for 3 h at room temperature. The primary anti-rabbit Hc1 antibody was diluted at 1:300 in 0.1 M PBS containing 2% normal goat serum. Incubation with primary antibodies lasted for 72 h. The tissues were washed three times for 15 min each in 0.1 M PBS, and subsequently incubated overnight with secondary antibody (Alexa fluor 488 Goat anti-rabbit antibody, Invitrogen, Carlsbad, CA, USA, 1:500 dilution) at 4°C. After washing three times, the tissues were dehydrated in an ascending ethanol series (50, 80, 100%), and then in an ascending methyl salicylas series (50, 100%). After mounting in neutral gum, fluorescence was detected using a Zeiss LSM 710 confocal microscope (Zeiss, Oberkochen, Germany).

dsRNA synthesis, RNAi and developmental analysis

dsRNAs of *GFP*, *Hc1* and *Hc2* were prepared using the T7 RiboMAX Express RNAi system (Promega) following the manufacturer's instructions. The primers for *Hc1* and *Hc2* dsRNAs had been optimized so that the synthesized dsRNAs for *Hc1* or *Hc2* were gene-specific and did not overlap with the region amplified in real-time quantitative PCR assays. The primers used for dsRNA preparation are listed in Table S1.

When eggs developed to the 5-day-old stage, they were immersed in 0.1% hypochlorous acid for 5 min to clear the chorion and to sterilize the egg surface (Bentley *et al*., 1979). Then, the eggs were cleaned by washing three times with saline water (NaCl 140 mM, KC1 5 mM, $CaCl_2.2H_2O$ 4 mM, $MgSO_4.7H_2O$ 2 mM, dextrose 55–65 mM, pH 7.2). Microinjections of dsRNAs were performed under an anatomical lens using a NANOLITER injector 2000 (World Precision Instruments, Sarasota, FL, USA) with a glass micropipette tip. A total of 63 nl of dsRNA at the desired concentrations was injected into the middle ventral part of the eggs. The eggs were then put on wet filter paper in sealed Petri dishes and maintained in an incubator at 30 $\pm$ 1°C. Two days later, embryonic development was examined and images of the embryos were captured under a microscope (Leica Application suite M205C, v. 3.3.0, Wetzlar, Germany). The whole developmental period of the locust embryos was classified into 27 distinct developmental stages according to the quantitative staging system described previously (Bentley *et al*., 1979). In addition, parts of the samples were frozen in liquid nitrogen for measurement of gene expression. This procedure was repeated on days two, four and six after dsRNA microinjection. New hatchlings were also counted to calculate egg hatchability. To validate the effect of RNAi on gene expression, 63 nl of 1 μg/μl dsRNA was injected and gene expression was measured using real-time quantitative PCR. To investigate the effect of different haemocyanin doses on embryonic development, we injected *Hc1* dsRNA at four titrations, ie 63 nl of 0.5, 1.0, 2.0 and 5.0 μg/μl dsRNA. Based on the dose response of *Hc1*, only 63 nl of 1 μg/μl dsRNA was injected for *Hc2*. At least 30 eggs were examined to determine the effect on embryonic development for each treatment. Five biological replicates of eight eggs each were used for expression analysis for each treatment.

Hypoxia treatments

In order to study the hypoxia response of embryos, eggs were maintained 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 could be controlled precisely. Differential oxygen partial pressure was achieved by inputting proportional normoxic air and pure nitrogen. Five-day-old eggs were exposed to composite air containing 5% oxygen for 4 days at 30 $\pm$ 1°C. Fifteen eggs were used in each treatment.

Data analysis and statistics

An independent *t*-test was performed to compare differences in gene expression. The effect of RNAi on embryonic development was evaluated by comparing the frequency distribution of the developmental stages using the Mann–Whitney *U*-test. Fisher's exact test was performed to compare the differences in abnormality frequency of embryos and hatchability of eggs between the treatment and control groups. Differences were considered statistically significant if $P < 0.05$. Data were analysed using the SPSS 16.0 software (SPSS, Chicago, IL, USA).

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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. Nucleotide sequences of the primers used in this study.

Figure S1. Multiple alignment of haemocyanin subunits 1 and 2 (Hc1 and Hc2) with those of two insects and one crustacean.

Figure S2. Gene expression of *haemocyanin subunits 1* and *2* (*Hc1* and *Hc2*) in 8-day-old eggs.

Figure S3. Validation of specificity of the primary antibody for haemocyanin subunit 1 (Hc1) by Western blot.

Figure S4. Effects of *haemocyanin subunits 1* and *2* (*Hc1* and *Hc2*) RNA interference (RNAi) on embryonic development.

Figure S5. Effects of *haemocyanin subunit 2* (*Hc2*) expression knockdown on embryonic development and egg hatchability.

Figure S6. Effects of hypoxia and *haemocyanin subunit 1* (*Hc1*) expression knockdown on embryonic development and egg hatchability.