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RESEARCH ARTICLE

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Hemocyanin and hexamerins expression in response to hypoxia in stoneflies (Plecoptera, Insecta)

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Abstract

Many freshwater ecosystems worldwide undergo hypoxia events that can trigger physiological, behavioral, and molecular responses in many organisms. Among such molecular responses, the regulation of the hemocyanin (Hc) protein expression which plays a major role in oxygen transportation within aquatic insects remains poorly understood. The stoneflies (Plecoptera) are aquatic insects that possess a functional Hc in the hemolymph similar to crustacean that co-occurs with a nonfunctional Hc protein, hexamerins (Hx). However, the role of both proteins during hypoxia remains undetermined. Here, we evaluated the effect of hypoxia on the expression of Hc and Hx proteins via a comparison between hypoxia and normoxia amino acid sequence variation and protein expression pattern within 23 stonefly species. We induced short-term hypoxia in wild-caught stoneflies species, sequenced the target region of Hc and Hx by complementary DNA synthesis, characterized the protein biochemistry using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, ultrafiltration, and polarographic fluorometric method, and amplified the genome region of the hypoxia-inducible factor (HIF) transcriptional response element that regulated Hc using genome walking library approach. We found a lack of Hc expression in all examined species during hypoxia conditions, despite recognition of the HIF gene region as a

possible regulatory factor of Hc, suggesting that compensatory responses as metabolic changes or behavioral tracheal movements to enhance respiratory efficiency could be possible mechanics to compensate for hypoxia. A short Hc-like novel isoform was detected instead in these 23 species, possibly due to either protein degradation or alternative splicing mechanisms, suggesting that the protein could be performing a different function other than oxygen transportation. Hx during hypoxia was expressed and exhibited species-level amino acid changes, highlighting a possible role during hypoxia. Our results demonstrate that hypoxia could enable a similar potential adaptive response of multiple species regarding specific physiological requirements, thereby shedding light on community behavior in stress environments that may help us to improve conservation practices and biomonitoring.

KEYWORDS

hemocyanin, hexamerins, HIF, hypoxia, Plecoptera

1 | INTRODUCTION

Dissolved oxygen (DO) concentrations are one of the most important ecological factors in determining habitat quality for aquatic biota (Burnett, 1997). DO is essential for the processes of respiration and metabolism in aquatic organisms, therefore fluctuating DO concentrations whether high (hyperoxia) or low (hypoxia) can result in changes to their distribution and survival (Burnett, 1997; Galic et al., 2019; Reid, 1961; Wilding et al., 2012; Wu, 2002). Between these two conditions, hypoxia (i.e., DO concentrations <2 mgO_2/L) has increased in duration and frequency, mainly due to anthropogenic impacts and climatic change endangering the effective oxygen uptake of aquatic organisms (Ekau et al., 2010; Friedrich et al., 2014). A better understanding of how organisms cope with hypoxia is an important component of predicting how organism distributions may change in response to environmental changes.

Among the aquatic biota, insects exhibit a remarkable array of adaptations that allow them to survive hypoxic conditions. The behavioral escape of damselflies (Apodaca & Chapman, 2004), an increase of spiracular opening in caddisflies (Philipson & Moorhouse, 1974), increase gill-beating rates in some mayfly species (Bäumer et al., 2000), and push-up behaviors of stoneflies (Genkai-Kato et al., 2000) allow them to cope with a higher demand of oxygen via compensatory processes. However, the most remarkable adaptation of aquatic insects to hypoxia involves molecular responses. Hypoxia appears to regulate the expression of genes and enzymes related to protection against low DO (Harrison et al., 2018) via the effect of the hypoxia-inducible factor (HIF) on the transcriptional expression. HIF alters the transcription of more than 300 genes associated to oxygen-related proteins (Spicer, 2014), thereby playing an essential role for both aquatic and nonaquatic insects during hypoxic conditions (Coon et al., 2017; Marden et al., 2013; Morin et al., 2005).

Two major proteins regulated by HIF play a major role in oxygen transportation within aquatic insects, the hemoglobin (Zebe, 1991), and the hemocyanin (Hc; Burmester, 2001). Among them, Hc is widely found in insects and belongs to a large protein superfamily that links arthropod and crustacean evolution (Burmester, 2001, 2002; van Holde & Miller, 1995; van Holde et al., 2001). Hc is freely dissolved in the hemolymph and is composed of six identical or similar subunits of around 75 kDa each (van Holde & Miller, 1995; Salvato & Beltramini, 1990). Each subunit contains three domains, with Domain II having a highly conserved region that can bind an O_2 molecule by the means of two Cu^+, coordinated by three histidines in two binding sites (van Holde & Miller, 1995; Linzen et al., 1985). Hc has been studied in aquatic insects (Pick et al., 2009); however, its role during hypoxia remains undetermined.

Here, we study the role of Hc during hypoxia and explore the presence of the HIF as a primary regulator of Hc in stonefly aquatic insects. Stoneflies (Plecoptera) were the first aquatic insects reported to have Hc (*P. grandis* Fochetti et al., 2006; *Perla marginata*, Hagner-Holler et al., 2004), and to date, the Hc has been found to form two different subunits: subunit 1 (Hc1) of 77 kDa and subunit 2 (Hc2) of 76.3 kDa (Amore et al., 2009). Stoneflies are taxonomically separated into 17 families (Fochetti & Tierno de Figueroa, 2008); however, a functional Hc has only been found in 11 species of the Perlidae and Perlodidae families (Amore & Fochetti, 2009). In addition, stoneflies are the only insect in which a functional Hc has been observed to co-occur with a nonfunctional Hc protein in the hemolymph. This nonfunctional Hc, named hexamerins (Hx; Amore & Fochetti, 2009; Hagner-Holler et al., 2007), is involved in metamorphosis, molting, reproduction, and energy production as a source of amino-acid storage (Burmester, 1999; Telfer & Kunkel, 1991), and has been linked to life-history changes and adaptation of individuals (Kvist et al., 2013). The role of both proteins (Hc and Hx) during hypoxia has not yet been explored. In general, stoneflies are sensitive to DO changes (Gamboa et al., 2017; Steward & Stark, 2002), but some species seem to be adapted to low DO conditions. For example, *Paraperlis frontalis* and *Isocapnia missouri* spend most of their life within hyporheic zones (i.e., the connection between the surface and groundwater where DO concentration can be lower than that surface water; Mugnai et al., 2015; Stanford & Gaufin, 1974; Stanford et al., 1994), while *Neoperla geniculata* lives up to 60 m deep where DO concentration is limited (Nishino, 2012). This outstanding plasticity of DO concentration adaptation among stonefly species shows a unique capacity to respond to hypoxic conditions.

In this study, we explored the effects of hypoxia on stoneflies nymphs by examining 23 species collected from an Alpine river. We carried out complementary DNA (cDNA) sequences and protein biochemistry to test whether stoneflies experimentally exposed to low DO express Hc, Hx, or both in response to hypoxia. We also compared Hc and Hx amino-acid sequence variation during normoxia and hypoxia to detect evolutionary changes associated with sequences variation and investigated the possible role of HIF in Hc regulation to provide hints on the functional adaptation in stoneflies.

2 | MATERIALS AND METHODS

2.1 | Hypoxia experiment

Twenty-three stonefly species (Table S1) nymphs were collected from the Tagliamento River, using D-frame nets (250 μm mesh) during Spring and Summer 2010. Between 20 and 25 individuals per species were collected (n = 476). Live nymphs were placed into 30-L aquaria filled with river water and natural substrate (e.g., cobbles and gravel) originating from the river for up to 3 h for acclimatization. Aquaria were equipped with an air stone and supplied with atmospheric oxygen. Individual stoneflies were then placed in smaller aquaria (~500 ml) with river water and an air stone connected to nitrogen gas (N_2) cylinder, and N_2 was then added to the small aquaria to induce hypoxia. The pH was measured with a pH meter (L0015632; Veto) to monitor that the levels of carbon dioxide were constant. We established the hypoxic status of each stonefly by measuring the oxygen concentration

in the water down to 2 mg/L (as suggested by Knight & Gaufin, 1966) and by observing the push-up behavioral response (i.e., stoneflies thoracic movement to enhance respiratory efficiency; Genkai-Kato et al., 2000). Specimens were placed inside the small aquaria, which was made airtight and flooded with nitrogen. For each individual, a range between 1 and 1.5 h of hypoxia exposure was implemented (as suggested by Flachsbarth et al., 2017). Hemolymph from live individuals was immediately withdrawn using a sterile syringe and diluted in RNAlater (QIAGEN). As a control, we stored the hemolymph of normoxic individuals experiencing the same conditions described above, but without the induction of hypoxia. All individuals used in these experiments were subsequently stored in 100% ethanol for species-level identification following the descriptions given by Fochetti and Tierno de Figueroa (2008). All samples were then stored at −20°C.

2.2 | Protein biochemistry

2.2.1 | Functional Hc

To observe if the Hc function of binding oxygen was affected by short-term hypoxia, an oxygen-binding properties analysis was performed. The analysis was conducted by ultrafiltration as previously describe Roxby et al. (1974) for all hypoxic and normoxic stonefly species (23 species, $n = 146$) using a Microcon filter (cutoff 100,000 Da; Millipore) following the manufacturer's instructions. The Hc oxygen-binding property was spectrometrically analyzed by reading the absorption spectrum from 260 to 420 nm on a Cary photometer. The absorption spectrum at 340 nm represents the signal of an oxygenated Hc (Markl & Decker, 1992). The deoxy spectrum was measured by reducing the sample with sodium dithionite.

2.2.2 | Protein molecular weight

The expression of the Hc and Hx was compared between hypoxia and normoxia conditions for all stonefly species (23 species, $n = 138$) to determine if the expression of the proteins was affected by short-term hypoxia. A sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method was conducted following the methods of Rochu and Fine (1984) to specific target Hc proteins. The ultrafiltrated sample from above was diluted with an equal volume of 100 mM Tris HCl, pH 7.5, 10 mM $MgCl_2$, and 5 mM $CaCl_2$, then centrifuged for 10 min at 10,000*g* at 4°C and frozen at −20°C until use. Samples were run in 7% polyacrylamide gels at 40 V for 2 h. Gels were stained with coomassie blue (Sigma-Aldrich). The SeeBlue Plus2 mixture was used as molecular weight markers. We targeted a band range between 76 and 83 kDa molecular mass, corresponding to 76−77 kDa molecular weight characteristic of oxygen-type Hc (Markl & Decker, 1992), and an 82.35 kDa corresponding to the predicted mass of nonoxygen Hx (Burmester, 1999).

2.2.3 | The Hc oxygen capacities

To observe if the Hc ability to bind oxygen changes due to short-term hypoxia, a polarographic fluorometric method to obtain oxygen-binding curves as proposed by Hagner-Holler et al. (2004) was implemented. Ten individuals of *P. marginata* of the Perloidea superfamily, with a functional Hc (Amore & Fochetti, 2009) and 10 *Nemoura cinerea* of the Nemouroidae superfamily, which lacks oxygen-binding activity (Amore & Fochetti, 2009) were chosen to measure differences between hypoxic and normoxic conditions. The measurements were carried out at the University of Mainz.

2.3 | Total RNA isolation and cDNA synthesis

RNA was extracted for all hypoxic and normoxic stoneflies species (23 species, *n* = 192) using an RNeasy Mini Kit (Qiagen GmbH) following the manufacturer's instructions. Reverse-transcription polymerase chain reaction (RT-PCR) reactions were carried out using a One Step-Kit according to the manufacturer's instructions (Qiagen GmbH) and two-step RT-PCR Kit (Promega) with oligo(dT) primers for amplifying the first strand of cDNA. For both reactions, the following primers were used: Hc coding region directly linked to the copper ion of ~600 amino acids (Domain II; Amore et al., 2009), forward 5′-gagggnsagttcgtntacgc-3′ and reverse 5′-gaanggyttgtggttnagrcg-3′; and Hx protein ~300 amino acids (Hagner-Holler et al., 2007), forward 5′-ccnccncccntaygarrtctaccc-3′ and reverse 5′-tcgtacttgggtccnaggaagac-3′. The resulting cDNA quality and quantity were visually checked using a 2% agarose gel. cDNA synthesis products were purified using the MinElute Gel Extraction Kit (Qiagen GmbH), and cloned into a pGEM T-Easy vector (Promega). Sequencing was performed via 3500xL (Applied Biosystems) automated sequencer. All sequence data reported here have been deposited into GenBank (MT532538–MT532556).

2.4 | Sequence analyses

Sequences were assembled and edited using CodonCode Aligner v 3.5 (Codon Code Corporation) and compared to the NCBI nucleotide database using BLASTN queries (http://blast.ncbi.nlm.nih.gov). Translated protein products were obtained using TRANSLATE (ExPASy Proteomics Server; http://www.expasy.org/), and aligned with Protal2DNA (http://bioweb2.pasteur.fr/soft-pasteur.html#protal2dna). Identical amino acid sequences were collapsed into a single sequence using CleanCollapse software (http://sray.med.som.jhmi.edu/SCRoftware/CleanCollapse).

Newly sequences obtained here, as well as those previously published for the stonefly, other insects, and crustacean obtained from GenBank (Table S1), were included in multiple alignments for Hc and Hx. The sequences were selected as described by Burmester (2001). A phylogenetic tree was reconstructed using MrBayes v. 3.1.2 (Ronquist & Huelsenbeck, 2003) with a WAG matrix (ProtTest v. 2.4; Abascal et al., 2005) and gamma distribution. Metropolis-coupled Markov chain Monte Carlo sampling was performed with one cold chain and three heated chains and run for 100,000 generations. Trees were sampled every tenth generation. Posterior probabilities were estimated on the final 20,000 trees (burn-in of 80,000 trees).

2.5 | Identification of HIF

Genomic DNA was extracted from individuals that had been preserved in 100% ethanol (above) using DNAeasy Tissue Kits (Qiagen GmbH), following the manufacturer's instructions. A genome walking library was constructed following a protocol adapted from Siebert et al. (1995). DNA was digested with the *Hae*II restriction enzyme (Takara Bio Inc.) for 20 min at 37°C, according to the manufacturer's instructions. Adapter ligation was performed with the following primers: long adapter 5′-GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT-3′, and short adapter 5′-H2N-cccgacca-PO4-3′ using a T4 ligase to create gene-walker libraries. Gene-specific primers were used to search for the presence of core (5′-CGTG-3′) and consensus (5′-rcgtg-3′ and 5′-brcgtgvb-3′, where b = g, t, c, and v = g, a, c) binding sites reported in the literature for the hypoxia response elements (HRE, Wenger & Gassmann, 1997) of HIF. HRE-specific primers were used for a touchdown PCR, AP1 (5′-GTAATACGACTCACTATAGGGC-3′) and AP2 (5′-ACTATAGGGCACGCGTGGT-3′). Two Hc-specific primers were used for secondary PCR (Hc region promotor: GSP1 forward, 5′-CTGGACAAAGCCTCCATCATGA-3′ and GSP1 reverse, 5′-TGTCCGTGGCACAAAG ATCTT-3′; and 3′-untranslated region [3′UTR] of Hc: GSP2 forward, 5′-gtgagattttcgctgagctggc-3′ and GSP2 reverse, 5′-atacatgagtccccctccaggt-3′) in two independent PCR reactions using AP1 and AP2 primers. PCR products were visualized on a 1.2% agarose gel, and individual bands were isolated using MinElute Gel Purification Kit (Qiagen

GmbH). PCR products were sequenced by Eurofins—Operon using the aforementioned primers. The HIF sequences obtained were analyzed using BLASTN against HIF sequences from GenBank.

3 | RESULTS

3.1 | Characterization of stonefly Hc

SDS-PAGE analysis of hemolymph from normoxic stoneflies detected a band ranging from 76 to 82 kDa (Figure 1), probably suggesting the occurrence of Hc. The absorption spectrum showed a peak at 340 nm that disappeared upon the addition of dithionite as a reducing agent (Figure 1), suggesting the presence of an oxygenated Hc. The Hc oxygen-binding curves compared between individuals of *P. marginata* and *N. cinerea* (see Section 2), showed reversible binding of O_2 of *P. marginata* with a saturation pressure of 8.03 ± 0.3 Torr, while no reversible binding was detected for *N. cinerea*. In the same analyses of hypoxic individuals, no band was detected at 76 kDa, no absorption spectrum read obtained, and no oxygen-binding property was observed.

We investigated Hc cDNA sequence variation in 23 stonefly species (Table S1) by sequencing and cloning the Hc Domain II. Sequences from normoxic individuals contained 218 amino acids (654 base pair [bp]) and exhibited an amino acid arrangement similar to the corresponding *P. marginata* sequences from GenBank (AJ555403; Hagner-Holler et al., 2004) as well as the presence of the oxygen-binding regions CuA and CuB (Figure 2). Hc cDNA sequence was detected for normoxic individuals of *P. marginata*, *P. grandis*, *Dinocras cephalotes*, and *Isoperla grammatica*, as reported previously (Amore & Fochetti, 2009), and newly detected here for *I. saccai*, compare with *I.andreinii*, *Besdolus ravizzarum*, and *Chloroperla susemicheli*. The other species exhibited no messenger RNA (mRNA) expression for Hc.

Sequences from hypoxic individuals of all 23 species were only 125 amino acids (375 bp) long and contained 17 amino acid changes (glutamine, serine, isoleucin, and threonine) compared to normoxic sequences (Figure 2). Within hypoxic sequences, the CuA oxygen-binding site contained two amino acid changes and the CuB oxygen-binding region was not present (Figure 2). A Bayesian phylogenetic analysis of the Hc sequences obtained herein along with other stonefly and arthropod Hc proteins (Table S2) recovered our hypoxic and normoxic stonefly sequences as a clade that the included published Hc subunit 1 from *P. marginata* and *P. grandis* (Figure 3a). Other clades included the Hc subunits 2 and 3–6, with amino acid similarities ranging from 40% to 95%.

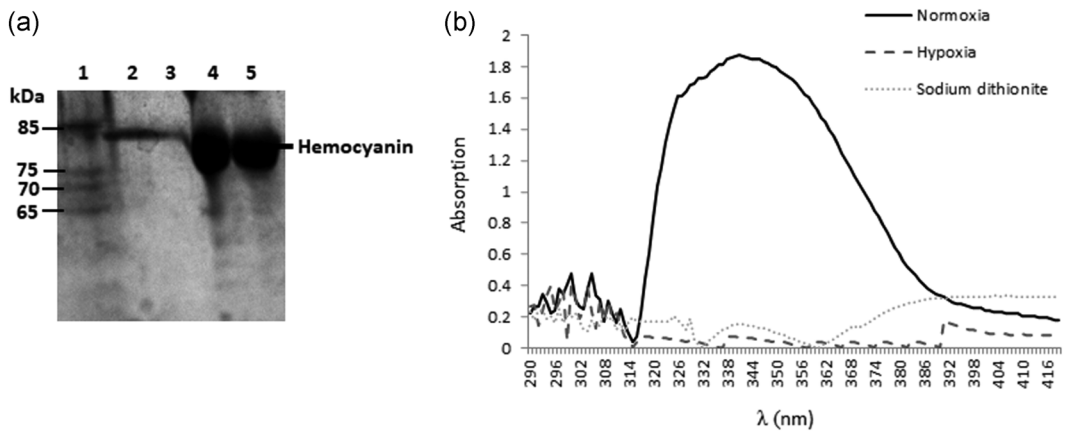

FIGURE 1 Protein biochemistry. (a) Identification of hemocyanin (Hc) protein in the hemolymph by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lane 1, protein standards; Lanes 2–3, samples from hypoxia; and Lanes 3–4, samples from normoxia. The Hc band is indicated. (b) The absorption spectrum of a purified Hc protein by ultrafiltration

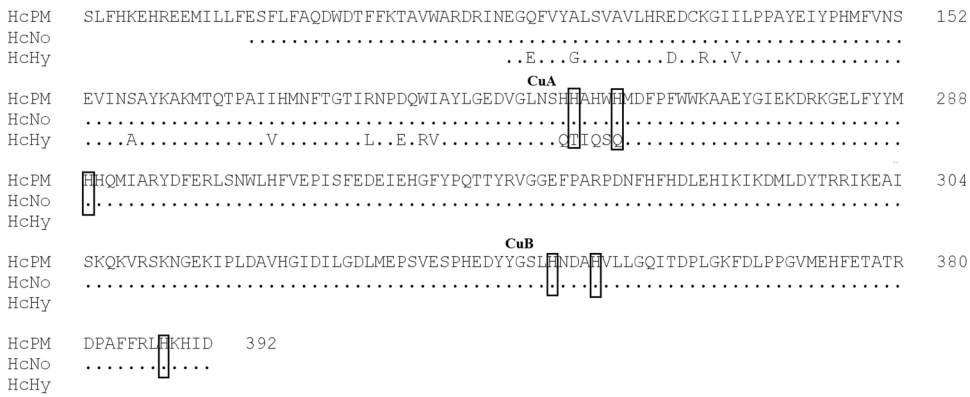

FIGURE 2 Comparison of hemocyanin (Hc) amino acid sequences obtained from normoxic (HcNo) and hypoxic (HcHy) stoneflies with a published normoxic sequence from *Perla marginata* (HcPM, GenBank accession no. AJ555403). Amino acid residues were deduced from complementary DNA nucleotide sequences and are shown only at positions where they differ from the published *P. marginata* sequence. Locations of the copper-binding histidines (CuA and CuB) are indicated with black rectangles

The possible role of HIF on Hc regulation was investigated on the isolated genomic DNA for all normoxic stonefly species (23 species) by genome walking library construction. To search for HIF-1 binding sites in the promoter and 3′-UTR regions of stoneflies Hc genes, we used the HRE core binding sequence 5′-CGTC-3′. In all species examined, two genomic regions were detected containing putative HIF-1 binding sites on Hc. A fragment with an average of 256 bp contained 5′-GCGTGGCGG-3′ and that of an average of 486 bp contained 5′-GCGTGC-3′ only. These fragments were homologous to noninsects species in the GenBank.

3.2 | Characterization of stonefly Hx

Hx cDNA sequence variation in 23 stonefly species by sequencing Hx protein revealed sequences from normoxic individuals were 241 amino acids (723 bp) and were similar to *P. marginata* mRNA for Hx1 (GenBank accession AM690365), while the sequences from hypoxic individuals were shorter (121 amino acids) and variable, containing a total of 26 amino acid substitutions (Figure 4). All the stonefly species examined contained a shorter sequence in hypoxic conditions as compared to that of the corresponding normoxic Hx. The most common amino acid changes were phenylalanine (22%) and isoleucine (19%).

Within the Bayesian phylogeny, our normoxic sequences from *P. marginata* and *P. grandis* formed a well-supported monophyletic clade with published (normoxic) *P. marginata* sequences of Hx1 (Figure 3b). These formed a sister group with the large clade of hypoxic Hx sequences obtained in the current study, which exhibited substantial genus- and species-level variation (Figure 3b). Also included in this large clade were published sequences from normoxic *Allocapnia vivipara* and *Taeniopteryx burksi* Hx type 1 mRNA (Hagner-Holler et al., 2007).

4 | DISCUSSION

Hypoxia alters the expression of genes that regulate proteins, depending on the physiological requirements of the species (Harrison et al., 2018). Here, we aimed to detect the response of Hc and Hx proteins to hypoxia within 23 stonefly species.

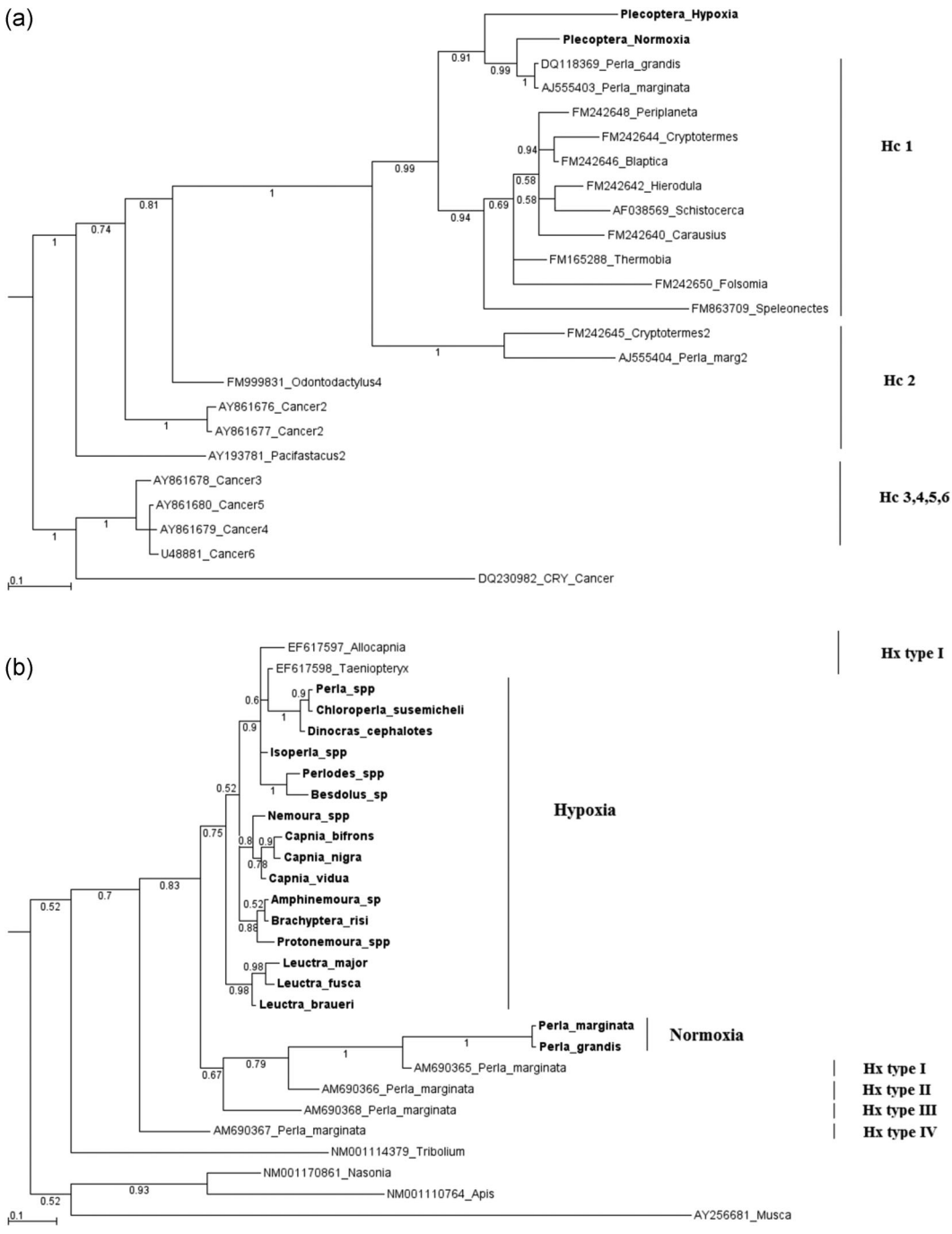

FIGURE 3 Bayesian phylogenetic reconstructions of (a) hemocyanin (Hc) and (b) hexamerins (Hx) using amino acid sequences and a WAG matrix. For both analyses, newly sequenced normoxic and hypoxic stoneflies are in bold font and terminal labels include GenBank accession codes. Bayesian posterior probabilities are shown above the branches. Vertical lines indicate membership in Hc domains or Hx type

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AM690365_Pm GSLYNMLRTVYGHYADPMYQYEVAPSVLEHFTTALRDPAYYTLYKRIDTLFKEYKKLMPEYTYDELTYPGVKVESV 456
PmarginataN ...........................................................................
EF617598_Tb .A...IM..IF..VT..TF..G.........E.........SM....EY..SSF.ENL.H......V.....I..L
Besdolus                                                                      .....I..L
Rhabdiopteryx                                                                 .....IDPF
Brachyptera                                                                   .....I..L
Dictyogenus                                                                   .....I..I
Chloroperla                                                                   .....I..L
Perla                                                                         .....I..L
Dinocras                                                                      .....I..L
Capnia                                                                        .....I..L
Capnioneura_sp1                                                               .....I..L
Capnioneura_sp2                                                               .....I..L
Nemoura                                                                       .....I..L
Isoperla                                                                      .....I..L
Isogenus                                                                      .....I..I
Perlodes                                                                      .....I..L
Protonemoura                                                                  .....I..L
Leuctra_sp1                                                                   .....I..I
Leuctra_sp2                                                                   .....I..I
Leuctra_sp3                                                                   .....I..I
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AM690365_Pm   EIEKLVTYFDNFDIDLDNAVDVGVIEDGQKVNIQARQMRLNHKPYTYKVKVVSDKAATSMVRVFLGPKYDFYGNEY 532
PmarginataN   ...............................................................................
EF617598_Tb   .V...I.F.............T....EF.......S.....AF...I..A..................SF.K.F
Besdolus      .V...I.............E..T....EF.......F.....RF...I..A.....N...........
Rhabdiopteryx .V...I.............E..T....EF.......F.....GF...I..A.....N...........
Brachyptera   .V...I...............S....EF.......F.....GF...I..A.................
Dictyogenus   .V...I.F.............T....EF.......F.............S.................
Chloroperla   .V...I.F.............T....EF.......Q.....AFN.....E...S.F...........
Perla         .V...I.F.............T....EF.......Q......FN.....E...S.F...........
Dinocras      .V...I.F.............T....EF.......Q.....AFN.....E.....F...........
Capnia        .V...I...........V....S....EF.......F......F.....A...S.Y...........
Capnioneurasp1.V...I.....L......V....S....EF.......F......F......P.....Y.......D.ST
Capnioneurasp2.V...I.....L......V....S....EF.......F......F......T.....Y.........ST
Nemoura       .V...I...........V....S....EF.......F......F......A...PLT...........
Isoperla      .V...I.............T....EF.......F.............S.................
Isogenus      .V...I.L...........T....EF.......F.............S.................
Perlodes      .V...I.F.........V....T....EF.......F.............T.................
Protonemoura  .V...I...............S....EF.......S.....AF...I..A.....H...........
Leuctra_sp1   .VD..I....H.......V....T....EFI.....N.....AFNF....Q.................
Leuctra_sp2   .VD..I.F..H.......V....T....EF.......N.....AFN.....Q.................
Leuctra_sp3   .VD..I.Y..H.......V....T....EF.......N......F......Q.................
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FIGURE 4 Comparisons of hexamerins amino acid sequences obtained from normoxic *Perla marginata* (PmarginataN) and 17 species of hypoxic stoneflies with published (normoxic) sequences of *P. marginata* (AM690365_Pm) and *Taeniopteryx burksi* (EF617598_Tb). Amino acid residues were deduced from complementary DNA nucleotide sequences and are shown only at positions where they differ from the published *P. marginata* sequence

Arthropod Hc is characterized by an absorption spectrum of 340 mm (Burmester, 2001), a conserved molecular weight of ca. 76–77 kDa (Markl & Decker, 1992), and an oxygen saturation pressure of 8 Torr (Hagner-Holler et al., 2004). The lack of Hc detection in any of the biochemical assessments performed indicated that Hc was not expressed during hypoxia within these stonefly species. The absence of respiratory proteins during short-term hypoxia has been observed previously in mammals (Wenger, 2000), marine crustaceans (Brouwer et al., 2004; Racotta et al., 2002), and insects such as *Drosophila melanogaster* (Gleixner et al., 2008), the collembolan *Folsomia candida* (Flachsbarth et al., 2017), and the beetle *Tribolium castaneum* (L. Wang et al., 2018) primarily caused by changes in the metabolic pathway. Hypoxia triggers a deficiency of ATP production due to insufficient DO supply (Harrison et al., 2018). To cope with ATP demands, the organisms switch from aerobic to anaerobic metabolism pathways (Verberk et al., 2013) via the creation of energy through the combustion of carbohydrates in the absence of oxygen (Contreras & Bradley, 2009). Lactate, alanine, succinate, malate, alpha-glycerol phosphate, glycerol, glycerol-3-P, acetate, and ethanol have been reported as carbohydrates present in insects due to anaerobic

metabolism during hypoxia (Harrison et al., 2018; Hoback & Stanley, 2001). Interestingly, the accumulation of carbohydrates inhibits HIF (Chinopoulus, 2013). HIF is involved in a conserved response to low oxygen stress that leads to the up- and downregulation of several genes involved in hypoxia tolerance (Storey & Storey, 2010; G. L. Wang & Semenza, 1993), and regulation of Hc expression is controlled by HIF (Head, 2010). We confirmed the presence of two putative HREs from HIF in the Hc gene, suggesting that these might play a role in the regulation of Hc within stoneflies, as previously observed in stoneflies (Malison et al., 2020). However, due to a lack of oxygen saturation in the hemolymph and Hc expression occurring during hypoxia, this could be an indication that anaerobic metabolic inhibits HIF, and, therefore, the expression of Hc. There is no information available concerning the mechanism underlying HIF induction within Hc gene regulation, and an accumulation effect of carbohydrates on the inhibition of HIF on hypoxic aquatic insects. Further studies are needed to understand potential Hc–HIF interactions and HRE function in hypoxia-mediated downregulation.

In addition to metabolism changes resulting from hypoxia, a behavior-compensatory response is often observed (Wu, 2002). An increase of oxygen permeability in the exoskeleton by a ventilation system (Hetz & Bradley, 2005) is a well-known behavior of stoneflies (Genkai-Kato et al., 2000). This thoracic movement known as push-up to enhance respiratory efficiency has to help stoneflies to increase oxygen from the critical concentration of 4–7 (Nagell, 1973) to 14 mgO_2/L (Gaufin et al., 1974), an average of 14% of oxygen saturation increase depending on body size, species, and water temperature (Knight & Gaufin, 1966). The rapid increase of oxygen uptake leads to suppression of oxygen-related protein expression as a mechanism of controlling oxygen production (Konz et al., 1998), as observed in fish (Richards, 2009) and *Drosophila melanogaster* (Gleixner et al., 2008). Therefore, the ability of the stonefly to rapidly achieve an increase of oxygen by alteration to a push-up behavior (which was observed for all species in this study, see Section 2) may lead to additional inhibition of Hc.

Despite Hc expression being undetected, a short Hc-like isoform was found in all species examined here under short-term hypoxia. This isoform was highly conserved among species, with 95% of amino acid sequence similarity when compared to normoxic Hc cDNA. Alternative gene splicing is a typical mechanism for pre-mRNA, resulting in a single gene coding for multiple proteins (Black, 2003). Hc is known to undergo alternative splicing (X. Zhao et al., 2012) due to immune responses. The shrimp *Litopenaeus vannamei* exhibited a Hc affected by alternative splicing resulting in a protein 89% shorter in comparison with wild Hc but still highly conservative with 98% amino acid similarity (S. Zhao et al., 2013). Meanwhile, the mollusks *Concholepas concholepas*, *Fissurella latimarginata*, and *Megathura crenulata* (Zhong et al., 2016) exhibited differential gene expression with shorter Hc sequence length due to an induced inflammatory signal. As a result of alternative splicing, Hc in arthropods has been recognized as a nonspecific immune molecule (Jiang et al., 2007) that displays antiviral and antimicrobial activities. To our knowledge, no studies have been conducted so far on stonefly Hc immune responses, therefore this current work could provide useful information for further studying the stonefly Hc novel isoform. Conversely, a shorter protein isoform could be a consequence of wild protein degradation. Experimental handling and human errors, as well as changes in temperature, pH, and cellular metabolic pathways, could lead to protein degradation (Lovric, 2011). On the basis of the detection of the normoxic Hc protein, we can exclude the possibility that experimental handling and human errors could be the causes of protein degradation. However, potential biochemical and metabolic changes of the cells due to stress or/and hypoxia can not be rejected. Further studies need to be conducted to identify the expression pathway mechanisms of Hc in stoneflies to clarify the role of Hc during critical oxygen conditions.

In addition to Hc responses to hypoxia, we aimed to evaluate Hx protein responses. Hx are Hc-derived proteins that have lost the ability to bind oxygen (Hagner-Holler et al., 2007). They function as storage proteins to provide amino acids and energy, cuticle formation, transport of hormones, and immune defense (Burmester, 1999). Our study found that Hx is expressed during hypoxia and even exhibits sequence variation among species. The diversification of normoxic stonefly Hx preceded the divergence of morphological taxonomic families (Hagner-Holler et al., 2007). In this study, the diversification of Hx during hypoxia shows species-specific sequence variations among six species (*Leuctra braueri*, *L. fusca*, *L. major*, *Capnia vidua*, *C. nigra*, and *Zwicknia bifrons*), suggesting a differing

diversification pattern from the remainder of the studied species. Similar diversification patterns were also observed in other normoxic insects (Telfer & Kunkel, 1991), particularly in ants where Hx diversification allows caste differentiation (Terrapon et al., 2014). Hypoxic Hx sequences showed high amino acid similarities (95%) with a Hx type 1 from the Genbank, and its expression was observed during hypoxic conditions due to hormone regulation during morphogenesis in insects (Metzger & Krasnow, 1999; Somervuo et al., 2014), suggesting that Hx proteins are associated with ecological and physiological requirements of individuals (Amore & Fochetti, 2009; Burmester, 1999). Our hypoxic Hx type 1 sequence contained a large amount of amino acid phenylalanine, which is a key residue in the regulation of oxygen (Hazes et al., 1993) in tissues. During the developmental stage of insect metamorphosis, episodes in which oxygen is lacking tend to occur (Mangum, 1985). The presence of Hx during molting cycles (Chandrasekar et al., 2009) and larval developmental (Somervuo et al., 2014) suggests its expression during the absence of oxygen. We demonstrate here that Hx expression occurs during hypoxia; however, future metabolism and physiological-cascade pathway experiments are necessary to obtain a complete understanding of the biochemical adaptation of Hx to stress environments.

In conclusion, our data show that the lack of Hc and species-specific Hx expression during hypoxia in multiple stonefly species may be associated with a potential remarkable compensatory response for short-term oxygen deficiency in the environment. A similar molecular response of Hc within multiple species during hypoxia suggests that environmental stress plays a stronger role in the adaptation of stoneflies than the specific physiological requirements of the species, as previously observed by Gamboa et al. (2017), which is especially important for community studies concerning climatic change adaptation. To further understand the influence of oxygen concentration on Hc and Hx expression, studies incorporating an oxygen concentration gradient should be conducted that may provide clearer insights into the genetic responses of stoneflies to short-term hypoxia.

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AUTHOR CONTRIBUTIONS

Maribet Gamboa: contributed in conceptualization (lead); data curation (lead); formal analysis (lead); funding acquisition (lead); investigation (lead); methodology (lead); project administration (lead); resources (lead); software (lead); supervision (lead); validation (lead); visualization (lead); writing original draft (lead); and writing review and editing (lead).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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