RESEARCH ARTICLE

Influence of Temperature, Hypercapnia, and Development on the Relative Expression of Different Hemocyanin Isoforms in the Common Cuttlefish *Sepia officinalis*

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ABSTRACT

The cuttlefish *Sepia officinalis* expresses several hemocyanin isoforms with potentially different pH optima, indicating their reliance on efficient pH regulation in the blood. Ongoing ocean warming and acidification could influence the oxygen-binding properties of respiratory pigments in ectothermic marine invertebrates. This study examined whether *S. officinalis* differentially expresses individual hemocyanin isoforms to maintain optimal oxygen transport during development and acclimation to elevated seawater pCO_2 and temperature. Using quantitative PCR, we measured relative mRNA expression levels of three different hemocyanin isoforms in several ontogenetic stages (embryos, hatchlings, juveniles, and adults), under different temperatures and elevated seawater pCO_2. Our results indicate moderately altered hemocyanin expression in all embryonic stages acclimated to higher pCO_2, while hemocyanin expression in hatchlings and juveniles remained unaffected. During the course of development, total hemocyanin expression increased independently of pCO_2 or thermal acclimation status. Expression of isoform 3 is reported for the first time in a cephalopod in this study and was found to be generally low but highest in the embryonic stages (0.2% of total expression). Despite variable hemocyanin expression, hemolymph total protein concentrations remained constant in the experimental groups. Our data provide first evidence that ontogeny has a stronger influence on hemocyanin isoform expression than the environmental conditions chosen, and they suggest that hemocyanin protein abundance in response to thermal acclimation is regulated by post-transcriptional/translational rather than by transcriptional modifications. *J. Exp. Zool. 317A:511–523, 2012.* © 2012 Wiley Periodicals, Inc.

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During the last decade, a growing body of evidence indicates that global warming and acidification of ocean surface waters impact marine organisms and ecosystems (Walther et al., 2002; Feely et al., 2004; Fabry et al., 2008; Melzner et al., 2009; Munday et al., 2009). The concept of oxygen- and capacity-limited thermal tolerance (Frederich and Pörtner, 2000; Pörtner, 2010) implies the onset of a decrease in whole-animal aerobic scope at low and high *pejus* thresholds, due to limitations of the functional capacities of circulatory and ventilatory systems to ensure oxygen supply. These limitations of the oxygen delivery system transfer to lower hierarchical levels and can lead to cellular, molecular, and biochemical disturbances (Pörtner, 2002). Accordingly, a recent study determined a limiting role of the cardiovascular system and the critical temperatures (7 and 26.8°C) by the onset of anaerobic metabolism in *Sepia officinalis* acclimated to 15°C (Melzner et al., 2006, 2007).

Hemocyanin, the extracellular respiratory pigment of *S. officinalis*, most likely plays a central role in the temperature tolerance of the whole organism in supporting the ventilatory and circulatory systems to cover cellular oxygen demand. In all species of the Cephalopoda investigated so far, the blue, copper-containing ring-shaped respiratory protein hemocyanin possesses a molecular mass of ca. 4 MDa and is expressed and synthesized in the branchial gland (van Holde and Miller, '95; Markl et al., 2001; Beuerlein et al., 2004). While some molluscs, for example, the sea hare *Aplysia californica*, possess only one type of hemocyanin, other species, for example, the abalone *Haliotis tuberculata*, the keyhole limpet *Megathura crenulata*, the freshwater cerithioid *Melanoides tuberculata*, and the cephalopods *Loligo pealei*, *Octopus dofleini*, and *S. officinalis* (van Holde and Miller, '85; Chignell et al., '97; Lieb, 2001; Altenhein et al., 2002; Lieb et al., 2010) express at least two distinct isoforms.

All subunits or isoforms, respectively, that have been analyzed so far, are encoded by distinct genes (e.g., Altenhein et al., 2002; Bergmann et al., 2007; Lieb et al., 2010). Commonly, every hemocyanin-holomolecule is formed by only one unique isoform building homo-multimeric hemocyanins (Harris et al., 2000; Gebauer et al., 2002). Those isoforms seem to be differentially expressed. Besides the common oxygen transport capacity, they may also have distinct functions of their own, which however are not yet known (Streit et al., 2005).

Recent DNA sequence analyses of the hemocyanin of *S. officinalis* from the North Sea, the English Channel, and the Bay of Biscay provide evidence for three hemocyanin isoforms (Melzner et al., 2007). This is the first evidence for the existence of a putative third isoform of hemocyanin of *S. officinalis* and might also be a result of thermal adaptation of the respiratory protein (Somero, 2005).

Ectothermic animals are able to alter gene expression in response to acclimation to changing abiotic conditions, that is, temperature, oxygen, or osmolarity (Somero, 2005). Modifications in the expression of different isoforms can either result in a change of the amount/activity of a protein, or in different properties of the protein with the same basic biochemical function (Schulte, 2004). Thus, protein modifications of hemocyanin could be one response towards changing temperature, similarly as found in gobies, where conformational modifications in lactate dehydrogenase (LDH) alter its thermostability (Fields et al., 2002).

Two out of the three known *S. officinalis* hemocyanin isoforms [*S. officinalis* hemocyanin type 1 (SofHc 1) and 2 (SofHc 2)] have already been sequenced (SofHc 1: GenBank accession no. **DQ388569**, SofHc 2: GenBank accession no. **DQ388570**), while the complete sequence of the SofHc 3-cDNA has yet to be published (Mark et al., unpublished). The calculated theoretical isoelectric points (p*I*) of SofHc 1 (p$I = 5.74$) and SofHc 2 (p$I = 5.89$) reveal one more acidic hemocyanin subunit and one more alkaline form (Melzner et al., 2007). Such a difference of 0.15 pH units most probably influences their pH dependence, and also their P_{50} values. This could be of particular interest when hemolymph pH changes and raises the question whether there is a difference in regulation and physiological function of the different hemocyanin types.

Among the Mollusca, cephalopods possess the highest levels of hemocyanin in the blood and rely strongly on the associated oxygen transport (Pörtner, '94a). The high pH sensitivity of oxygen transport by the respiratory pigment hemocyanin is depicted by a large Bohr effect in cephalopods, describing a reduced oxygen-affinity with decreasing pH (Brix et al., '81; Pörtner, '90a, '94b). Additionally, temperature affects the oxygen affinity of hemocyanin in that increasing ambient temperature leads to a decreased oxygen affinity and a change of cooperativity of the pigment (Pörtner, '90a; Zielinski et al., 2001). Therefore, it is very important for cephalopods to regulate hemolymph pH in relation to ambient temperatures.

One mechanism to adjust extracellular pH is described by the α-stat hypothesis (Reeves, '72), which projects a lowering of pH by −0.015 to −0.020 pH units per °C in extracellular (pH_e) and intracellular (pH_i) body fluids with increasing temperature. According to this hypothesis, pH regulation in the body compartments follows the p*K*' shift of histidine with temperature and thereby keeps the dissociation equilibria of histidyl residues

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constant over a wide range of temperatures (Reeves, '85; Seibel and Walsh, 2003). A balanced expression of the acidic versus alkaline hemocyanin isoforms could be a possibility to maintain optimal oxygen transport despite changing environmental conditions (temperature, pCO_2, pO_2), similar to the situation in cod (*Gadus morhua*) hemoglobin (Petersen and Steffensen, 2003). Hypercapnia induced changes in the extracellular acid–base status could induce a higher degree of protonation of the histidine residues of hemocyanin. The two hemocyanin isoforms possess different amounts of histidine residues. Whereas histidines comprise 120 of all amino acids of SofHc 1 (in total, SofHc 1 contains 168 His, including six copper-complexing residues per *functional unit*, i.e., 48 His), the amino acid sequence of SofHc 2 contains 131 (in total 179 His) histidine residues, leaving SofHc 2 with an estimated 10% higher physiological buffering capacity. In this case, a shift in relative expression towards SofHc 2 would be useful under acidic conditions.

The ongoing acidification of ocean surface waters (ocean acidification), mainly driven by anthropogenic CO_2 emissions (Doney et al., 2009), has the potential to strongly affect marine organisms by decreasing the pH of their extra- and intracellular body fluids (Fabry et al., 2008). In the cephalopod *S. officinalis*, it has recently been shown that pH_i can be fully and pH_e partially compensated, primarily by means of active bicarbonate accumulation (Gutowska et al., 2010). This strong pH regulatory capacity in cephalopods is probably related to the high pH sensitivity of their extracellular respiratory pigment (Brix et al., '81).

Cephalopod hemocyanin oxygen transport might be even more endangered by hypercapnia in embryonic stages, as the egg wall in this lecithotrophic species represents a considerable barrier to the diffusion of respiratory gases (O_2 and CO_2). Particularly towards the end of embryonic development, Gutowska and Melzner (2009) measured very low pO_2 (<60,000 µatm) and up to 10-fold higher pCO_2 values (>4,000 µatm, pH 7.2) in the egg fluid (perivitelline fluid, PVF) surrounding the developing cuttlefish embryo. As adult hemolymph pCO_2 are typically found to be 2,000–3,000 µatm higher than those of the ambient medium (Gutowska et al., 2010), it is reasonable to expect pCO_2 values of up to 6,000–7,000 µatm in embryonic cephalopod plasma at a PVF pCO_2 of 4,000 µatm, and a further increase in PVF pCO_2 by 1,000–2,000 µatm within the next 100–300 years due to ocean acidification.

Knowledge about the effect of such high pCO_2 levels on the highly pH-sensitive hemocyanin during embryonic/juvenile development in cephalopods is very limited. Currently, only a few studies have investigated the developmental changes between different hemocyanin subunits or molecules and their expression in invertebrates (Terwilliger and Terwilliger, '82; Wache et al., '88; Durstewitz and Terwilliger, '97). While many adult marine vertebrates and invertebrates are good regulators of extracellular acid–base disturbances at moderate pCO_2 levels (Melzner et al., 2009), recent studies started highlighting the higher vulnerability of earlier life stages, for example, in fish or crustaceans, already at CO_2 concentrations predicted for the near future (Walther et al., 2010; Nilsson et al., 2012). Embryonic stages are of particular importance for the recruitment of the animals' population and thus their capability to compensate higher pCO_2 levels can be seen as a "bottleneck" in the light of ongoing ocean acidification. Accordingly, adult *S. officinalis* can maintain the same growth rate under 4,000–6,000 µatm CO_2 as under control conditions (Gutowska et al., 2008), while embryos already showed reduced growth rates at 3,700 µatm CO_2 (Hu et al., 2011a).

We hypothesize that hemocyanin expression in the common cuttlefish *S. officinalis* is influenced by the most important abiotic factors of climate change, temperature, and CO_2. We suggest that a differential expression of hemocyanin isoforms provides a means to respond to changes in these factors to maintain optimal oxygen transport. To study our hypothesis, we used quantitative PCR (qPCR) to analyze relative mRNA expression patterns of the three hemocyanin isoforms. In our study, we concentrated on (1) hemocyanin expression between different life stages (embryos, hatchlings, juveniles, and adults), (2) the effect of acclimation to higher pCO_2 on hemocyanin expression in different life stages, and (3) on the effect of thermal acclimation on hemocyanin expression in adult animals.

MATERIALS AND METHODS

CO_2-Experiments: Animal Collection and Acclimation

Early Lifestages. Eggs of *S. officinalis* were collected in the Oosterschelde (Netherlands) in May 2009, for the analysis of the effect of CO_2 on embryos, hatchlings, and juveniles over a short time-period. A batch of these eggs was kept at present atmospheric CO_2 concentrations of 380 µatm to serve as control; another batch of the cuttlefish eggs was acclimated for 6 weeks to 4,000 µatm CO_2 at the Helmholtz Centre for Ocean Research (GEOMAR, Kiel, Germany). The pCO_2 of 4,000 µatm is a value predicted for shallow coastal waters within the next century (Thomsen et al., 2010), and, thus, was used by Hu et al. (2011a) for their acclimation study from which the samples were obtained. Detailed acclimation and seawater physicochemical conditions are displayed in Table 1, and by Hu et al. (2011a).

The pH was measured daily with a National Bureau of Standards (NBS) buffer calibrated WTW 340i pH meter and SenTix81 electrode (WTW, Weilheim, Germany). Dissolved inorganic carbon was measured using gas chromatography, following a protocol modified from Lenfant and Aucutt ('66) and Pörtner ('90b). Seawater carbonate chemistry was calculated with the CO2SYS software (Lewis and Wallace, '98), using the measured pH and dissolved inorganic carbon values.

Adults. Eggs of *S. officinalis* were collected in the Oosterschelde (Netherlands) in July 2005. They were reared to maturity at the Alfred Wegener Institute (Bremerhaven, Germany). In this part of the study, it was intended to analyze mechanistic responses of

Table 1. Seawater physicochemical conditions during different CO_2 acclimations of embryos, hatchlings, and juveniles at 15°C.

Conditions	Embryos and hatchlings, 380 µatm CO_2	Embryos and hatchlings, 4,000 µatm CO_2	Juveniles, 380 µatm CO_2	Juveniles, 4,000 µatm CO_2
pH	7.96 ± 0.02	7.28 ± 0.03	8.21 ± 0.08	7.39 ± 0.09
pCO_2 (µatm)	710 ± 40	3713 ± 29	503 ± 88	3089 ± 37
DIC (mmol/kgSW)	2301 ± 25	2529 ± 48	2939 ± 36	2646 ± 52
T (°C)	14.6 ± 0.5	14.6 ± 0.5	14.8 ± 0.2	14.8 ± 0.2
S (psu)	34.9 ± 0.1	34.9 ± 0.1	33.1 ± 0.2	33.1 ± 0.2
Duration	6 Weeks	6 Weeks	7 Weeks	7 Weeks

DIC, dissolved inorganic carbon; pCO_2, partial pressure of CO_2; SW, seawater.
Values are mean ± SEM.

adult *S. officinalis* towards high pCO_2 levels (beyond pCO_2 ranges currently expected by ocean acidification scenarios). The adult animals were acclimated for 6 weeks to 10,000 µatm CO_2, at constant temperature (16.2 ± 0.1°C) and salinity (30–32 psu), control animals to 380 µatm under the same conditions. Animals in all experimental groups were fed with either live *Crangon crangon* or *Palaemon* sp.

Temperature-Experiments (Adults): Animal Collection and Acclimation

Eggs of *S. officinalis* were collected in the English Channel (Luc Sur Mer, France) in June 2007 at a local temperature of 15°C. They were transported to and raised in a closed, re-circulating aquarium system at the Alfred Wegener Institute, on a diet of mysids (*Neomysis integer*) and *C. crangon* at constant temperature (16 ± 0.1°C), salinity (30–32 psu), and pH (>8.0). Adult animals were acclimated to 11 and 21°C for 6–8 weeks before sampling at mature age, with 16°C being the control group.

Sampling Procedure

The body mass of the animals acclimated to different temperatures was the following: at 11°C 121.67 ± 16.82 g (standard error of the mean, SEM), at 16°C 61.60 ± 7.2 g (SEM), and at 21°C 179.77 ± 24.20 g (SEM, $N = 6$ per treatment). The adult CO_2 acclimated ($N = 6$) and control animals ($N = 4$) weighed between 403 and 577 g. The weight of the CO_2-acclimated juveniles (4,000 µatm) and their respective control animals ranged from 3.43 to 5.33 g ($N = 8$, control/treatment).

Before sampling, all animals were anesthetized by transferring them into seawater with 2.5% ethanol. The animals were then removed from the seawater, sacrificed by decapitation, and the mantle was opened by a ventral incision.

In case of the juveniles, the two whole gills were sampled in late-stage embryos and 2-day-old hatchlings. During the time course of CO_2 acclimation (4,000 µatm, 42 days) of the juveniles, gill tissue (ca. 1.5 mm in length) was taken at three time points, 48 hr/2, 10, and 42 days from eight specimens per treatment, respectively (eight control, eight CO_2 acclimated; see Hu et al. (2011a) for further details).

In case of the adults (CO_2/temperature acclimation), gill tissue and branchial gland were sampled, while only branchial gland tissue was used for RNA isolation later on. Hemolymph was collected from the *vena cava cephalica* and stored at −20°C. In all cases, the sampled tissue was shock-frozen in liquid nitrogen and stored at −80°C until used for the determination of mRNA expression patterns for hemocyanin isoforms.

RNA Preparation, PCR, and Sequencing

For total RNA extraction, 25–30 mg of the branchial gland was homogenized in a glass potter using 600 µL lysis buffer (Qiagen GmbH, Hilden, Germany). The spin-column extraction followed the RNeasy Mini Kit (Qiagen GmbH). DNA contaminations were removed using the DNA-free™ kit (Applied Biosystems, Darmstadt, Germany). cDNA synthesis was performed with the High-Capacity Reverse Transcription Kit Protocol (Applied Biosystems). The 20 µL final volume reaction contained 0.5 µg RNA to provide the same amount of cDNA in each template. qPCR primers were developed based on control PCRs at cDNA and gDNA levels, using a standard three-step protocol with hemocyanin-specific and degenerate primers (Lieb, 2001).

PCR products were separated in a standard 1% agarose gel and bands were extracted using the QIAQuik Gel Extraction Kit Protocol (Qiagen GmbH). Purified PCR products were cloned either into "pCR4-Topo" (Invitrogen, Carlsbad, Germany) or "pGemT-easy" vectors (Promega, Madison, WI, USA). The plasmid inserts were sequenced using the commercial service of Eurofins MWG GmbH (Martinsried, Germany).

Sequence Analysis

Alignments were created by MacVector® 9.0.2 (MacVector Inc., Cambridge, UK), consensus sequences with AssemblyLIGN™ 1.0.9 (Oxford Molecular Group, Oxford, UK). Database searches were

performed using standard sequence analyses tools (BLASTN and BLASTX) available at NCBI (http://www.ncbi.nlm.nih.gov/).

Primer for Real-Time Quantitative PCR (qPCR)

Highly purified salt-free primer for hemocyanin isoform 1 (GenBank accession no. **DQ388569**), hemocyanin isoform 2 (GenBank accession no. **DQ388570**), and hemocyanin isoform 3 (GenBank accession no. **JN392726**) were designed with the Primer Express Software for Real-Time PCR, version 3.0 (Applied Biosystems) and generated by MWG Eurofins GmbH with the following nucleotide sequences (fwd, forward; rev, reverse):

SofHc 1, fwd: CTTTTCGAGTTTACCAGCTCTTGTT
SofHc 1, rev: CCTGCAACGTCAATATATGAGTGAT
SofHc 2, fwd: TCTCCCGTTTTTGGTAACTGAAC
SofHc 2, rev: TGTCAGCAACATCAATATAACCATGA
SofHc 3, fwd: TGCTCCGTCAACCAATGTCC
SofHc 3, rev: TCCAGGGCATCTCGTTTTCAC

Conditions for all PCRs were optimized in the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems) with regard to concentrations of template and forward/reverse primer. qPCR reactions were conducted in MicroAmp® Optical 96-well plates (Applied Biosystems) in 20 µL reactions containing SYBR® Green PCR Master Mix (Applied Biosystems) and 2 µL cDNA (4 ng reverse transcribed total RNA) as PCR template. The following PCR program was used in the ABI 7500 System (according to the 7500 SDS Software; Applied Biosystems): activation (10 min at 95°C), amplification 40× (15 sec at 95°C for denaturing, 1 min at 60°C for annealing, with a single fluorescence measurement).

To verify that the reaction yielded only a single product, a melting curve (60–95°C with a heating rate of 0.1°C/sec and a continuous fluorescence measurement) was performed after the PCR cycles. The melting curves resulted in single product-specific melting temperatures of 73.5°C for SofHc 1, 74.2°C for SofHc 2, and 75.9°C for SofHc 3.

qPCR Analysis

The efficiency of the qPCR was calculated for the different primer pairs according to the equation $E = 10^{(-1/\text{slope})}$ using a standard-curve with five dilution steps of the respective primer pair. High qPCR efficiencies were determined for all three isoforms (SofHc 1, 1.94; SofHc 2, 1.91; and SofHc 3, 1.90). Cycle thresholds (C_t) of the qPCR were detected with the Applied Biosystems 7500 System SDS Software, Version 1.3 (Applied Biosystems). Further analyses of the data were performed using MS Excel. For comparing relative expression results between treatments, the delta-Cq quantification model was used. The basic principle of this model is that a difference (delta) in quantification cycle values between two samples is transformed into relative quantities using an exponential function with the efficiency of the PCR reaction as its base (Schefe et al., 2006). As a result, the highest relative quantity of all genes (hemocyanin isoforms) is set to 1.0.

Hemocyanin Content Measurement

Frozen blood samples of the adult experimental animals were thawed and centrifuged at 15,000*g* for 20 min at 4°C to remove cell debris. The supernatant was diluted 1:100 with stabilization buffer (50 mM Tris, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 150 mM NaCl, pH 7.4). Hemolymph protein content was detected with a Pharmacia-LKB-Biochrom-4060 UV–Visible Spectrophotometer (Amersham Pharmacia Biotech, London, UK) by ultraviolet absorption at 280 nm. In the hemolymph of *S. officinalis*, the extracellular hemocyanin amounts to about 95% of the total protein (Senozan et al., '88), thus the photometrically determined overall protein content could be used to estimate hemocyanin content.

Statistical Analyses

All data were tested for normality (Kolmogorov–Smirnov) and outliers using Nalimov's test (Noack, '80). The statistical difference of the investigated acclimations to their respective control conditions was evaluated by a one-way ANOVA followed by post hoc test (Tukey) or by unpaired *t*-test (Newman–Keuls). A $P < 0.05$ was considered to be significant. Data are presented as means ± SEM. For an estimation of the impact of acclimation effects and developmental stage on mRNA expression, a canonical correspondence analysis was applied (CCA with Brodgar, Highland Statistics Ltd., Newburgh, UK).

RESULTS

Relative Composition of Hemocyanin mRNA

In this part of the study, we intended to analyze if the relative composition of hemocyanin isoforms changes between life stages and as a result of acclimation. Therefore, we calculated the percentage of isoforms 1–3 of total hemocyanin transcript for each life stage and each acclimation condition (Table 2).

In adult animals, hemocyanin composition comprised significantly lower amounts of SofHc 1 (lowest: 15.14% in adults, 10,000 µatm CO_2; highest: 20.73% in adults, 380 µatm CO_2) than of SofHc 2 (79.22% in adults, 380 µatm CO_2; 86.16% in adults, 10,000 µatm CO_2). Relative abundance of the three isoforms was not altered by the different acclimation treatments (change in temperature, higher pCO_2).

In contrast, in embryos, hatchlings and early juveniles, hemocyanin mRNA comprised almost equal parts of isoforms 1 and 2 (SofHc 1: 52.18–54.28% and SofHc 2: 42.47–47.74%). Thus, during ontogeny *S. officinalis* clearly displays a change in hemocyanin isoform composition with decreasing amounts of SofHc 1 with ongoing development.

In comparison to the two other isoforms, the amount of SofHc 3 mRNA was generally low and lowest with 0.024% in 11°C adults.

Table 2. Relative mRNA expression of each hemocyanin isoform (% of total hemocyanin expression) at the different acclimations.

Treatment	mRNA expression (%)		
	Isoform 1	Isoform 2	Isoform 3
Embryos 380 µatm CO_2	52.62	47.24	0.136
Embryos 4,000 µatm CO_2	54.28	45.56	0.154
Hatchlings 380 µatm CO_2	54.28	45.57	0.144
Hatchlings 4,000 µatm CO_2	57.41	42.47	0.123
Juveniles 2 days 380 µatm CO_2	54.20	45.73	0.064
Juveniles 2 days 4,000 µatm CO_2	53.93	46.01	0.062
Juveniles 10 days 380 µatm CO_2	52.25	47.67	0.073
Juveniles 10 days 4,000 µatm CO_2	52.18	47.74	0.077
Juveniles 42 days 380 µatm CO_2	53.09	46.87	0.042
Juveniles 42 days 4,000 µatm CO_2	52.89	47.07	0.040
Adults 380 µatm CO_2	20.73	79.22	0.051
Adults 10,000 µatm CO_2	15.14	84.80	0.058
Adults 11°C	20.61	79.36	0.024
Adults 16°C	16.75	83.15	0.096
Adults 21°C	17.05	82.89	0.058

This was significantly lower than in the early life stages (a maximum of 0.154%, embryos 4,000 µatm pCO_2).

mRNA Expression Patterns of Individual Hemocyanin Isoforms

In this part of the study, we followed the individual changes in relative abundance of each hemocyanin mRNA isoform, between life stages and between treatments. In contrast to the relative composition analysis (see above), this analysis investigates the course of mRNA expression of the individual hemocyanin isoforms with ontogeny and as a result of the acclimations.

Embryos. Eggs of *S. officinalis* were acclimated to 4,000 µatm CO_2 and hemocyanin expression was measured in gill tissue immediately before hatching (stage 30 embryos; Lemaire, '70). In comparison to the control eggs kept at 380 µatm, the mRNA expression (given in relative quantities) of SofHc 1 and 2 was significantly lower (SofHc 1 was reduced by 27.1%, SofHc 2 by 31.8%) in embryos exposed to a pCO_2 of 4,000 µatm. In contrast, for SofHc 3 no significant difference in mRNA expression was observed between embryos maintained at 380 µatm (control) and at 4,000 µatm CO_2 (Fig. 1).

Hatchlings and Juveniles. In this part of the study, we compared hemocyanin expression in embryos, hatchlings, and the juveniles of the time series experiment in order to investigate potential ontogenetic effects (Fig. 1).

In contrast to the embryos, the acclimation to a pCO_2 of 4,000 µatm did not lead to a lower expression of SofHc 1 and 2 in

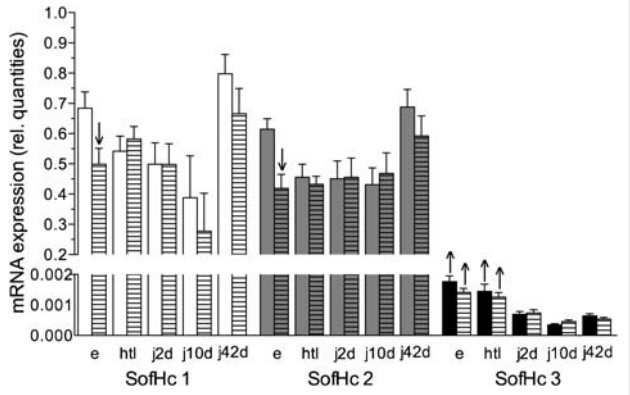

Figure 1. Hemocyanin expression in different developmental states acclimated to a pCO_2 of 4,000 µatm. mRNA expression of SofHc 1, 2, and 3 in gill tissue of embryos ('e', $N = 7–9$), hatchlings ('htl', $N = 10$), and juveniles ('j') sampled after 2, 10, and 42 days ($N = 7–9$). Solid white, gray, and black bars depict acclimation to control pCO_2 (380 µatm), striped bars are 4,000 µatm CO_2 acclimations. Changes of mRNA expression are shown in relative quantities (mean ± SEM). Indicates significantly lower expression than in control embryos. ↑ Depicts a significantly higher expression in embryos and hatchlings (pCO_2 4,000 µatm and control) than the juvenile stages (2, 10, and 42 days; ANOVA, $P \geq 0.05$). SofHc 1/2/3 = *Sepia officinalis* hemocyanin isoform 1/2/3.

hatchlings and juveniles. A linear regression analysis of mRNA expression in embryos, hatchlings, and juveniles (2, 10, and 42 days) revealed a significant increase of SofHc 2 expression with development under high pCO_2, but not in the control group (data not shown). For SofHc 3, a linear regression analysis of mRNA expression levels showed a significantly decreased expression from embryos to juveniles (42 days) in both acclimated and control groups (data not shown). The time-series experiment itself (sampling after 2, 10, and 42 days of exposure to a pCO_2 of 4,000 µatm) did not reveal an increase or decrease in gene expression of SofHc 3 under control conditions or at higher pCO_2 acclimation. However, for SofHc 3, both embryo and hatchling control as well as pCO_2 acclimated groups displayed a significantly higher mRNA expression relatively to juveniles (Fig. 1).

Adults (CO_2 Acclimation). Adult *S. officinalis* were acclimated for 6 weeks to a pCO_2 of 10,000 µatm. In comparison to their control group (pCO_2 of 380 µatm), mRNA expression (in the branchial gland) of the single hemocyanin isoforms did not change significantly (SofHc 1: downregulation by 8.4%, SofHc 2: upregulation by 34.28%, SofHc 3: upregulation by 42.47%; Fig. 2).

Adults (Temperature Acclimation). Under control conditions, total hemocyanin mRNA expression in the branchial gland of adult *S. officinalis* was composed of 16.7% SofHc 1-mRNA, 83.2% SofHc 2-mRNA, and 0.096% SofHc 3-mRNA (see Table 2). Figure 3 displays the quantitative change in isoform (1/2/3) mRNA expression after acclimation to 11/21°C in relative quantities versus the control (16°C). Acclimation to 11°C led to a significant reduction in expression in all isoforms in comparison to the control group. SofHc 1 was downregulated by 36.7% and SofHc 2 by 38.1%. Acclimation to 21°C did not induce any significant adjustment of SofHc 1 or 2 expression, respectively. SofHc 3 was significantly downregulated in both acclimation treatments (11 and 21°C): mRNA expression was reduced by 62.4% at 11°C and by 78.2% at 21°C, respectively (Fig. 3).

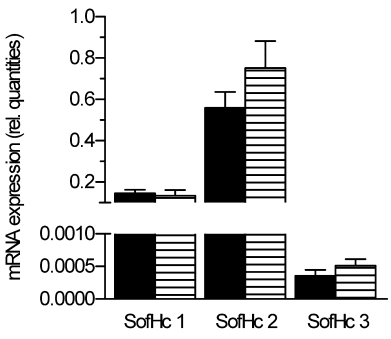

Figure 2. Hemocyanin expression in adult *S. officinalis* after acclimation to 10,000 µatm CO_2. mRNA expression of SofHc 1, 2, and 3 in the branchial gland of adult cuttlefish (N = 4–6). Black bars represent control conditions, 380 µatm CO_2, striped bars indicate 10,000 µatm CO_2 acclimation. Changes of mRNA expression are given in relative quantities (mean ± SEM). SofHc 1/2/3 = *Sepia officinalis* hemocyanin isoform 1/2/3.

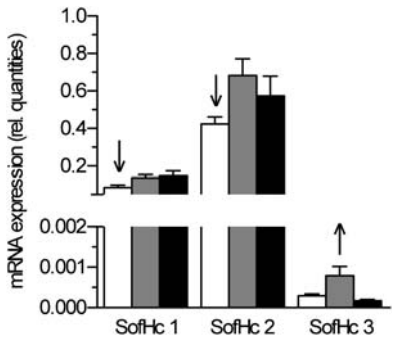

Figure 3. Hemocyanin expression in temperature acclimated adult *S. officinalis*. mRNA expression of SofHc 1, 2, and 3 in the branchial gland of adult *S. officinalis* (N = 6). White bars: 11°C acclimation, gray bars: 16°C acclimation (control), black bars: 21°C acclimation. mRNA expression is given in relative quantities (mean ± SEM). ↓ Indicates significantly lower expression than at 16°C. ↑ Indicates significantly higher expression than at 11 and 21°C for the given isoform (t-test, $P \geq 0.05$). SofHc 1/2/3 = *Sepia officinalis* hemocyanin isoform 1/2/3.

Table 3. Protein concentration in the hemolymph of adult *S. officinalis* under different temperature acclimations and higher pCO_2 acclimation.

Treatment	Protein concentration (mg/mL)
Adults 11°C	134.90 ± 3.26
Adults 16°C	144.39 ± 2.45
Adults 21°C	135.47 ± 7.94
Adults 380 µatm CO_2	135.58 ± 8.82
Adults 10,000 µatm CO_2	131.82 ± 8.61

Hemocyanin Protein Concentrations

For analyzing whether changes in mRNA expression were also visible at the protein level, protein content was measured spectrophotometrically at 280 nm in native hemolymph. Hemocyanin protein content was similar in all treatments (Table 3).

DISCUSSION

Hemocyanin mRNA Composition; Physiological Role of Isoform 3

This study addresses the effect of acclimation to ocean acidification and warming on the relative hemocyanin expression in the common cuttlefish *S. officinalis*. In the following sections,

we discuss the changes in hemocyanin isoform composition in different developmental stages, in response to higher pCO_2 and changing temperature, as well as the resulting functional consequences.

We observed a clear shift in the hemocyanin composition in *S. officinalis* with ontogeny. In adult animals, total hemocyanin mRNA comprised the greatest part of isoform 2 (up to 70%). Gielens et al. (2000) found a protein ratio of ca. 1:1 for isoforms 1 and 2 in adults. In the present study, isoform 1 to 2 transcript ratios of about 1:4 were determined in adults, while in embryos, hatchlings, and juveniles, stable ratios of 1:1 were found. This could be interpreted as a clear sign for transcriptional regulation of isoform expression during development and is supported by studies of, for example, Decleir and Richard ('70), Decleir et al. ('71), and De Wachter et al. ('88), who detected a gradual conversion from embryonic hemocyanins ("pre-hemocyanins"; Decleir et al., '71; Wolf and Decleir, '80) towards adult hemocyanins after hatching.

Accordingly, the relative amount of isoform 3 was higher in embryos and hatchlings (0.14% of all three isoforms in embryos, 16°C, 380 µatm pCO_2) than in the adults of our study (0.1% of all three isoforms in adults, 16°C, 380 µatm pCO_2; Table 2), reflecting the disappearance of embryonic hemocyanin during development. This suggests a more important role during embryonic development than in the late-stage embryos, juveniles, or adults investigated in our study, and might be related to the challenging ambient conditions during embryogenesis (gradually increasing pCO_2, decreasing pH within the egg; Melzner et al., 2009). This was supported by the CCA (Fig. 4), where the negative correlation of SofHc 3 to developmental stage clearly indicates a time-dependent decrease in transcript level, followed by an increase of SofHc 1 and 2 during development from embryos towards adults.

In the crab *Cancer magister*, hemocyanin is also found to be gradually changing from embryonic to adult hemocyanin isoforms (Terwilliger and Dumler, 2001), and specific hemocyanin isoform expression profiles are found in the abalone *Haliotis asinina*, depending on their developmental stage (Streit et al., 2005). In crustaceans, Terwilliger and Terwilliger ('82) and Wache et al. ('88) found shifts in hemocyanin subunit ratios during development at the protein level. In vertebrates, shifts from embryonic towards adult isoforms occur for several proteins within the first month post-hatching. Such a regulatory pattern has also been demonstrated for myosin heavy chain classes in the common carp, *Cyprinus carpio* (Cole et al., 2004; Nihei et al., 2006).

The presence of specific embryonic, respiratory pigments in *S. officinalis* could be a possible adaptation to high (and increasing) pCO_2 and resulting shifts in extracellular acid–base parameters during embryonic development. However, possible post-translational modifications are not known, and the isoform 3 protein still needs to be identified unequivocally at the biochemical level to elucidate whether the third hemocyanin isoform proves to be an embryonic "pre-hemocyanin."

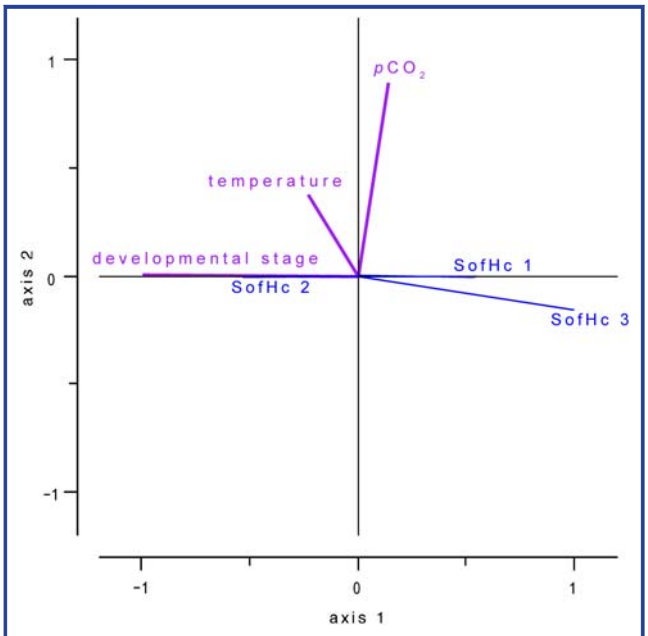

Figure 4. Canonical correspondence analysis: effect of developmental stage, ambient temperature, and pCO_2 on the expression of SofHc 1, SofHc 2, and SofHc 3. Length of the lines indicate the importance of the different effects on mRNA expression. The position of each gene in relation to the center of the plot reflects its relative correlation with the variables temperature, pCO_2, and developmental stage. SofHc 1/2/3 = *Sepia officinalis* hemocyanin isoform 1/2/3.

In case of the adults, the relative amount of SofHc 3 was significantly higher at 16°C than at 11 or 21°C (0.1%, Table 2). Thus, SofHc 3 mRNA expression appears to be highest in the optimal temperature range of *S. officinalis* (von Boletzky, '83). Owing to the extremely low expression level of SofHc 3 in juvenile and adult *S. officinalis* in comparison to the two other hemocyanin isoforms, its physiological function in oxygen transport may be negligible and remains unclear at present in these life stages.

The two major hemocyanin isoforms of *S. officinalis* examined in this study possess different p*I* (SofHc 1 more acidic, SofHc 2 more alkaline), and are most likely characterized by different pH and thermal sensitivities, as well as by different proton buffering capacities, respectively, and thus could constitute a mechanism to optimize oxygen affinity to changing ambient temperature and pCO_2. Durstewitz and Terwilliger ('97) and Schulte (2004, for a review) concluded that differential isoform expression might be, for example, a response to ongoing development or to changing environmental conditions. Yet, in adult cuttlefish, our results

indicate that hemocyanin isoforms 1 and 2 ratio (Table 2) was not altered by temperature or CO_2 acclimation.

Similar findings have been obtained using *C. magister* as a model (Terwilliger and Dumler, 2001). A differential regulation of the two mainly expressed isoforms could be physiologically mediated via post-translational modifications of the hemocyanin protein, namely selective recognition and sequestration by differential glycosylation (Lieb et al., 2000). Similarly, this mechanism regulates shifts in hemocyanin isoform proportions in individuals of the abalone *H. tuberculata* (Keller et al., '99) or the keyhole limpet *Megathura crenulata* (Lieb et al., 2000), depending on the physiological condition of the animals. Such differential glycosylation has in part also been demonstrated for *S. officinalis* by Gielens et al. (2004). Therefore, differential glycosylation appears as a conceivable way to adjust hemocyanin function to different environmental conditions in *S. officinalis*.

Hypercapnia Effects on Hemocyanin Expression in Embryos

Hemocyanin mRNA expression (SofHc 1 and 2) in cuttlefish embryos was significantly decreased under elevated seawater pCO_2 (4,000 µatm; Fig. 1). Under normocapnia, pCO_2 in the PVFs of *S. officinalis* eggs increases along with embryonic growth, reaching 4,100 µatm in late embryonic stages. This corresponds to embryonic hemolymph pCO_2 values of 6,000–8,000 µatm, thereby being much higher than in hatchlings and later developmental stages (Gutowska and Melzner, 2009). Thus, embryos are surrounded by CO_2 concentrations up to 10-fold higher than those of ambient seawater (380 µatm CO_2). Ambient pCO_2 is adding almost linearly to the high PVF pCO_2 leading to 8,000 µatm inside a cuttlefish egg at an ambient seawater pCO_2 of 4,000 µatm (Hu et al., 2011a). Considering this, late embryonic stages of *S. officinalis* might be especially vulnerable to the additional hypercapnic stress caused by ocean acidification.

Although cephalopod embryos possess epidermal ionocytes on yolk and skin epithelia to actively secrete acid equivalents via sodium–hydrogen exchanger-based proton secretion pathways, it can be expected that embryonic acid–base regulatory capacities are lower than those of adults (Hu et al., 2011b). Unfortunately, hemolymph acid–base status and hemocyanin content of embryos have not been studied so far due to methodological limitations. The effect of lower mRNA expression in embryos under 4,000 µatm CO_2 (Fig. 1) on total hemocyanin content therefore remains unclear.

Hu et al. (2011a) demonstrated a delay in embryonic growth when cephalopod eggs were exposed to a pCO_2 of 4,000 µatm, resulting in slower development and a smaller hatching size. Such hypercapnia-induced developmental delays have also been reported for several other invertebrate species (Dupont et al., 2008; Kurihara and Ishimatsu, 2008; Walther et al., 2010; Stumpp et al., 2011). Similarly, Marsh et al. (2000) reported large changes in gene expression during ontogeny, while enzyme activities remained constantly high. Thus, a developmental delay could well affect the timing and magnitude of mRNA expression levels and vice versa, explaining the lower mRNA expression of SofHc 1 and 2 found in the embryos acclimated to higher pCO_2 (Fig. 1). A similar, very strong downregulation (up to 80%) of transcripts coding for putative acid–base transporters was associated with a delayed differentiation of the embryonic gill under elevated seawater pCO_2 (Hu et al., 2011a).

Hypercapnia Effects on Hemocyanin Expression in Hatchlings and Juveniles

In contrast to the embryos, the expression levels of all hemocyanin isoforms were not significantly affected in hatchlings under elevated pCO_2 (4,000 µatm; Fig. 1). Hatchlings of *S. officinalis* show physiological conditions already very similar to juveniles and adults (Fioroni, '90), and might possess similarly high acid–base regulation capacities as adult *S. officinalis*, for which constant metabolic rates were observed under acutely elevated pCO_2 of 6,000 µatm (Gutowska et al., 2008). The shift in pH_e therefore may not have been drastic enough to induce a higher expression of isoform 2 to support extracellular buffering.

In the study on early juveniles of *S. officinalis* acclimated to a pCO_2 of 4,000 µatm for 42 days, a linear regression analysis of isoforms 2 and 3 revealed a similar change of mRNA expression over time in control and acclimated animals (Fig. 1). Apparently, changes in mRNA expression can be referred to as a developmental phenomenon (ontogentic effect and/or delayed growth), but not to an effect of acclimation to increased pCO_2 (Hu et al., 2011a; Stumpp et al., 2011; Windisch et al., 2011).

For a comprehensive picture, we used the CCA (Fig. 4) to provide an overview of the different effects (temperature, pCO_2, developmental stage) on mRNA expression of the three hemocyanin isoforms. The small angle between developmental stage and SofHc 2 indicates strong positive correlation, while SofHc 1 and 3 are negatively correlated. This supports the hypothesis of a continuously decreasing SofHc 3 expression with development, with a concomitant increase of SofHc 2 (cf. Table 2 and Fig. 1). The CCA demonstrates that developmental, rather than changes in abiotic conditions (reflected by the almost rectangular angle of the variables temperature and pCO_2 to the three isoforms), are the most important factors shaping hemocyanin expression. Among the abiotic factors, pCO_2 has a more pronounced effect than temperature, indicated by the relative length of the arrows.

Hypercapnia Effects on Hemocyanin Expression in Adults

When adult individuals of *S. officinalis* were acclimated for 6 weeks to 10,000 µatm CO_2, this acclimation did not elicit significant changes in total mRNA expression or protein concentration of hemocyanin (Fig. 2 and Table 2). Similarly, hemolymph protein concentration (mainly comprised of hemocyanin) in the blue crab *Callinectes sapidus* was suggested not to be adjusted for acid–base regulation purposes under hypercapnia (Henry and Wheatly, '92). In adult *S. officinalis*, extracellular

acid–base disturbances at an ambient pCO_2 of 6,000 µatm can be partially compensated through high capacities for rapid, active bicarbonate accumulation, and thus hemocyanin function and oxygen loading in the gills are maintained (Gutowska et al., 2010). No change in protein concentration would hence be necessary, which corroborates our findings.

Temperature Effects on Hemocyanin Expression in Adults

Several studies have addressed direct changes in gene expression in response to temperature acclimation, highlighting the profound temperature effect on the whole transcriptome (e.g., cold-induced cDNA upregulation in the carp *C. carpio*; Gracey et al., 2004). Changes in mRNA concentration or shifts in gene expression can often be observed in genes and proteins involved in acclimation responses, for example, transcripts of heatshock proteins, apoptosis, oxidative stress, or shifts in enzyme synthesis for repair of damaged proteins (Somero, 2005; Voolstra et al., 2009). Examples include the expression of different myosin heavy chain isoforms in adult carp (*C. carpio*) depending on cold or warm acclimation (Goldspink, '95; Imai et al., '96), or the thermal response of LDH isoforms found in salmonids and goldfish (Schulte, 2004).

In our study, the significantly reduced mRNA expression of SofHc 1, 2, and 3 at 11°C (Fig. 3) did not correspond to the stable protein concentration in the hemolymph of *S. officinalis* after acclimation to increased/decreased temperature (Table 3). This indicates that cold exposure of *S. officinalis* leads to a specific decrease only in hemocyanin transcript abundance. Lower transcript concentrations without a concomitant decrease in protein concentration may be caused by enhanced transcript stability in the cold (Martin et al., '93; Podrabsky and Somero, 2004). This may be paralleled by either enhanced protein stability or adjustments in translational capacity during thermal acclimation (McCarthy et al., '99; Storch et al., 2003). Higher RNA translation capacities thus seem necessary to support high rates of protein synthesis to maintain protein levels (post-transcriptional control; Lackner and Bahler, 2008). Similarly, our data revealed different transcript levels, but constant protein levels. Thus, the present work provides additional evidence that the regulation of hemocyanin expression in cuttlefish would rather happen at translational or post-translational level than at transcriptional level.

CONCLUSION

In this study, we analyzed differential hemocyanin expression in the common cuttlefish *S. officinalis* under different acclimation conditions and developmental stages. In summary, neither acclimation to temperature nor to hypercapnia significantly influenced the expression ratio between hemocyanin isoforms 1 (SofHc 1) and 2 (SofHc 2) in adult *S. officinalis* (c.f. Table 2). Only the earlier ontogenetic stages were characterized by relative changes in the isoform composition of the total hemocyanin mRNA pool following hypercapnic acclimation. We thus suggest that differential expression is strongly related to ontogeny, rather than elicited by abiotic factors in the ambient medium. Embryos showed higher expression variability under increased pCO_2, indicating that they might be more susceptible than adults because of the already high constitutive pCO_2 in the egg capsules.

Although temperature (warm and cold acclimation) led to a general decrease in hemocyanin transcript in adult animals, no changes in protein concentration could be found. For a regulation in response to changing environmental conditions, different post-translational patterns at this stage seem more likely than changes in expression ratios of the individual hemocyanin isoforms. Consequently, plasticity through regulatory and functional shifts may be small, as the gene expression and translation patterns of hemocyanin are already optimized to function under a wide range of environmental conditions.

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