ISJ 12: 29-37, 2015 ISSN 1824-307X

RESEARCH REPORT

Silencing two main isoforms of crustacean hyperglycemic hormone (CHH) induces compensatory expression of two CHH-like transcripts in the red swamp crayfish *Procambarus clarkii*

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Accepted January 14, 2015

Abstract

RNA interference has frequently been applied to modulate gene function in organisms. With the aim of creating new autocidal methods based on neuro-endocrine disruptors for invasive populations of *Procambarus clarkii*, we silenced the Crustacean Hyperglycemic Hormone (CHH) by injecting the corresponding dsRNA. CHH is a pleiotropic hormone that primarily regulates the mobilization of energy reserves and plays a pivotal role in stress responses. Here, we describe two experiments aimed at testing whether CHH silencing significantly alters important physiological aspects. The first experiment investigates the effects of CHH silencing at the glycemic and transcriptomic level in the eyestalk. The second experiment explores the long-term effects of CHH silencing and the effects on mortality and moulting rates. Osmotic deficits and mortality were recorded in specimens injected with CHH dsRNA, whilst controls were injected with GFP dsRNA. After 20 days, despite still silenced for CHH, individuals that survived recovered a strong hyperglycemic response after serotonin injection due to the compensatory effect of two peptides belonging to the crustacean neurohormone CHH protein family.

Key Words: *Procambarus clarkii*; crustacean hyperglycemic hormone (CHH); RNA interference; stress; survival

Introduction

The red swamp crayfish is an invasive species widely distributed worldwide (ISSG, 2012). Its burrowing behaviour and high fertility make it very competitive and often winning against native species which inhabit the same habitat. The policies applied to contrast the red swamp crayfish invasion into new habitats so far have been unsuccessful. The use of autocidal methods appears to be a promising strategy. In fact, autocidal approaches are based on the target species' biology and therefore do not cause environmental contamination and do not impact non-target species (Gherardi and Angiolini, 2004). Silencing key hormones via baits would allow selective disturbance of the target alien species and reduce adverse effects on native species, even those closely related to the alien species. In addition, this method is potentially easily applicable year round. The method is also relatively inexpensive, compared to the costs of trapping, the approach used so far to restrain the spreading of invasive species.

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The crustacean hyperglycemic hormone (CHH) controls many fundamental physiological functions such as glucose mobilization from glycogen depots during stress responses, moulting, reproduction and osmoregulation (Brown, 1934; Scharrer, 1952; Huberman and Aguilar, 1989; De Kleijn, 1994; Chung and Webster, 2003; Lorenzon, 2005; Lorenzon *et al.*, 2005; Katayama *et al.*, 2013; Turner *et al*., 2013) and behavioural responses, such as aggression (Aquiloni *et al.*, 2012) and anxiety (Lok *et al.*, 1977). In the crayfish *Pontastacus leptodactylus*, CHH injection causes a short term increase in glucose levels and its reduction through eyestalk ablation resulted with a decrease to basal levels (Mosco *et al.,* 2012, Lebaupain *et al.,* 2012). Recent studies highlighted that CHH specifically modulates ionic and metabolic homeostasis in the blue crab *Discoplax celeste* (Turner *et al*., 2013) and a variety of other functions involving, for example, inhibition of ecdysteroid (Chung and Webster, 2003), methyl farnesoate (Borst *et al*., 2001) and ovarian protein synthesis (Khayat *et al.*, 1998; Avarre *et al*., 2001). Furthermore, the involvement of two CHHs in the production of primary urine and in its branchial reprocessing was recently demonstrated (Turner *et al.*, 2013). CHHa increases Na^+ uptake at the gills in the very dry

Table 1 Experimental plans of experiment 1 and 2

Experiment 1

Groups	1st Day	2nd Day	4th Day
GFPi	G – dsGFP	Ser – G*	Ser – G* – X
CHHi	G – dsCHH	Ser – G*	Ser – G* – X

Experiment 2

Groups	1st Day	20th Day	26th Day
GFPi	G – dsGFP	Ser – G*	X
CHHi	G – dsCHH	Ser – G*	X

G: glycemia measurement, G*: glycemia measurements after 1, 2, 4, and 8 h from the serotonin injection, dsGFP or dsCHH: double strand GFP or CHH injection (2.5 µg/g body weight), Ser: Serotonin injection: Experiment 1: $1x10^{-8}$ mol/g body weight and Experiment 2: $2x10^{-8}$ mol/g body weight, X: sacrifice of specimens for eyestalk RNA extractions.

(pre-wet) season and CHHb significantly increases Na^+ uptake at the gills in the wet season. Noteworthy, recombinant CHH induces cellular and humoral responses in *Litopenaeus vannamei* infected with *Vibrio harveyi*, resulting in a higher survival rate in this group compared to controls (*Wanlem et al.*, 2011). Recombinant CHH also induces CHH expression in *P. clarkii* hemocytes (Kung *et al*., 2013). These data suggest an immune-related role for CHH. Serotonin (5-HT) injection depletes endogenous CHH peptide reserves and elevates glucose level both in *P. leptodactylus* and in *Squilla mantis* (Lorenzon *et al*., 2005). These results corroborate the held view that 5-HT has a strong hyperglycemic effect through CHH release from the medulla terminalis X organ-sinus gland complex (MTXO-SG), mediated by modulation of X-organ cells electrical activity (Sáenz *et al*., 1997).

RNA interference (RNAi) is a very effective technique to inhibit expression of genes targeting specific sequences (Manoharan, 2003). This procedure has been widely applied to develop new therapies (Kim and Rossi, 2007; Yu *et al*., 2014), to formulate novel drugs and vaccines (Lundstrom, 2014), to understand important and diversified physiological mechanisms (Denlinger and Armbruster, 2014; Liu *et al*., 2014), to control pathogens (Md Ali *et al.*, 2013; Dinh *et al.,* 2014) and invasive species (Bandaranayake and Yoder, 2013; Deng *et al*., 2013; Wynant *et al.*, 2014). Two CHHs were found in the *P. clarkii*'s eyestalk transcriptome with different expression levels. CHH1 resulted to be 6X more expressed than CHH2 (Manfrin *et al.*, 2014). Aiming at creating new pest management strategies based on the use of autocidal molecules administered via baits, we decided to explore the functional aspects of silencing this pivotal pleiotropic neurohormone. The combination of CHH transcript-silencing and CHH peptide depletion by serotonin was expected to cause a decrease in glucose levels. In this study, an unexpected hyperglycaemia was recorded. The new CHH-like transcripts previously found in *P. clarkii*'s eyestalk transcriptome were up-regulated in eyestalks of experimental *P. clarkii*. It is difficult to explain the function and mode of action of these two CHHs, but the CHH depletion, along with the other CHH-like transcripts, could affect the survival of *P. clarkii* in autocidal-based methods.

Materials and Methods

Experimental designs

Experiments were designed to evaluate 1-short-term effects of CHH triggered by the dsCHH injection (Experiment 1) and 2-long-term effects of CHH and effect of dsCHH injection on the survival and moult rate of the individuals (Experiment 2). Experiment 1: two groups of *Procambarus clarkii* males (10 individuals per group) were used in a four-day time course experiment as described in Table 1. About 2.5 µg/g body weight of Green Fluorescence Protein (GFP) or Crustacean Hyperglycemic Hormone (CHH) double strand RNA were injected at the beginning of the experiment to crayfish of the control and treated group, respectively. Serotonin (5-HT) was injected ($1x10^{-8}$ mol/g body weight, Sigma-Aldrich), at day 2 and day 4. Hemolymphatic glycemic levels were measured before the serotonin injections and 1, 2, 4 and 8 h after 5-HT injection. Mortality and moulting events were recorded daily. RNAs extracted from the eyestalks collected on the last day of experiment were subjected to qRT-PCR in order to prove the RNAi-affected CHH-silencing. Experiment 2: 10 control *P. clarkii* males and 15 dsCHH RNA-injected males were used in a twenty-six-day experiment aimed at examining the long-term effects of CHH-RNAi injections on both glycemia and survival rate. As described above, 2.5 µg/g body weight of GFP or CHH double strand RNAs were injected at the beginning of the experiment. Mortality was recorded on a daily basis for 20 days. On the 20th day $2x10^{-8}$ mol/g body weight of serotonin were injected to each individual of both groups. Hemolymphatic glycemic levels were measured before and 1, 2, 4

and 8 h post serotonin injections as in experiment 1. The individuals' health was checked every day until the 26th day and on this day total RNA was extracted from the eyestalks of both groups. The plan of experiment 2 is presented in Table 1.

Animal husbandry

All the *P. clarkii* individuals used in this study were collected from a drain inside the "Bonifica del Brancolo" (45°46' N, 13°30' E, GO, Italy). They were all adults, in intermoult and at non-reproductive stage. Specimens were acclimatized for a week in 120 L tanks provided with closed circuit filtered, and thoroughly aerated tap water at ~18 °C, and fed fish pellets (Sera granular, Heisenberg, Germany) three times a week. Each male was maintained in an individual cage within the same tank to preserve the same environmental conditions for all experimental specimen.

Ethical note

The following experimental procedures comply with the current Italian law. No specific permits were required for this study, as it did not involve endangered or protected species. Individuals were maintained in appropriate laboratory conditions to guarantee their welfare and responsiveness. After the experiments were completed, crayfish were euthanized by hypothermia.

Double-strand RNA synthesis

Specific primers amplifying a Green Fluorescence Protein contained in the cloning vector pEGFP-N1 (GenBank accession number U55762) and the Crustacean Hyperglycemic Hormone (GenBank accession number AB027291) were used. Both dsGFP and dsCHH primers fused with T7 5'-tail sequence (underlined in Table 2) were designed with Primer3 (Untergrasser *et al.*, 2012) and checked for secondary structures and possible hairpin formation with Oligocalc (Kibbe, 2007). Their sequences, along with the resulting amplicon sizes, are reported in Table 2. Standard PCRs were performed by Go Taq (R) G2 DNA polymerase (Promega), following these thermal conditions: 95 °C for 2', 35 cycles at 95 °C for 30", 57 °C for 30" and 72 °C for 45" with a final extension step at 72 °C for 5'. The resulting amplicons were agarose gel purified using E.Z.N.A. Gel extraction kit (Omega Bio Tek) and used as templates for the double-strand RNAs synthesized with the TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific). The dsCHH RNA probe was designed to silence the two *P. clarkii* CHH genes CHH1 and CHH2 (Manfrin *et al*., 2014), both involved in the hyperglycemic activity stimulation. The double-stranded RNA codifying the Green Fluorescent Protein (dsRNA-GFP) has been widely used as non-specific control in a variety of RNA interference studies (RNAi) (Westenberg *et al*., 2005; Ponprateep *et al*., 2012).

Injection and hemolymph withdrawal

Double-stranded RNAs were suspended in crustacean saline solution 0.5M (NaCl 14.5 g, $CaCl_2$ 0.72 g, $MgSO_4$ 3.18 g, KCl 0.35 g, HEPES 5mM, $NaHCO_3$ 0.5 g at pH 7.4 in a final volume of 1.2 L) with a final volume of 100 µL/animal.

Table 2 Primer sequences and their relative amplicon sizes used to synthesize double strand-specific RNAs and to evaluate CHH isoforms gene expression

	ID primer	5'-3' sequence	Amplicon size
Set of primers used for dsRNA synthesis	dsGFP FOR	TAATACGACTCACTATAGGGCACATGAAGCAGCACGACTTC	304 bp
	dsGFP REV	TAATACGACTCACTATAGGGGTTCACCTTGATGCCGTTCTTC	
	dsCHH FOR	TAATACGACTCACTATAGGGTCAGCTTCCTCTCCCAAGAC	302 bp
	dsCHH REV	TAATACGACTCACTATAGGGTACTTGCCGACAGTTTGGAC	
Set of primers used in qRT-PCR	EF1-α FOR	AGATCTGAAACGTGGTTTTGTT	186 bp
	EF1-α REV	TCAATCTTTTCCAGAAGTTCGT	
	β-actin FOR	AGGGCGTGATGGTTGGTAT	100 bp
	β-actin REV	CCGTGCTCAATGGGATATTT	
	CHH FOR	GCTTGACCGAGTGTGTGAAG	171 bp
	CHH REV	TACTTGCCGACAGTTTGGAC	
	CHHop FOR	CCGGCTCCTTCTACAAAATC	65 bp
	CHHop REV	AGTACGTCAACTGCCAAGGC	
	CHHip FOR	GAAACGGAATGCAGAAAAGG	70 bp
	CHHip REV	GCAGGAAAAGGTCGGATACA	

Injections and hemolymph withdrawal were performed through the abdominal hemolymph sinuses. To evaluate hemolymphatic glucose content, hemocytes were pelleted from the sampled hemolymph and the serum was kept on ice for later glucose measurement, which was performed using a glucose oxidase method (glucose liquid mono reagent, Hospitex diagnostics, Italy). The normal distribution of glycemic levels was verified with a Shapiro-Wilk test and homogeneity of variance across groups was checked with a Bartlett test. The null hypotheses from both tests could not be rejected. Hence, differences of glucose levels among the experimental groups were tested using non-parametric statistics, Kruskal-Wallis rank sum test with post-hoc Wilcoxon rank sum test pairwise comparisons with Bonferroni correction. Box and whiskers plots were drawn with the boxplot command of R.

Gene expression level evaluation

Total RNA from eyestalks was extracted by homogenization in TriReagent RNA isolation solution (Sigma-Aldrich) and purified with RNeasy MinElute Cleanup Kit (Qiagen). Reverse transcription and real time PCR reactions were accomplished by using the Go Taq R 2 step RT-qPCR System (Promega) and the PCR amplifications were performed in triplicates for each RNA sample using the CFX96 Real-Time PCR detection system (Bio Rad) mounted on c1000 Thermal cycler (Bio-Rad) with the following thermal profile: 95 °C for 2', 38 cycles at 95 °C for 15", 57 °C for 30" and 72 °C for 20", and a final melting curve analysis from 65°C to 95°C with an increment of 0.5 °C every 5". Elongation factor 1-alpha (EF1-α) and β-actin were selected as candidate reference housekeeping genes. Amplification efficiencies were estimated by LinReg v12.1 (Ramakers *et al.*, 2003). Gene expression stability for the references EF1-α and β-actin was tested considering as output the comprehensive ranking values derived from the comparison of Delta Ct (Silver *et al*., 2006), Best Keeper (Pfaffl *et al*., 2004), Normfinder (Andersen *et al.*, 2004) and Genorm (Vandesompele et al., 2002) software. Data were computed using the Bio Rad CFX Manager software (version 3.0.1224.1015) and the statistical analysis performed using REST-2009 (Pfaffl *et al.*, 2002).

Survival analysis

The effects of silencing on survival were assessed using the treatment as variable. Both exponential and Weibull parametric models with censoring were chosen because some individuals outlived the experiment. Statistical analyses were performed using R version 3.0.2 with library 'survival' (R Core Team, 2013; Therneau, 2014).

Results and Discussion

Glycemic effects

The amount of 5-HT to be injected in the animals was assayed through a pilot trial. During the trial we injected $1x10^{-8}$ mol/g of 5-HT and measured the glycemic levels in hemolymph 0, 1, 2, 4 and 8 hours after the injection. After an h, glycemic levels were around 120 mg/dL, after 2 h around 160 mg/dL and at 4 h around 40 mg/dL (data not shown). This amount of 5-HT was then considered adequate to be injected in experimental groups, in order to deplete eyestalk endogenous CHH reserves, according to Lorenzon and colleagues (2005). In experiment 1, 24 h after the injection of dsRNA both dsCHH- and dsGFP-injected groups showed a hyperglicemic peak at 2 h post serotonin injection with a mean glycemia of 160.71 ± 9.01 mg/dL (dsCHH) and 135.04 ± 12.03 mg/dL (dsGFP). On the 4^{th} day of the experiment the peak of glycemia of dsCHH-injected group was recorded at 2 h post serotonin injection with a mean glycemia of 79.14 ± 12.27 mg/dL, whereas the dsGFP-injected animals showed a glycemic peak of 90.41 ± 15.23 mg/dL 4 h post serotonin injection. No significant differences were recorded between the 2 experimental groups (Fig. 1A). These results were possibly a consequence of the short duration of experiment 1. It was therefore assumed that the silencing duration was too short to reduce the CHH transcript level. In fact, the effects of dsCHH-RNA were evaluated after 4 days from the dsRNA injection and the amount of 5-HT was not enough to deplete the major amount of endogenous eyestalk CHH reserves. As a consequence, the duration of experiment 2 was expanded to 20 days and the amount of 5-HT injected in the 25 animals was doubled. The glycemic peak was recorded at 2 hours post serotonin-injection with a mean value of 89.54 ± 17.57 mg/dL for the dsCHH-injected group and 52.96 ± 16.66 mg/dL for dsGFP-injected animals and no significant differences were recorded (Fig. 1B). Considering the 2 experiments together, there is a bias in the data collected on day 2 because the mean glycemia at time 0 is rather high due to the stress of dsRNA injection. In order to compare the peak at 2 hours (2 h) post serotonin injection in the 2 experimental groups at different days we decided to subtract the glycemia value at time 0 to the value of 2 h for each animal. This mean glycemia of dsCHH varies significantly among day 2, 4 and 20 (Kruskal-Wallis, p = 0.01) and on day 4 it is significantly lower than that on the day 2 (pairwise comparisons using Wilcoxon rank sum test, p = 0.004) but no significant differences were recorded in mean glycemia between day 2 and 20 (Figs 1A, B). The means of glycemia at peak time (2 h) of dsGFP do not differ among day 2, 4 and 20 (Figs 1A, B).

CHH silencing

Three individuals from each of the CHHi and GFPi experimental groups were used to evaluate CHH expression level, as well as of the above mentioned CHHop and CHHip genes using the EF1A as reference gene normalized to the GFPi group (Fig. 2). For both experiments, we were able to confirm the efficacy of the CHH1-2-RNA interference. However, the upregulation of CHHop and CHHip identified in the transcriptome of *P. clarkii* (Manfrin *et al*., 2014) was observed only after 26 days of CHH1-2 silencing.

We observed an up-regulation of CHHop (6 times higher than the GFPi group) and a highly

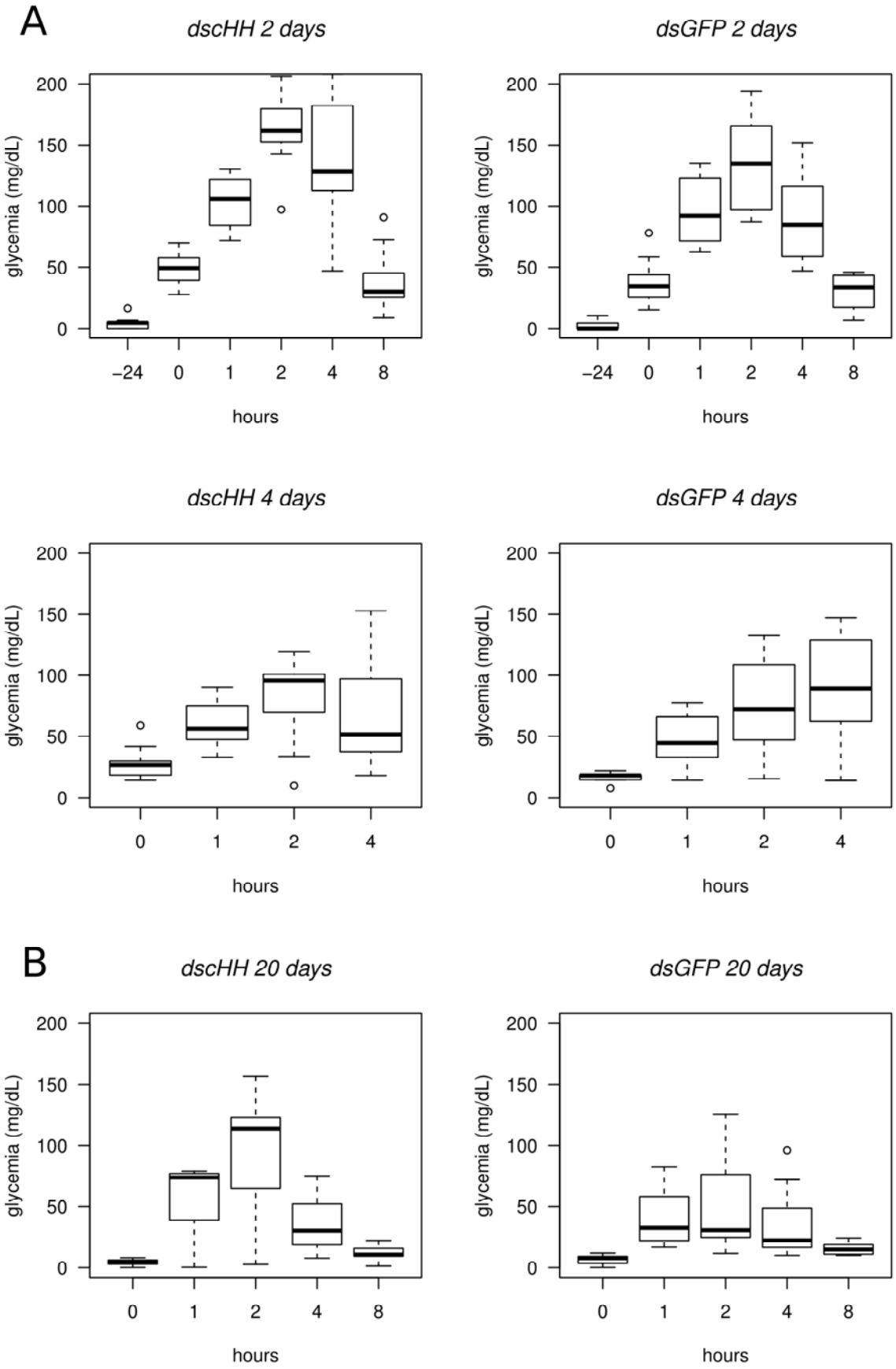

Fig. 1 Box plots of glycemia levels recorded in both the experiment 1 (A) and experiment 2 (B). In the Time point axis -24 indicates withdrawal led the day before the 5-HT injection, 0 represents the withdrawal done just before the 5-HT injection and the others time-points are the hours when hemolymph was collected.

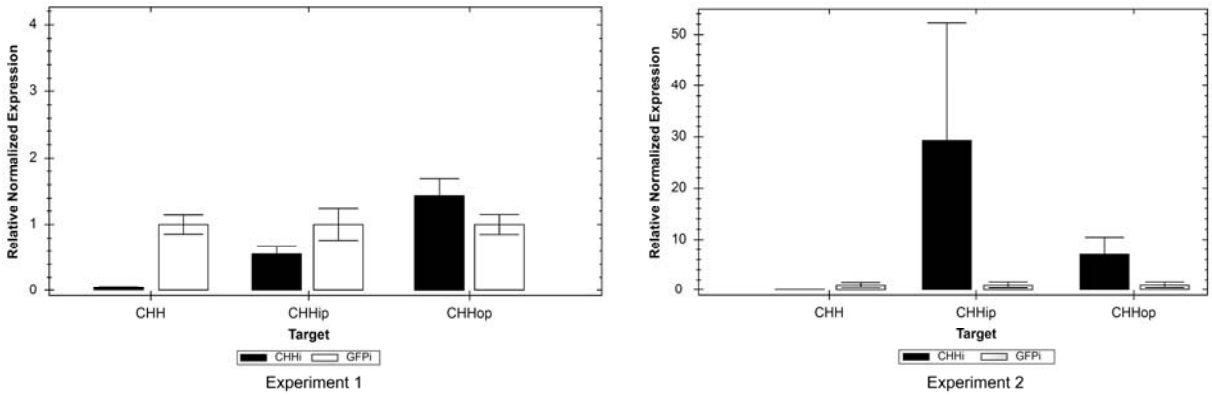

Fig. 2 Relative expression of CHH and CHH-like transcripts in eyestalks detected by qRT-PCR. CHHi: CHH interference group and GFPi: Green Fluorescent Protein interference for both the two experiments. The ΔΔCt method was applied by using GFPi group and EF1-α as calibrator samples. CHH1-2, CHHop (CHH homologous *Procambarus*) and CHHip (CHH Immune-related *Procambarus*).

significant over-expression of CHHip (80 times higher than the GFPi group, p = 0.01) in the eyestalk of *P. clarkii*. A remarkable ability of decapods is their long-term survival rate even when bilaterally eyestalk-ablated. A possible explanation of this phenomenon is that the animals compensate the lack of eyestalk neuropeptides via their expression or the expression of peptides of the same family in ectopic tissues. Similarly, the ability to perform a normal hyperglycemic response after 20 days of CHHs silencing followed by serotonin application suggests that other CHH family members could restore the hyperglycemia even when CHH1-2 are selectively silenced. Indeed, the induced expression of CHHop and CHHip after 20 days of CHH1-2 silencing may be this compensating factor.

Due to the relatively low sequence similarity between CHH1-2 and CHHop-ip we hypothesized that their involvement in compensating glycemia is a secondary function. Our results led us to suppose the possible involvement of CHHop-ip in the immune and stress responses. In fact, their up-regulation during experiment 2 was detected only in the specimens that survived the dsCHH-RNA injection, since almost half of *P. clarkii* males died in the 10 days following the injection.

Another important aspect is the absence of moulting events in both experimental groups, in contrast with the shedding episodes recorded in specimens collected from the same area, during the same days and maintained at the same laboratory conditions, but not challenged with any treatment. This finding is likely to be related to the stress triggered by the injection of double-stranded RNA.

Survival analysis

During experiment 1, 5 animals belonging to the dsCHH-injected group died, as well as 3 from the dsGFP-injected group. The higher number of specimens per experimental group in experiment 2 allowed more accurate evaluation of survival rate, and this is presented in Figure 3. Following the Kaplan-Meier estimation, the fraction of subjects living during the time course of the experiment after the dsGFP or dsCHH injection was recorded, highlighting the higher mortality caused by the dsCHH injection.

The highest mortality rate was reached between day 5 and 10 in the dsCHH RNA injected group. After 20 days from the beginning of the experiment, about 53 % of the specimens belonging to the dsCHH group and 70 % of the specimens from the dsGFP group were still alive. No significant difference was detected between the two groups, even though osmotic deficits were recorded only in individuals challenged with dsCHH RNA.

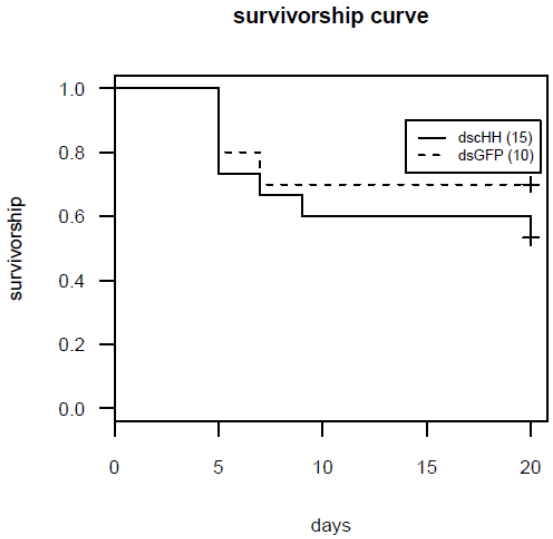

Fig. 3 Survival analysis. dsCHH represents the group of *P. clarkii* specimens (15) injected with dsCHH-RNA and dsGFP represents the animals injected with dsGFP-RNA (10 individuals). + indicates the end of the observation and the presence of alive animals at this time.

Fig. 4 Osmotic deficit recorded in deceased *P. clarkii* specimens following dsCHH- injection. The detachment of the carapace from the first pleon tergite is visible in this picture.

Specimens found deceased following the dsCHH injection, as documented in Figure 4, showed indeed a detachment of the carapace from the first pleon tergite, with the underlying membranous cuticle and epidermis becoming visible. This is an indication of osmotic deficit. *In vivo* experiments on shrimps (Nagabhushanam and Jyoti, 1977; McNamara *et al*., 1990) and crabs (Kamemoto et al., 1966; Kamemoto and Ono, 1969; Kato and Kamemoto, 1969; Kamemoto and Tullis, 1972; Heit and Fingerman, 1975; Davis and Hagadorn, 1982) demonstrated that the eyestalk ligation or ablation increases water influx. Similarly, CHH silencing produces an increase in hemolymph volume, that can lead to death.

These findings highlight the fundamental role of CHH in osmoregulatory processes suggesting that they might be controlled by a neuroendocrine mechanism, as already reported for other crustacean species (*e.g.,* Serrano *et al*., 2003, Turner *et al.*, 2013).

The second scenario refers to the specimens that were able to face the CHHs silencing, as shown by the glycemic levels presented in Figure 1, where a compensatory effect was activated. Glycemia increased along the time course experiment, to slowly decrease after 2 h from the initial 5-HT injection. What is responsible for the increased glycemic level if the two main CHHs produced in the eyestalks are silenced? It is our opinion that there are many other molecules related to the CHH family which were not silenced by our dsRNA probes due to the difference in nucleotide sequences. They may have compensated the lack of the known CHHs. To investigate this hypothesis further, we tested the expression level of two other CHH transcripts, CHHop and CHHip. After 26 days, both of them resulted up-regulated in the eyestalk of *P. clarkii* survived to the dsCHH-RNA treatment and significantly less expressed in specimens injected with dsGFP-RNA.

Little is known about the possible involvement of the CHH-superfamily in the immune response, but this study represents the first analysis from this point of view. Silencing the main CHHs in the red swamp crayfish outlined two different scenarios: 1- death in the specimens not able to face the lacking of the CHH, 2- survival of *P. clarkii* individuals able to activate secondary responses involving other CHH-superfamily members. The modes of action and intermediates messengers associated to these pathways are still unknown, but CHH depletion, along with other CHH-like transcripts, could impact the survival of *P. clarkii* in autocidal-based methods.

Acknowledgements

We thank the anonymous referees whose comments refined our thinking on the subject and Dr Marconi V, who assisted us in English editing. This study was supported by the European Project LIFE10 NAT/IT/000239 RARITY and by the Progetti di Ricerca di Interesse Nazionale (PRIN) 2010-11 Prot. 20109XZEPR_002, Ministero dell'Istruzione, dell'Università e della Ricerca.

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