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德國蜚蠊昇海藻糖激素於生殖與抗氧化逆境反應之角色
Functional Roles of Hypertrehalosemic Hormone Related
to Reproduction and Antioxidative Stress Responses in the
German cockroach *Blattella germanica* (L.)



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本論文係黃佳欣君 (F94632007) 在國立臺灣大學昆蟲學研究所完
成之博士學位論文，於民國一百年十一月十一日承下列考試委員審查
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摘要

昇海藻糖激素是一種胜肽荷爾蒙，屬於降脂激素家族，其主要功能是促使昆蟲體內海藻糖的生成。本論文研究是在探討德國蜚蠊昇海藻糖激素於生殖與抗氧化逆境反應的功能。該研究分別選殖出昇海藻糖激素與其受器基因，接著利用核糖核酸干擾技術來靜默基因表現以研究其功能。本研究首先證實昇海藻糖激素調控昆蟲體內海藻糖的恆定性，當昇海藻糖激素基因或其受器基因受到核糖核酸干擾而抑制後，德國蜚蠊血淋巴中海藻糖濃度顯著地降低。此外，處女雌蟲血淋巴海藻糖濃度在每個生殖週期有逐漸上升的趨勢，而且與卵巢發育具有正相關性；經實驗驗證，昇海藻糖激素具有調節生殖週期內海藻糖濃度上升的角色。昇海藻糖激素也會影響雌蟲排出卵鞘的行為，因為處女雌蟲體內的昇海藻糖激素或其受器基因受到靜默後，卵鞘排出的時間出現顯著性的延遲。

另一方面，該論文研究尚且探討德國蜚蠊昇海藻糖激素具有對抗氧化逆境的功能。該研究使用殺草劑巴拉刈注射到 10 日齡的雄蟲體內，不僅會造成雄蟲體內的氧化壓力上升並且後來會促使雄蟲死亡。當正常的雄蟲在接受固定濃度的巴拉刈注射後，過半的致死蟲數發生於第 4 天；然而，額外添加不同濃度的昇海藻糖激素 (10, 40, 80 pmole) 都能夠顯著地延後死亡率的發生，過半的致死蟲數則達第 7 天以後。此外，巴拉刈的致死作用對於昇海藻糖激素及其受器基因受到靜默而降低表現的雄蟲反而有更劇烈的影響，處理藥劑後的第二天就有半數以上的雄蟲死亡。接下來我進行血淋巴中脂質代謝分析作為昆蟲體內氧化壓力的一項指標。分析的研究結果顯示，將昇海藻糖激素與巴拉刈同時注射到雄蟲體內能夠維持丙二醛 (malondialdehyde) 濃度在正常量的水準；但是對於昇海藻糖激素與其受器基因受到靜默的雄蟲，外加注射的昇海藻糖激素並無法降低氧化逆境的傷害產生，其丙二醛的總量則呈現顯著性地升高。最後，本研究還發現昇海藻糖激素能夠在外加注射巴拉刈的狀態下提高粒線體細胞色素氧化酶第一次單元基因 (*cytochrome c oxidase subunit I*) 與細胞色素基因 (*CYP4G19*) 的表現量。上述的兩個基因曾經被指出與昆蟲抗氧化能力反應相關，關於昇海藻糖激素抵抗氧化壓力的機制亦在本論文研究中進一步討論。

關鍵詞：降脂激素，海藻糖，排卵，氧化壓力，昇海藻糖激素受器，核糖核酸干擾，巴拉刈

Abstract

A hypertrehalosemic hormone (HTH) peptide has been identified in the adipokinetic hormone (AKH) family to induce mobilization of carbohydrate in many insects. The aim of this study was to investigate the functions of hypertrehalosemic hormone in relation to reproduction and antioxidative stress response in the German cockroach *Blattella germanica*. I have isolated the *Blage-HTH* and its receptor (*Blage-HTHR*) genes, and thus studied their function through RNA interference (RNAi) to knockdown their expressions. The conventional role of HTH mediating trehalose homeostasis was observed in both sexes, in which the hemolymph trehalose was significantly reduced by RNAi-mediated knockdown of either *Blage-HTH* or *Blage-HTHR* genes. Especially, *Blage-HTH* regulated the increase of hemolymph trehalose in parallel with the ovarian development in the virgin females. Furthermore, *Blage-HTH* would assist the oviposition process because the ootheca production was delayed in the virgin females with either *Blage-HTH* or *Blage-HTHR* silenced.

In addition, I have unveiled another function of *Blage-HTH* against oxidative stress in *B. germanica*. Paraquat (PQ), a bipyridilium herbicide used in this study to elicit oxidative stress, causes a detrimental effect on the lifespan of 10-day-old adult males. Co-injection of synthetic HTH peptides at 10, 40, and 80 pmol concentrations significantly reduced the speed of mortality increase, with median survival time attained about 7 days in contrast to 4 days without HTH injection. However, PQ treatment caused a rapid mortality increase, with median survival time attained on 2 days, in the male adults whose *Blage-HTH* and *Blage-HTHR* were silenced simultaneously. I further examined the lipid peroxidation as an indicator of oxidative stress in the hemolymph after PQ treatment. Results showed that co-injection of HTH peptide maintained a normal level of lipid peroxidation, but it was not rescued by HTH in *Blage-HTH* and

Blage-HTHR knockdown specimens. Finally, *Blage-HTH* also stimulated the expression of *COXI* (cytochrome c oxidase subunit I) and *CYP4G19* (cytochrome P4504G19) in the fat body under oxidative stress elicited by PQ. The mechanism how *Blage-HTH* potentiates antioxidant protection has been discussed in the present study.

Keywords: Adipokinetic hormone, Trehalose, Oviposition, Oxidative stress, Hypertrehalosemic hormone receptor, RNA interference, Paraquat



Table of contents

口試委員會審定書	i
誌謝	ii
摘要	iii
Abstract	iv
Table of contents	vi
Chapter I. General introduction	1
References	6
Chapter II. RNA interference unveils functions of the hypertrehalosemic hormone on cyclic fluctuation of hemolymph trehalose and oviposition in the virgin female <i>Blattella germanica</i>	11
Chapter III. Functional characterization of hypertrehalosemic hormone receptor in relation to hemolymph trehalose and to oxidative stress in the cockroach <i>Blattella germanica</i>	13
Chapter IV. General discussion and conclusion	15
References	19
Appendices	21

Chapter I.

General introduction

One of the notable issues in insect endocrinology is on the neuropeptide research because neuropeptides mediate a variety of physiological and behavioral events in insects (Gade and Hoffman, 2005; Mercier et al., 2006; De Loof, 2008). Neuropeptides in the adipokinetic hormone (AKH) family, which are synthesized, stored and released by corpora cardiaca (CC) (Goldsworthy et al., 1972), have been intensively isolated nearly 50 different forms and characterized their function in relation to energy metabolism in insects for five decades (Gade, 2009). Common structural characteristics of AKH peptides are 8-10 amino acids long and start with a pyroglutamate residue blocking the N-terminus, an aromatic amino acid (phenylalanine or tyrosine) at position 4, a tryptophan at position 8, a glycine at position 9 (if present), and end by a amide group blocking the C-terminus. Peptides that can stimulate lipid mobilization are given the name of adipokinetic hormone, while peptides in AKH family which increase the level of hemolymph trehalose in the species in which they were isolated are referred to as hypertrehalosemic hormone (HTH). The principle action of AKH peptides on energy substrate released into the hemolymph, either lipids, trehalose, or a combination, depends on the insect species and the times during the metabolic needs (Schooley et al., 2005).

The action of AKH peptide is through a G protein-coupled membrane receptor with seven transmembrane segments. The identification of AKH receptors (AKHR) in insects has remarkably lagged behind. The insect AKHR was first identified from the fruit fly *Drosophila melanogaster* and the silkworm *Bombyx mori* (Staubli et al., 2002). The obese phenotype of *Akhr* mutant of *D. melanogaster* that shows the metabolic

abnormality in accumulation of energy reserves in the fat body reveals the important function of AKH on regulation of energy homeostasis (Gronke et al., 2007; Bharucha et al., 2008). Recently, the advance of whole genome sequencing provides more sequence information of AKHR in insects. In Africa malaria mosquito *Anopheles gambiae*, the supreme action of AKH is required a high expression level of its receptor because the mosquitoes whose AKHR expression is silenced fail to mobilize the glycogen reserves in the fat body after injection of Anoga-AKH-I (Kaufmann and Brown, 2008). Furthermore, the expression of *AKHR* in the dorsal unpaired median neurons has been demonstrated to mediate the action of Peram-AKH-I on stimulating locomotion in *Periplaneta americana* (Wicher et al. 2006). On the cellular mechanisms of AKH signaling, the *in vitro* study reports that the *Bombyx* AKH brings about a dose- and time-dependent internalization of AKHR via the clathrin-coated pit pathway (Huang et al., 2011).

More recently, the insect AKH peptides have been proposed to share a common ancestral peptide with the gonadotropin-releasing hormone (GnRH) in other metazoans (Lindemans et al., 2009). The same authors demonstrated a delay in time of egg-lying process and a decrease in progeny in the nematodes *Caenorhabditis elegans* after RNAi-mediated knockdown of the *Ce-AKH-GnRH* and *Ce-GnRHR* transcripts. GnRH is a pivotal peptide hormone to govern the reproductive process such as release of gonadotropins and behavior via the control of both endocrine and neural pathways in vertebrates (Okubo and Nagahama, 2008). The finding of *Bombyx* AKH activating one of the GnRH receptor of the amphioxus, a most basal chordate, also supports a common ancestor between GnRH and AKH signaling systems (Tello and Sherwood, 2009). In addition, the two AKH receptors of *D. melanogaster* and *B. mori* are regarded to be structurally and evolutionarily related to vertebrate GnRH receptors (Staubli et al.,

2002). However, the canonical role of insect AKHs is the energy mobilization but only few studies suggest the involvement of AKHs on reproduction by finding the expression of *AKH* mRNA (Kaufmann and Brown, 2006; Abdel-Latief and Hoffmann, 2007) and *Aedae-AKHR* mRNA (Kaufmann et al., 2009) in the insect ovary. So far, the functional examination of AKH peptides in reproductive processes remains an uncertain conclusion in insects.

The female German cockroach *Blattella germanica* has a cyclic reproduction to produce an ootheca in every cycle regardless a successful mating or not (Roth and Stay, 1962). During each reproductive cycle, the terminal oocytes become mature by uptake of vitellogenin which is an energy consuming process since less growing of oocytes after injection of trehalase inhibitor in the cockroaches (Kono et al., 2001). In addition, the virgin female *B. germanica* displays a mate-finding behavior to increase locomotor activity which masks the locomotor circadian rhythm in a few days before oviposition (Lee and Wu, 1994; Lin and Lee, 1996). Therefore, I presume that the virgin female *B. germanica* would require a higher energy demand during a reproductive cycle for physiological and behavioral needs. Trehalose is the major sugar in the hemolymph of most insects that is used as energy source by tissues like muscles, ovaries, etc. (Thompson, 2004). A question that arises is whether the level of hemolymph trehalose is increased for energy needs during reproductive cycle in the virgin female *B. germanica*. And next question is whether HTH peptide regulates the changes of hemolymph trehalose in related to those reproductive processes.

RNA interference (RNAi), the biological mechanism by which a introduced double-stranded RNA (dsRNA) is diced into a pool of 21-nucleotide small interfering RNA (siRNA) duplexes, induces gene silencing by targeting complementary mRNA for degradation, is one of the milestone discoveries in biological sciences for the past 15

years (Ketting, 2011; Meister and Tuschl, 2004). Systemic RNAi has become the most potent tool to unveil the gene functions in non-model insects because of its advantages including fast and maneuverable approach, higher versatility of silencing, stage-specific survey and so on (Belles, 2010). Among the non-model insects studied, the German cockroach *B. germanica* is particularly sensitive toward RNAi and has been studied many gene functions in diverse biological levels *in vivo* using this tool successfully (Belles, 2010). Consequently, RNAi provides a great opportunity to examine the roles of HTH in *B. germanica* through a reverse functional approach to silence its expression.

In order to examine the question if HTH mediates level of hemolymph trehalose during reproductive cycle in female *B. germanica* or not, I approached it using the molecular method by cloning of *Blage-HTH* and its receptor (*Blage-HTHR*) genes, thereby through RNAi to study their function. In chapter II, a sexual dimorphism in the changes of hemolymph trehalose were examined in *B. germanica* and the fluctuation of hemolymph trehalose showed a positive correlation with the terminal oocyte length in the virgin females. The further RNAi experiments of *Blage-HTH* and *Blage-HTHR* have demonstrated the critical role of *Blage-HTH* on the regulation of carbohydrate homeostasis. Additionally, silencing either *Blage-HTH* or *Blage-HTHR* resulted in a delay of ootheca production in the virgin females. These results revealed the roles of *Blage-HTH* involving in the reproductive processes in the female *B. germanica*.

AKH peptides exert numerous biological activities in addition to their well-known metabolic role (Kodrik, 2008). Generally, AKHs stimulate most catabolic reactions such as mobilization of energy reserves in the fat body while inhibit the anabolic reactions including RNA and protein synthesis at the same time. Furthermore, AKHs are considered as stress response hormones because some of their functions are associated to the stress conditions such as starvation (Lee and Park, 2004), pathogen (Goldsworthy

et al., 2002, 2003), oxidative stress (Kodrik et al., 2007, Vecera et al., 2007), insecticide treatment (Candy, 2002; Kodrik and Socha, 2005) and photophase interruption (Kodrik et al., 2005). However, the mechanism how AKH peptides exert their action in those stress conditions remains an open question even though some information about the signaling cascade of AKHs inducing energy mobilization in the fat body has been reviewed (Van der Horst et al., 2001; Gade and Auerswald, 2003).

Although a wealth of information has been available about the action of AKHs in relation to stress response at biochemical levels, information about molecular support has not been approached in this field yet. In Chapter III, I focused on the detailed examination of Blage-HTH against oxidative stress using *B. germanica* as a model through a forward method by injection of HTH peptide as well as a reversed method through interrupting Blage-HTH signaling pathway using RNAi under oxidative stress. These results unveil the role of Blage-HTH against oxidative stress at both biochemical and molecular levels. Moreover, I examined the other gene expressions related to oxidative stress response with the combination of paraquat, HTH, and RNAi treatments. Finally, a further discussion underlying the molecular mechanism how Blage-HTH potentiates an antioxidant protection in the cockroach *B. germanica* has been approached in this study. The goals of this study were to provide an experimental paradigm in molecular approach for the functional study of peptide hormones and provide the fundamental research for comprehensively understanding the pleiotropic roles of HTH peptides in this cockroach. The findings from these studies uncover the Blage-HTH functions in relation to reproduction and oxidative stress response and provide a possibility of application for pest control in the future.

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Chapter II.

RNA interference unveils functions of the hypertrehalosemic hormone on cyclic fluctuation of hemolymph trehalose and oviposition in the virgin female *Blattella germanica*

The main focus of this chapter is to investigate the functions of hypertrehalosemic hormone (Blage-HTH) in relation to reproductive processes in the virgin female *Blattella germanica*. Blage-HTH mediates increase of hemolymph trehalose in parallel to ovarian development as well as ootheca extruded at the time of oviposition during a reproductive cycle in the virgin females. These results provide a first functional examination of the insect AKHs involving in the reproductive processes to support a recent hypothesis about the evolution of AKHs from a superfamily of GnRH neuropeptides.

The question that arose at the beginning of project was whether the energy demand increases in a reproductive cycle of the virgin female *B. germanica* because the virgin females display a robust ovarian development and locomotion. The preliminary results showed a positive correlation between the fluctuations of hemolymph trehalose and the terminal oocytes length in the virgin females. Next, I have isolated Blage-HTH gene and performed RNAi to study its function. The virgin females whose Blage-HTH were silenced showed a delayed increase of hemolymph trehalose in contrast to dsEGFP-treated control. The potential role of hemolymph trehalose increase in assistance to vitellogenin uptake into growing oocytes during a reproductive cycle was discussed (Appendix 1). Of note, those dsHTH-treated females also delayed the time of ootheca production, and a few (10%) specimens produced a malformed ootheca. The unexpected finding of Blage-HTH RNAi in delay of ootheca production is

corresponding to another study of Ce-AKH-GnRH-like peptide mediating egg-laying behavior in *C. elegans* in 2009. Accordingly, the present study unveils a novel function of Blage-HTH in the reproductive processes of the female German cockroach.

This work has been published in the Journal of Insect Physiology (see Appendix 1 for more details) and could be accessed via the following citation:

Huang, J. H., and Lee, H. J., 2011. RNA interference unveils functions of the hypertrehalosemic hormone on cyclic fluctuation of hemolymph trehalose and oviposition in the virgin female *Blattella germanica*. *J. Insect Physiol.* 57, 858-864.



Chapter III.

Functional characterization of hypertrehalosemic hormone receptor in relation to hemolymph trehalose and to oxidative stress in the cockroach *Blattella germanica*

Nowadays, the advent of genomic era, many tools of molecular biology provide much assistance to understand the molecular mechanisms of biological events. Insect AKHs have been proposed as a stress hormone through the biochemical methodology previously. Here, I have conducted one of the most breakthrough tool, RNAi, to study the function of Blage-HTH against oxidative stress.

In the present study, I first isolated a hypertrehalosemic hormone receptor (Blage-HTHR) cDNA in *B. germanica*. Comparing the protein sequences of known AKHRs in insects, those receptors are likely to evolve fast within each insect order because the intracellular C-terminal region shows a distinct variety of sequences shared in the same order (Appendix 2, supplementary Figure S2). For verifying this novel sequence as the receptor of Blage-HTH functionally, I conducted RNAi to silence its expression and thereby examine the hypertrehalosemia of exogenous Blage-HTH in the specimens. Injection of 10 pmol Blage-HTH could induce a 113% increase of hemolymph trehalose 2 hr after treatment in dsEGFP controls but a significantly less level (49%) in dsHTHR-treated cockroaches. In addition, the levels of the hemolymph trehalose were significantly lower in the both sexes of *B. germanica* whose HTHR was silenced. These results confirm that Blage-HTH mediates the carbohydrate homeostasis in *B. germanica* through its receptor obtained in the present study.

Next, I investigated the question whether Blage-HTH potentiates an antioxidant protection in *B. germanica* through forward method by injection of Blage-HTH and

reversed method using RNAi to silence Blage-HTH and Blage-HTHR simultaneously. Paraquat (PQ), a bipyridium herbicide, was injected into male *B. germanica* to elicit the lethal oxidative stress in the present study. Co-injection of varied concentration of Blage-HTH with PQ resulted in a delay of mortality increase for 3 days at least. However, the males with a disrupting Blage-HTH singling by RNAi-silencing both Blage-HTH and Blage-HTHR showed a short median survival time (MST) attained on day 2 after PQ treatment in contrast to the control's MST attained on day 5. Furthermore, the lipid peroxidation, an indicator of oxidative stress, was increased significantly 4 hr after PQ treatment. However, lipid peroxidation was maintained in a normal level in the dsEGFP-treated males but increased in the dsHTH and dsHTHR-treated males after co-injection of Blage-HTH and PQ. These results proves the role of Blage-HTH against oxidative stress through reducing the oxidative damages such as lipid peroxidation.

This work has been accepted in *Frontiers in Experimental Endocrinology*, a specialty of *Frontiers in Endocrinology*, in participation of the research topic “Molecular mechanisms underlying insect behaviors: receptors, peptides, & biosynthetic pathways” and be accessed via the following citation:

Huang, J. -H., Belles, X., and Lee, H. -J., 2011. Functional characterization of hypertrehalosemic hormone receptor in relation to hemolymph trehalose and to oxidative stress in the cockroach *Blattella germanica*. *Front. Endocrin.* 2, 114. doi: 10.3389/fendo.2011.00114.

Chapter IV.

General discussion and conclusion

This study unveils that Blage-HTH plays two roles on reproduction in female *B. germanica*. One is to regulate the elevation of hemolymph trehalose, which is used for ovarian development and locomotion. Another is to promote oviposition process. In addition, Blage-HTH behaves as a stress hormone, which is released at the period of high stress, to relieve oxidative stress in particular.

AKH peptides are considered as the general endocrine regulator of energy homeostasis in insects (Lorenz and Gade, 2009). In chapter II, we have discussed the possible role of Blage-HTH on daily orchestrated regulation of anabolic and catabolic reactions during reproductive cycle in the female *B. germanica* because Blage-HTH displays a contradictory effect on the syntheses of trehalose and vitellogenin. Additionally, the hemolymph trehalose fluctuated in the 6-day-old females treated with dsEGFP, but the level of hemolymph was not fluctuated and significant lower in the females treated with dsHTHR (Appendix 3). These results of Blage-HTHR RNAi support our previous hypothesis postulating that Blage-HTH mediates daily fluctuation of hemolymph trehalose. The endogenous circadian clock mediating rhythmic AKH release in the hemolymph is also inferred from the daily changes of AKH titer in fire bug *Pyrrhocoris apterus* under both light-dark photoperiod and constant darkness (Kodrik et al., 2005) as well as higher AKH titer in cricket *Gryllus bimaculatus*, during early scotophase than during early photophase (Lorenz and Gade 2009). Therefore, Blage-HTH is most likely to release at different time within a day to regulate the allocation of energy metabolism during reproduction in female *B. germanica*.

More recently, insect AKH peptides are proposed in the superfamily of gonadotropin releasing hormone (GnRH) which is a critical regulator of reproduction in vertebrates and their receptors could share a common ancestral origin in the deep of Bilaterian animals (Roch et al., 2011). Equivalently, Blage-HTH regulating reproductive events was found in parallel since Blage-HTH RNAi resulted in a delay of oviposition in virgin female *B. germanica* in chapter II. Furthermore, similar effect on delaying oviposition was observed in Blage-HTHR RNAi (Appendix 4) and possible action of Blage-HTH was proposed to stimulate the contraction of oviduct muscles with *Blage-HTHR* mRNA expression (in chapter III). In addition, the involvement of AKH peptides on reproductive processes was presumed according to the mRNA of *AKHR* gene in the ovary of mosquito *Anopheles gambiae* (Kaufmann and Brown, 2006) and *Aedes aegypti* (Kaufmann et al., 2009). In summary, those results suggest that insect AKHs mediates the reproduction and are functionally related to the GnRH in other metazoa.

In chapter III, the involvement of Blage-HTH in antioxidant capability was demonstrated through injection of HTH peptides and interruption of Blage-HTH signaling by RNAi in male *B. germanica* under oxidative stress (injection of paraquat, PQ). The antioxidant capability of Blage-HTH is likely a quick response because the response of signaling cascades to this peptide by cockroach trophocytes is demonstrated within few minutes (Steele et al., 2001) and the induction of locomotion is about 1 hr after AKH I injection in *P. americana* (Wicher et al., 2006). Accordingly, the levels of lipid peroxidation in the hemolymph maintained 4 h post co-injection PQ and HTH in dsEGFP controls revealed the quick response of HTH against oxidative stress. Similar effect of AKH on glutathione efflux is reported 4 hr post oxidative stress treatment in *P. apterus* (Vecera et al., 2007). However, PQ could exert detrimental effect on survival in

both normal and RNAi-treated cockroaches. Oxidative stress might cause some degrees of non-reversible damage to cockroaches.

We further to examine the possible mechanism of Blage-HTH against oxidative stress by verifying gene expression in the fat body of *B. germanica*. Results showed that co-injection of HTH peptide resulted in a up-regulation of cytochrome c oxidase subunit I (*CoxI*) and a cytochrome P450 (*CYP4G19*) genes 4 hr after PQ treatment (Appendix 5). RNAi mutant of *D. melanogaster* with inactivated mitochondrial respiratory complex IV including CoxI shows a decrease in PQ resistance (Copeland, et al., 2009). Moreover, the cytochrome P450 genes become up- and down-regulated in response to PQ treatment in *D. melanogaster* (Girardot et al., 2004). Subsequently, these results suggest that the molecular mechanism of Blage-HTH against oxidative stress could induce or maintain the expression of *CoxI* and *CYP4G19* genes in the fat body of *B. germanica*.

Why does Blage-HTH have an antioxidant capability? The intrinsic effect of Blage-HTH elevates the synthesis of trehalose in the fat body. The synthesis of trehalose is an energy consuming process (Becker et al., 1996). The reactive oxygen species (ROS) generated in the mitochondrial electron chain during aerobic respiration is well known in mammals as well as discovered in *D. melanogaster* recently (Sanz et al., 2010). It appears that higher mitochondrial respiration would produce more endogenous free radicals to cause oxidative stress. In addition, AKH peptides also stimulate the locomotor activity in many insects (Kodrik et al. , 2000; Lorenz et al., 2004; Wicher et al., 2006). Higher locomotor activity requires more energy expenditure in the muscles, which could counter more endogenous free radical produced by mitochondria at the mean time. In order to relieve the damage of endogenous ROS, the signaling cascades of HTH peptide might evolve to potentiate antioxidant response in

insects.

In summary, this study has revealed the pleiotropic function of Blage-HTH with respect to reproduction and oxidative stress responses in the German cockroach *B. germanica* through experimental demonstration at molecular level. Meanwhile, this study provides an open window to study the molecular mechanism how AKH peptides exert a pleiotropic function in insects as well as the basic information for potential applications in insect pharmacology such as development of peptide mimics as insecticides.



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Appendices

The appendix section includes one published paper (Appendix 1), one accepted manuscript (Appendix 2), and three unpublished data (Appendices 3 - 5).





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RNA interference unveils functions of the hypertrehalosemic hormone on cyclic fluctuation of hemolymph trehalose and oviposition in the virgin female *Blattella germanica*

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ABSTRACT

Hypertrehalosemic hormone (HTH) is a neuropeptide within the adipokinetic hormone (AKH) family that induces a release of trehalose from fat body into hemolymph in a number of insects. In this study, we first showed that female adult German cockroach, *Blattella germanica*, displayed a cyclic fluctuation of hemolymph trehalose levels correlated to the maturation of oocytes in the reproductive cycle. After cloning the *HTH* cDNA from the German cockroach (*Blage-HTH*), expression studies indicated that *Blage-HTH* mRNA showed the cyclic changes during the first reproductive cycle, where peak values occurred in 8-day-old virgin female cockroaches, which were going to produce oothecae. The functions of *Blage-HTH* were studied using RNA interference (RNAi) to knockdown its expression. Adult virgin females of *B. germanica* injected with *Blage-HTH* dsRNA increased hemolymph trehalose levels in the late period of vitellogenesis more slowly than control. Furthermore, RNAi of *Blage-HTH* delayed oviposition time and some (10%) individuals did not produce the first ootheca until 15 days after eclosion, whereas the control group produced ootheca before 9 days in all cases.

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1. Introduction

Hypertrehalosemic hormone (HTH) is a neuropeptide that belongs to the adipokinetic hormone/red pigment-concentrating hormone (AKH/RPCH) family, which regulates energy metabolism by stimulating the mobilization of energy substrate in the fat body (Gäde et al., 1997). The initial discovery of these hormones in the corpora cardiaca (CC) showed that they served as hyperglycemic factors, by inducing trehalose increase in the hemolymph of the American cockroach, *Periplaneta americana* (Steele, 1961). Peptides of the AKH/RPCH family are usually synthesized and stored in the CC (Van der Horst et al., 2001), and nearly 50 different peptide variants have been identified in diverse numbers of insect species (Gäde, 2009). AKH peptides are 8–10-amino acids long, with a pyroglutamate blocked N-terminus and an amidated C-terminus, aromatic residues at positions 4 and 8, and a glycine residue at position 9, when present (Gäde, 2009).

Insect AKHs have pleiotropic functions in various insect species (Schooley et al., 2005; Kodrik, 2008). Besides the primary role of inducing mobilization of energetic compounds, insect AKHs possess myotropic effects, thus they accelerate heartbeat rhythms in cockroaches (Scarborough et al., 1984; Keeley et al., 1991) and

the contraction rate of leg muscles in locusts (O'Shea et al., 1984) as well as general locomotion in several species (Kodrik et al., 2000; Lorenz et al., 2004; Wicher et al., 2006). Experiments of genetic manipulation of AKH gene expression in *Drosophila melanogaster* point to roles related to locomotion and hemolymph trehalose titer in response to starvation stress (Lee and Park, 2004; Isabel et al., 2005).

Lorenz and Gäde (2009) regard the AKHs as general regulators of energy homeostasis, which not only control the energy metabolism underlying locomotion, but also participate in other energy-demanding processes, such as development and reproduction. In the cricket, *Gryllus bimaculatus*, for examples, there is an orchestration of anabolic and catabolic reactions in fat body during oogenesis (Lorenz and Anand, 2004), and AKHs have been proposed as the triggers of catabolic reactions in the fat body that produce energetic substrates to be incorporated into the growing oocytes of *G. bimaculatus* (Lorenz and Gäde, 2009). On the other hand, many studies indicate that the involvement of AKH peptides in reproductive processes is the inhibition of vitellogenesis which is a huge anabolic process in the fat body (Carlisle and Loughton, 1979, 1986; Moshitzky and Applebaum, 1990; Comas et al., 2001; Lorenz, 2003). Some previous studies also suggest a role of AKHs related to reproduction by finding the expression of AKH genes (Kaufmann and Brown, 2006; Abdel-Latif and Hoffmann, 2007) and *Aedae-AKH* receptor mRNA (Kaufmann et al., 2009) in the ovary. Furthermore, AKH-like peptides in the

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nematode *Caenorhabditis elegans* are involved in egg-laying behavior through a structurally vertebrate-like gonadotropin-releasing hormone (GnRH) receptor (Lindemans et al., 2009).

Females of the German cockroach, *Blattella germanica*, follow several reproductive cycles in their adult life and produce an ootheca enclosing the mature eggs, either fertilized or unfertilized, in each reproductive cycle (Roth and Stay, 1962). Virgin females of *B. germanica* display both calling behavior and robust locomotor activity for several days before oviposition (Lee and Wu, 1994; Lin and Lee, 1996). Increased locomotion is related to mate-searching behavior, since the females of *B. germanica* reduce locomotion immediately after a successful mating (Lin and Lee, 1998). We can presume that a high energy demand is needed to support robust mate-searching behavior, and trehalose is the major sugar in the hemolymph that is used as an energy source by tissues like muscles and ovaries (Thompson, 2004). Moreover, the involvement of trehalose in vitellogenin intake by developing oocytes has been demonstrated in cockroaches (Kono et al., 2001). Therefore, mobilization of trehalose should be expected to increase before oviposition in the female *B. germanica*.

In the present study, the hemolymph trehalose titers of *B. germanica* were determined during reproductive cycles, and results show that the fluctuations of hemolymph trehalose levels parallel the ovarian growth. Then, the *Blage-HTH* gene was cloned in *B. germanica*, and its physiological function was investigated by RNA interference (RNAi) to which this cockroach species is particularly sensitive (Bellés, 2010). RNAi-mediated knockdown of *Blage-HTH* gene expression delayed the increase of hemolymph trehalose and the production of the first ootheca in virgin female *B. germanica*.

2. Materials and methods

2.1. Insects

The German cockroach, *B. germanica* (L.), colony was reared in environmental chambers under 28 °C and L:D = 16:8 h conditions. Dog chow and water were provided *ad libitum*. Newly emerged adults were collected within 24 h and separated by sex. Detailed information about rearing has been given previously (Lee and Wu, 1994).

2.2. Cloning of *Blage-HTH* in *B. germanica*

Total RNA was isolated with TRIzol[®] reagent (Invitrogen, Carlsbad, CA) from brain–corpora cardiaca–corpora allata (Br–CC–CA) complexes of adult *B. germanica* females, following the manufacturer's protocol. We used Superscript III reverse transcriptase (Invitrogen) and the oligo(dT)₂₀ primer to generate cDNAs. The first primer set which was designed based on the known AKH sequences of other insects, including *Blaberus discoidalis* (U35277), *P. americana* (AY622321), and *Bombyx mori* (AB298930), and was as follows: forward, 5'-TGT GTG AGG CTC AGG TGA ACT T-3'; and reverse, 5'-TGA GAA TTT TTC ACA TTC CA-3'. The amplified fragment (159 bp) was subcloned into the pGEMT-easy vector (Promega) and sequenced. To complete the *Blage-HTH* cDNA sequence, GeneRacer™ Kit (Invitrogen) was applied to accomplish rapid amplification of cDNA ends (RACE), according to the manufacturer's protocol. For 5'-RACE, the reverse primer was 5'-CAC ATT CCA GCA GTT TCT GA-3', and for 3'-RACE, the forward primer was 5'-GTA CAT GTA CAG TGC AAT GC-3'. All PCR products were subcloned into pGEMT-easy vector (Promega) and sequenced. BLAST analyses indicated that the sequence obtained in *B. germanica* was *B. discoidalis* prepro-hypertrehalosemic hormone homologue (GenBank: U35277.1) and we called it as *B. germanica* prepro-hypertrehalosemic hormone (*Blage-HTH*, GenBank accession no. FJ943774).

2.3. Quantitative real time PCR analysis

To quantify the mRNA levels of *Blage-HTH*, we followed a quantitative real-time PCR (qRT-PCR) approach. Total RNA was extracted from 3 adult female brain–CC–CA complexes with TRIzol[®] reagent (Invitrogen, Carlsbad, CA). DNase I (Promega) was used to remove the genomic DNA contamination. About 1 µg of total RNA of each sample was reverse transcribed into cDNA with oligo(dT)₂₀ primer using Superscript III reverse transcriptase (Invitrogen). Expression of the housekeeping gene, *actin* (AJ862721), was used as a reference, and the primer pairs were as follows: forward, 5'-TTG TGC TGG ACT CTG GTG AC-3', and reverse, 5'-ACG ATT TCT CGC TCT GCT GT-3'. The primers to study the *Blage-HTH* gene were: forward, 5'-TTG GTA GTT GTG GTG GCT CTA GCA-3', and reverse, 5'-CCA GCA GTT TCT GAG CTT CAC TCT-3'. The efficiency of each primer set was first validated by constructing a standard curve through four serial dilutions. The qRT-PCR reactions were carried out in triplicate in a Bio-Rad iCycler iQ5 Real-Time PCR detection system (Bio-Rad), using SYBR Green Realtime PCR Master Mix (#QPK-201; Toyobo Co., Osaka, Japan). A control without template was included in all batches. The PCR program began with a single cycle at 95 °C for 3 min, 40 cycles at 95 °C for 15 s and 60 °C for 60 s. Afterwards, the PCR products were heated to 95 °C for 15 s, cooled to 55 °C for 15 s and increasing temperature 0.5 °C/min in order to measure the dissociation curves and determine a unique PCR product for each gene. We followed a method based on Ct (threshold-cycle) to measure the gene expression levels according to the Pfaffl mathematical model (Pfaffl, 2001). The results were analyzed using the software associated with the instrument. The values were normalized with the values of *actin* in contrast to the values of 1-day-old samples. Results of 6 biological independent samples were used to calculate the mean ± SEM.

2.4. RNAi and semiquantitative RT-PCR analysis

The *Blage-HTH* double-stranded RNA fragment (dsRNA) was prepared following the PCR-template method using MEGAscript RNAi Kit (Ambion, Inc.). The *Blage-HTH* cDNA of the German cockroach was first cloned into the pGEMTeasy vector (Promega, Madison, WI), then a 330 base pair (bp) fragment targeting to the whole ORF of *Blage-HTH* mRNA (Fig. 2A) was amplified by PCR. The primers, which contained the T7 promoter sequence (TAATAC-GACTCACTATAGGGAGACCAC), used in this PCR reaction were as follows: forward primer, 5'-AGC TCC TAC ATC CCA CGT GTT-3', and reverse primer, 5'-TGT ACA TGT ACT GTG CAA TGC A-3'. The MEGAscript RNAi Kit (Ambion) was used to generate the dsRNA following the manufacturer's protocol. The dsRNA solution was stored at –80 °C until use. A dose of 3 µg of the 332-bp dsRNA targeting the *Blage-HTH* gene was injected into the abdomen of the newly emerged female adults. As the dsRNA control, we used the enhanced green fluorescence protein gene applied of the same dose. The *Blage-HTH* gene expression in the brain–CC–CA complex was determined by semiquantitative RT-PCR within the following days after dsRNA treatment.

To investigate the effect of RNAi, *Blage-HTH* expression in the cockroaches was determined by semiquantitative RT-PCR based on the ORF of the *Blage-HTH* gene. Thus, the 25 µl PCR mixture for amplifying the *Blage-HTH* fragment included 1 µl of cDNA, 10 pmol of forward primer (5'-CTG CCA TTC AAC TGG AAG ACG A-3'), 10 pmol of reverse primer (5'-AGC ATT CCC CAC TCA TAC ATA CAA AAT C-3'), and 5 µl of *Taq* polymerase mixture (Protech). The PCR (30 cycles) was performed as follows: denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 30 s. In PCRs for amplifying the *actin* gene fragment were performed in parallel. The PCR products were separated and visualized on a 1.5% agarose gel.

2.5. Trehalose determination

Trehalose in the hemolymph was determined with the method of Parrou and François (1997), slightly modified. Hemolymph was obtained from the hind leg coxa and a 5 μ l volume from two individuals was collected using a pipetman. The hemolymph sample was placed in a polyethylene centrifuge tube containing 200 μ l 0.25 M Na₂CO₃ buffer, it was then vortexed for 1 min, and incubated at 96 °C for 2 h to inactivate all enzymes and to convert glucose into its reductive form. Then, 120 μ l of 1 M acetic acid and 480 μ l 0.25 M Na-acetate (pH 5.2) were added, and the solution was centrifuged at 12,000 rpm, 24 °C for 10 min. An amount of 100 μ l of supernatant was incubated overnight at 37 °C with 2 μ l porcine kidney trehalase (Sigma, #T8778) in order to convert trehalose into glucose. The amount of glucose in 50 μ l of the above solution was measured using the Glucose Assay Kit (Sigma, GAGO-20). Glucose concentration was corrected by deducting the glucose amount of the same supernatant prepared under the same conditions in the absence of trehalase.

2.6. Statistical analysis

The results in the graphs represent the means of measurements \pm SEM. Statistical analysis was performed using the computing environment R (The R Foundation for Statistical Computing, 2009). Significances of the results were evaluated using unpaired *t*-test (Figs. 1A and 4B), one-way ANOVA ($\alpha = 0.05$) with Fisher's LSD multiple comparisons post-test (Fig. 3B), and Wilcoxon test (Fig. 4C). Correlation between the hemolymph trehalose and basal oocytes length in virgin female *B. germanica* was analyzed by linear regression using basic regression command, GLM, in R.

3. Results

3.1. Daily fluctuation of hemolymph trehalose

Fluctuation of the trehalose titer in the hemolymph in adult *B. germanica* after imaginal molt is shown in Fig. 1A. Trehalose titer in the virgin females was about 4 μ g/ μ l after imaginal molt and steadily increased almost 2-fold (7.5 μ g/ μ l) on day 8, when the ootheca was formed. When the unviable oothecae were dropped on day 9, trehalose titer became similar to that of individuals at the early stage of the reproductive cycle. Then, hemolymph trehalose titer of virgin females increased again while the next reproductive cycle proceeded. Regression analysis showed a positive correlation between hemolymph trehalose and basal oocyte length (Fig. 1B) in virgin female *B. germanica* ($r = 0.98$, $P < 0.0001$). Interestingly, a successful mating advanced the decreased response of the trehalose titer in the hemolymph (Fig. 1A). When virgin females were mated on day 5, the hemolymph trehalose titer showed significant differences with that of virgin females on day 7 (*t*-test, $P < 0.05$) and 8 (*t*-test, $P < 0.001$). Moreover, the hemolymph trehalose of the females carrying an ootheca did not increase, and showed significant differences with that of virgin females who were into the next reproductive cycle on days 17 and 19 (*t*-test, $P < 0.001$). In contrast, the adult virgin male *B. germanica* displayed a relative low and constant trehalose level after emergence in the first days of adult life (Fig. 1A).

3.2. Cloning and characterization of Blage-HTH

Blage-HTH cDNA (GenBank accession no. FJ943774) of *B. germanica* was cloned and sequenced following a two-steps PCR strategy, first amplifying a fragment of the specific sequence with primers based on the conserved motifs of other HTH

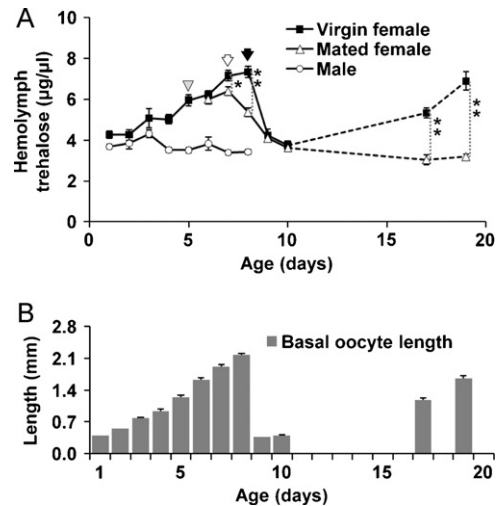


Fig. 1. (A) Daily changes of hemolymph trehalose levels in adults of *Blattella germanica*. The curve with solid squares represents the hemolymph trehalose concentration of virgin females ($n = 10$ –16) and the solid arrow indicates the time of oviposition for more than 50% of the virgin females. The curve with open triangles represents the hemolymph trehalose concentrations of mated females who mated with sexually mature male adults (10-day-old) on day 5 (marked with a gray triangle), and the open arrow indicates the time of oviposition for more than 50% of the mated females ($n = 12$). The curve with open circles represents the hemolymph trehalose concentration of males ($n = 10$). (B) The growth of basal oocyte length in virgin females of *Blattella germanica* ($n = 10$). All values represent the mean \pm SEM; significant differences of hemolymph trehalose titer between virgin and mated females are indicated by asterisks (*t*-test, $*P < 0.05$; $**P < 0.001$).

sequences, and then using 5'- and 3'-RACE approach to complete the sequence (Fig. 2A). The *Blage-HTH* mRNA contained 77 nucleotides in the 5' untranslated region (UTR) before the open reading frame (ORF), which extends from the start codon ATG at position 78 to the stop codon TGA at position 296. The ORF was followed by a 3'-UTR of 189 nucleotides including the potential polyadenylation signal AATAAA (Beauvoing et al., 2000) found at position 439, and a 20-nucleotide poly (A) tail. The predicted amino acid sequence of *Blage-HTH* peptide was QVNFSPGWGT, which was identical to the HTH peptide previously isolated from the cockroach (Gäde and Rinehart, 1990). The HTH-precursor-related-peptide (HPRP) for the putative *Blage-HTH* C-terminal peptide had two cysteine residues (Fig. 2B) predicted to form disulfide bonds for homodimerization during storage in the CC (O'Shea and Rayne, 1992). In addition, alignment among known AKH prepropeptide sequences in the Order Blattaria showed that *Blage-HTH* shared a high percentage of identical residues with *B. discoidalis* HTH (72.2%; U35277) and *P. americana* AKH-II (57.7%; AY622321) (Fig. 2B).

3.3. Expression of Blage-HTH

The expression of *Blage-HTH* was studied in different tissues including brain–CC–CA complex, ventral nerve cord, midgut, fat body, ovary, oviduct, and accessory gland of 6- and 7-day-old adult virgin female *B. germanica*. The brain–CC–CA complex was the only tissue expressing the *Blage-HTH* gene (Fig. 3A). The expression pattern of the *Blage-HTH* gene in virgin females of *B. germanica* after imaginal molt is shown in Fig. 3B. Expression gradually increased after emergence and reached at a peak on day 8 (one-way ANOVA, $F_{66,4,606} = 6.0588$, $P = 0.01646$; LSD post hoc test). When the unfertilized ootheca was formed by the virgin female, the highest expression level of the *Blage-HTH* gene immediately dropped to the same level as the beginning of reproductive cycle.

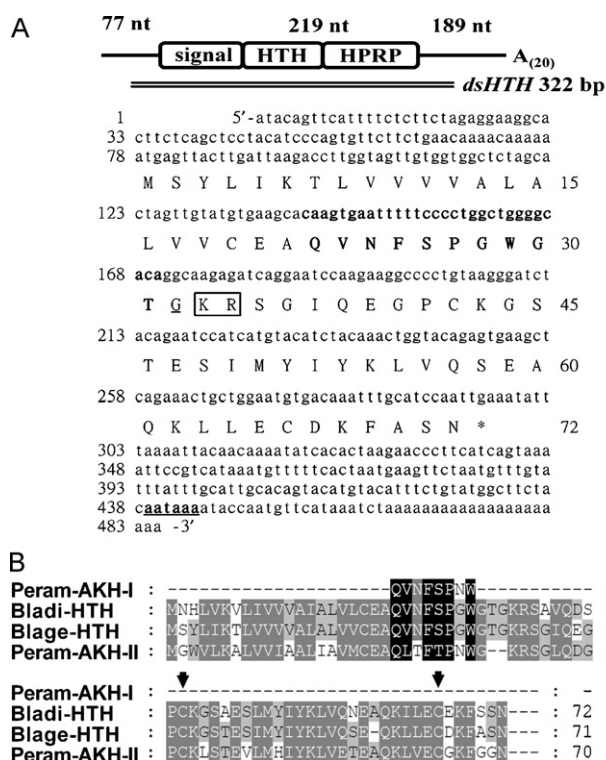


Fig. 2. (A) Nucleotide sequence and deduced amino acid sequence for *Blattella germanica* HTH (Blage-HTH) with flanking 5'- and 3'-UTR sequences. The upper diagram shows the *Blage-HTH* gene organization which consists of a 77-nucleotide 5'-untranslated region, 219 nucleotides encoding the HTH polypeptide precursor, HTH-precursor-related-peptide (HPRP) a 189-nucleotide 3'-untranslated sequence, and polyadenylated tail. In the prepropeptide sequence, signal peptide is in italics, and Blage-HTH is shown in bold type with the underlined glycine residue required for amidation and the potential dibasic cleavage residues boxed, followed by the HPRP. *The stop codon. A possible polyadenylation signal is shown in both bold type and underlined in the nucleotide sequences at 3'-UTR. (B) Comparison of deduced amino acid sequences on known HTH peptide among different species of cockroaches. The level of sequence similarity is indicated by black shading for 4 identity, gray shading with white letter for 3 identity, and gray shading with black letter for 2 identity. The two conserved Cys residues shown by arrows are thought to be involved in inter-molecular disulfide bonds for homo- or heterodimerization between these peptides during storage in cells (O'Shea and Rayne, 1992). Peram-AKH-I: *Periplaneta americana* AKH I (P84259), Bladi-HTH: *Blaberus discoidalis* hypertrehalosemic hormone (U35277), Blage-HTH: *Blattella germanica* hypertrehalosemic hormone (FJ943774), and Peram-AKH-II: *P. americana* AKH II (AY622321).

3.4. Functional studying of *Blage-HTH*

The study of the functional roles of *Blage-HTH* was approached with RNAi experiments. Concerning transcript lowering, the less expression of *Blage-HTH* was observed one day after dsRNA injection, but it became undetectable from day 2 after the dsRNA treatment to, at least, day 12 (Fig. 4A).

Once the *Blage-HTH* expression was knocked down, trehalose levels in the hemolymph were monitored (Fig. 4B). On days 5 and 6, the hemolymph trehalose titer of dsHTH-treated females remained at the same level as the controls (dsEGFP-treated). On day 7, however, the titer increased in controls but remained at the same levels in dsHTH-treated specimens; thus, on that day, hemolymph trehalose titer was significantly lower in dsHTH-treated specimen than in controls (*t*-test, $P = 0.003$). On day 8, the levels in dsHTH-treated increased, and those of control stabilized. The data indicate that the increase of hemolymph trehalose level increased more slowly in dsHTH-treated than in controls.

The silencing effect of *Blage-HTH* gene on ootheca production is shown in Fig. 4C. Most of the control (dsEGFP-treated) females started to produce an ootheca on day 8, whereas the treated

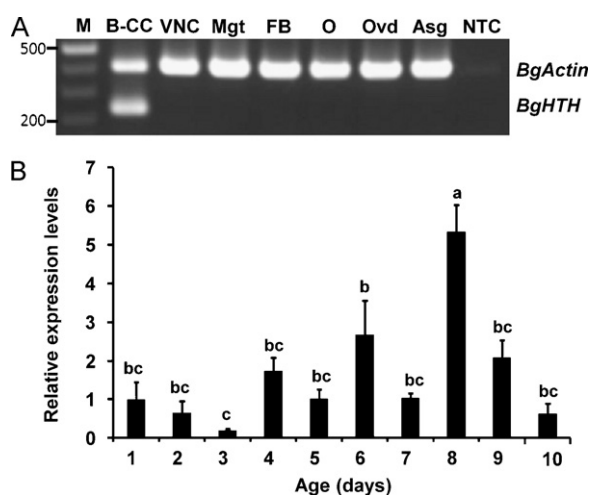


Fig. 3. Expression of *Blage-HTH* transcript in female adult *Blattella germanica*. (A) RT-PCR analysis of *Blage-HTH* expression (*BgHTH*, 232 bp) in different tissues: brain-CC-CA complex (B-CC), ventral nerve cord (VNC), midgut (Mgt), fat body (FB), ovary (O), oviduct (Ovd), and accessory gland (Asg) of 6- or 7-day-old adult females. The *actin* gene (*BgActin*, 409 bp) was used as control. M: 100 bp DNA marker; NTC: non-template control. (B) Expression pattern of *Blage-HTH* mRNA in the brain-CC-CA complex during the first reproductive cycle of female *B. germanica*. Quantitative Real-time RT-PCR analysis of *Blage-HTH* expression level was normalized against *actin* gene. The data represent the mean \pm SEM ($n = 6$) of relative transcription levels normalized in comparison to 1-day-old female for the *Blage-HTH* gene in the different ages. Different letters on the figure indicate significant differences (ANOVA, $P < 0.05$ and followed by LSD post hoc test).

(dsHTH-treated) females delayed the time of oviposition significantly (Wilcoxon test, $P < 0.01$). Some (10%) of the dsHTH-treated females did not oviposit until day 15. Furthermore, a few individuals (7.9%) treated with dsHTH produced short deformed oothecae (Fig. 4D).

4. Discussion

Given that reproductive processes require huge amounts of energy, the energy metabolism must be an important trait in the life history of adult insects (Lorenz and Gäde, 2009). We have monitored the fluctuations of hemolymph trehalose titers after imaginal molt and characterized functions of the *Blage-HTH* gene during the reproductive cycle in female *B. germanica*. In *B. germanica*, adult females undergo several gonadal maturation cycles accompanied with vitellogenesis and sexual receptive behaviors including sex pheromone release and robust locomotion in contrast to the adult males, which display no such cyclic reproductive activities (Lee and Wu, 1994; Schal et al., 1997). The fluctuation of hemolymph trehalose between female and male *B. germanica* also shows this sexual dimorphism (Fig. 1A). The dynamics of hemolymph trehalose in the female *B. germanica* suggests that energy demand increases during the reproductive cycle and that trehalose supplies, at least in part, this energy. Moreover, the parallel relationship between hemolymph trehalose and ovarian development was observed in virgin females of *B. germanica*, whereas mated females did not increase their hemolymph trehalose during the period of carrying an ootheca, which inhibits ovarian growth (Schal et al., 1997) (Fig. 1A). During oogenesis, the insect oocytes accumulate various nutrients from hemolymph such as carbohydrates, lipids, and vitellogenin (Vg) in particular (Raikhel and Dhadialla, 1992; Martin et al., 1996; Raikhel et al., 2005). The Vg uptake by oocytes could be suppressed by injection of the trehalase inhibitor, validoxylamine A (VAA), as shown in several insect species including *B. germanica* (Tanaka et al., 1998; Kono et al., 1999). The uptake of Vg by the maturing oocytes is considered as an energy-demanding process according

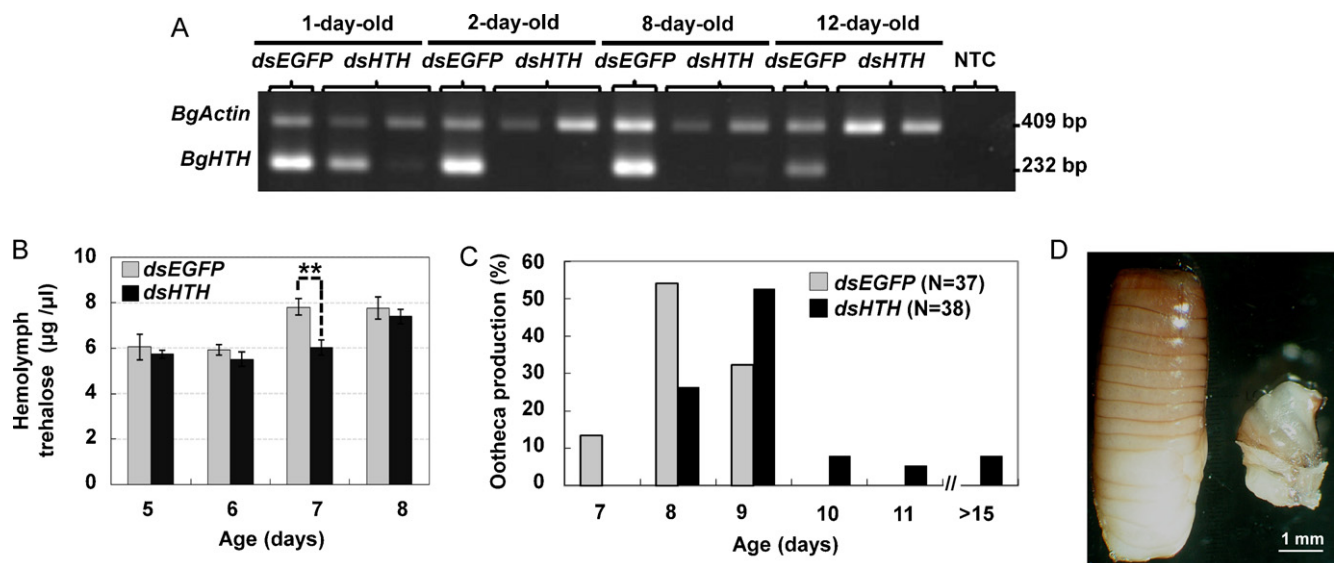


Fig. 4. RNAi of *Blage-HTH* in virgin females of *Blattella germanica*. (A) Silencing effect of *Blage-HTH* expression by the injection of *Blage-HTH* dsRNA. Total RNA from the brain-CC-CA complex was extracted and then amplified by RT-PCR. *BgHTH*: partial cDNA of *Blage-HTH* gene (232 bp); *BgActin*: partial cDNA of *actin* gene (409 bp); dsEGFP: double stranded RNA of enhanced green fluorescence protein; dsHTH: double stranded RNA of *Blage-HTH*. (B) RNAi-mediated knockdown effect of *Blage-HTH* on hemolymph trehalose levels. Values with vertical bars express the mean \pm SEM ($n = 8$). **Significant differences statistically at $P < 0.01$ (t -test). (C) Effect of RNAi of *Blage-HTH* on the timing of ootheca production during the first reproductive cycle. *Significant delay of ootheca production by injection dsHTH RNA (Wilcoxon test, $P < 0.01$). (D) Short and malformed ootheca produced by dsHTH-treated females. The scale bar corresponds to 1 mm.

to the further demonstration of reducing Vg content by impairing the activity of trehalase in the ovary of the cockroach *P. americana* (Kono et al., 2001). The steadily increase of hemolymph trehalose during the reproductive cycle in the female *B. germanica* can therefore be assumed as an energy fuel for the process of oocyte maturation.

The manifest function of HTH is to trigger biosynthesis of trehalose from glycogen of the fat body in the Order Blattaria (Scarborough et al., 1984; Gäde, 1989) and likely in response to the increase of hemolymph trehalose in the virgin female of *B. germanica*. In order to study the function of *Blage-HTH* as the upstream regulator of trehalose on reproduction, we successfully cloned the *Blage-HTH* gene in *B. germanica* and inhibited its expression by systemic RNAi. The treatment with dsHTH triggering a rapid reduction of the *Blage-HTH* mRNA results in a delayed increase of hemolymph trehalose (Fig. 4). The results imply that the continuous synthesis of *Blage-HTH* assists the increase of hemolymph trehalose during the reproductive cycle in the virgin female *B. germanica*. However, the later increase of hemolymph trehalose on day 8 might be caused by other hypertrehalosemic factors such as octopamine (Thompson, 2004) or few *Blage-HTH* transcripts proceeding since the RNAi method only diminishes the expression level of the gene in general.

The AKH peptides work as stress hormones by triggering energy mobilization as well as general locomotion, while inhibiting anabolic synthesis of expensive compound such as vitellogenin (Kodrik, 2008). Synthesized *Blage-HTH* has been demonstrated to reduce vitellogenesis in the fat body of *B. germanica* (Comas et al., 2001). Our deduction of continuous biosynthesis of *Blage-HTH* inducing the catabolic reaction of hemolymph trehalose during reproductive cycle appears to contradict the increase of anabolic vitellogenesis at the same time in the fat body of female *B. germanica* (Martin et al., 1996). One of possible scenario is the release of *Blage-HTH* peptides under circadian control to provide a daily orchestrated regulation of anabolic and catabolic reactions. Lorenz and Gäde (2009) have proposed that the release of AKH at different time within a day helps allocate the energy metabolism and triggers the mobilization of energetic compounds that are incorporated into growing oocytes.

Expression studies show a highest peak of *Blage-HTH* mRNA level in virgin females of *B. germanica* on day 8, when it produces an ootheca in the first reproductive cycle (Fig. 3B). The continuous synthesis of AKHs is important for the rapid release of AKHs contained within secretory granules (Harthoorn et al., 2002), whereas older secretory granules become non-releasable 8 h after synthesis (Sharp-Baker et al., 1996). In addition, the age-dependent release of HTH peptide is parallel to both transcription and translation levels in the CC of *B. discoidalis* (Sowa et al., 1996; Lewis et al., 1997). Consequently, we consider that the occurrence of *Blage-HTH* transcript peak would result in more release of the peptide from CC in *B. germanica*.

Blage-HTH function is also involved in the process of oviposition, which occurs at the highest levels of *Blage-HTH* mRNA expression (Fig. 3B), because the RNAi-mediated knockdown effect on *Blage-HTH* mRNA brings about a delay in ootheca production in virgin females of *B. germanica* (Fig. 4C). The myotropic effect of AKHs on muscle contraction has been implied in several insects and summarized by Kodrik (2008). Hence, it could be hypothesized that the surge of *Blage-HTH* stimulates the muscle contraction of oviducts to release mature eggs during oviposition. On the other hand, a few ds*Blage-HTH*-treated females produced short deformed oothecae (Fig. 4D), but those cockroaches retained most matured oocytes in the ovarioles (personal observation). It also suggests that the malformation is related to the process of oocytes extruded itself.

More recently, the insect AKH peptides have been proposed to share a common ancestral peptide with the gonadotropin-releasing hormone (GnRH) in other metazoans (Lindemans et al., 2009). This hypothesis is also supported by the finding that one of four GnRH receptors of the amphioxus, the most basal chordate, is activated by AKH of *B. mori* (Tello and Sherwood, 2009). In addition, the two AKH receptors of *D. melanogaster* and *B. mori* are regarded to be structurally and evolutionarily related to vertebrate GnRH receptors (Staubli et al., 2002). GnRH is a pivotal peptide hormone to govern reproductive process as such as release of gonadotropins and behavior via the control of both endocrine and neural pathways in vertebrates (Okubo and Nagahama, 2008). Our present study suggests that insect AKH

peptides participate in reproductive processes including energy utilization and oviposition. Similar function of GnRH peptides on oviposition has been suggested by the fact that Oct-GnRH increases spontaneous contractions of the oviduct in *Octopus vulgaris* (Iwakoshi-Ukena et al., 2004), and the gamete release of *Ciona intestinalis* is induced by injection of its GnRH peptides (Terakado, 2001). Furthermore, the nematode *C. elegans* treated with dsRNAs to knockdown the expression of the AKH-like peptide exhibits a delay in time of egg-laying and a decrease in progeny (Lindemans et al., 2009). Taken together, we postulate that the signaling system of ecdysozoan AKHs and GnRHs in other metazoans is functionally related within the context of reproductive processes, especially in oviposition.

In the present report, we show that the function of *Blage-HTH* is related to the cyclic fluctuation of hemolymph trehalose and oviposition in virgin females of *B. germanica*. Interestingly, null mutants of *Drosophila* with AKH synthesis ablated do not show the fatal effects on development and reproduction (Lee and Park, 2004; Isabel et al., 2005), suggesting a role for AKHs as an auxiliary system proposed previously (Lorenz and Gäde, 2009). Taking into consideration that we observed no impaired reproduction by RNAi-mediated knockdown of *Blage-HTH* gene in our data, we regard *Blage-HTH* as a coordinator of the fine-tuning in the normal reproductive physiology in female *B. germanica*.

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Appendix 2

Functional characterization of hypertrehalosemic hormone receptor in relation to hemolymph trehalose and to oxidative stress in the cockroach *Blattella germanica*

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Running title: Functional characterization of Blage-HTHR

Abstract

Hypertrehalosemic hormone (HTH) is a peptide hormone that belongs to the AKH/RPCH (adipokinetic hormone/red pigment concentrating hormone) family, which exerts pleiotropic actions related to catabolic reaction and stress response. AKH peptides have been demonstrated to participate in stress response including oxidative stress in several insects. In order to study the signaling pathway of HTH involved in anti-oxidative stress, we have characterized a hypertrehalosemic hormone receptor cDNA in *Blattella germanica* (*Blage-HTHR*) in structural and in functional terms using RNA interference (RNAi). *Blage-HTHR* is expressed in various female adult tissues (brain-CC-CA, ventral nerve cord, midgut, fat body, oviduct), but maximal expression is observed in the fat body. RNAi-mediated knockdown of *Blage-HTHR* expression results in a significantly lower level of hemolymph trehalose, even though HTH is exogenously administered. Paraquat elicits lethal oxidative stress in *B. germanica*, and co-injection of paraquat and HTH reduces this detrimental effect and extends the median survival time. Interestingly, the “rescue” effect of HTH on mortality caused by paraquat is diminished in specimens with depleted expression of *Blage-HTH* and *Blage-HTHR*. Finally, lipid peroxidation in the hemolymph increases 4 h after paraquat treatment, in comparison with control specimens or with HTH-treated specimens. However, lipid peroxidation induced by paraquat was not “rescued” by HTH in *Blage-HTH* and *Blage-HTHR* knockdown specimens. Our results demonstrate that HTH acts as a stress hormone mediating anti-oxidative protection in *B. germanica*, and that its receptor, *Blage-HTHR*, is essential for this action.

Keywords: adipokinetic hormone, paraquat, RNA interference, hypertrehalosemic hormone receptor, German cockroach.

1. Introduction

The AKH/RPCH (adipokinetic hormone/red pigment concentrating hormone) family is one of the extensively characterized group of neuropeptides whose members play important roles on energy homeostasis, as well as on other physiological and

50 behavioral processes in insects (Schooley et al., 2005). AKH peptides are 8- to 10- amino
51 acid long, with a pyroglutamate blocked N-terminus and an amidated C-terminus,
52 aromatic residues at positions 4 and 8, and a glycine residue at position 9, when present
53 (Gäde, 2009). Among them, the peptides that primarily induce trehalose efflux into
54 hemolymph are referred to as a hypertrehalosemic hormone (HTH).

55
56 Recently, AKHs have been related to stress response, through mechanisms involving
57 the stimulation of catabolic reactions while inhibiting synthetic reactions by increasing
58 available energy (Kodrik, 2008). Earlier studies have related the release of AKHs as a
59 result of insecticide poisoning through recording the increase of metabolites such as
60 trehalose and lipids in the hemolymph of the cockroach *Periplaneta americana* (Granett
61 and Leeling, 1972) and the locust *Schistocerca gregaria* (Samaranayaka, 1974). Direct
62 measurement of AKH titer increase after insecticide exposure has been further reported in
63 a number of insects, including the locust *Schistocerca gregaria* and the bug *Pyrrhocoris*
64 *apterus* (Candy, 2002, Kodrik and Socha, 2005, Velki et al., 2011). In addition, AKHs
65 participate in anti-oxidative mechanisms by increasing reduced glutathione efflux and
66 decreasing protein carbonyl levels in response to treatment with paraquat (PQ), a
67 bipyridilium herbicide that is currently used to elicit oxidative stress in animals through
68 redox-cycling reactions (Hassan, 1984); it has been used in this way, for example, in the
69 potato beetle *Leptinotarsa decemlineata* (Kodrik et al., 2007) and in *P. apterus* (Vecera et
70 al., 2007).

71
72 Oxidative stress is a type of stressor that causes neurodegeneration of brain cells
73 under severe stress condition (Chaudhuri et al., 2007); it also affects a number of
74 physiological processes, including an adaptive compensatory specific response of the
75 organism that is activated to sustain homeostasis (Valko et al., 2007). Reactive oxygen
76 species, such as superoxide anion radicals, hydroxyl radical and hydrogen peroxide,
77 represent a class of molecules that are derived from the normal metabolism of oxygen in
78 the majority of aerobic organisms. Reactive oxygen species arise as endogenous
79 by-products of metabolic reactions in the cells, and from exogenous exposure to
80 environmental pollutants, ionizing radiation and others. Excess of reactive oxygen
81 species and/or deficit of scavenging antioxidants results in oxidative stress, which may
82 impair correct regulation of lipids, proteins and DNA (Halliwell and Gutteridge, 2007).

83
84 Most of the previous studies show that AKHs are involved in antioxidant protection
85 mechanisms used biochemical experimental approaches such as monitoring AKH titer
86 changes or exogenously applying AKH under oxidative challenges (Kodrik et al., 2007;
87 Vecera et al., 2007). Thus, little information is available at the molecular level on the
88 relation of AKH with antioxidant protection mechanisms. In this context, we aim to
89 provide molecular evidence that the signaling pathway of HTH is involved in the
90 antioxidant protection mechanisms, using the cockroach *Blattella germanica* as model.
91 Previously, we had cloned the hypertrehalosemic hormone (*Blage-HTH*) cDNA in *B.*
92 *germanica* (Huang and Lee, 2011). In the present study we report the cloning of the
93 cDNA of the hypertrehalosemic hormone receptor (*Blage-HTHR*) and the effect of PQ
94 when both *Blage-HTH* and *Blage-HTHR* expression are silenced by RNA interference
95 (RNAi) in *B. germanica*, a species that is particularly sensitive to this knockdown
96 technique (Belles, 2010).

97 98 **2. Materials and Methods**

99

100 2.1 Insects

101 A colony of the German cockroach, *B. germanica* (L.), was maintained in
102 environmental chambers at 28°C under L:D = 16:8 h conditions. Dog chow and water
103 were provided *ad libitum*. Nymphs and adults were reared in groups, but adults were
104 separated by sex to keep them in virgin state. Detailed information about rearing has been
105 described previously (Lee and Wu, 1994).

106

107 2.2 RNA extraction and reverse transcription to cDNA

108 All RNA extractions were performed using TRIzol Reagent (Invitrogen) following
109 the supplier's protocol. Possible genomic DNA contamination was removed by DNase I
110 (Promega) treatment. One µg of total RNA was reverse transcribed using High Capacity
111 cDNA Reverse Transcription Kits (ABI).

112

113 2.3 Cloning of the HTH receptor

114 The first primer set was designed based on the known AKH receptor sequences of
115 other insects, including *P. americana* (DQ217786), *Apis mellifera* (AY898652),
116 *Tribolium castaneum* (DQ422965) and *Bombyx mori* (AF403542). The primer sequences
117 were as follows: 5'-TCC ACG TGG AGG AGC ACC C-3' forward; and, 5'-TTC TTC
118 CAS STG GAG GRR CAC CCC-3' reverse. The amplified fragment (420 bp) was
119 subcloned into the pGEMT-easy vector (Promega) and sequenced. To complete the
120 sequence, the GeneRacer Kit (Invitrogen) for rapid amplification of cDNA ends (RACE)
121 was used following the manufacturer's protocol. For 5'-RACE, the reverse primer was
122 5'-GAC TCA GCT GTA CCA GTT CTG GTA CAG GA-3', and for 3'-RACE, the
123 forward primer was 5'-GTC TGA ACT GAG CTG ATA TCT GCG A-3'. All PCR
124 products were subcloned into pGEMT-easy vector (Promega) and sequenced.

125

126 2.4 Phylogenetic analysis

127 The amino acid sequences of all known insect AKH receptors were obtained from
128 GenBank and aligned using the Clustal X 1.18 program (Thompson et al., 1997). The
129 resulting alignment was manually edited using the GeneDoc program (Nicholas et al.,
130 1997) and analyzed by the Neighbor-joining method for construction of a phylogenetic
131 tree. The AKH receptor of the insect species with the GenBank accession numbers are
132 *Drosophila melanogaster* (AAF52426), *Anopheles gambiae* (ABD60146), *Aedes aegypti*
133 (CAY77164 and CAY77166), *Glossina morsitans* (AEH25943), *Bombyx mori*
134 (AAL95712), *Manduca sexta* (ACE00761), *Apis mellifera* (AAX83121), *Nasonia*
135 *vitripennis* (NP_001161243), *Tribolium castaneum* (ABE02225), *Acyrtosiphon pisum*
136 (XP_001945436), *Pediculus humanus* (EEB15485), *Periplaneta americana*
137 (ABB20590), and *Blattella germanica* (ADL60118). Five-letter abbreviations used for
138 these AKH receptors and species are indicated in Fig. 1B. Phylogenetic trees based on the
139 resulting alignment were constructed using MEGA version 5 (Tamura et al., 2011). The
140 gonadotropin-releasing hormone receptor of *Caenorhabditis elegans* (Cael-GnRHR,
141 GenBank accession number NP_506566) was used as outgroup. The robustness of
142 phylogenetic analysis was evaluated by bootstrapping using 1000 replicates.

143

144 2.5 *Blage-HTHR* expression analysis

145 We performed semiquantitative RT-PCR to investigate the distribution of
146 *Blage-HTHR* in different tissues of 7-day-old adult female *B. germanica* and to assess the
147 effects of RNAi. Thus, the 25 µl PCR mixture for amplifying the *Blage-HTHR* fragment
148 included 1 µl of cDNA, 10 pmol of forward primer (5'-GAC TCA GCT GTA CCA GTT
149 CTG GTA-3'), 10 pmol of reverse primer (5'-GGG AAA TGT CTT GTG AAC CAG

150 GTC-3'), and 5 μ l of Taq polymerase mixture (Protech). The PCR (30 cycles) was
151 performed as follows: denaturation at 94°C for 30 s, annealing at 58°C for 30 s, extension
152 at 72°C for 1 min. Expression of *BgActin* (GenBank EU514491) was used as reference.
153 PCR products were separated and visualized on a 1.5% agarose gel.

154
155 Quantitative real-time PCR measurements were carried out in triplicate in Applied
156 Biosystems StepOnePlus real-time PCR systems with Fast SYBR Green Master Mix
157 (Applied Biosystems). The primers used to measure *Blage-HTHR* mRNA were as follows:
158 5'-TCT ATC GGG ACA ACA GCA ACC AGA-3' forward, and 5'-TGC TTC AGG AAC
159 TTC ACT CAC AGT-3' reverse. The efficiency of this primer set was first validated by
160 constructing a standard curve through five serial dilutions. The expression levels were
161 normalized to *BgActin* expression using 2(-Delta Delta C(T)) method (Pfaffl, 2001). The
162 PCR program began with a single cycle at 95°C for 3 min, 40 cycles at 95°C for 15 s and
163 60°C for 30 s. Afterwards, PCR products were heated to 95°C for 15 s, cooled to 60°C for
164 30 s and increasing the temperature 0.5°C/min in order to measure the dissociation curves
165 and to determine a unique PCR product for each gene. A template-free control was
166 performed in each batch.

167 2.6 RNA interference

168 The *Blage-HTHR* cDNA of the German cockroach was first cloned into the
169 pGEMTeasy vector (Promega, Madison, WI). Then, a 528 base pair (bp) fragment
170 targeting the 3'-UTR of *Blage-HTHR* mRNA was amplified by PCR. The primers (which
171 contained the T7 promoter sequence: TAATACGACTCACTATAG) used in this PCR
172 reaction were as follows: 5'-TGG ATA GAC CAG CAG TCT GCT GAA-3' forward
173 primer, and 5'-GGG AAA TGT CTT GTG AAC CAG GTC-3' reverse. The MEGAscript
174 RNAi Kit (Ambion) was used to generate the dsRNA following the manufacturer's
175 protocol. The dsRNA solution was stored at -20°C until use. A dose of 1.5 μ g of the 528
176 bp dsRNA targeting the *Blage-HTHR* mRNA was injected into the abdomen of newly
177 emerged adults. As dsRNA control, the enhanced green fluorescence protein (EGFP)
178 sequence was used at the same dose. *Blage-HTHR* expression in the fat body was
179 determined by semiquantitative RT-PCR within the following days after dsRNA
180 treatment.

181
182
183 To investigate the role of HTH against oxidative stress, we injected 1.5 μ g of both,
184 dsHTHR and dsHTH, the latter being prepared according to the protocol previously
185 described (Huang and Lee, 2011), into newly emerged male *B. germanica*. *Blage-HTHR*
186 expression in the fat body and *Blage-HTH* expression in the whole head were determined
187 by semiquantitative RT-PCR at the 10th day after dsRNA treatment.

188 2.7 Trehalose determination

189 Trehalose in the hemolymph was determined following the protocol previously
190 described (Huang and Lee, 2011). Briefly, 5 μ l hemolymph was obtained from the hind
191 leg coxae. Then, the hemolymph was incubated overnight at 37°C with 2 μ l porcine
192 kidney trehalase (Sigma) to convert trehalose into glucose. The amount of glucose in 50
193 μ l of the above solution was measured using the Glucose Assay Kit (Sigma). Glucose
194 concentration was corrected by deducting the amount of glucose from the same
195 supernatant prepared under identical conditions but without trehalase.

196 2.8 Oxidative stress treatments

197
198 The insects were individually injected with 1 μ L solution of 20 mM PQ (paraquat:
199

200 1,1'-dimethyl-4,4'-bipyridilium dichloride hydrate) to cause oxidative stress, or with 1
201 μ L Ringer saline as control. To study the effect of HTH against oxidative stress, the
202 insects were injected with 1 μ L solution containing synthetic HTH peptide
203 (pGlu-Val-Asn-Phe-Ser-Pro-Gly-Trp-Gly-Thr-NH₂) of *B. germanica* at various
204 concentrations (10, 40, and 80 pmol) in combination with 20 nmol PQ (1:1 in volume).
205 The number of dead cockroaches were recorded every day. All chemicals used in the
206 experiments and assays were obtained from Sigma-Aldrich Chemical Company (St.
207 Louis, MO, USA) unless otherwise stated.

208

209 2.9 Lipid peroxidation measurement

210 We used lipid peroxidation as specific test to measure the level of oxidative stress. A
211 total of 15 μ l hemolymph from a pool of 8 specimens was collected at 4 h post inoculation
212 of oxidative stress treatment and diluted with 30 μ l PBS containing 1% butylated
213 hydroxytoluene to prevent further oxidation of lipid during further processing. The
214 diluted hemolymph was shaken vigorously and centrifuged (10,000 \times g for 10 min at 4 $^{\circ}$ C).
215 To determine the levels of malondialdehyde as indicator of lipid peroxidation, the
216 supernatants were processed using the OxiSelect TBARS (thiobarbituric acid reactive
217 substances) assay kit (Cell Biolabs) following the manufacturer's instructions.

218

219 2.10 Statistical analysis

220 The results in the figures represent the mean \pm SEM. Statistical analyses were
221 performed using computing software R. Statistical significance of results were evaluated
222 using unpaired t-test between two groups and one-way ANOVA followed by Tukey's
223 HSD post-hoc test for multiple comparisons. The analyses of survival curves were carried
224 out using Kaplan-Meier test for median survival time, the Log-rank test for testing the
225 differences among treatment groups, and the Kruskal-Wallis rank sum test for post-test
226 calculations.

227

228 3. Results

229

230 3.1 Cloning and expression pattern of *Blage-HTH* receptor

231 Cloning of the hypertrehalosemic hormone receptor (*Blage-HTHR*) cDNA of *B.*
232 *germanica* was accomplished by a RT-PCR approach, combining the use of degenerate
233 primers based on AKHR conserved motifs to obtain a partial sequence, and 5'-RACE and
234 3'-RACE experiments to complete it. These amplifications rendered a full-length cDNA
235 of 1,769 bp encoding a predicted 406 amino acid protein (*Blage-HTHR*; GenBank
236 accession number GU591493; Figure S1). According to BLAST searches, cDNA
237 sequence of *Blage-HTHR* was very close (71.0% identity, 81.5% similarity) to *P.*
238 *americana* adipokinetic hormone receptor (*Peram-AKHR*; GenBank accession number
239 ABB20590). In addition, the alignment of receptor proteins of the two cockroach species
240 showed values of 62.4% identity and 72% similarity (Figure 1A). The deduced amino
241 acid sequence of *Blage-HTHR* exhibits the typical structural features of G
242 protein-coupled receptors including an extracellular N-terminus, seven transmembrane
243 regions, and an intracellular C-terminus.

244

245 Neighbor-joining analysis of insect AKH receptor sequences gave a tree (Figure 1B)
246 showing that *Blage-HTHR* clusters with *Peram-AKHR* from *P. americana*, as expected.
247 Indeed, these two cockroach receptors have an additional consensus sequence in the
248 intracellular C-terminus that is characteristic of them, and which is not present in other
249 insect AKH receptor sequences (Figure S2). The sister group of the cockroach node is

250 composed by phylogenetically distal hemimetabolous species (Phthiraptera, Hemiptera)
251 and basal holometabolous species (Coleoptera, Hymenoptera), and these two nodes have
252 the more modified holometabolous species (Lepidoptera and Diptera) as sister group. In
253 general, the topology of the tree approximately follows the phylogenetic relationships of
254 the insect orders currently established.

255

256 The expression of *Blage-HTHR* was detected and quantified in various tissues and
257 stages (Figure 2). Concerning the adult, the expression was observed in practically all
258 studied tissues, but the strongest expression was found in the fat body (Figure 2A).
259 Conversely, expression in the ovary was practically absent, and that in the accessory
260 glands was very faint. Comparing different life cycle stages (Figure 2B), expression was
261 very low in embryos and relatively low in the first instar nymph. Values were much
262 higher in the last instar (N6) nymph, and in the adult stage of both sexes.

263

264 3.2 RNAi-mediated knockdown of *Blage-HTHR* reduces the trehalosemic effect of HTH

265 We first studied the function of *Blage-HTHR* in *B. germanica* in relation to trehalose
266 regulation by depleting its expression by RNAi. *Blage-HTHR* mRNA was measured in
267 the fat body 1, 5 and 10 days after the treatment with dsHTHR, and results (Figure 3A)
268 showed that expression was clearly reduced in comparison with controls
269 (dsEGFP-treated) on days 1 and 5, and it was practically undetectable on day 10, either in
270 females as well as in males. The function of *Blage-HTHR* as HTH receptor was illustrated
271 by the significant reduction of trehalose titers in the hemolymph after injection of HTH in
272 dsHTHR-treated females (Figure 3B). The injection of 10 pmol of HTH resulted in the
273 increase of hemolymph trehalose up to 113% 2 h later in dsEGFP-treated females. In
274 contrast, the hemolymph trehalose increased significantly less (49%) in dsHTHR-treated
275 females 2 h after the HTH injection. These results suggest that the remarkable increase of
276 hemolymph trehalose elicited by HTH requires a high expression of its receptor,
277 *Blage-HTHR*.

278

279 The titer of hemolymph trehalose displayed a steady increase and peaked at day 8
280 in the first reproductive cycle of female adults, either in controls (dsEGFP-treated) as in
281 specimens treated with dsHTHR (Figure 3C), although the levels are lower in the latter.
282 The hemolymph trehalose pattern is coincident with that of ovarian development. In adult
283 males, the hemolymph trehalose levels practically do not fluctuate, either in controls
284 (dsEGFP-treated) as in specimens treated with dsHTHR (Figure 3D); again, the levels
285 were significantly lower in dsHTHR-treated specimens (Figure 3D).

286

287 3.3 Role of HTH as reliever of oxidative stress

288 Subsequently, we studied the potential roles of HTH as reliever of oxidative stress.
289 Given that female *B. germanica* exhibits a varied ability against oxidative stress during
290 the reproductive cycle (unpublished data), we chose only males to conduct the following
291 experiments. The injection of 20 nmol of PQ on 10-day-old male adults caused a
292 mortality that rapidly increased with time, thus median survival time was attained on day
293 4 (Figure 4A). However, exogenous HTH at 10, 40, and 80 pmol concentrations
294 significantly reduced the speed of mortality increase, with median survival time attained
295 around day 7 (Figure 4A), thus, the protective effect of HTH did not show significant
296 differences among the 3 concentrations used.

297

298 To further assess the possible protective role of HTH against oxidative stress, the
299 expression of *Blage-HTHR* after PQ treatment was studied (Figure 4B). Injection of PQ

(20 nmol) caused a significant reduction of the *Blage-HTHR* expression in the fat body 4 h later. Mean value of *Blage-HTHR* mRNA was also decreased in the specimens injected with PQ and 40 pmol HTH, which extended the life span under the provoked oxidative stress. But the level of *Blage-HTHR* in PQ co-injected with HTH was not significantly different from that in either control or PQ treatment.

3.4 RNAi-silenced HTH signaling reduces anti-oxidative protection

We further assessed the protective role of HTH on oxidative stress in specimens where *Blage-HTH* and *Blage-HTHR* mRNA levels had been simultaneously depleted by RNAi. A treatment of 1.5 µg of dsHTH and 1.5 µg of dsHTHR (dsHTH/R) was administered to newly emerged males of *B. germanica*, and the corresponding expression of *Blage-HTH* and *Blage-HTHR* was undetectable 10 days later in the tissues where these transcripts are most expressed: *Blage-HTH* in the head and *Blage-HTHR* in the fat body (Figure 5A). There were no lethal effects when the expression of both genes was knocked down, as all specimens survived in apparently healthy conditions at least during 25 days after the dsRNA injections. However, PQ treatment elicited a faster mortality in the specimens knockdown for *Blage-HTH* and *Blage-HTHR* (median survival time was attained on day 2 after PQ treatment), than in controls (dsEGFP-treated) (median survival time attained on day 5) (Figure 5B). Moreover, a dose of 40 pmole of HTH significantly relieved the detrimental effect elicited by PQ in control specimens (median survival time attained on day 8), but this “rescue” effect of HTH was milder in the specimens knockdown for *Blage-HTH* and *Blage-HTHR* (median survival time attained on day 3) (Figure 5B).

Finally, the anti-oxidative stress function of *Blage-HTH* and *Blage-HTHR* was further demonstrated by measuring malondialdehyde as indicator of lipid peroxidation under oxidative stress caused by PQ, using specimens where *Blage-HTH* and *Blage-HTHR* expression had been depleted by RNAi, and applying exogenous HTH. Results (Figure 5C) show that without PQ, the malondialdehyde titer in the group of specimens knockdown for *Blage-HTH* and *Blage-HTHR* was higher than that of the dsEGFP-treated group. PQ elicited and increased the titer of malondialdehyde in both groups, but differences between them were not statistically significant. Finally, exogenous HTH significantly counteracted the effects of PQ in the dsEGFP-treated group, but not in the group of *Blage-HTH* and *Blage-HTHR* knockdowns.

4. Discussion

About 50 peptides belonging to the AKH family have been identified in insects (Gäde, 2009) since the first hyperglycemic factor was discovered in the cockroach *P. americana* (Steele, 1961). The physiological function of these peptides on energy homeostasis as well as on signal transduction at the cellular level have been thoroughly reviewed by a number of authors (Van der Horst et al., 2001, Gäde and Auerswald, 2003, Schooley et al., 2005). However, studies at molecular level of AKH receptors have been conducted more recently in the fruit fly *D. melanogaster* and in the silkworm *Bombyx mori* (Staubli et al., 2002). In cockroaches, only the AKH receptor cDNA of *P. americana* has been characterized (Hansen et al., 2006, Wicher et al., 2006). The present contribution reports the characterization of the HTH receptor in another cockroach, *B. germanica*, which is structurally close to that of *P. americana* AKH receptor (Figure 1).

Alignment of all known AKH/HTH receptor protein sequences shows a large region

350 of evolutionary conserved residues in the transmembrane domain, but a highly diversified
351 region in the C-terminus, which is associated to intracellular signaling (Figure S2). The
352 phylogenetic analysis indicates that the intracellular C-terminus has considerably
353 diverged in different insect orders, and this might have derived in different signal
354 transduction mechanisms in different orders. Interestingly, an additional sequence at the
355 beginning of the intracellular C-terminus in cockroaches might be related to the export of
356 the receptor to the cell membrane, as an equivalent sequence in the C-terminal end of the
357 AKH receptor of *B. mori* serves for this function (Huang et al., 2011).

358

359 The most abundant expression of *Blage-HTHR* in the fat body among the tissues
360 tested (Figure 2A) is in agreement with the fact that HTH is the major regulator of energy
361 metabolism in insects. This is also supported by the increasing expression level of
362 *Blage-HTHR* from nymphal to adult stage which coincides with increasing locomotion
363 which occurs in parallel (Yang et al., 2009), and which requires increasing energy
364 expenditure. Our previous results showed that depletion of *Blage-HTH* expression results
365 in a delay of oviposition in virgin females of *B. germanica* (Huang and Lee, 2011). In the
366 present study, we observed *Blage-HTHR* expression in the oviduct, but not in ovary
367 (Figure 2A), which suggests that *Blage-HTH* release could stimulate the contraction of
368 oviduct muscles during basal oocyte extrusion. Indeed, a myoactive effect of AKHs on
369 the dorsal vessel has been demonstrated in *P. americana* (Scarborough et al., 1984).

370

371 The canonical function of *Blage-HTHR* as HTH receptor in *B. germanica* has been
372 demonstrated by injecting HTH in specimens where *Blage-HTHR* had been depleted by
373 RNAi, and observing that mobilization of trehalose in the hemolymph was impaired
374 (Figure 3B). Equivalently, the malaria mosquito *Anopheles gambiae* with lowered
375 expression of AKH receptor (Anoga-AKHR) also fails to mobilize the glycogen reserves
376 in the fat body after injection of Anoga-AKH-I (Kaufmann and Brown, 2008). However,
377 the occurrence of a significant elevation of hemolymph trehalose in dsHTHR-treated
378 group was likely considered as incomplete silencing of *Blage-HTHR* expression because
379 RNAi does not completely knockout gene expression. In addition, studies *in vitro*
380 demonstrate that reduced expression of the *Bombyx* AKHR on the cell membrane results
381 in lower levels of intracellular cAMP (cyclic adenosine monophosphate) formation after
382 AKH stimulation (Huang et al., 2011). Therefore, a maximal action of HTH would
383 require an adequate expression of its receptor to trigger signal transduction.

384

385 RNAi-mediated depletion of *Blage-HTHR* mRNA was found to decrease
386 hemolymph trehalose levels in the adult of both sexes (Figure 3C and 3D), although the
387 female displays fluctuations of trehalose related to the reproductive cycle. Our present
388 results of *Blage-HTHR* RNAi (Figure 3C) supports our previous hypothesis postulating
389 that HTH induces hemolymph trehalose mobilization during the reproductive cycle in
390 virgin females (Huang and Lee, 2011). The results suggest that HTH plays a critical role
391 in carbohydrate homeostasis, as occurs in AKH-cell-deficient *D. melanogaster* that has
392 relatively low hemolymph trehalose levels (Lee and Park, 2004).

393

394 The involvement of AKH peptides in anti-oxidant protection in insects was inferred
395 from the increase in AKH titer after exposure to oxidative stress (Kodrik et al., 2007,
396 Vecera et al., 2007). The same authors showed that exogenous AKH application enhanced
397 the efflux of reduced glutathione to the hemolymph and maintained the normal levels of
398 protein carbonyl, a marker of oxidative stress. In the present study, we have fully
399 demonstrated the protective role of HTH upon oxidative stress under different

400 experimental approaches, first, through experiments of co-injection of PQ and HTH on
401 control specimens (Figure 4A), and secondly, in specimens with the expression levels of
402 *Blage-HTH* and *Blage-HTHR* depleted by RNAi (Figure 5B). These results provide a new
403 and relevant information supporting that Blage-HTH potentiates a stress hormone on
404 anti-oxidative stress response in *B. germanica*. The promoter of oxidative stress used in
405 our experiments has been PQ, which acts on the redox-cycling reactions, thus eliciting
406 free radicals as well as causes oxidative modification of macromolecules, such as lipid
407 peroxidation (Hassan, 1984). Accordingly, we examined lipid peroxidation in the
408 hemolymph 4 h after PQ treatment (Figure 5C). Levels of lipid peroxidation in the
409 hemolymph increased significantly after PQ injection in dsEGFP and dsHTH/R groups.
410 However, the co-injection of HTH maintained the lipid peroxidation at a normal level in
411 controls but not in the *Blage-HTH* and *Blage-HTHR* knockdowns, which suggests that
412 HTH lowers the oxidative damage in *B. germanica*. A likely mechanism might be that
413 HTH induces the efflux of glutathione into the hemolymph, as demonstrated in other
414 insect AKHs (Kodrik et al., 2007, Vecera et al., 2007). However, the significant reduction
415 of *Blage-HTHR* expression after PQ treatment (Figure 4B), suggests that the oxidative
416 stress directly impairs its expression. The less severe reduction of *Blage-HTHR*
417 expression observed after co-injecting PQ and HTH (Figure 4B) might be the outcome of
418 downstream response induced by HTH to relieve the oxidative damage. Moreover,
419 genome-wide analysis in *D. melanogaster* after PQ treatment have shown that
420 cytochrome P450 genes become up- and down-regulated (Girardot et al., 2004), and it has
421 been additionally reported that HTH stimulates cytochrome P450 expression in the
422 cockroach *Blaberus discoidalis* (Lu et al., 1995). Therefore, we further speculate that
423 HTH might induce the expression of P450 genes, thus contributing to decrease the
424 oxidative stress in *B. germanica*.

425

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433

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533

534

535 **Figure legends**

536

537 Figure 1. (A) Alignment of the deduced amino acid sequences of the *Blattella germanica*
538 HTH receptor (Blage-HTHR, GenBank GU591493) with the *Periplaneta americana*
539 AKH receptor (Peram-AKHR, GenBank ABB20590). Identical amino acid residues are
540 highlighted in grey. The seven-transmembrane α -helices are indicated by TM1-7. The
541 dotted-line rectangle indicates an insertion present in both cockroach species. (B)
542 Neighbor joining tree of AKH receptor insect sequences. The tree was generated using
543 MEGA 5 with 1000 bootstrap replicates. The evolutionary distance is given in units of the
544 number of amino acid substitutions per site. The abbreviated names of insect AKHR are:
545 Acypi-AKHR (*Acyrtosiphon pisum*), Aedae-AKHR-1 and Aedae-AKHR2 (*Aedes*
546 *aegypti*), Anoga-AKHR (*Anopheles gambiae*), Apime-AKHR (*Apis mellifera*),
547 Blage-HTHR (*Blattella germanica*), Bommo-AKHR (*Bombyx mori*), Drome-AKHR
548 (*Drosophila melanogaster*), Glomo-AKHR (*Glossina morsitans*), Manse-AKHR
549 (*Manduca sexta*), Nasvi-AKHR (*Nasonia vitripennis*), Pedhu-AKHR (*Pediculus*
550 *humanus*), Peram-AKHR (*Periplaneta americana*), and Trica-AKHR (*Tribolium*
551 *castaneum*). Gonadotropin-releasing hormone receptor of *Caenorhabditis elegans*
552 (Caeel-GnRHR) was used as outgroup.

553

554

555 Figure 2. (A) Semiquantitative RT-PCR analysis (*Blage-HTHR*, 790 bp) in various tissues:
556 brain-CC-CA complex (B-CC), ventral nerve cord (VNC), midgut (Mgt), fat body (FB),
557 ovary (O), oviduct (Ovd), and accessory gland (Asg) of 7-day-old adult females. *BgActin*
558 expression (365 bp) was used as a reference. (B) Quantitative real-time RT-PCR analysis
559 of *Blage-HTHR* expression in the whole body of cockroaches at various developmental
560 stages. N1 and N6 represent first and last (sixth) nymphal instars, respectively. Male and
561 female are 7-day-old adults. The *Blage-HTHR* mRNA was normalized against *BgActin*
562 expression. Data represent the mean \pm SEM (n = 3) of relative transcription levels
563 normalized in comparison with embryo.

564

565

566 Figure 3. Effects of silencing *Blage-HTHR* on hemolymph trehalose levels in *Blattella*
567 *germanica*. dsHTHR or dsEGFP were injected in newly emerged adults. (A)
568 Semiquantitative RT-PCR analysis showing the down-regulation of *Blage-HTHR*
569 expression in the fat body one day after injection of the corresponding dsRNA. (B)
570 Silencing effect of *Blage-HTHR* on hemolymph trehalose levels after treatment with
571 HTH (10 pmol) (n = 16) 0 and 2 h after HTH treatment. * indicates significant differences
572 between 0 and 2 h after HTH treatment ($P < 0.05$, two-tailed student t-test). (C) Pattern of
573 hemolymph trehalose in dsHTHR-treated adult females during the first reproductive
574 cycle (n = 8, except at the age of 4 days, where n = 4). (D) Pattern of hemolymph
575 trehalose in dsHTHR-treated adult males (n = 6). * ($P < 0.05$) is using two-tailed student
576 t-test.

577

578

579 Figure 4. (A) Survival curve of male adults of *Blattella germanica* after treatment with
580 paraquat (PQ) and HTH (H). SA: injection with saline (control); PQ: injection with 1 μ l
581 of 20 mM PQ; 10H, 40H, and 80H: injection with various concentrations (pmol) of HTH.
582 The median survival time (days) is indicated in the parenthesis of each treatment group.
583 (B) Quantitative real-time PCR analyses on mRNA expression of *Blage-HTHR* in fat

584 body of adult male of *B. germanica* 4 h after the treatment. Expression levels were
585 calculated after normalizing against *BgActin* expression in each sample. Results are
586 indicated as the mean \pm SEM (n = 3 per treatment group).

587

588

589 Figure 5. Effects of double RNAi of *Blage-HTH* and *Blage-HTHR* against oxidative
590 stress in male adults of *Blattella germanica*. (A) The effect of double injection of dsHTH
591 and dsHTHR on the gene expression 10 days after the treatment. (B) Effect of *Blage-HTH*
592 and *Blage-HTHR* RNAi on survival rate under PQ treatment. E: injection of dsEGFP; H:
593 double injection of dsHTH and dsHTHR on newly emerged adults. SA, PQ, and 40H as
594 described in Figure 3. The median survival time (days) is indicated in the parenthesis of
595 each treatment group. (C) Lipid peroxidation 4 h after PQ treatment. MDA:
596 malondialdehyde as an indicator of lipid peroxidation. Results are presented as the mean
597 \pm SEM (n = 4 per treatment group). Different letters on the histogram indicate significant
598 differences within the same dsRNA treatment. Two-tailed t-test was performed between
599 different dsRNA injections.

600

601

602 Supplementary Figure S1. Nucleotide sequence of cDNA encoding the open-reading
603 frame for *B. germanica* HTHR with flanking 5'- and 3'-UTR sequences.

604

605 Supplementary Figure S2. Alignment of the AKH receptor protein sequences from
606 insects and gonadotropin-releasing hormone receptor of *Caenorhabditis elegans* used in
607 the phylogenetic analysis. The abbreviated names of sequences used are provided in
608 Figure 1B.

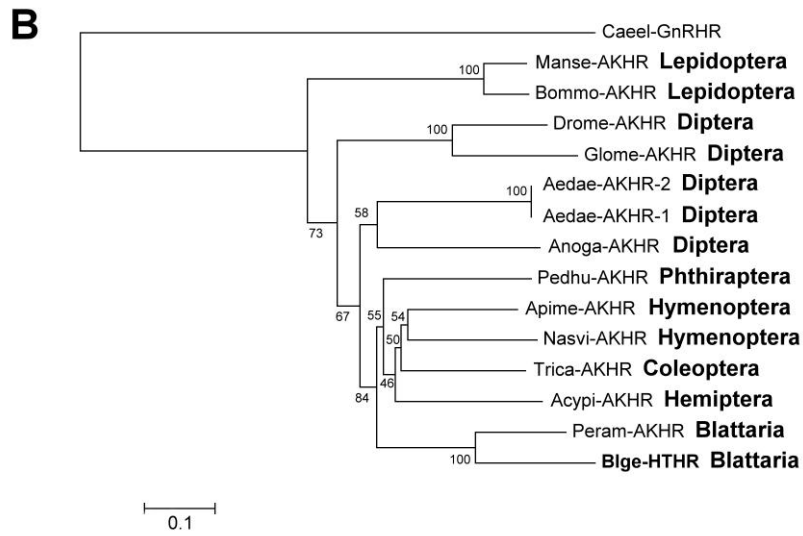


Figure 1

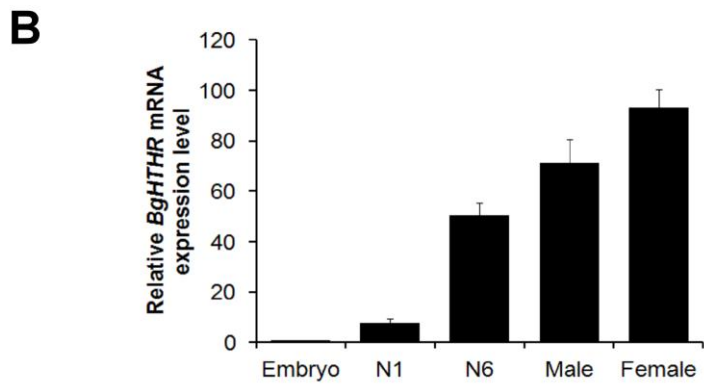
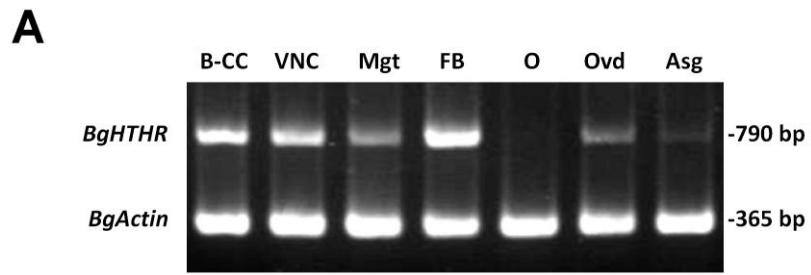


Figure 2

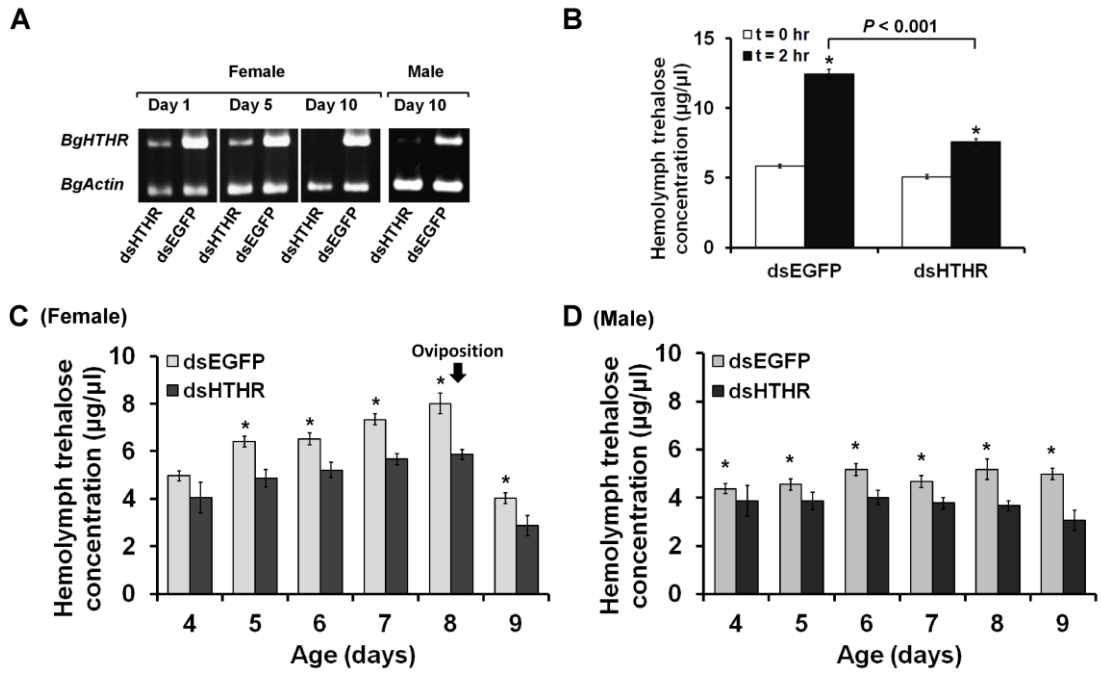


Figure 3

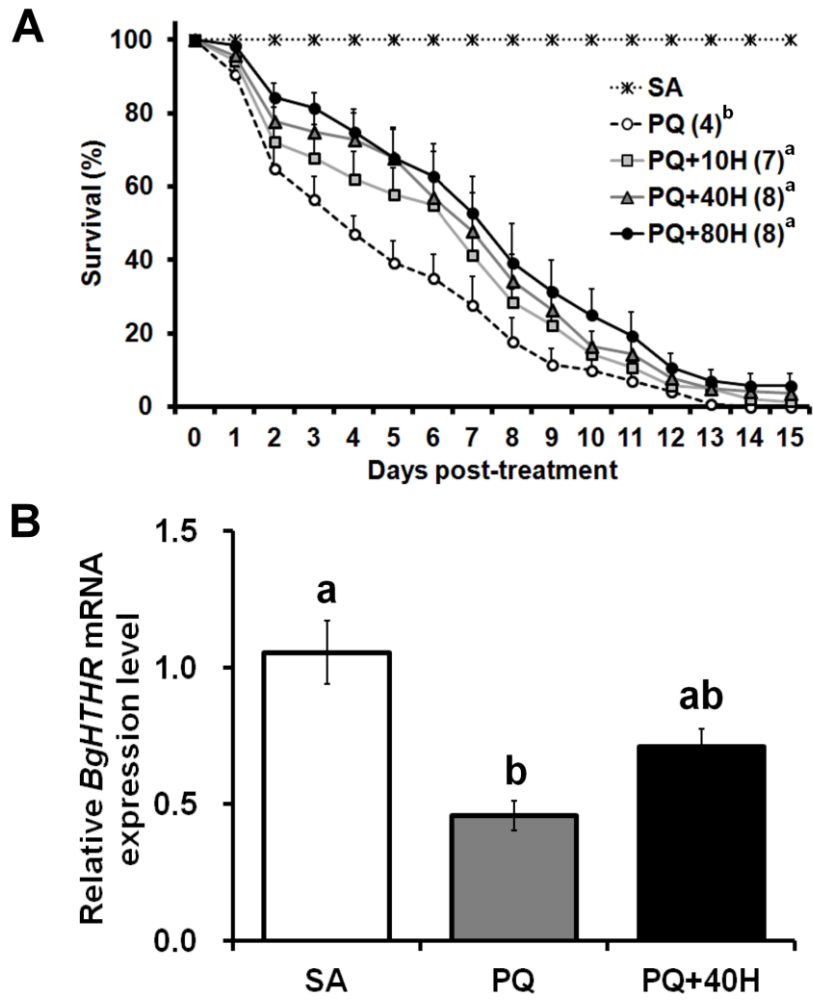


Figure 4

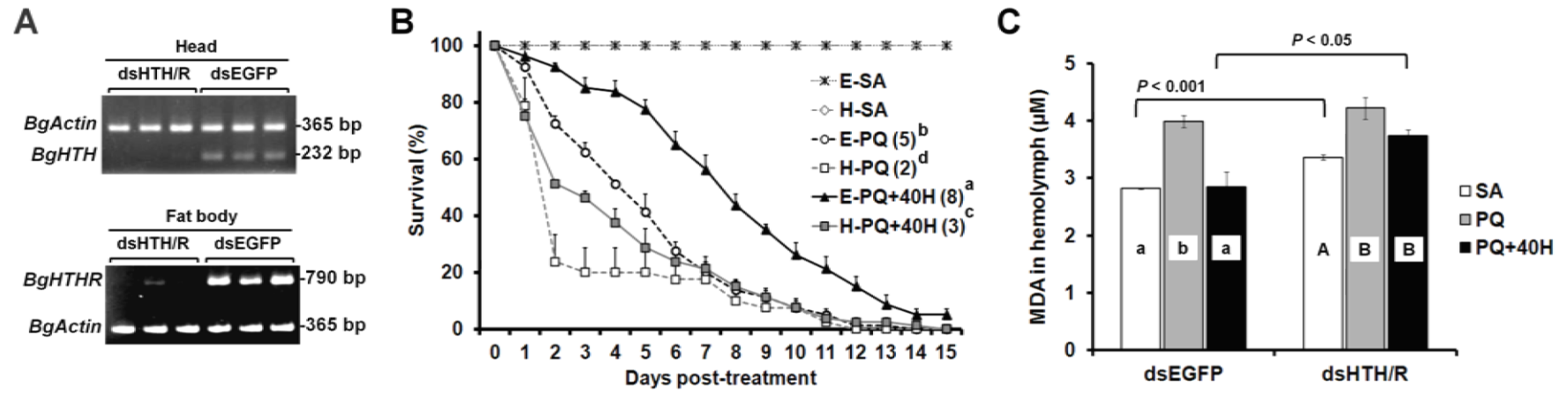
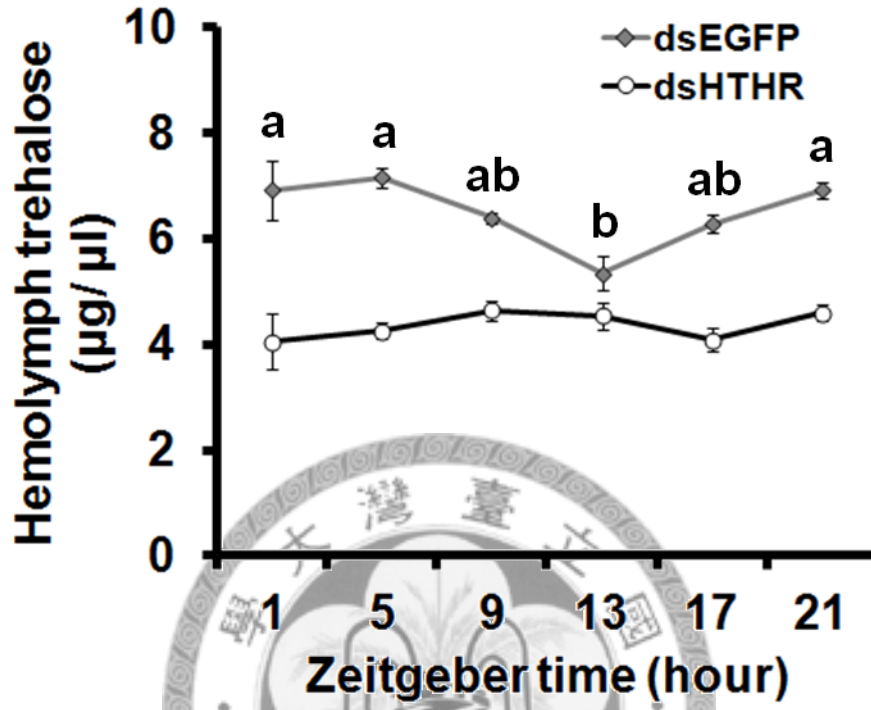


Figure 5

1 5' -AATATGAATCTAACAAGCTATCATACTTCT
31 CTTATGGCTGGAGGAGGCGGAAAAATCTGCAGTGTAGAAAAAGCGATAACATTCTAAGAACAACTGAATGATTAGACAAATCAAATGT
121 TCGAGAATTTATTCAACGAAGATTGAAGTGATGAACGAATTAGTTCTAACAGTGAAATGGAAGAGACGAAGTGATGATTTCGCTTAGTGAA
211 ACAGTGGCATGGAAGAATTTGGGTGTTGGTAATCTTGATTCTTTCAACTGTCAACATTTTGACATGAGCATTTGACAGGAACCCAAG
301 **ATG**ACGACTACAGAGCTGCCCCGTGAGCAGCAGCTGACTGAGGACATGACTTTTGGCTCCATACATAAGTTATGTATCGCCACGTATTGC
M T T T E L P R E Q Q L T E D M T F G S I H K L C I A T Y C
391 GTTCTCATGACTGTATCTGCAATTGGCAACATCACGGTCTGGTCAACATACTCAAGAGACGCCGCAACCTTCGTTTTGGGAACAACACTAC
V L M T V S A I G N I T V L V N I L K R R R N L R F G N N Y
481 ATGTTTCATGCATCTTGCCATAGCAGACCTATTGGTGACTTTTCTCATGATGCCGCTAGAAAATCGGCTGGAATGCAACAGTGTCTTGGAGA
M F M H L A I A D L L V T F L M M P L E I G W N A T V S W R
571 GCCGGTGACGCTGCTTGACAGAGTGATGTCGTTCTTCAGGATATTCGGCCTCTATCTGTCTAGCTTCGTAATTGTGTGCATCAGTCTGGAC
A G D A A C R V M S F F R I F G L Y L S S F V I V C I S L D
661 CGCTGTTTCGCCATTCTAAGGCCCATGTGCAACGTCGTCACGTCGCAAAACGCAGCAGAGTAATGCTGACCACTGCCTGGTCATTGGCC
R C F A I L R P M S N V V N V A K R S R V M L T T A W S L A
751 ACTGTTTGTAGTTTACCTCAGGTGTTTCATCTTCCACGTACAACAGCATCCCCTTTTTCATGGTATGAGCAGTGTGGACTTCGACATG
T V C S L P Q V F I F H V Q Q H P V F T W Y E Q C L D F D M
841 TTTCC**GACTCAGCTGTACCAGTTCTGGTA**CAGGATATTAATATGTTTCTAGTGTACGGTTTCCCACTCCTCGTCATTTTCATCTCATA
F P T Q L Y Q F W Y R I L N M V L V Y G F P L L V I F I S Y
931 GCCTGTATCCTCACAGAGATTTTTTCGAGATATCAGCTCAGTTCAGACGAAAACCTCCGGAGATCGAGCCTTGTGTTCTGAACAGAGCC
A C I L T E I F R R Y Q L S S D E N F R R S S L V F L N R A
1021 AAAAAATAGGACGCTCAAAATGGCCATCATAATATTCGTAGTGTCTTTCATCTGCTGGACTCCCTACTATGTGATGTGTCTCTGGTAC**TGG**
K N R T L K M A I I I F V V F F I C W T P Y Y V M C L W Y W
1111 **ATAGACCAGCAGTCTGCTGAAAAGGTTGACTTGC**CGCGTGAGAAAAGGCCTGTTCTGTTTGCCTGTGTACCAATTCCTGTATGAACCCGATT
I D Q Q S A E K V D L R V R K G L F L F A C T N S C M N P I
1201 **GTGTACGGTACTTCAACTTCCGTT**CAGGACGGGAAGTGGTTATGGTGAACAAGACCAGGGCAGCAGTTACAGCATCATCAAATAACT
V Y G Y F N F R S G R G S G Y G A T R P G Q Q L Q H H Q I T
1291 **GCATTGAGCAATAACTCAACGGGAGTTAACAGCCGAAGGGGAAGCAACTGCAGCAGCA****TCTATCGGGACAACAGCAACCAGAGCATGTCC**
A L S N N S T G V N S R R G S N C S S I Y R D N S N Q S M S
1381 **TGGAATCGCCGAAGCAGCCATGAAACAGAAATGCACGCCAATAACAATCGAGACGAAAATCACTTACACCCCAACTCAGCTGCGAACCAC**
W N R R S S H E T E M H A N N N R D E N H L H P N S A A N H
1471 **AATTTGCGAACTACAGTATCTACTGTGAGTGAAGTTCTGAAGCAAGATGA**GGTTTACTCCTTGTGCTTCGAAATTATACTATCAGTGAT
N L R T T V S T V S E V P E A R *
1561 **AAACATTGCCAATGTAATTAACATTAATGTTCTAGTCTAAGACTTGACATGACCTGGTTCACAAGACATTTCC**AAGAGAGTGAACAAA
1651 ACAATTGAATTGGTATAAACATGTGTTAAACTAATGTACAATACACTCACTCAACAGTTTTACACAAAAATAAATTTTATTATTTTGT
1741 CCACTAAAAAAAAAAAAAAAAAAAAAAAAAAAAA-3'

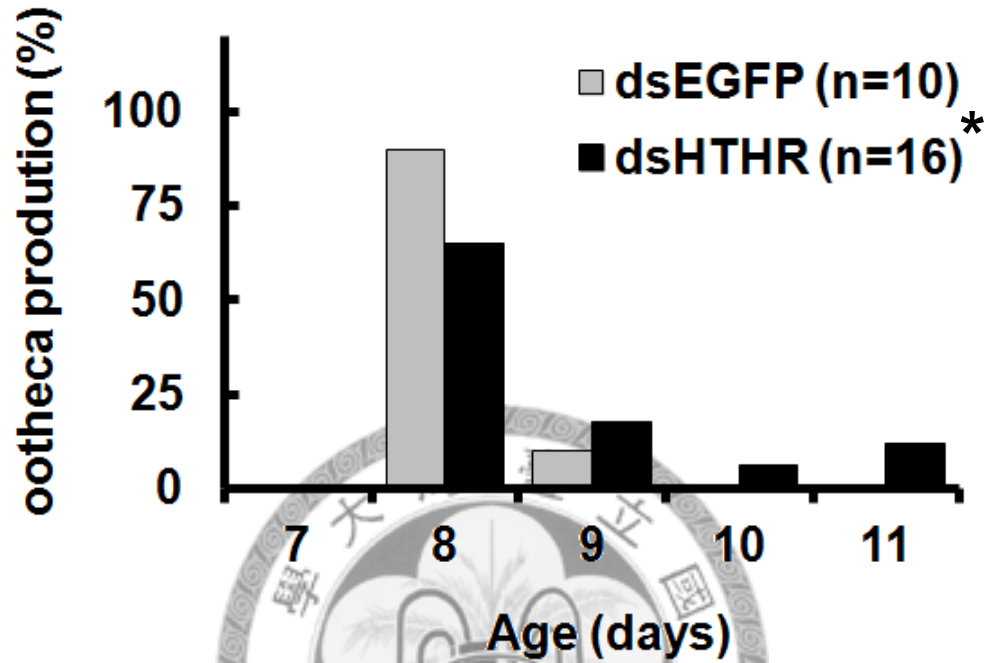
Supplementary Figure S1. Nucleotide sequence of cDNA encoding the open-reading frame for *B. germanica* HTHR with flanking 5'- and 3'-UTR sequences (GenBank accession number GU591493). Start and stop codon are indicated by yellow background. The primer pair for qRT-PCR is shown in underlined bold type. The primer pair for semiquantitative PCR is boxed. The fragment encompassed by the dsHTHR used in the RNAi experiments is shown in green background.

Appendix 3



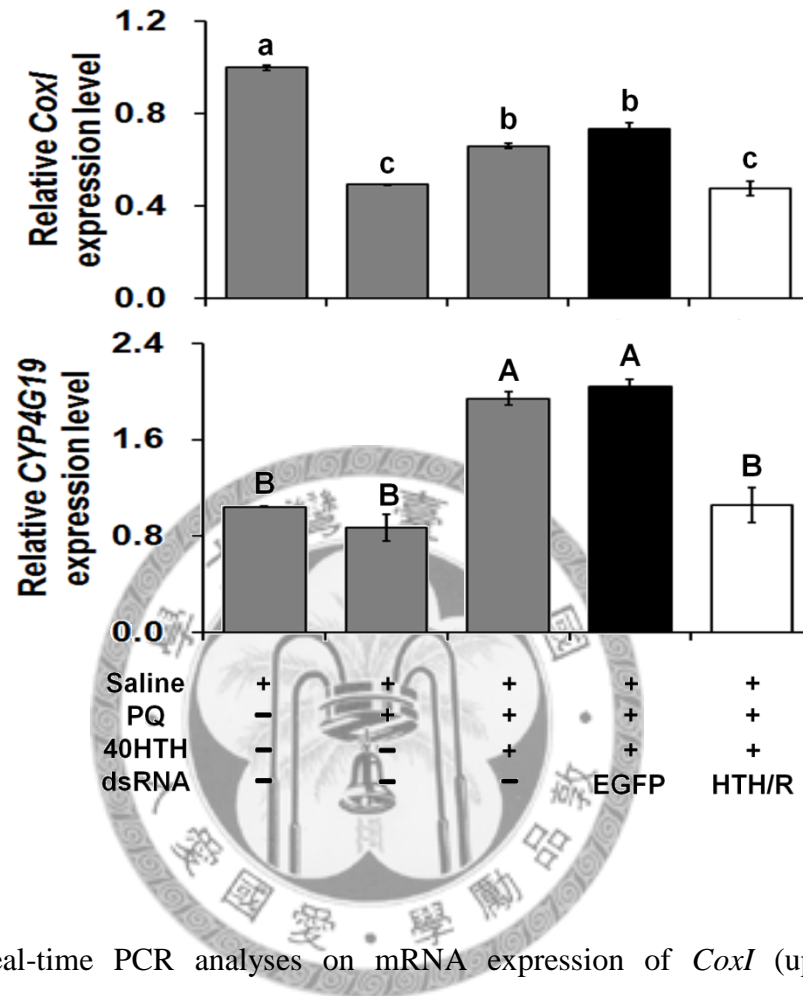
Daily fluctuation of hemolymph trehalose in 6-day-old virgin female *Blattella germanica*. The value represents mean \pm S.E.M. ($n = 7$). Different letters on the line plot indicate significant differences in dsEGFP treatment (one way ANOVA, $P < 0.05$, followed by Tukey's HSD post-hoc test). All time points in dsHTHR treatment are no significant difference (one way ANOVA, $P = 0.39$).

Appendix 4



Effect of RNAi of *BgHTHR* on the timing of ootheca production during the first reproductive cycle. *: significant delay of ootheca production by injection of 3 μ g dsHTHR (Wilcoxon test, $P < 0.05$).

Appendix 5



Quantitative real-time PCR analyses on mRNA expression of *CoxI* (upper) and *CYP4G19* (lower) in fat body of male adult of *B. germanica* 4 hr after treatments. The levels of gene expression were calculated after normalizing against *Actin* in each sample. N = 3 per treatment group; one-way ANOVA, $P < 0.01$, followed by Tukey's HSD post-hoc test. *CoxI*: cytochrome c oxidase subunit I (GenBank: AY176057). *CYP4G19*: cytochrome P450 monooxygenase CYP4G19 (GenBank: AY176056)