論 文 の 内 容 の 要 旨

論文題目　Endocrine network essential for energy homeostasis in the two-spotted cricket, *Gryllus bimaculatus*

（フタホシコオロギにおけるエネルギー恒常性に重要な内分泌系ネットワーク）

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## Introduction

In insects, diets from outside provide the entire energy required for multiple living processes. Due to the instability of environment, insects have to acclimate to various conditions. Then, ‘what to eat’ and ‘how to eat’ become unavoidable discussion in insects. ‘Self-selection’ is brought out to describe the fundamental eating strategy of insects, which tells a close relationship between appetite and dietary need. The ‘self-selection’ enables us to study the mechanism of metabolic and endocrinal regulation of feeding behavior in insects. During the life history of insects, collaborative regulation of food intake and energy expenditure happens all the time for actively maintaining a stable condition, which has been described as energy homeostasis by Cannon in 1930.

Energy homeostasis is achieved via a highly coordinated communication between the central nervous system (CNS) signals and circulating homeostatic signals, by which neuro-modulatory factors are well-documented for their roles associated with feeding behavior. Cross-talk or communications among neuropeptides, especially the cerebral-peripheral communication, have gained increasing researches in regulatory determinants of energy homeostasis (Fig. 1). Associated literatures of various insects generated a schematic endocrine network (endocrinet) composed of the feeding behavior-related neuro-modulatory factors. However, answers to questions pertaining to energy homeostatic metabolism so far have been mostly addressed around one or two signals, whereas much more neuropeptides are demonstrated with collaborative roles in regulating energy homeostasis. Hence, a comprehensive understanding of neuropeptides that contribute to energy homeostatic network is imperative.

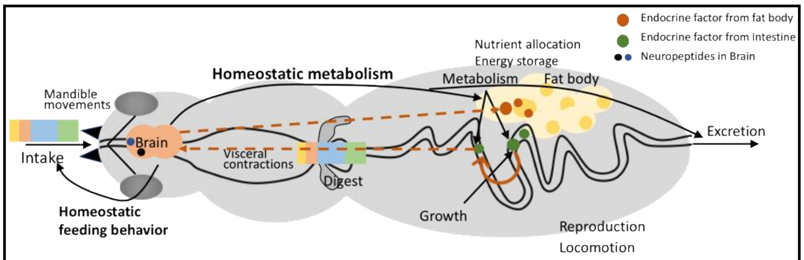


**Fig. 1** Schematic diagram of mechanism of energy homeostatic regulation by endocrine factors across the whole body in insects.

## Objectives

In this study, a prototype of endocrine network (endocrinet) in the two-spotted cricket *Gryllus bimaculatus* will be described by evaluation of neuropeptides in regulation of energy homeostasis, in which neuropeptides are considered as nodes, and their communications are considered as linkages. Screening of the core-regulators among neuropeptides and their characterization will be performed to prove the role of ‘corpora cardiaca (CC)’ as the energy homeostatic regulation center. Communication between screened neuropeptides will be addressed to further analyze their mode of action on metabolism and feeding behavior.

## Results

**Virtual network analysis and real hormone screening of neuropeptides in energy homeostasis**

Bibliometric mapping of science is the quantitative method for visually representing scientific literatures, which provides an authentic co-occurrence relative network based on according literatures. Here, I used VOSViewer to provide an overview of the structure of endocrinet in insects. Over 1132 literatures with associations of feeding behavior regulated by endocrine modulators from PubMed were extracted to provide relationships of co-localization, co-evolution, receptor-sharing, and releasing regulation of endocrine factors (Fig. 2). The generated Adipokinetic hormone (AKH)-oriented virtual network proving the role of AKH as a core regulator is well-researched in multiple insects. To elaborate the endocrinet in *G. bimaculatus*, I identified 32 neuropeptides from transcriptomic data. Transcriptional levels of identified neuropeptides were measured in three different nutrient states, satiety, starvation and refeeding. For analyses of feeding activity, three additional parameters were measured; ① weight of diet tablet for food intake; ② numbers of feces for excretion; ③ first-bite time after 24-hour starvation for feeding motivation.

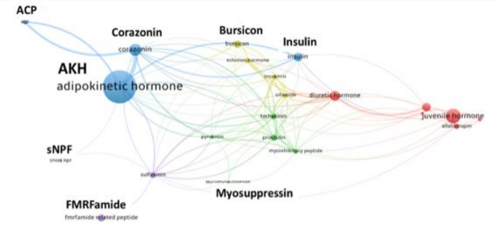


**Fig. 2** Virtual neuropeptide network by bibliometric mapping of endocrine modulatory literatures. Size of bubble represents occurrences; Thickness of line represents co-occurrence.

| Starvation/Refed | Food intake | Excretion | First-bite |
|---|---|---|---|
| AKH | AKH | AKH | ACP |
| ACP | ACP | ACP | Corazonin |
| CCAP | Allatostatin-A | CCAP | MIP 1 |
| FMRFamide | CCHamide-1 | CCHamide-1 | |
| MIP | CCHamide-2 | DH44 | |
| Myosuppressin | DH44 | FMRFamide | |
| Short NPF | FMRFamide | MIP 1 | |
| | Myosuppressin | | |
| | MIP 2-4,7-10 | | |

**Table. 1** Neuropeptides involved in regulation of feeding behaviors. Listed neuropeptides exhibited significant alterations.

Neuropeptides are listed in Table 1 with their significant alterations in the above four parameters. AKH and AKH/Corazonin-related peptide (ACP) were nominated as candidates of core-regulators in energy homeostasis. Both AKH and ACP reduced food intake and excretion, and elevated carbohydrate and lipid concentrations in hemolymph. For discovering the potential downstream endocrinal signals in peripheral tissue and brain under the regulation of AKH/ACP, transcriptional levels of eight GPCRs and 17 neuropeptides were measured after treatment of mature peptides and dsRNAs of AKH/ACP. In total, six GPCRs and seven neuropeptides responded to AKH/ACP signals (Fig. 3).

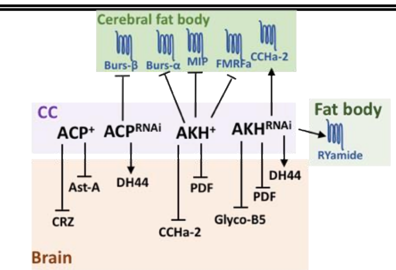


**Fig. 3** AKH and ACP signals, as key-regulators, delivered 'information' between tissues

**Potential of corpora cardiaca as a homeostatic endocrinal center**

Based on the experimental evidence of AKH and ACP's predominant expression in CC, I next analyzed transcriptomic data of CC by RNA sequencing. 100 pairs of CCs were dissected, and RNA was extracted and purified for RNA-seq. In total, 23022 contigs were *de novo* assembled by CLC workbench. GO, CoG and KEGG analyzes were performed for preparing the transcriptome of CC in *G. bimaculatus*. 77 GPCRs were observed in CC by comparing with GPCRs in other insects. Peptidome analysis of CC in different nutritional states was also performed by MALDI-TOF MS.

**Energy homeostatic regulation of two neuropeptides, Crustacean cardioactive peptide (CCAP) and Myosuppressin (MS)**

Application of CCAP resulted in the inhibition of food intake, elevation of circulating carbohydrate, enhancement of food storage in the crop. The amount of MS in CNS was significantly decreased with CCAP application. Furthermore, the colocalization of MS receptor (MSR) and CCAP-producing cells was confirmed in the anterior midgut of *G.*

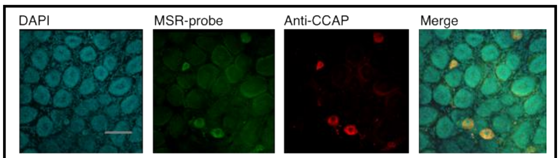


**Fig. 4** Colocalization of MSR and CCAP in anterior midgut

*bimaculatus* (Fig. 4). MS showed counteractive effects on CCAP-inhibited food consumption and excretion, while no additional effects between CCAP and MS were observed due to no additional effects in $CCAP^{RNAi}/MS^{+}$ crickets or $CCAP^{+}/MS^{RNAi}$ crickets (Fig. 5 AB). This counteraction was also observed in controlling lipid and carbohydrate levels in the hemolymph. To further understand this counteraction, I examined their abilities of lipid and carbohydrate mobilizations by knockdown both of AKH receptor (AKHR) (Fig. 5 CD) and Insulin receptor substrate (IRS) (Fig. 5 EF), which have been observed in other insects as core-regulators in energy homeostasis. Consequently, data indicated that neither AKHR nor IRS is essential for CCAP's functions on lipid and carbohydrate mobilization. Besides, MS appeared to function dependent of both AKHR and IRS. To elucidate how CCAP and MS regulate carbohydrate mobilization, transcriptional levels of the enzymes involved in trehalose biosynthesis and metabolism were measured. The knockdown of MS receptor (MSR) significantly altered transcriptional level of trehalose phosphate synthetase (TPS), whereas no alteration was observed by knockdown of CCAP receptor (CCAPR). Overall, I found a novel counteractive regulation between CCAP and MS on carbohydrate and lipid mobilization, in which the function of MS is mediated by the AKH/Insulin signal, and CCAP is independent of AKH/Insulin signal.

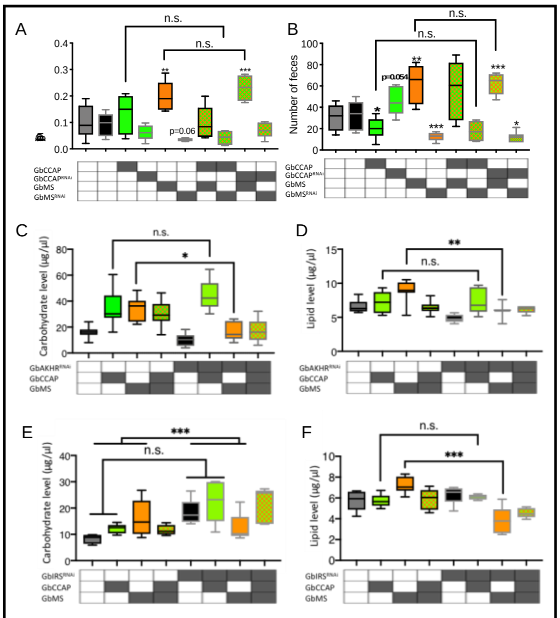

**Fig. 5** (A,B) Counteraction of CCAP and MS in food intake and excretion. (C~F) Influences of CCAP and MS on lipid and carbohydrate mobilization by knockdown of AKHR and IRS. (n=6~8, mean+S.D., Tukey's test $^{*}p<0.05$, $^{***}p<0.001$, n.s.: not significant.)

**Effects of circulating sugars change neuropeptidyl and non-neuropeptidyl factors in endocrine network**

To evaluate the effects of trehalose and glucose by their loading tests on neuropeptides, I performed *ex vivo* incubation of CNS and Gut and oral application with a high concentration of trehalose. As a result, trehalose did not alter the expression level of CCAP, while glucose in circulation increased the expression level of CCAP in CNS and dietary glucose decreased the expression level of CCAP in Gut. Both circulating and dietary trehalose increased expression levels of MS in CNS and Gut. Furthermore, the actual amounts of all neuropeptides in CNS after injection of high concentrated trehalose and glucose were measured by MALDI-TOF-MS (Fig. 6). Except for CCAP and MS, alterations were observed in the amounts of allatoregulatory peptides. Also, transcriptome analysis of anterior midgut after trehalose and glucose injections was performed, several non-neuropeptidyl factors were differentially expressed, including peroxiredoxin-like protein, chymotrypsin and several unknown genes. These findings led to the idea of next studies on energy homeostasis.

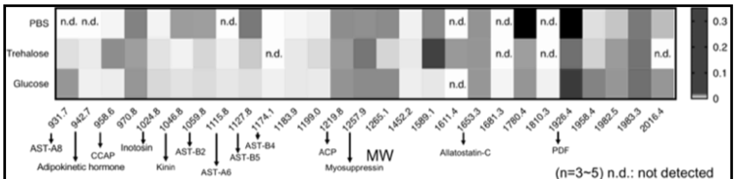

**Fig. 6** Measurement of neuropeptides by application of high concentration trehalose and glucose using MALDI-TOF-MS. Data were normalized by internal standard authentic peptide.

## Discussion

In this study, observation on feeding behavior and measurements of circulating energy were utilized for the evaluation of neuropeptides' roles. The simulation of the bibliometric network contributed to the discovery of the AKH's central role in regulating energy homeostasis in insects. Comprehensive identification of neuropeptides in *G. bimaculatus* contributes to the entire evaluation of endocrine network. An overall evaluation of feeding control by physiological aspects promises the validity of AKH, ACP, CCAP as energy homeostasis regulators.

So far, except for storing brain-synthesized neuropeptides, the role of CC remains poorly understood. Here, I proved that CC is an 'energy homeostatic endocrine center' by respond to upstream neuropeptide signals and deliver signals to peripheral tissues by circulation. AKH/ACP-oriented homeostasis was illuminated by transcriptional alterations that caused by AKH/ACP peptide application and knockdown. Also, the maintenance of circulating lipids observed in knockdown of AKH or ACP revealed a possibly compensatory role between AKH and ACP.

However, mechanisms of transcriptional alterations of GPCRs in the fat body by AKH or ACP stimulation remain unknown. Two possibilities can be considered; 1) Those receptors were altered by corresponding ligands directedly released from CC; 2) Receptor of AKH, which is only expressed in fat body, mediates the alterations of expression levels of those receptors. Collectively, the role of CC as a signal-mediating sensor is plausible because of the abundance of GPCRs and predominant roles of AKH/ACP in energy homeostasis.

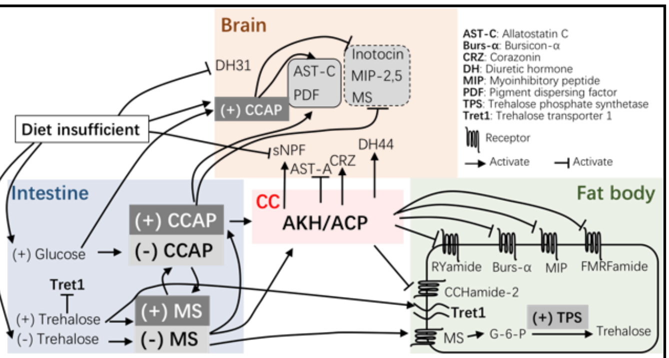

**Fig. 7** CC-oriented endocrinet model in diet insufficient state among cerebral and peripheral organs, composed of neuropeptides and their communications.

To date, CCAP and MS are two neuropeptides that influence the release of AKH proved by the *in vitro* experiments in *Locusta migratoria*. In this study, the release of MS was also proved to be regulated by CCAP. Thus, the endocrine circuit among AKH, ACP, CCAP, and MS is emerging in cricket (Fig. 7). To respond to external stimulus, counteraction between CCAP and MS in energy homeostasis and amount alteration of several peptides in CNS were observed. Transcriptional levels of short Neuropeptide F, Allatostatin-A, Corazonin, Diuretic hormone 44 in brain and several neuropeptides receptors in fat body were altered by manipulation of AKH/ACP signals. Together with distribution of their expression sites, a simulated organ-across endocrinet was generated. Starvation resulted in a decrease of trehalose and an increase of glucose concentration in hemolymph, which followingly inhibited MS's expression and activated AKH's expression. Once AKH is activated to release, trehalose is mobilized from fat body to hemolymph to maintain trehalose homeostasis. Based on the negation of CCAP's function of carbohydrate mobilization via AKH/Insulin signals (Fig. 5 C~F). I, thus, proved that CCAP generates a new endocrine pathway that independent of the well-documented AKH/Insulin pathway. Interestingly, a recent study showed that CCAP in *Drosophila melanogaster* regulates energy homeostasis via NPF signals, which is different but not contradicting to this study. The discovery of multiple downstream signals of CCAP provided a direction for studying CCAP in cricket and enhanced the significance of the endocrinet in regulating energy homeostasis.

This study described a prototype of endocrinet among cerebral and peripheral organs based on neuropeptides and their regulatory roles in feeding behavior and carbohydrate and lipid mobilization, which can be considered as a milestone in neuropeptide and energy homeostasis researches. However, dynamics of metabolism occur all the time according to external environments, which might be difficult to observe in real-time.