<table>
<thead>
<tr>
<th>Title</th>
<th>Regulatory mechanisms of cell population in the midgut of the American cockroach, Periplaneta americana, under starvation (飢餓条件下におけるワモンゴキブリ中腸細胞数の調節機構)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author</td>
<td>Moon Soo Park</td>
</tr>
<tr>
<td>Degree</td>
<td>博士（学術）</td>
</tr>
<tr>
<td>Date of Degree</td>
<td>2008-09-25</td>
</tr>
<tr>
<td>Resource Type</td>
<td>Thesis or Dissertation / 学位論文</td>
</tr>
<tr>
<td>Report Number</td>
<td>甲4424</td>
</tr>
<tr>
<td>URL</td>
<td><a href="http://www.lib.kobe-u.ac.jp/handle_kernel/D1004424">http://www.lib.kobe-u.ac.jp/handle_kernel/D1004424</a></td>
</tr>
</tbody>
</table>

※当コンテンツは神戸大学の学術成果です。無断複製・不正使用等を禁じます。著作権法で認められている範囲内で、適切にご利用ください。
Regulatory mechanisms of cell population in the midgut of the American cockroach, *Periplaneta americana*, under starvation

Moon Soo Park

Graduate School of Science and Technology, Kobe University
August, 2008
Regulatory mechanisms of cell population in the midgut of the American cockroach, *Periplaneta americana*, under starvation

飢餓条件下におけるワモンゴキブリ中腸細胞数の調節機構

Moon Soo Park

Graduate School of Science and Technology, Kobe University
August, 2008
Starvation suppresses cell proliferation that rebounds after refeeding in the midgut of the American cockroach, *Periplaneta americana*

## Chapter 2

### 2.1 INTRODUCTION

#### 2.2 MATERIALS AND METHODS

- **2.2.1 Insects**
- **2.2.2 Starvation stress and refeeding**
- **2.2.3 Length of midgut**
- **2.2.4 Injection of BrdU**
- **2.2.5 Immunohistochemistry and diameter of midgut**
- **2.2.6 Specificity of antibodies**
- **2.2.7 Evaluation of cell proliferation activity**
- **2.2.8 Statistical analysis**

### 2.3 RESULTS

- **2.3.1 The size of midgut**
- **2.3.2 Structure of midgut and specificity of the antibodies**
- **2.3.3 Structure of midgut and cell proliferation activity under starvation**
- **2.3.4 Cell proliferation activity after refeeding**
- **2.3.5 Effect of nutrients on cell proliferation activity**

### 2.4 DISCUSSION

- **2.4.1 Effect of starvation on midgut size**
- **2.4.2 Cell proliferation activity under starvation and refeeding**
- **2.4.3 Effect of nutrients on the cell proliferation activity**
- **2.4.4 Survival strategies of midgut under starvation**

### 2.5 REFERENCES
Chapter 3
Starvation induces apoptosis in the midgut stem cell clusters of Periplaneta americana: A histochemical and ultrastructural study

3.1 INTRODUCTION .................................................................................................................................................. 34
3.2 MATERIALS AND METHODS
  3.2.1 Insects ................................................................................................................................................................. 36
  3.2.2 Starvation stress ..................................................................................................................................................... 36
  3.2.3 Light microscopy .................................................................................................................................................... 36
  3.2.4 Histochemistry for TUNEL-assay ....................................................................................................................... 37
  3.2.5 Quantification of TUNEL-positive signals .......................................................................................................... 38
  3.2.6 Immunohistochemistry for active caspase 3 ....................................................................................................... 39
  3.2.7 Electron microscopy ............................................................................................................................................. 40
3.3 RESULTS
  3.3.1 Structure of the cockroach midgut ..................................................................................................................... 41
  3.3.2 TUNEL-assay ......................................................................................................................................................... 41
  3.3.3 Immunohistochemistry for the localization of active-caspase 3 ......................................................................... 42
  3.3.4 Ultrastructural observation of the midgut ........................................................................................................... 43
3.4 DISCUSSION
  3.4.1 TUNEL-assay ......................................................................................................................................................... 48
  3.4.2 Immunohistochemistry for active-caspase 3 ....................................................................................................... 48
  3.4.3 Ultrastructural observation of midgut .................................................................................................................. 49
  3.4.4 Survival strategies of the midgut against starvation ........................................................................................... 51
3.5 REFERENCES .......................................................................................................................................................... 53

Chapter 4
GENERAL DISCUSSION ............................................................................................................................................. 58
REFERENCES ............................................................................................................................................................... 61
ACKNOWLEDGEMENT .................................................................................................................................................. 64
Chapter 1: GENERAL INTRODUCTION

Dietary resources are not always available to insects, which forces insects to endure starvation. Insects must have countermeasures against periodic or non-periodic starvation. When insects encounter periodic starvation during winter or dry season, some enter diapause to survive this period. However, sometimes starvation comes in a non-periodic manner and then insects must undergo drastic changes not only in behaviors but also in metabolism and related physiological events for survival. Effects of non-periodic starvation on insects have been studied in various aspects of their life from behavior to development (Nijhout, 1975; Barcay and Bennett, 1991; Shintani et al., 2003). In the tobacco hornworm, *Manduca sexta*, starvation decreased the weight and the head size of larvae. The developmental delay under starvation increased the total number of molts (Nijhout, 1975). When larvae of the yellow-spotted longicorn beetle, *Psacothea hilaris*, were under-nourished, the body weight became only half that of control animals feeding on nutritious diets after the 3rd ecdysis (Shintani et al., 2003). Males of the German cockroach, *Blattella germanica* increased the velocity of locomotion, distance traveled, and proportion of time in motion when they were starved (Barcay and Bennett, 1991). However, the effects of the starvation on internal organs have not been sufficiently studied. Storing organs must provide the energy to support
basic physiology, and other organs may reduce their sizes to minimize energy consumption under starvation. The reduction of the organ sizes may be derived from the reduction of cell proliferation or increase of cell death. In the intestinal tract of mammals, starvation decreases not only the weight of tissues (Chaudhary et al., 2000) but also cell proliferation activity in tissues (Konishi et al., 1996; Chaudhary et al., 2000; Habold et al., 2004), and increases cell death in tissues (Boza et al., 1999; Jeschke et al., 2000; Chappell et al., 2003). However, the intestinal tract in insects may not continue to take these strategies endlessly because insects have evolved and adapted to environmental adversity and this is the only organ that can digests foods and absorbs nutrients. Therefore, the intestinal tract must keep a minimal functional level for restoration immediately after refeeding took place.

Insect intestinal tract consists of 3 parts: the foregut, midgut, and hindgut. The foregut stores foods ingested, and hindgut collects water from undigested food stuffs. The midgut is the only organ that digests foods, and absorbs nutrients. It should be affected more seriously than other two parts. Studies on the effects of starvation on insect midgut and the regulatory mechanism of cell population under starvation must clarify the overall survival strategies of insects not only under starvation but also other stress conditions. Cell proliferation (Blackburn et al., 2004, Palli and Parthasarathy 2008) and
cell death (Jiang et al., 1997; Lee et al., 2002; Uwo et al., 2002; Wu et al., 2006; Parthasarathy and Palli, 2007ab) in insect midgut have been studied using cell biology for metamorphosis of lepidoptera or diptera. Cockroaches and lepidoptera may have the different regulatory mechanism of cell population in the midgut because the midgut of lepidoptera have 4 types of cell, columnar cell, endocrine cell, stem cell, and goblet cell, but cockroach midgut does not have goblet cells.

In the present investigation, effects of non-periodic starvation and refeeding were studied on the midgut of male adult of *Periplaneta americana* in terms of organ atrophy, cell proliferation, and cell death. Chapter 2 deals with organ atrophy and cell proliferation. The effect on the cell proliferation activity was evaluated based on the immunohistochemical detection of thymidine analog, bromodeoxyuridine (BrdU) uptake, and mitotic marker, phospho-histone H3. Chapter 3 deals with cell death. The effect on cell death was evaluated based on the TUNEL-assay. Immunohistochemistry for active-caspase 3 and electron microscopy were employed to clarify the feature of cell death that occurred in the midgut under starvation. Chapter 4 investigates hierarchical defense mechanism and regulatory mechanism of midgut cell population under starvation based on the reports of other scientists and our preliminary experiments.
REFERENCES


Uwo, M.F., Ui-Tei, K., Park, P., Takeda, M. 2002. Replacement of midgut epithelium in

Chapter 2: Starvation suppresses cell proliferation that rebounds after refeeding in the midgut of the American cockroach, *Periplaneta americana*

2.1 INTRODUCTION

Effects of starvation on the intestinal tract of *Periplaneta americana*, on atrophy, have been known experientially, but not been established experimentally. If starvation reduces the size of intestinal tract, the reduction may be due to reduced cell proliferation because the weight and cell proliferation in the intestinal tracts of mammals reduce under starvation (Konishi et al., 1996; Chaudhary et al., 2000; Habold et al., 2004). *P. americana* may also reduce cell proliferation in the intestinal tracts under starvation because the tissue is unnecessary under starvation and the reduction of cell proliferation contributes to the reduction of energy consumption. However, the intestinal tract must maintain their minimal functions including cell proliferation to restore the normal and best condition immediately after refeeding. Midgut, the only tissue digesting foods and absorbing nutrients must adopt the regulation system to support the lifer of cockroaches. Cell proliferation in the midgut has been intensively studied when insects undergo metamorphosis (Baldwin and Hakim, 1991; Waku and Sumimoto, 1970). Some midgut
cell proliferation stimulators, α-arylphorine and ecdysone, were identified (Blackburn et al., 2004; Parthasarathy and Palli, 2008) but effects of starvation on midgut cell proliferation or regulatory mechanism of cell population under starvation have not been studied sufficiently.

This study investigates the effects of starvation and refeeding on the sizes and the cell proliferation activity in the midgut of a representative hemimetabolic insect, *P. americna*. Two antibodies were used to evaluate cell proliferation activity; anti-bromodeoxyuridine (BrdU) and anti-phospho-histone H3. BrdU is a thymidine analog and taken up into chromatin under the synthetic phase (S-phase) of the cell cycle. Phospho-histone-H3 appears under the mitotic phase (M-phase), especially at metaphase and the value calculated from this antibody directly indicates mitotic activity (Gurley et al., 1978; Ajiro et al., 1996). What factor stimulates cell proliferation activity was also investigated by feeding the starved cockroaches with representative nutrients, casein (protein), starch (carbohydrate), and cooking oil (lipid), as well as a non-nutrient diet (talc).
2.2 MATERIAL AND METHODS

2.2.1 Insects

Stock colonies of *Periplaneta americana* were maintained in mass on an artificial diet (MF, ORIENTAL YEAST CO. LTD, Tokyo, Japan) and water provided *ad libitum* at 25 °C. Newly emerged adult males collected from the colonies were kept in plastic containers (30.0 × 16.5 × 13.5 cm) for one week. Each container housed 15 to 18 cockroaches that had a constant access to the artificial diets and water on a light-dark regimen (12 hr light: 12hr darkness).

2.2.2 Starvation stress and refeeding

The cockroaches were transferred from the containers to small plastic cups (φ10.0 × H4.5 cm) and kept individually for several weeks at 25°C under the same light-dark cycle. For starvation experiments cockroaches were kept without food and the control cockroaches with food. For refeeding experiments, the cockroaches starved for 4 weeks were divided into two groups. One group was refed and the other group starved for control. All cockroaches were provided with water.

2.2.3 Length of midgut
All the fed and starved cockroaches were dissected in phosphate buffer (PBS, pH 7.4) at 4°C under CO₂ anesthesia. After dissection, each midgut was stretched to its maximum length on a rubber mat and the length was measured. The length from the base of the caeca to the base of the Malpighian tubules was defined as the length of midgut. The mean length was calculated from 24 to 33 samples per treatment group.

2.2.4 Injection of BrdU

BrdU was purchased from Roche Diagnostics Corporation (Basel, Switzerland). Fifty micrograms of BrdU in 10 μl of PBS was injected into each cockroach 2 h before dissection. The puncture made by injection was sealed with an instant adhesive, Aron-alpha (TOAGOSEI CO. LTD, Tokyo, Japan).

2.2.5 Immunohistochemistry and diameter of midgut

All cockroaches, with or without BrdU injection, were dissected 5 h after lights on, under CO₂ anesthesia in PBS (4°C). The midgut was stretched and fixed in the Bouin’s solution on a rubber mat after dissection. Five to 10 min later, it was transferred into a new Bouin’s solution (10 ml) and placed on a shaker at 4°C overnight. After the fixation, the tissues excised at the center of midgut were dehydrated with an ethanol series and
then exposed to xylene for dealcoholation. The tissues were embedded in paraffin and kept overnight at 60°C. Sections of 8 μm were cut from the paraffin blocks by using a rotary microtome. Sections were mounted on 3-aminopropyltriethoxysilane-coated glass slides and dried up overnight at 37°C.

Sections were dewaxed in xylene, passed through an ethanol series, and then rinsed in distilled water twice for 5 min and PBS containing 0.5% Tween 20 (PBT) for 5 min. The sections were incubated with blocking solution: BSA-contained PBT for 1 h at room temperature. After the blocking, the sections were incubated overnight at 4°C with monoclonal mouse antibody against BrdU which was diluted 200 times in blocking solution (Roche Diagnostics Corporation). After the incubation, the sections were rinsed in PBT 3 times for 5 min and then incubated with biotinylated anti-mouse IgG (VECTASTAIN ABC KIT PK-6102, Vector Laboratories, Burlingame, USA), diluted 200 times in blocking solution, for 1 h at room temperature. They were rinsed in PBT 3 times for 5 min each and incubated with avidin-horseradish peroxidase complex (VECTASTAIN ABC KIT PK-6102) for 30 min at room temperature. After rinsed with PBT they were exposed to 3, 3’-diaminobenzidine (DAB) solution for 10 min. Positively reacted areas of the sections became brown in color. To identify the negative nuclei, the sections were counterstained blue with Mayer's hemalum solution. The
diameter of the midgut was measured from digital images of these sections under microscope (BX50 and DP70, Olympus Corporation, Tokyo, Japan). The mean diameter was calculated from 8 sections per treatment group. One section was prepared from a single animal.

Sections derived from samples that did not take up BrdU were incubated with an antibody solution against phospho-histone H3 diluted 2000 times in blocking solution (Upstate) as a primary antibody to detect M-phase nuclei. Anti-rabbit IgG was incubated as secondary antibody (VECTASTAIN ABC KIT PK-6101) and DAB reaction was allowed for 2 min 10 s.

2.2.6 Specificity of antibodies

Specificity of the antibody against BrdU was tested as follows; 1) sections of the midgut from cockroaches injected with BrdU were incubated with the antibody, 2) those from cockroaches without BrdU injection were incubated with the antibody, and 3) those from cockroaches injected with BrdU were incubated with normal mouse IgG (CHEMICON International Inc, Temecula, USA). The antibody and normal mouse IgG were the same protein concentration. Specificity of the antibody against phospho-histone H3 was also tested against normal rabbit IgG (Upstate).
2.2.7 Evaluation of cell proliferation activity

To evaluate cell proliferation activity one formula based on cross-sections of midgut incubated with antibody of BrdU or phospho-histone H3 was adopted: (total number of positive reactive nuclei in the nidi per section / total number of positive and negative reactive nuclei in the nidi per section) × 100 (%). The number of both positively and negatively reactive nuclei in the nidi were counted under microscope (BX50, Olympus Corporation, Tokyo, Japan). The cell proliferation activity was calculated based on the mean of 6-8 sections. One section was prepared from one sample. The values were arcsine-transformed for statistical analysis.

2.2.8 Statistical analysis

Two-way ANOVA or Fisher’s PLSD was used for statistical analysis using StatView 5.0 software Japanese version (SAS Institute Inc., USA). Statistical difference was defined as $p<0.05$. 
2.3 RESULTS

2.3.1 The size of midgut

Difference in the length and diameter of midgut between the fed and the starved cockroaches was easily detected after one week, but longer starvation did not further affect both the length and diameter (Fig. 1ab). For length, two-way ANOVA showed a statistical difference between conditions (fed and starved) (d.f.=1, 216, $F=89.862$, $p<0.0001$), but not between weeks (1, 2, 3, and 4 th week) (d.f.=3, 216, $F=1.650$, $p=0.1789$). No interaction was detected between feeding condition and week (d.f.=3, 216, $F=1.016$, $p=0.3867$). In diameter, the results of statistical analysis by two-way ANOVA were similar to those in length (d.f.=1, 56, $F=19.732$, $p<0.0001$ for the effect of condition, d.f.=3, 56, $F=0.057$, $p=0.9821$ for the effect of week, and d.f.=3, 56, $F=0.461$, $p=0.7104$ for interaction between condition and week).

2.3.2 Structure of midgut and specificity of the antibodies

The midgut consists of two layers (Fig. 2a). The outer layer is consisted of muscles cells, trachea and collagen fibers. The inner layer is an epithelium that consists of three different cells: columnar cells, endocrine cells, and stem cells. The columnar cells have microvilli on the lumenal side and are cylindrical shape. Paracrine or endocrine cells are
intercalated between the columnar cells. Nests of stem cells (nidi) are located at the basal side of the epithelium at regular intervals.

Only sections of cockroaches that were injected with BrdU and incubated with antibody of BrdU had positive reactivity only in the nidi (arrows of Fig. 2a). Other sections that had no BrdU (Fig. 2b) or were incubated with normal mouse IgG (data not shown) showed no-reactivity. Sections incubated with anti-phospho-histone H3 also had positive reactivity only in the nidi (arrowheads of Fig. 2c), but those incubated with normal rabbit IgG at the same protein concentration had no-reactivity (Fig. 2d).

2.3.3 Structure of midgut and cell proliferation activity under starvation

While normally fed cockroaches showed many BrdU-positive nuclei in the cells of nidi (Fig. 3a), starved cockroaches for 4 weeks showed fewer positive nuclei in the cells of nidi (Fig. 3b). The starved cockroaches showed many small condensed nuclei in the nidi and the outer layer subsided into the epithelium than in the fed cockroaches.

Cell proliferation activity evaluated using antibody against BrdU started declining after 2 weeks of starvation and showed 10.9% after 4 weeks of starvation (Fig. 3c). The cell proliferation activity of the cockroaches starved for 4 weeks was about a half that of the cockroaches fed for 4 weeks (21.8%). Two-way ANOVA of the cell proliferation
activity showed statistical differences between conditions (fed and starved) (d.f.=1, 56, $F=22.773$, $p<0.0001$) and between weeks (1, 2, 3, and 4 th week) (d.f.=3, 56, $F=4.261$, $p=0.0088$). Interaction was detected between condition and week (d.f.=3, 56, $F=4.240$, $p=0.0090$).

2.3.4 Cell proliferation activity after refeeding

Cell proliferation activity after refeeding was evaluated using antibody of BrdU or phospho-histone H3 (Fig. 4ab). Cell proliferation activity based on BrdU-uptaking nuclei of the cockroaches starved for 4 weeks increased sharply in a day after refeeding and a 10% overshoot level over the control (continuously fed group) was maintained for at least 2 more days (Fig. 4a). Two-way ANOVA of the cell proliferation activity showed a statistical difference between conditions (fed, starved, and refed) (d.f.=2, 62, $F=59.639$, $p<0.0001$), but not between days (1, 2, and 3 rd day) (d.f.=2, 62, $F=0.793$, $p=0.4568$). No interaction was detected between condition and day (d.f.=4, 62, $F=1.405$, $p=0.2427$).

Cell proliferation activity based on immuno reactive nuclei against phospho-histone H3 of the cockroaches starved for 4 weeks was also about a half that of the cockroaches fed for 4 weeks (Fig. 4b). Refeeding restored the cell proliferation activity in a day, but
a super-rebound that overshot the level of the continuously fed cockroaches was observed after 2 days. Two-way ANOVA for the cell proliferation activity showed statistical differences between conditions (fed, starved, and refeeding) (d.f.=2, 60, $F=34.610$, $p<0.0001$) and between days (1, 2, and 3 rd day) (d.f.=2, 60, $F=4.310$, $p=0.0178$). Interaction was also detected between condition and day (d.f.=4, 60, $F=2.557$, $p=0.0477$).

2.3.5 Effect of nutrients on cell proliferation activity

All foods: casein, starch, and oil, restored cell proliferation activity based on BrdU-uptaking nuclei in the cockroaches starved for 4 weeks (Fig. 5). The cell proliferation activity after oil feeding was higher than that after casein or starch feeding, and close to that of standard food refeeding. However, there was no significant difference in the cell proliferation activity among these three foods ($p>0.05$, Fisher’s PLSD). Non-nutrient food; i.e., talc slightly stimulated the cell proliferation activity, but it was not significantly different from the starved controls ($p=0.2453$, Fisher’s PLSD).
Fig. 1 Effect of starvation on the midgut length (a) and diameter (b). Difference in both the length and diameter between fed cockroaches (open circles) and starved ones (filled circles) is easily detected after one week, but longer starvation does not further affect both the length and diameter. Each point represents the mean of at least 24 samples for the length and the mean of 8 samples for the diameter. Error bars indicate S.E.M.
Fig. 2 Immunohistochemical reactivity in the midgut and specificity of antibodies. (a) Nidi are seen at the basal sides of columnar cells and muscle layers are located beneath the nidi. The midgut injected with BrdU shows BrdU-uptaking nuclei only in nidi cells under the incubation with BrdU antibody (arrows). (b) Negative control of Fig. 2a, i.e., the midgut not injected with BrdU shows no reactivity against BrdU antibody. (c) Phospho-histone-H3 like products are seen only in nidi cells (arrowheads). (D) Adjacent section shown in Fig. 2c incubated with normal rabbit IgG show no reactivity. All non-immuno-reactive nuclei were counterstained with hematoxylin in blue. cc, columnar cell; l, lumen; ml, muscle layer; mv, microvilli; nd, nidi. Bar indicates 50 μm.
Fig. 3 Cell proliferation activity based on BrdU-uptaking nuclei under starvation. Cockroaches fed for 4 weeks show many BrdU-uptaking nuclei in nidi (a), but cockroaches starved for 4 weeks show few ones in nidi (b). The starved cockroaches also show many condensed small nuclei in the nidi (arrowheads) and rough muscle layers. The cell proliferation activity of starved cockroaches (filled circles) begins to reduce after 2 weeks and become about half as high as that of fed controls (open circles) after 4 weeks (c). Means ± S.E.M. N=8. Bars indicate 50 um.
Fig. 4 Cell proliferation activity after refeeding based on BrdU-uptaking nuclei (a) or immuno reactive nuclei against phospho-histone H3 (b). (a) The cell proliferation activity of the cockroaches starved for 4 weeks restores and overshoots that of the cockroaches fed continuously in a day after refeeding. (b) The cell proliferation activity of the starved cockroaches restores in a day and overshoots that of continuously fed cockroaches in 2 days after refeeding. ●; fed cockroaches, ■; starved cockroaches, ▲; re-fed cockroaches. Means ± S.E.M. N=7-8.
Fig. 5 Effect of nutrients on cell proliferation activity based on BrdU-uptaking nuclei one day after refeeding. All foods: casein, starch, and oil restore the cell proliferation activity of the cockroaches starved for 4 weeks. Non-nutrient food: talc stimulates the cell proliferation activity slightly. Fed: cockroaches kept with food for 4 weeks and one day. Stv, those kept without food for 4 weeks and one day. Refed, those kept without food for 4 weeks and had standard food for one day. Different letters indicate statistical difference (p<0.05, Fisher's PLSD). Means ± S.E.M. N=6-8.
2.4 DISCUSSION

2.4.1 Effect of starvation on midgut size

The length and diameter in the midgut of starved cockroaches were shorter than those of fed ones after one week of starvation, but further reduction both of them was not observed under further starvation (Fig. 1ab). Mammals showed the reduction both in size and mitotic rate in the intestinal tract including small intestine and colon under starvation (Chaudhary et al., 2000). Therefore, atrophy of the midgut under starvation may be derived from the reduction of cell proliferation.

2.4.2 Cell proliferation activity under starvation and refeeding

Cell proliferation activity based on BrdU-uptaking in the nidi began to reduce after 2 weeks of starvation and showed half level of the continuously fed control after 4 weeks of starvation (Fig. 3c). Refeeding restored the cell proliferation activity. This is supported by increased BrdU-uptaking (Fig. 4a) and the number of immuno-reactive nuclei against phospho-histone H3 (Fig. 4b). Both indexes showed the cell proliferation activity overshot the level of the fed control. However, the two indexes showed a slight difference in time course. It may be derived from the lag of in M-phase from S-phase. Rats increase the S-phase span of intestinal cells under long starvation and then starved
cockroaches may also increase the S-phase span of midgut stem cells, and gradually restoring the increased span to normal span after refeeding.

These results show that cockroaches adjust their midgut cell proliferation activity according to the nutritional changes, starvation and refeeding as in mammals (Fig. 3c and 4). The overshooting after refeeding may be derived from the change in energy consumption and energy efficiency in the midgut under starvation. Cockroaches reduce the size of midgut and cell proliferation activity in the midgut to reduce energy consumption and keep the minimum function of the midgut at low energy consumption under starvation. They may also increase energy efficiency for cell proliferation. The reduction of energy cost and increase of energy efficiency induce the overshooting in the cell proliferation activity under refeeding. Overeating during refeeding could be the reason for the overshooting because all refed cockroaches showed more filled crops than that of normal fed ones (data not shown). Zudaire et al. (2004) showed that food intake affected the cell proliferation activity using antibody of PCNA in the midgut of *Locusta migratoria*. However, one week of starvation could not reduce the cell proliferation activity in the midgut of *P. americana* (Fig. 3C). Further experiments were required to establish the relationship between food intake and cell proliferation activity. In *P. americana*, the overshooting may contribute to a quick recovery of the digestive
tract damaged by starvation because rat also restores damaged jejunal epithelium soon after refeeding (Habold et al., 2004).

The effect of starvation on the cell proliferation activity (Fig. 3c) can not explain the reduction in the midgut sizes sufficiently (Fig. 1ab) because the cell proliferation activity began to reduce after 2 weeks of starvation while the size reduction started within a week of starvation. Starvation probably induces atrophy in midgut at several steps, and what factor first induces the atrophy remains unknown. Other evaluation systems are required for the total cell number, the size of epithelial cells and muscles.

2.4.3 Effect of nutrients on the cell proliferation activity

All the representative nutrients examined, but talc, stimulated the cell proliferation activity of the cockroaches starved for 4 weeks (Fig. 5). This result matches polyphagous feeding of cockroach. Cooking oil (31.7%) stimulated the cell proliferation activity more than the other food sources (casein=27.2% and starch=26.3%) nearly up to the rebound level (37.1%). This result may be derived from the higher calorie per gram levels of lipids (9 kcal) than that of other nutrients (4 kcal). However, cooking oil (9 kcal / g) stimulated the cell proliferation activity less than artificial diet (3.6 kcal / g). The cell proliferation activity may be stimulated by not only total calorie but also
specific nutrients or nutritional balance because the artificial diet was the mixture of lipid and protein. Zudaire (2004) also showed that nutritional balanced food increased the cell proliferation activity more than non-balanced food.

2.4.4 Survival strategies of midgut under starvation

Our results showed that starved cockroach reduces the size of midgut and the cell proliferation activity. The midgut switches off the metabolism to reduce the energy consumption in the tissue for survive until refeeding (Fig. 3c). After refeeding, the cell proliferation activity is accelerated to restore the normal midgut functions (Fig. 4). The starved cockroach may undergo a series of metabolic switches not only the reduction of cell proliferation, but also the increase of cell death in the midgut for survival because cockroaches starved for 4 weeks showed the small and condensed nuclei in the nidi of the midgut (Fig. 3ab). The nuclear condensation that was not found in the nidi of fed cockroaches may be derived from chromatin condensation in the nuclei of apoptosis, or apoptotic bodies. The increase of cell death induces the reduction of midgut cell population and the appearance of cell debris that provides engulfing cells with energy. TUNEL assays showed that starvation stimulated apoptosis in the intestinal tracts of mammals (Boza et al., 1999; Jeschke et al., 2000; Chappell et al., 2003), but the
appearance of apoptosis in crypts, the sites of cell proliferation under starvation, has not been studied sufficiently (Premoselli et al., 1998). Chapter 3 investigates the effect of starvation on cell death in the midgut to clarify the survival strategies in the midgut under starvation.
2.5 REFERENCES


Blackburn, M.B., Loeb, M.J., Clark, E., Jaffe, H., 2004. Stimulation of midgut stem cell proliferation by Manduca sexta α-arylborin. Archives of Insect Biochemistry and


Premoselli, F., Sesca, E., Binasco, V., Caderni, G., Tessitore, L., 1998. Fasting/re-feeding before initiation enhances the growth of aberrant crypt foci induced
by azoxymethane in rat colon and rectum. International Journal of Cancer 77, 286-294


Zhang, J., Iwai, S., Tsugehara, T., Takeda, M., 2006. MbIDGF, a novel member of the imaginal disc growth factor family in *Mamestra brassicae*, stimulates cell proliferation in two lepidopteran cell lines without insulin. Insect Biochemistry and Molecular Biology 36, 536-546


Chapter 3: Starvation induces apoptosis in the midgut stem cell clusters of *Periplaneta americana*: A histochemical and ultrastructural study

3.1 INTRODUCTION

Chapter 2 showed that starvation induced the reduction of organ size and cell proliferation activity that rebounded after refeeding in the midgut of *Periplaneta americana*. It also reported that the midgut of cockroaches starved for 4 weeks showed subsidences of basal mucosa into the epithelium in a complex manner. Many small condensed nuclei appeared in the stem cell clusters, termed nidi (Fig. 3b of Chapter 2). Apoptosis also shows nuclear condensation with chromatin concentration (Kerr, et al., 1972 and Clarke, 1990). These results propose a hypothesis that starvation induces cell death in the nidi of the midgut and the cell death appears to occur by apoptosis. In the intestinal tracts of mammals, TUNEL assays showed that starvation stimulated apoptosis in the epithelium (Boza et al., 1999; Jeschke et al., 2000; Chappell et al., 2003), but the appearance of apoptosis in crypts, the sites of cell proliferation under starvation, has not been studied sufficiently (Premoselli et al., 1998). Studies on cell death in insect midgut have been developed in the collapse of larval epithelium of
lepidoptera or diptera during metamorphosis (Jiang et al., 1997; Lee et al., 2002; Uwo et al., 2002; Wu et al., 2006; Parthasarathy and Palli, 2007ab), but the effect of starvation on cell death in the midgut have not been studied.

This chapter investigated the effects of starvation on cell death in the midgut epithelium of *P. americana* using the TUNEL method. Immunohistochemistry for active-caspase 3 and electron microscopy were employed to determine whether the cell death in the nidi was apoptosis or not. Studies on cell death under starvation shall contribute to clarify the survival strategies of insect midgut under starvation.
3.2 MATERIALS AND METHODS

3.2.1 Insects

Stock colonies of *P. americana* were maintained in mass on an artificial diet (MF, ORIENTAL YEAST CO. LTD, Tokyo, Japan) and water provided *ad libitum* at 25°C. Newly emerged adult males collected from the colonies were kept in plastic containers (30.0 × 16.5 × 13.5 cm) for one week. Each container housed 15 to 18 cockroaches that had a constant access to the artificial diet and water on a light-dark regimen (12 h light: 12 h darkness).

3.2.2 Starvation stress

The cockroaches were transferred from the containers to small plastic cups (φ10.0 × H4.5 cm) and kept individually for several weeks at 25°C under the same light-dark cycle. For starvation experiments, cockroaches were kept without food but water was provided. The control cockroaches were kept with and water.

3.2.3 Light microscopy

All the fed and starved cockroaches were dissected in phosphate buffer (PBS, pH 7.4) at 4°C under CO₂ anesthesia. After dissection, the midguts were collected and stretched on a rubber mat and then poured with 4% paraformaldehyde (PFA) in Milloning’s
phosphate buffer (M-PBS) at pH 7.4. Five min later, tissues cut out from the central part of midgut were transferred into a bottle containing fresh PFA and kept for 4 h at 4°C. After the fixation, the tissues were dehydrated in an ethanol series and then exposed to xylene for dealcoholation. The tissues were embedded and kept overnight in paraffin at 60°C. Sections of 8 μm thickness were cut from the paraffin blocks using a rotary microtome. Sections were mounted on 3-aminopropyltriethoxysilane-coated glass slides and dried up overnight at 37°C.

3.2.4 Histochemistry for TUNEL-assay

ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (S7101, CHEMICON INTERNATIONAL Inc.) was used to microscopically examine cell death in the midgut of *P. americana* using the TUNEL-assay. Dewaxed sections were rinsed in PBS for 5 min and then incubated with Proteinase K (20 μg/ml, Sigma, in PBS) for 15 min at room temperature. The sections were rinsed in distilled water twice for 2 min each and incubated with 3% H₂O₂ solution for 5 min. The sections were rinsed in PBS for 5 min and terminal deoxynucleotidyl transferase (TdT) was added. The reaction mixture containing TdT was incubated in a humidified chamber at 37°C for 1 h and the reaction was stopped in stop solution. After rinsed in PBS 3 times for 5 min each, the sections
were incubated with digoxigenin antibody for 30 min at room temperature. After rinsing in PBS 3 times for 5 min each, the sections developed a brown color in 3, 3'-diaminobenzidine (DAB) solution. Negative nuclei were also counterstained in blue with hematoxylin. Sections for the negative control were also incubated with reaction buffer without TdT.

3.2.5 Quantification of TUNEL-positive signals

To compare the incidence of cell death in the midgut between the fed and starved cockroaches, the density of positive signals of TdT in the columnar cells or the nidi was calculated from the TUNEL-assay data. The numbers of intensely positive signals in the columnar cells or the nidi were counted under a microscope. The area of columnar cells or nidi was determined by a point counting method (Weibel, 1979 and 1980). A transparent sheet with a grid lattice of 1855 (35×53) points was put on the photographed images of midgut sections and then the number of intersecting points was determined to measure the areas. The density of positive signals in the columnar cells or in the nidi is shown as mean values derived from 4 to 6 samples with the standard error of the mean. The starved group was compared statistically with the fed controls at the same time points by Mann-Whitney's U-test (StatView 5.0 software Japanese version, SAS
Institute Inc., USA). Statistical difference was defined as $p < 0.05$.

3.2.6 Immunohistochemistry for active caspase 3

Sections derived from the midgut of cockroaches fed for 4 weeks or starved for 4 weeks were dewaxed in xylene, passed through an ethanol series, and then rinsed in PBS twice for 5 min each. The sections were incubated in 0.01 M citrate buffer at pH 6.0 at 80°C for 1 h. Thereafter, they were placed at room temperature for 2 h. The sections were rinsed in distilled water twice for 5 min each and in PBS with 0.5% Tween 20 (PBT) for 5 min. The sections were incubated with blocking solution (PBT containing BSA) for 1 h at room temperature. After blocking, the sections were incubated with affinity purified rabbit antibody against active-caspase 3 (AB3623, CHEMICON INTERNATIONAL Inc), which was diluted 50 times in the blocking solution, overnight at 4°C. After incubation, the sections were rinsed in PBT 3 times for 5 min each and then incubated with biotinylated anti-rabbit IgG (VECTASTAIN ABC KIT PK-6101, Vector Laboratories, Burlingame, USA), which was diluted 200 times in the blocking solution, for 1 h at room temperature. The sections were rinsed in PBT 3 times for 5 min each and incubated with avidin-horseradish peroxidase complex (VECTASTAIN ABC KIT PK-6101) for 30 min at room temperature. After rinsing with
PBT and then exposed to DAB solution for 20 min, and positively reacting areas of the sections became brown in color. The sections were also counterstained with hematoxylin. Sections used for the negative control were also incubated with blocking solution but without the primary antibody.

3.2.7 Electron microscopy

Midguts dissected from cockroaches fed for 4 weeks or starved for 4 weeks were fixed in 2.5% glutaraldehyde (GA) in M-PBS. Five min later, the midgut was cut into small pieces of 2 mm in length, transferred into a new bottle, and fixed in fresh GA at 4°C overnight. The samples were rinsed in M-PBS 3 times for 10 min each and fixed with buffered 1% OsO₄ for 1 h at 4°C. The sections were then rinsed in distilled water for 1 min, dehydrated in ethanol, and embedded in Quetol 812 resin mixture. Ultrathin sections were cut from the resin blocks with an ultra-microtome MT-1 (Ivan Sorvall Inc.) using a histo-diamond knife or diamond knife (Diatome, Switzerland). The sections were stained with 4% uranyl acetate for 10 min and lead citrate for 10 min. The sections were then observed using a Hitachi H-7100 electron microscope at 75 kV.
3.3 RESULTS

3.3.1 Structure of the cockroach midgut

The midgut of *P. americana* consists of two layers (Fig. 1a). The outer layer is connective tissues consisting of muscles, teacheae and collagen fibers. The inner layer is an epithelium that consists of three different cell types: columnar cells, endocrine or paracrine cells, and stem cells. The columnar cells have microvilli on the lumenal side and are cylindrical in shape. Paracrine or endocrine cells are intercalated between the columnar cells and their nuclei are basally located (data not shown). Nests of stem cells (nidi) are seen on the basal side of the epithelium at regular intervals.

3.3.2 TUNEL-assay

Two different types of positive signals from the TdT enzyme were observed in the columnar cells; one was in the nuclei (black arrows of Fig. 1a) and the other was in the apical cytoplasm (white arrow of Fig. 1a). The sizes of positive signals in the apical areas were equal to or smaller than those of the nuclei in the columnar cells. Cockroaches starved for 3 or 4 weeks showed positive signals not only in the columnar cells, but also in the nidi (arrowheads of Fig.1b). The midgut of both fed and starved cockroaches, when incubated without TdT enzyme solution, showed no signals (data not
Quantitative analysis showed that starvation increased TUNEL-positive signals both in the columnar cells and in the nidi. In the columnar cells the density of the positive signals began to exceed that of fed cockroaches after 2 weeks of starvation (Fig. 1c). In the nidi the density of the signals in the fed insects remained at low values, but those of the starved insects began to increase after 2 weeks ($p < 0.05$) and showed significant increases after 4 weeks of starvation (Fig. 1d).

3.3.3 Immunohistochemistry for the localization of active-caspase 3

The midgut of cockroaches starved for 4 weeks was further examined immunohistochemically by targeting active-caspase 3 and using electron microscopy to clarify the features of cell death in the nidi under starvation. The midgut of cockroaches fed for 4 weeks was also examined as a control.

The midgut of cockroaches fed for 4 weeks showed active-caspase 3-like immunohistochemical reactivity only in the apical areas of columnar cells (arrows of Fig. 2a). The sizes of the immuno-positive granules were equal to or smaller than the sizes of the nuclei in the columnar cells. Cockroaches starved for 4 weeks showed reactivity not only in the columnar cells but also in the nidi (arrow and arrowheads of
Fig. 2b). The reactivity in the nidi was seen as granules or localized in the cytoplasm around the nucleus. The midguts of both fed and starved cockroaches incubated without the antibody showed no reactivity (data not shown).

3.3.4 Ultrastructural observation of the midgut

Columnar cells of cockroaches fed for 4 weeks had rich cytoplasm with abundant rough endoplasmic reticulum (RER), mitochondria, and various sizes of secondary lysosomes (Fig. 3a). Some secondary lysosomes contained the debris of organelles (Fig. 3b). The nidi consisted of multilayered packed cells with a small cytoplasmic volume. The cytoplasm contained many mitochondria, little RER, and few secondary lysosomes (Fig. 3c). Connective tissues surrounded the epithelial tube. The tissues were made of collagen fibers, ground substances, fibroblasts, muscles, and tracheas. Smooth basal laminae were deposited outside the epithelium.

The majority of columnar cells of cockroaches starved for 4 weeks did not show significant structural differences from those of cockroaches fed for 4 weeks, but some columnar cells of the starved cockroaches collapsed and their organelles including nuclei, mitochondria, and RER, were released into the lumen (Fig. 3d). The nuclei seem to be intact, but euchromatin was rarely observed and heterochromatin was more
condensed than that of the fed cockroaches (Fig. 3ad). The nidi of the starved cockroaches showed different structures from those of the fed cockroaches. The papilla-shaped nidi appeared with plenty of secondary lysosomes, specific cytoplasmic fragments, aggregated intracellular tubules, and wider intercellular splits between the nidus cells (Fig. 3ef). The cytoplasmic fragments had split nuclei with condensed chromatin (arrows of Fig. 3e). Moreover, mitotic division was also rarely observed in the nidus (Fig. 3g). Basal laminae of the starved cockroaches were more complex and rougher than those of the fed ones (Fig. 3e).
Fig. 1 Localization (ab) and quantification (cd) of TUNEL-positive signals in the midgut of fed or starved cockroaches. a) Cockroaches fed for 4 weeks show positive signals in the nuclei (black arrows) and in apical areas (white arrow) of the columnar cells. b) Cockroaches starved for 4 weeks show positive signals not only in the columnar cells (arrow) but also in the nidi (arrowheads). cc: columnar cell, l: lumen, ml: muscle layer, mv: microvilli, nd: nidus (a nest of stem cells). Bars indicate 50 μm. Density of the positive signals in the columnar cells and nidi begin to increase after 2 weeks of starvation (cd) and that in the nidi show a significant increase after 4 weeks starvation (d). ○: fed cockroaches, ●: starved cockroaches. N=4-6. Data are presented as means ± S.E.M. * Significantly different from fed controls at the same time point, p < 0.05.
Fig. 2 Active-caspase 3-like immunohistochemical reactivity in the midgut of cockroaches fed or starved for 4 weeks. a Cockroaches fed for 4 weeks show positive reactivity as granules only in the apical areas of columnar cells (arrows). b Cockroaches starved for 4 weeks show positive reactivity not only in the columnar cells (arrow) but also in the nidi (arrowheads). cc: columnar cell, l: lumen, ml: muscle layer, mv: microvilli, nd: nidus (a nest of stem cells). Bars indicate 50 μm.
Fig. 3 Columnar cells and nidi in the midgut of cockroaches fed (a, b, and c) or starved (d, e, f, and g) for 4 weeks. 

a The columnar cells have a large cytoplasmic area with abundant rough endoplasmic reticulum, mitochondria, and various sizes of secondary lysosomes. Some secondary lysosomes contain the debris of organelles (arrow of Fig. 3b). 

b The nidi consist of aggregates of multilayered small cells. The basal laminae are smooth at the basal side of the nidi. 

c The nuclei, mitochondria (arrows), and denatured rough endoplasmic reticulum (arrowheads) released from the collapsed columnar cells are found in the lumen above the microvilli. 

d Papilla-shaped nidi show many cytoplasmic fragments containing split nuclei with condensed chromatin (arrows) and a large volume of aggregated intracellular tubules. The basal laminae are more complex than those of the fed cockroaches. A secondary lysosome (f) and mitotic division (g) are seen in other nidi. 

e Collagen fiber, f; lumen, mc; muscle, nc; nucleolus nd; nidi, rer; rough endoplasmic reticulum, sl; secondary lysosome, t; trachea. 

Bars of Fig. 3a, c, d, and e indicate 5 μm and those of Fig 3b, f, and g indicate 2 μm.
3.4 DISCUSSION

3.4.1 TUNEL-assay

The TUNEL-assay detects apoptosis in tissues. The midgut of fed cockroaches showed TUNEL-positive signals in columnar cells (Fig. 1a), but that of cockroaches starved for 4 weeks showed signals not only in columnar cells but also in nidi (Fig. 1b). Quantitative analysis showed that starvation increased the density of signals both in the columnar cells and nidi (Fig. 1cd). In the nidi of fed cockroaches the signals were at extremely low density, but the midgut showed a significant increase in the density of signals after 4 weeks of starvation (Fig. 1d). These results show that starvation induces apoptosis not only in the columnar cells but also in the nidi. Starvation for 4 weeks accelerated apoptosis in the nidi dramatically.

3.4.2 Immunohistochemistry for active-caspase 3

Generally, the caspase family induces typical apoptotic features: condensation of chromatin, blebbing of cytoplasm, and internucleosomal degradation of DNA. The caspase family is divided into two categories: initiator caspases (caspase 8 and 9) and effector caspases (caspase 3 and 7). Extracellular apoptotic signals are mediated by caspase 8, while internal apoptotic signals derived from mitochondria are mediated by
caspase 9. Both signals activate effector caspases such as caspase 3 and activated caspase 3 induces apoptotic features (Lockshin and Zakeri, 2004).

Immunohistochemistry for active caspase 3 showed that the midgut of control cockroaches fed for 4 weeks had positive reactivity as granules in the apical areas of columnar cells (Fig. 2a). Secondary lysosomes containing debris of organelles were seen occasionally at the same positions by electron microscopy (Fig. 3ab). Active-caspase 3 may relate to the digestion of cell debris in the secondary lysosomes of columnar cells because caspase 3 is involved in digestion of the cytoskeleton under metamorphosis (Martin and Baehrecke, 2003). Cockroaches starved for 4 weeks had positive reactivity not only in the columnar cells but also in the nidi (Fig. 2b). These results showed that starvation induced the expression of an apoptotic stimulator: active-caspase 3 in the nidi of the midgut. This leads to the conclusion that the TUNEL-positive signals in the nidi of starved cockroaches represent apoptosis (Fig. 1bd).

3.4.3 Ultrastructural observation of midgut

In 1972, the concept of apoptosis was proposed by Kerr from data obtained from light and electron microscopy. Apoptosis has several morphological features including the
condensation of nuclear chromatin and separation from neighboring cells. Apoptotic cells undergo cytoplasmic fragmentation and are engulfed by neighboring cells. The cytoplasmic fragments of apoptotic bodies show marked condensations of both nuclei and cytoplasm. This background proves electron microscopy to be one of the most effective methods of diagnosis for apoptosis.

Columnar cells in the midgut of cockroaches fed or starved for 4 weeks did not show nuclei with markedly condensed chromatin (Fig. 3ad). Endo and Nishiitsutsuji-Uwo (1982) found dark intact columnar cells containing dark nucleus without markedly condensed chromatin in the midgut of *P. americana* and concluded that these were degenerating cells and would be left into the lumen. Similar dark cells with degenerating cytoplasm and nuclei were defined as apoptotic in the villus tips of the small intestine in mice (Potten and Allen, 1977). However, the collapse of some columnar cells releasing nuclei into the lumen of cockroaches starved for 4 weeks might be different from the cell death reported by Endo and Uwo (1982) because the released nuclei were not dark and seemed to be intact (Fig. 3d). The collapse may not be programmed cell death but necrosis and indicates the collapse of homeostasis in the midgut cell populations.

Nidi in the midgut of cockroaches starved for 4 weeks showed some cytoplasmic
fragments containing nuclear fragments with condensed chromatin (Fig. 3e) but those of cockroaches fed for 4 weeks did not show any of these cytoplasmic fragments (Fig. 3c). The morphological features of these fragments were similar to those of apoptotic bodies shown by Kerr (1972).

These results show that starvation induced cell death not only in the columnar cells but also in the nidi of the midgut (Fig. 1). The cell death in the nidi appeared to be apoptosis based on the data obtained from immunohistochemistry and electron microscopy (Fig. 2b and Fig. 3e), but the apoptotic bodies in the nidi (Fig. 3e) might be derived from the columnar cells. At least, starvation for 4 weeks induced several events related to cell death in the nidi.

3.4.4 Survival strategies of the midgut against starvation

Chapter 2 showed that the midgut of starved cockroaches reduced cell proliferation activity and this chapter showed that of starved cockroaches increased cell death both in the columnar cells and nidi. Under starvation these reduce the total cell population and saves energy consumption in the midgut. The cell debris, including apoptotic bodies that appeared after cell death, could induce the appearance of secondary lysosomes and supply energy to engulfing cells and the whole body (Fig. 3f). The appearance of
apoptotic bodies in nidi after 4 weeks of starvation (Fig. 3e) may be the last measure to supply energy for the midgut to sustain a minimal level of function including cell proliferation (Fig. 4ab of Chapter 2 and Fig. 3g). Indeed, the nidi showed a high-cell proliferation activity after refeeding (Fig. 4ab of Chapter 2).
3.5 REFERENCES


Chapter 4: GENERAL DISCUSSION

A sequence of defense mechanisms under starvation and their adaptive significance

We concluded that the reduction of cell proliferation activity and an increase in cell death in the midgut of *P. americana* under starvation were adaptive responses to reduce energy consumption and to produce new energy for survival, respectively. However, the reasons are unknown why the reduction of cell proliferation and the increase of cell death appeared after 2 weeks of starvation. Bede et al. (2007) showed that caterpillars metabolize glucose derived from foods into glycogen and then store it in their fat body. During short-term starvation, the stored glycogen is metabolized into trehalose and then released into the hemolymph to maintain the hemolymph levels of trehalose. However, if starvation continues for a long term, the levels begin to reduce because stored glycogen becomes depleted. In our preliminary experiments, the total protein or dry weight of fat body of *P. americana* dropped drastically in one or 2 weeks of starvation. Survival rates of *P. americana* began to decrease over 2 weeks of starvation. These results may explain why cell proliferation activity drops after 2 weeks of starvation followed by cell death in the midgut of *P. americana* (Fig. 3c of Chapter 2 and Fig 1c of Chapter 3). During the first week of starvation, cockroaches use nutrients stored in their fat body to sustain the normal digestive condition of the midgut. However, the nutrients
are used up after 2 weeks of starvation and then the midgut forms the second and third defense lines; the reduction of cell proliferation, followed by increased cell death. The reduced cell proliferation or increased cell death save energy consumption or production of energy. Further elongation of starvation must force further mobilization of energy derived from the apoptosis at nidi. However, the present results showed that the midgut maintained the minimum ability of cell proliferation under harsh starvation (Fig. 3bc of Chapter 2 and Fig. 3g of Chapter 3). Refeeding intensified the cell proliferation not only to restore but also to overshoot the original level (Fig. 4ab). This suggests that cell proliferation and cell death in the midgut are regulated under starvation by nutrients or factor in the fat body through hemolymph. BrdU injected into hemolymph was taken into the nuclei of the nidi through basal membrane (Fig. 2a of Chapter 2) and α-arylphorin isolated from the fat body of *M. sexta* stimulated the cell proliferation in the midgut stem cell culture of *H. virescens* (Blackburn et al., 2004). These show a possibility of the appearance of the regulation system using fat body through hemolymph.

The midgut of *P. americana* may undergo autophagy when starved. Appearance of autophagy producing nutrients from digesting cytoplasm enclosed by membrane are symptomatic morphological feature for autophagy is induced under starvation both in
mammals and *Drosophila melanogaster* (Schworer et al., 1981; Mortimore et al., 1983; Rusten et al., 2004; Scott et al., 2004; Lum et al., 2005; Martinet et al., 2005, Fabre et al., 2007). Clarke (1990) mentioned that it can be difficult to tell whether autophagic structure is autophagic or heterophagic (or both) and cockroaches starved for 4 weeks show autophagic structure: secondary lysosome not only in the columnar cells but also in the nidi (Fig. 3df). These show a possibility that the midgut of the starved cockroach recycles nutrients not only from cell debris but also cytoplasm by autophagy. Autophagy has been suggested as the first and apoptosis as the second countermeasures against starvation *in vitro* in mammals (González-Polo, et al., 2005; Fabre et al., 2007). Further experiments, electron microscopic observation, measurement of the epithelium area, and biochemical detection of autophagy, are required.

This study demonstrated a series of distinct processes taken for survival strategy in the midgut of *P. americana* against starvation, particularly cell proliferation and cell death in the midgut. This study also documented how nutritional condition regulated cell proliferation by refeeding experiments. The regulation system indicates that insect has highly organized and integrated defense mechanism against starvation. Further experiments clarify more specific defense mechanism and regulatory factors mobilized against starvation.
REFERENCES


ACKNOWLEDGEMENT

I would like to express my sincere and warm gratitude to my professor Dr. Makio Takeda for his valuable advices, repeated reading of the manuscript, keen understanding, strong support and precious time during my study under his kind personality that allowed me to work in a comfortable environment. His encouragement and support in all matters other than the academic life are beyond the words of thanks and appreciation.

I thank Dr. Marcia J. Loeb for critical reading of the manuscript and Dr. Shintaro Goto for technical assistance of microscopic observations.

My thanks are due to professor Dr. Pyoyun Park and the staff of his laboratory: Cell Structure and Regulation for critical comments not only for electron microscopic observations but also for publication of these data.

Finally I am also thankful to all fellow students of the Laboratory of Insect Science, Kobe University for their support and cooperation.