The Effects of Calcium, Phosphorus, Magnesium, Sodium, and Zinc in Improving the Depression of Gonadal Development in Growing Male Rats Kept under a Disturbed Daily Rhythm—Investigations Based on the $L_{16}(2^{15})$-Type Orthogonal Array

Miho HANAI* and Takatoshi ESASHI**

Division of Applied Food Research, National Institute of Health and Nutrition, 1–23–1 Toyama, Shinjuku-ku, Tokyo 162–8636, Japan

(Received March 22, 2006)

Summary The purpose of this study was to clarify the effects of nutrients on the gonadal development of male rats kept under constant darkness as a model of disturbed daily rhythm. This experimental protocol was designed based on the $L_{16}(2^{15})$-type orthogonal array, which can examine six factors. Five minerals (calcium (Ca), phosphorus (P), magnesium (Mg), sodium (Na), and zinc (Zn)) were selected as experimental factors, and the dietary content of these minerals was normal (AIN-76 diet) or 1/3.5 of the normal content. Lighting conditions (constant darkness or normal lighting) were also added as a factor. Four-week-old rats (Fischer 344 strain) were kept under constant darkness or normal lighting (12-h light/dark cycle) for 4 wk. After 4 wk, the gonadal weights and serum testosterone content were evaluated. The lighting condition, Ca, Mg, and Na, and the interactions between the lighting condition and Ca, and Mg and Na were observed to affect the testes weight. Among the constant darkness groups (D-groups), the highest value for testes weight was observed under the normal-Ca, normal-Mg, and normal-Na diet, and the lowest value was observed under the low-Ca, normal-Mg, and low-Na diet. Among the normal lighting groups (N-groups), the highest value for testes weight was observed under the low-Ca, normal-Mg, and low-Na diet. Among the D-groups, the highest value for serum testosterone was observed under the normal-Ca, normal-Mg, and low-Na diet. Among the N-groups, the highest value was observed under the low-Ca, normal-Mg, and low-Na diet. It became clear that the amount of dietary Ca necessary for the gonadal development of rats increases when rats are kept under constant darkness as a model of disturbed daily rhythm compared with the normal lighting condition.

Key Words constant darkness, disturbed daily rhythm, gonad, mineral, orthogonal array

Recently, the number of people living under conditions of disturbed daily rhythm has been increasing due to the globalization of business and social activities as well as the diversification of the forms of labor. Such irregularities in daily rhythms can adversely affect bioregulatory mechanisms, resulting in an abnormal diurnal rhythm that can impede biological activities structurally and functionally. The process by which such disorders are induced is, in theory, dependent upon the nutritional condition of the individual. However, there are no basic data on the nutritional aspects of maintaining or promoting health under disturbed daily rhythms.

We have been using the condition of constant darkness as a model of disturbed daily rhythm (1, 2). In general animal studies, the lighting condition is 12-h light/12-h dark, with the light working as an entrainer of circadian rhythm formation. It is thought that constant darkness causes a disturbed daily rhythm because there is no light information acting as an external factor for daily rhythm and circadian rhythm formation. It has been observed that rats kept under constant darkness develop disturbances in their feeding and motor-activity rhythms, and suffer from altered rhythms of hormone secretion and enzyme activity (3–5). Sakai has reported that rats kept in constant darkness show a decrease in pituitary gland weight and depression of gonadal development (6, 7). Esashi et al. have observed that the delivery rate of rats kept in constant darkness decreases, and the depression of gonadal development of rats kept in constant darkness is accelerated by a low-protein diet (8, 9). The gonad has high sensitivity against constant darkness and nutrients. We have therefore focused on gonadal development and have examined the effect of
nutrients on the gonads of rats kept under constant darkness.

The effects of protein, methionine, vitamins, minerals, and oil on the gonads of rats kept under constant darkness have previously been reported (1). The results have shown that the depression of gonadal development in rats kept under constant darkness is mitigated by a normal-protein, methionine-added, low-vitamin, low-mineral, and normal-oil diet, and is accelerated by a low-protein, methionine-added, normal-vitamin, normal-mineral, and low-oil diet. The purpose of the present study was to clarify the effects of various minerals on gonadal development.

Five kinds of minerals, calcium (Ca), phosphorus (P), magnesium (Mg), sodium (Na), and zinc (Zn), were selected as factors, and the effect of these five minerals and the interaction between the lighting condition and these minerals were evaluated. The present study was carried out using an orthogonal array, which is one type of experimental design (10, 11) and was applied in a previous study we have already reported (1, 2, 12).

METHODS

1. Animals. Forty-eight Fischer strain (F344) male rats (purchased from Charles River Japan Inc., Kanagawa, Japan, at 3 wk of age) were preliminarily maintained for 1 wk on the AIN-76 purified diet (13), and then divided into experimental groups. There were a total of 16 groups with three rats in each group. No differences were found in the mean body weights of rats from each of the 16 groups. The rats were kept under constant darkness (D-groups) or normal lighting (12-h light/dark cycle, N-groups) for 4 wk. Food intake and body weight were recorded every other day. The care of rats kept under constant darkness was carried out by lighting a red lamp for approximately 2 h; the light was for photographs and is not known to cause a phase variation of circadian rhythm. The rats were housed in individual, stainless-steel, wire-mesh-bottomed cages at 22±1°C and humidity of 55±5%, in a room free from specific pathogens. Food and distilled water were provided to all rats ad libitum.

Animals were maintained in accordance with the Guidelines for the Care and Use of Laboratory Animals.

2. Diets. Five minerals (Ca, P, Mg, Na, and Zn) and the lighting condition were selected as a factor. Therefore, the experimental protocol and composition of the diets were designed based on the $L_{16}^{(2^{15})}$-type orthogonal array, which can examine six factors (10, 11). The examined factors and their levels are shown in Table 1.

Eight types of experimental diets were prepared based on the $L_{16}^{(2^{15})}$-type orthogonal array (Table 2). The mineral contents were normal (AIN-76 diet) or 1/3.5 of the normal content. Normal and low levels of minerals are shown as level 1 and level 2, respectively. Level 2 was not deficient in nutrients, simply low-level; the pilot study confirmed that this quantity did not result in noticeable deficiency diseases.

The eight diets were given to rats kept under constant darkness or normal lighting conditions; i.e., there were eight D-groups and eight N-groups for a total of 16 groups.

3. Analysis. After 4 wk of treatment, the rats were decapitated, and the blood and gonadal organs (testes, epididymides) were collected. The weights of gonadal organs were measured. Blood was centrifuged (3,000 rpm, 15 min, 4°C) and the serum obtained was stored at −20°C until analysis. The serum testosterone concentrations were measured by a radioimmunoassay kit (CIS Diagnostic Co., Tokyo, Japan).

4. Statistical analysis. Statistical analysis followed the original method for the orthogonal array table (10, 11). The software (JUSE-QCAS/V6.0) developed by the Japan Technology Training Institute Co., Ltd. (Tokyo, Japan) was used for analysis.

First of all, the effects of factors and interactions between factors on gonadal organ weights and serum testosterone concentrations were determined by ANOVA. The estimated values of gonadal organ weight and serum testosterone concentrations were calculated only in accordance with the factors that showed significant differences ($p<0.05$). Therefore, the values in the tables are estimated values and have a statistical difference.

RESULTS

The functioning of the gonadal organ in the growing period is in direct proportion to the weight of the organ (14). Therefore, in this study, the weights of the gonadal organs were compared by absolute value, not by the value per 100 g body weight; and the high weight of the gonadal organ was evaluated as being suitable.

1. Results of ANOVA

The effects of dietary mineral content on gonadal organ weight, serum testosterone concentration, body weight, and total food intake are shown in Table 3.

The effects of the lighting condition, Mg, and Na on gonadal organ weight, body weight, and total food intake were observed. The interaction of the lighting condition and Ca was observed to affect the gonadal organ weight, serum testosterone concentrations, body weight, and total food intake. Additionally, the interaction of Mg and Na was observed to affect the gonadal organ weight and serum testosterone concentrations.

2. Estimated values of body weight, total food intake, gonadal organ weight, and serum testosterone concentration

The factors (lighting condition and five kinds of min-
erals) that had a significant effect on body weight, total food intake, gonadal organ weight, and serum testosterone concentration, and these estimated values are shown in Tables 4–8.

The average and SD of these analyzed values for each group are also shown as a reference value (Appendix 1).

2–1. Body weight and total food intake. The lighting condition, Ca, P, Mg, and Na, and the interaction of the lighting condition and Ca were observed to affect the body weight (Tables 3 and 4). The highest value for body weight (183.3 g) was observed in rats maintained under normal lighting and a low-Ca, low-P, normal-Mg, and normal-Na diet, and the lowest value (134.0 g) was observed in rats maintained under constant darkness and a low-Ca, normal-P, low-Mg, and low-Na diet. In the D-groups, the highest value for body weight (160.8 g) was observed in rats on the normal-Ca, low-P,

### Table 2. Composition of the diets.

| Ingredients            | Diet No.   |
|------------------------|------------|
|                        | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  |
| Milk casein            | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 |
| L-Methionine           | 0.3| 0.3| 0.3| 0.3| 0.3| 0.3| 0.3| 0.3|
| Soybean oil            | 5  | 5  | 5  | 5  | 5  | 5  | 5  | 5  |
| Vitamin mixture¹       | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |
| Mineral mixture²       | 3.5| 3.5| 3.5| 3.5| 3.5| 3.5| 3.5| 3.5|
| Cellulose fiber        | 5  | 5  | 5  | 5  | 5  | 5  | 5  | 5  |
| Choline bitartrate     | 0.2| 0.2| 0.2| 0.2| 0.2| 0.2| 0.2| 0.2|
| Sucrose                | 62.9|63.1|64.1|64.4|63.4|63.3|63.7|63.5|
| CaHPO₄                 | 1.25|1.25|0   |0   |0   |0   |0   |0   |
| NaCl                   | 0.19|0   |0.19|0   |0   |0.19|0   |0.19|
| K₂C₃H₂O₇·H₂O           | 0.55|0.55|0.55|0.55|0.55|0.55|0   |0   |
| K₂SO₄                 | 0.08|0.08|0.08|0.08|0.08|0.08|0   |0   |
| MgO                    | 0.06|0   |0.06|0   |0   |0.06|0   |0.06|
| CaCO₃                  | 0   |0   |0   |0   |0.93|0.93|0   |0   |
| KH₂PO₄                 | 0   |0   |0   |0   |0   |0   |1.26|1.26|
| ZnO                    | 0   |0.004|0   |0   |0.004|0.004|0   |0.004|

1AIN-76ᵀᴹ(7) vitamin mixture.
2Calcium, phosphorus, magnesium, sodium, zinc, and potassium contents are 1/3.5 of AIN-76ᵀᴹ(7) mineral mixture.
3Calculated value.

### Table 3. Effects of minerals on reproductive organ weight, serum testosterone level, body weight and total food intake.

| Lighting condition¹ | Ca (B) | P (C) | Mg (D) | Na (E) | Zn (F) | A×B | A×C | A×D | A×E | A×F | D×E |
|---------------------|--------|-------|--------|--------|--------|-----|-----|-----|-----|-----|-----|
| Testes (g)          | **²   | **   | **    | **    | *     |     |     |     |     |     |     |
| (g/100 g BW)        |        |       |       |        |        |     |     |     |     |     |     |
| Epididymides (g)    | **    | *    | **    | **    | *     |     |     |     |     |     |     |
| (g/100 g BW)        |        |       |       |        |        |     |     |     |     |     |     |
| Testosterone (ng/mL)| **    |       | **    | **    | *     |     |     |     |     |     |     |
| Body weight (g)     | **    | *    | *     | **    | **    |     |     |     |     |     |     |
| Total food intake (g)| **  | **  | *    | **    | **    |     |     |     |     |     |     |

¹Lighting condition: normal lighting or constant darkness.
²*: p<0.05, **: p<0.01.
normal-Mg, and normal-Na diet (Table 4). The interaction of the lighting condition and Ca showed that the effect of Ca levels in the diet differed according to the lighting condition. Specifically, body weight increased in response to changing to a low-Ca diet from a normal-Ca diet in the N-groups (8.5 g increase, Table 4), but was reduced by this change in the D-groups (5.8 g reduction, Table 4).

On the other hand, the lighting condition, Ca, P, Mg, and Na, and the interaction of the lighting condition and Ca were observed to affect the total food intake (Tables 3 and 5). The highest value for total food intake (350.7 g) was observed in rats maintained under normal lighting and a low-Ca, low-P, normal-Mg, and normal-Na diet, and the lowest value (281.4 g) was observed in rats maintained under constant darkness and a low-Ca, normal-P, low-Mg, and low-Na diet. In

### Table 4. The estimated value of combinational effect of minerals on body weight.

| Factor level ABCDE | Estimated value (g) | Factor level ABCDE | Estimated value (g) |
|--------------------|---------------------|--------------------|---------------------|
| 111112             | 168.9               | 21111              | 155.0               |
| 111112             | 158.9               | 21112              | 149.4               |
| 111212             | 163.7               | 21121              | 149.8               |
| 112112             | 153.7               | 21211              | 139.8               |
| 112122             | 174.8               | 21212              | 160.8               |
| 112122             | 164.7               | 21212              | 150.8               |
| 112222             | 169.6               | 21222              | 155.7               |
| 121111             | 159.6               | 22111              | 145.6               |
| 121122             | 177.5               | 22112              | 149.2               |
| 121212             | 167.4               | 22121              | 139.2               |
| 121222             | 172.3               | 22122              | 144.1               |
| 122111             | 162.3               | 22211              | 134.0 minimum       |
| 122122             | 183.3 maximum       | 22212              | 151.1               |
| 122212             | 173.3               | 22221              | 145.1               |
| 122222             | 178.2               | 22222              | 150.0               |
| 122222             | 168.1               | 22222              | 139.9               |

1: Lighting condition, 2: normal level, 3: constant darkness or low level.

### Table 5. The estimated value of combinational effect of minerals on total food intake.

| Factor level ABCDE | Estimated value (g) | Factor level ABCDE | Estimated value (g) |
|--------------------|---------------------|--------------------|---------------------|
| 111112             | 333.9               | 21111              | 324.0               |
| 111122             | 325.1               | 21121              | 315.2               |
| 111222             | 308.6               | 21122              | 298.7               |
| 112112             | 345.3               | 21211              | 335.4               |
| 112122             | 328.8               | 21212              | 318.9               |
| 112222             | 316.5               | 21222              | 326.6               |
| 121111             | 319.9               | 22111              | 301.0               |
| 121122             | 339.3               | 22112              | 306.7               |
| 121211             | 322.8               | 22121              | 290.2               |
| 121212             | 330.5               | 22122              | 297.9               |
| 121222             | 313.9               | 22212              | 281.4 minimum       |
| 122111             | 350.7 maximum       | 22212              | 318.1               |
| 122122             | 334.1               | 22222              | 310.6               |
| 122212             | 341.9               | 22222              | 309.3               |
| 122222             | 325.3               | 22222              | 292.8               |

1: Lighting condition, 2: normal level, 3: constant darkness or low level.
Appendix 1. The analyzed values of body weight, total food intake, organ weight, and serum testosterone concentration of each group.

| Lighting condition | Diet No. | Mineral levels of each diet | Body weight (g) | Total food intake (g) | Testes (g) | Epididymides (mg) | Serum testosterone (mg/mL) |
|-------------------|---------|-----------------------------|-----------------|----------------------|------------|-----------------|--------------------------|
| Normal lighting   | 1       | 1 | 1 | 1 | 1 | 175.4±1.4 | 342.0±4.4 | 1.830±0.187 | 211.0±54.3 | 0.365±0.574 |
|                   | 2       | 1 | 1 | 2 | 2 | 146.4±1.9 | 304.1±5.5 | 1.539±0.349 | 167.3±44.4 | 0.597±0.552 |
|                   | 3       | 2 | 2 | 1 | 1 | 180.5±1.1 | 354.9±11.1 | 1.967±0.260 | 235.0±69.7 | 2.019±1.547 |
|                   | 4       | 2 | 2 | 2 | 1 | 169.6±2.7 | 321.6±5.9 | 1.705±0.179 | 185.7±26.1 | 1.759±1.389 |
|                   | 5       | 2 | 1 | 2 | 1 | 163.5±5.0 | 326.0±3.3 | 1.534±0.124 | 165.0±15.7 | 2.226±1.273 |
|                   | 6       | 1 | 2 | 2 | 1 | 171.7±6.7 | 335.7±15.3 | 1.623±0.141 | 175.7±19.3 | 1.109±0.103 |
|                   | 7       | 2 | 1 | 1 | 2 | 1 | 170.1±6.7 | 324.8±17.5 | 1.756±0.230 | 192.3±20.0 | 1.814±1.173 |
|                   | 8       | 2 | 1 | 2 | 1 | 1 | 171.0±10.5 | 327.9±19.4 | 1.803±0.133 | 207.0±30.5 | 1.736±1.330 |
| Constant darkness | 1       | 1 | 1 | 1 | 1 | 153.1±13.8 | 321.2±23.9 | 1.098±0.480 | 120.7±42.7 | 1.087±0.955 |
|                   | 2       | 1 | 1 | 2 | 2 | 140.6±4.2 | 299.7±9.4 | 1.056±0.639 | 112.3±59.9 | 0.001±0.000 |
|                   | 3       | 2 | 2 | 1 | 1 | 151.4±9.7 | 310.4±24.3 | 1.188±0.377 | 122.3±40.0 | 0.051±0.087 |
|                   | 4       | 2 | 2 | 2 | 1 | 143.1±9.2 | 301.7±15.0 | 0.763±0.370 | 76.0±27.4 | 0.405±0.669 |
|                   | 5       | 1 | 2 | 1 | 2 | 148.8±6.2 | 313.0±12.5 | 1.024±0.329 | 106.7±22.1 | 2.486±1.037 |
|                   | 6       | 1 | 2 | 2 | 1 | 158.6±5.3 | 334.4±29.6 | 1.074±0.231 | 107.7±30.2 | 2.095±1.348 |
|                   | 7       | 2 | 1 | 1 | 2 | 1 | 141.6±3.4 | 295.1±4.8 | 0.615±0.100 | 63.3±8.7 | 0.838±0.660 |
|                   | 8       | 2 | 1 | 2 | 1 | 1 | 142.2±9.4 | 291.7±11.9 | 0.849±0.362 | 86.3±39.8 | 1.652±1.430 |

Mean±SD (n=3).

1: Normal level, 2: low level.

The effects of Ca and the interaction of the lighting condition and Ca on testes weight were the same as those on body weight. But the effects of Mg and Na on testes were different from those on body weight. P was observed to have an effect on body weight, but not on testes.

2–2. Testes weight. The lighting condition, Ca, Mg, and Na as well as the interactions of the lighting condition and Ca, and Mg and Na were observed to affect the testes weight (Tables 3 and 6). The highest value for testes weight (1.990 g) was observed in rats maintained under normal lighting and a low-Ca, normal-Mg, and normal-Na diet, and the lowest value (0.747 g) was observed in rats maintained under constant darkness and a low-Ca, normal-Mg, and low-Na diet. In the D-groups, the highest value for testes weight (1.245 g) was observed in rats on the normal-Ca, normal-Mg, and normal-Na diet (Table 6).

The interaction of the lighting condition and Ca showed that the testes weight increased when changing to a low-Ca diet from a normal-Ca diet in the N-groups (0.177 g increase, Table 6), but was reduced by this change in the D-groups (0.210 g reduction, Table 6). In addition, the interaction of Mg and Na showed that the effects of Na differed according to Mg levels in the diet. Specifically, the testes weight fell dramatically with the change to a low-Na diet from a normal Na diet when the diet included normal Mg (0.288 g reduction, Table 6); in contrast, there was only a slight reduction with a low-Mg diet (0.072 g reduction, Table 6).

The effects of Ca and the interaction of the lighting condition and Ca on testes weight were the same as those on body weight. But the effects of Mg and Na on testes were different from those on body weight. P was observed to have an effect on body weight, but not on testes.

2–3. Epididymides weight. The lighting condition, Ca, Mg, and Na as well as the interactions of the lighting condition and Ca, Mg, and Na were observed to affect the epididymides weight (Tables 3 and 7). The highest value for the epididymides weight (231.4 mg) was observed in rats maintained under normal lighting and a low-Ca, normal-Mg, and normal-Na diet, and the lowest value (72.9 mg) was observed in rats maintained under constant darkness and a low-Ca, normal-Mg, and low-Na diet. In the D-groups, the highest value for the epididymides weight (138.2 mg) was observed in rats on the normal-Ca, normal-Mg, and normal-Na diet (Table 7).

The interaction of the lighting condition and Ca showed that the epididymides weight increased in response to changing to a low-Ca diet from a normal Ca diet in the N-groups (25.3 mg increase, Table 7), but was reduced by this change in the D-groups (24.8 mg reduction, Table 7). The interaction of Mg and Na showed that the epididymides weight was greatly reduced by changing to a low-Na diet from a normal-Na diet when the diet included normal Mg (40.4 mg reduction, Table 7), but was reduced slightly by this change when the diet included low levels of Mg (8.8 mg reduction, Table 7).

The effects of minerals on epididymides were the same as those on the testes.

2–4. Serum testosterone concentrations. The lighting
condition, Ca, Mg, and Na as well as the interactions of the lighting condition and Ca, and Mg and Na were observed to affect the testosterone concentrations (Tables 3 and 8). The highest value for testosterone concentration (2.408 ng/mL) was observed in rats maintained under normal lighting and a low-Ca, normal-Mg, and low-Na diet, and the lowest value (0.162 ng/mL) was observed in rats maintained under constant darkness and a low-Ca, low-Mg, and low-Na diet. In the D-groups, the highest value for testosterone concentration (1.933 ng/mL) was observed in rats on the normal-Ca, normal-Mg, and low-Na diet (Table 8).

The interaction of the lighting condition and Ca showed that the testosterone concentrations increased in response to changing to a low-Ca diet from a normal-Ca diet in the N-groups (0.757 ng/mL increase, Table 8), and were reduced in response to this change in the D-groups (0.681 ng/mL reduction, Table 8). In addition, the interaction of Mg and Na showed that the testosterone concentrations increased in response to changing to a low-Na diet from a normal-Na diet when the diet included normal Mg (0.960 ng/mL increase, Table 8), but were reduced by this change when the diet included low levels of Mg (0.957 ng/mL reduction, Table 8).

The effects of Ca on testosterone concentrations were the same as those on testes weight. But the effects of interaction of Mg and Na on testosterone were different from those on testes weight.

**DISCUSSION**

We have previously reported that the depression of gonadal development of rats kept under constant darkness is mitigated by feeding a low-mineral diet (1). In the present study, an experiment was carried out to clarify which minerals come into play for the development of gonadal organs. Leathem has reported that Cu, P, Zn, manganese, molybdenum, and iodine affect gonadal development (15). Among the five kinds of minerals used in the present study, Ca, Mg, and Na showed effects on gonadal development, while P and Zn did not. In other words, whether the content of P and Zn in the diet is normal or 1/3.5 the normal level, there are no apparent effects on gonadal development.

1. Effects of calcium

The relation between Ca and gonadal development was found to vary according to the lighting condition. When calcium levels in the diet were low, the testes weights were high in the N-groups, while they were low in the D-groups. These results were the same as those regarding the effects of Ca on body weight. Therefore, it is thought that the effect of Ca on gonadal development was accompanied by body weight gain. In addition, the effects of Ca on body weight and total food intake were found to be similar; it is therefore thought that the result of body weight was accompanied by total food intake.

The review of calcium and appetite reported that rats have the ability of having a suitable amount of nutrients. For example, the Ca-deprived rats chose the Ca-supplemented diet between Ca-free and Ca-supplemented diets (16). In the present study, the food intake of rats kept under normal lighting condition was increased by a low-Ca diet. It is thought that these rats tried to get the appropriate amount of Ca. On the other hand, the food intake of rats kept under constant darkness decreased when fed the low-Ca diet. It is not clear whether the mechanism responsible for the influence of dietary Ca level on food intake is changed by the lighting condition. It has been known that the mechanism of self-selected intake regulation (hypothalamus-cerebral limbic system) matures with the rhythm regulatory system during the growth period (17). It is thought that the mechanism of self-selected intake regulation of rats kept under constant darkness did not mature. Further research is needed to interpret this result.

On the other hand, it has been reported that Ca absorption in the rat intestine displays a daily rhythm and the absorption is influenced by lighting conditions and mealtime (18, 19). In addition, we have reported that the effects of dietary mineral content on calcium balance in rats kept under constant darkness differs from that of rats kept under normal lighting (2, 12). Additionally, in the same paper, we reported that the amounts of Ca absorption and retention in rats kept under constant darkness are lower than those of rats kept under normal lighting. In the present study, serum Ca concentration was lower in the D-groups compared with the N-groups fed the normal-Ca diet or low-Ca diet (1.94 μg/mL reduction, all data are not shown). Similarly, the amounts of Ca absorption and retention were lower in the D-groups compared with the N-groups fed the normal-Ca diet or low-Ca diet (absorption: 4.70 mg/d reduction, retention: 2.05 mg/d reduction, all data are not shown). Therefore, these results indicate low testes and epididymides weights in rats fed a normal-Ca diet in the N-groups, but high weights in rats fed a normal-Ca diet in the D-groups; suggesting that the amount of absorbed Ca was the proper quantity in the D-groups. In addition, the low testes and epididymides weights in rats fed a low-Ca diet in the D-groups suggests the presence of a Ca deficiency in the D-groups.

2. Effects of magnesium and sodium

The results show the interaction of magnesium and sodium on gonadal development in both the D-groups and N-groups. These results were observed in the gonad only. It is therefore thought that the effect is specific on development.

According to Kato et al. (20), the nucleobase transport system in Sertoli cells, which provide nutrients and metabolic precursors to spermatogenic cells, is dependent on the extracellular Na concentration. The uptake of purine (guanine) and pyrimidine (uracil) nucleobases was time and concentration dependent, and uracil uptake was mediated by extracellular Na. In the present study, gonadal weight was reduced by a low-Na diet. It is thought that the amount of nucleobase transport is reduced by a low concentration of extracellular Na under a low-Na diet. On the other hand, Mg, an intra-
cellular mineral, acts as a coenzyme of various reactions in the body and regulates the concentration of intracellular and extracellular minerals (21). In the present study, the gonadal weight was greatly reduced by changing to a low-Na diet from a normal-Na diet when the diet included normal Mg. However, in a low-Mg diet, the gonadal weight was only slightly reduced. It is thought that the nucleobase transport system is maintained under normal Na and Mg by keeping the appropriate concentration of extra- and intra-cellular minerals, such as Na and Mg. Under a low-Na, -Mg diet, the nucleobase transport system is disturbed by an abnormal balance of extra- and intra-cellular Na and Mg concentrations. Unfortunately, the present study does not include data on Na and Mg concentrations in the testsis or Sertoli cells. Further research is needed.

The effects of magnesium and sodium and their interactions on gonadal development have never been reported. It is a novel observation, and further study is needed to clarify the relevant mechanisms of action.

3. Effects of zinc

There have been many reports regarding the relation between gonad and Zn (22, 23). Zinc deficiency induces a reduction in gonadal organ weight and abnormal sperm formation. However, in the present study, effects of Zn on testes and epididymides were not observed. The Zn content of the diets in the present study were normal level (content of analyzed value: 36–40 ppm) and low level (1/3.5 of normal, content of analyzed value: 15–16 ppm). In the meantime, previous reports showing the effects of zinc deficiency have used diets with a Zn content of 0.7 ppm (22) or 1.2 ppm (23). These levels are markedly lower than those of the present study. As such, no effects of Zn deficiency were observed in the present study.

4. The relation between gonadal organ weight and serum testosterone concentrations

We have previously reported that serum testosterone concentrations increase in response to a low-mineral diet in D-groups (1). In the present study, the minerals influencing serum testosterone concentrations were Ca, Mg, and Na. In the D-groups, serum testosterone levels were high with a normal-Ca, normal-Mg, and low-Na diet, and dietary P and Zn showed no effects on serum testosterone. These results suggest that dietary Na levels are associated with results indicating that a low-mineral diet leads to an increase in serum testosterone levels.

Furthermore, the results regarding interactions of the lighting condition and dietary Ca on testes and epididymides weights were the same as those for the serum testosterone concentrations. Specifically, the serum testosterone concentrations and the testes and epididymides weights increased in response to the low-Ca diet in the N-groups, and increased in response to the normal-Ca diet in the D-groups.

Gonadal development depends on testosterone, and the function of testosterone is expressed when testosterone or dehydrotestosterone (DHT) combines with the androgen receptor. Moreover, the function is influenced by the amount of androgen receptor and the binding capacity to androgen receptor. Additionally, the binding ability of the testosterone-, DHT-Receptor complexes to DNA, and the activity of transcription also influences the functional expression of testosterone (20). Therefore, results showing that the effects of dietary Ca on testes and epididymides are the same as those on serum testosterone suggest that the functional expression of testosterone on both testes and epididymides is not inhibited by dietary Ca.

In addition, we found that serum testosterone was decreased by a low-Ca diet in the D-groups. This decrease is thought to explain, in part, why the depression of gonadal development is induced by a low-Ca diet.

The effects of the interaction between Mg and Na on testes and epididymides weights were found to be different from those on serum testosterone. Remarkable differences were found regarding the effects of Na on the normal Mg diet. Specifically, the change to a low-Na diet from a normal-Na diet in a normal-Mg diet decreased the testes and epididymides weights, but increased the serum testosterone concentrations. These results suggest that the mechanisms of dietary Mg and Na on testes and epididymides weights are different from those for testosterone biosynthesis.

The Leydig cells synthesize testosterone, and the Sertoli cells control the entry and exit of nutrients, hormones, and other chemicals into the tubules of the testis. Sertoli cells are related to testis maturation and spermatogenesis (24). It has been reported that nucleotides and Ca ions are involved in the regulation of steroid biosynthesis, and steroid synthesis enzyme activity is modulated by Ca and Mg in the Leydig cells (25). Previous studies show that the voltage-dependent potassium conductance of Leydig cells is influenced by Na and Ca ions (26, 27). Our study shows that the dietary Ca, Mg, and Na influence gonadal weight and serum testosterone. Dietary Ca has a similar effect on gonadal weight and serum testosterone. Meanwhile, the effect of dietary Mg and Na on gonadal weight and serum testosterone differed. It is thought that the physiologically relevant concentration of Ca is similar in both the Leydig cells and Sertoli cells. However, the concentration of Mg and Na is different between the two.

Moreover, the ratio of Mg and Na in the diet may influence the functional expression of testosterone on testes and epididymides. More studies are needed to clarify these mechanisms.

In the present study, the depression of gonadal development in rats maintained under constant darkness as a model of disturbed circadian rhythm was mitigated by a normal-Ca, normal-Mg, and normal-Na diet, and was accelerated by a low-Ca, normal-Mg, and low-Na diet. It was also clarified that the effects of dietary Ca are different according to the lighting condition, and that the amount of Ca required for gonadal development in rats maintained under constant darkness is more than that in rats maintained under normal lighting conditions.

The results of the present and previous studies (1)
clearly indicate that other minerals which were not examined in the present study, for example, potassium, iron, or trace elements, are related to the previous result: the depression of gonadal development in rats maintained under constant darkness is mitigated by low-mineral diets. More research regarding the other minerals is needed.

REFERENCES

1) Hanai M, Kubo K, Esashi T. 2003. The effects of nutrients in improving the depression of gonadal development in growing male rats kept under disturbed daily rhythm—Investigations based on the L8(2^7)-type orthogonal array—. J Nutr Sci Vitaminol 49: 388–396.
2) Hanai M, Esashi T. 2000. Effect of dietary mineral levels and their interactions on calcium balance in male rats with a disturbed circadian rhythm—investigations based on the L8(2^7)-type orthogonal array—. Nippon Eiyo Shokuryo Gakkaishi (J Jpn Soc Nutr Food Sci) 53: 139–148 (in Japanese).
3) Esashi T, Suzuki K, Takahashi S, Suzue R, Nakamura A, Innami S. 1977. Effects of dietary casein content, L-tryptophan supplementation, melatonin injection and continuous darkness on the diurnal food intake rhythm of male rat. Eiyogaku Zasshi (J J Nutr Diet) 35: 183–192 (in Japanese).
4) Takahashi Y. 1991. Mammals. Endocrinology mechanism. In: Chronobiology Handbook (Chiba Y, Takahashi K, eds), p 112–129. Asakura Syoten Co. Ltd., Tokyo (in Japanese).
5) Depres-Brummer P, Bourin P, Pages N, Metzger G, Levi F. 1997. Persisitent T lymphocyte rhythms despite suppressed circadian clock outputs in rats. Am J Physiol 273: R1891–R1899.
6) Sakai T. 1963. Activities of hypophysis, testis and pineal body in rats housed in continuous darkness. J Physiol Soc Japan 25: 140–145 (in Japanese).
7) Sakai T. 1963. Reproductive organs in rats housed in continuous darkness. J Physiol Soc Japan 25: 133–139 (in Japanese).
8) Esashi T, Nakamura K, Tsuchiya K, Tamura Y, Shiomotomai A, Yokota F, Hayami H. 1973. The effect of illumination and darkness on the birth rate of rat. Eiyogaku Zasshi (J J Nutr Diet) 31: 195–198 (in Japanese).
9) Esashi T. 1994. Light deficit and nutrition. Eiyogaku Zasshi (J J Nutr Diet) 52: 211–222 (in Japanese).
10) Taguchi G. 1998. Table of orthogonal arrays. In: Experimental Design (Taguchi G. ed), 3rd ed, p 143–158. Maruzen Co. Ltd., Tokyo (in Japanese).
11) Taguchi G, Konishi S. 1987. How to Allocate Experimental Factors to the Table of Orthogonal Arrays, p 2–10. JUSE Press Ltd., Tokyo (in Japanese).
12) Hanai M, Esashi T. 1999. Effects of dietary mineral levels and their interactions with calcium, phosphorus, magnesium and zinc balance in male rats. —Investigations based on a L8(2^7)-type orthogonal array—. Nippon Eiyo Shokuryo Gakkaishi (J Jpn Soc Nutr Food Sci) 52: 193–199 (in Japanese).
13) American Institute of Nutrition. 1977. Report of the American Institute of Nutrition ad hoc committee on standards for nutritional studies. J Nutr 107: 1340–1348.
14) Dhar JD, Setty BS. 1990. Changes in testis, epididymis and other accessory organs of male rats treated with anandron during sexual maturation. Endocr Res 16: 231–239.
15) Leathem JL. 1970. Nutrition. In: The Testis 3 (Johnson AD, Gomes WR, Vandemark NL, eds), p 169–205. Academic Press, New York.
16) Tordoff MG. 2001. Calcium: taste, intake, and appetite. Physiol Rev 81: 1567–1597.
17) Kubo K. 1984. Central mechanism of feeding behavior and circadian rhythm. In: Expression Mechanism of Biological Rhythms (Kawakami M, Takasaka M, eds), p 68–95. Rikogakusha Publishing Co. Ltd., Tokyo (in Japanese).
18) Worbel J, Nagel G. 1972. Diurnal rhythm of active calcium transport in rat intestine. Experimenta 35: 1581–1582.
19) Shinoda H, Stern PH. 1992. Diurnal rhythm in Ca transport in Ca transfer into bone, Ca release from bone, and bone resorbing activity in serum of rats. Am J Physiol 262: R235–R240.
20) Kato R, Maeda T, Akaite T, Taimai I. 2006. Characterization of novel Na^+-dependent nucleobase transport systems at the blood-testis barrier. Am J Physiol Endocrinol Metab 290: E968–E975.
21) Fleet JC, Cashman KD. 2001. Magnesium: Present Knowledge in Nutrition (Bowman BA, Russell RM, eds), eighth ed, p 292–301. ILSI Press, Washington DC.
22) McClain CJ, Gavalter JS, Van Thiel DH. 1984. Hypogonadism in the zinc-deficient rat: Localization of the functional abnormalities. J Lab Clin Med 104: 1007–1015.
23) Merker HJ, Gunther T. 1997. Testis damage induced by zinc deficiency in rats. J Trace Elem Med Biol 11: 19–22.
24) Sakuma Y. 2000. Reproductive grand. Development and function of reproduction. In: Review of Medical Physiology (Hoshi T, Okada Y, Kawahara K, Sugasato T, Kumanada K, Kurosawa M, Sakuma Y, Sató T, Suzuki Y, Takawka Y, Nakamura Y, Fukuda K. tr), 19th ed, p 442–452. Maruzen Co. Ltd., Tokyo (in Japanese).
25) Valencia-Sanchez A, Ortega-Corona BG, Dominguez-Vargas O. 1993. Effective of calcium and magnesium on testicular sulfatase activity. Arch Androl 30: 129–136.
26) Desaphy JF, Rogier C, Joffre M. 1996. Modulation of K^+ conductances by Ca^2+ and human chorionic gonadotrophin in Leydig cells from mature rat testis. J Physiol 295: 23–25.
27) Desaphy JF, Joffre M. 1996. Inhibitory effect of internal sodium and Hepes on the voltage-dependent potassium conductance of rat Leydig cells. Biochem Biophys Acta 1285: 9–13.