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Reduced juvenile hormone synthesis in mosquitoes with low teneral reserves reduces ovarian previtellogenic development in *Aedes aegypti*

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Summary

We investigated the relationship among nutritional reserves, previtellogenic ovary development and juvenile hormone (JH) synthesis in *Aedes aegypti* female mosquitoes. By raising larvae under different nutritional regimes, two adult phenotypes (large and small females) were generated, which differed significantly in size at eclosion (measured by wing length). We measured the total amount of protein, lipids and glycogen in newly emerged (teneral) large and small females. Teneral reserves were significantly lower in small females. Maximum previtellogenic ovary development occurred only if enough teneral nutrients were present. Maximum previtellogenic ovary development was stimulated in small females with low teneral nutrients by topically applying a JH analog. The biosynthetic activity of *Ae. aegypti* corpora allata (CA) was studied *in vitro* using a radiochemical method. JH synthesis was significantly reduced in females emerged with low teneral reserves and stimulated by sugar feeding. These results establish that the CA synthesizes enough JH to activate ovary maturation only in the presence of large nutrient reserves.

Key words: *Aedes aegypti*, mosquito, juvenile hormone, corpora allata, nutrition, ovary.

Introduction

Oogenesis in mosquitoes is a nutrient-limited process, triggered only if sufficient reserves are available (Briegel, 1990; Wheeler, 1996). The primary follicles of *Aedes aegypti* are undifferentiated at adult emergence and, during the next 60 h, they grow to double their size and reach the maximum previtellogenic length or resting stage (Hagedorn et al., 1977). This ovaric previtellogenic phase of development is initiated only when nutrients are appropriate (Gwadz and Spielman, 1973). Females must have some mechanism to restrain ovary development if nutrients are not available, and a hormonal control system activated by suitable nutritional stimuli is very appropriate.

Juvenile hormone (JH) is the key hormone regulating previtellogenic ovarian development in mosquitoes (Hagedorn, 1994; Klowden, 1997). In newly emerged adult female mosquitoes (teneral females), an increase in JH signals that ecdysis of the adult has finished and reproductive processes should begin. JH levels in *Ae. aegypti* increase during the first day after adult emergence (Shapiro et al., 1986). This initial rise in JH is essential for reproductive maturation. JH acts on several tissues, including ovaries, fat body and midgut, making them competent to perform their adult-specific functions (Klowden, 1997).

JH is synthesized and released from the corpora allata (CA), a pair of endocrine glands with nervous connections to the brain (Stay, 2000). An increase in rates of JH biosynthesis is responsible for the rise in JH levels observed during the first day after eclosion (Li et al., 2003a).

Is there a relationship between the increase of JH synthesis in the CA and the presence of nutrient reserves? In this study, we report that low teneral nutrient reserves reduced JH biosynthesis and ovarian previtellogenic development in *Ae. aegypti* females.

Materials and methods

Chemicals

Juvenile hormone III (JH) was from ICN (Irvine, CA, USA). The juvenile hormone analog methoprene was from Zoecon Co. (Palo Alto, CA, USA).

Insects

Aedes aegypti L. of the Rockefeller strain were reared at 28°C and 80% relative humidity under a photoperiod of 16 h:8 h light:dark. The larval diet was a 10% solution of bovine liver powder (ICN, Aurora, OH, USA). Five hundred larvae were raised in pans (23 cm×35 cm×13 cm) containing 1 liter of distilled water. Large or small mosquitoes were obtained by changing the amount of diet added to the pan. Large mosquitoes were produced when larvae were reared in

pans adding the following amount of diet: 1.5 ml on days 1, 3, 4, 5 and 6 and 0.75 ml on day 2. Small mosquitoes were produced when larvae were reared in pans with the following diet: 0.75 ml on days 1, 3, 5 and 7. Under these rearing conditions, most larvae pupate at day 8. Virgin adult females were offered a cotton wool pad soaked in water or a 15% sucrose solution. We refer to the cotton wool pad sucrose-fed females as 'sugar-fed'.

Wing length and ovarian development measurements

Ovaries were isolated by tearing the soft cuticle between the fifth and sixth abdominal sternites, pulling off and placing the terminal segments in a drop of saline. Ovary and wing lengths were measured under a dissecting microscope using an ocular micrometer. Wing length, measured at adult emergence, describes the distance between the point of articulation and the wing tip, excluding the fringe scales (Nasci, 1990).

Microseparation and analysis of nutrients

Microseparation of glycogen, lipids and proteins from the same mosquito samples was accomplished as described by Van Handel (1965) and modified by Zhou et al. (2004). Protein data were obtained using the BCA protocol (Pierce, Rockford, IL, USA). Glycogen was measured using the hot anthrone protocol (Van Handel, 1985a) and lipids using vanillin as described by Van Handel (1985b). All biochemical analyses were carried out using triplicate groups of five females.

In vitro *radiochemical assay for CA activity*

Preparation of CA complexes (CA attached to the corpora cardiaca and connected to the brain) from adult *Ae. aegypti* females has been previously described (Li et al., 2003a). Rates of JH biosynthesis were estimated by the *in vitro* radiochemical assay, as described by Feyereisen and Tobe (1981) and Feyereisen (1985) and modified by Li et al. (2003a). The glands were incubated in 100 µl M-199 assay medium with labeled L-[methyl-^{3}H]methionine (specific activity 2.96–3.11 TBq mmol^{-1}; ~80–84 Ci mmol^{-1}; Amersham Pharmacia, Piscataway, NJ, USA). The final concentration of methionine in the medium was 50 µmol l^{-1} and the specific activity was 0.56 TBq mmol l^{-1} (15 Ci mmol l^{-1}). Under these conditions, the incorporation of L-[methyl-^{3}H]-methionine into JH was linear for at least 6 h (Li et al., 2003a). At the end of the experimental period, incubations were terminated by the addition of 100 µl 1% EDTA, and 100 µl methanol containing 25 µg of unlabeled JH as carrier and internal standard. The incubation medium and the gland were extracted together with 1 ml of hexane and separated by thin-layer chromatography (TLC). After TLC separation [developed in 2:1 (v/v) hexane:ethyl acetate], the JH band was detected under UV light, cut, put into 10 ml scintillation cocktail overnight and assayed for ^{3}H. The quantities of JH produced in the experiment were calculated from the specific activity of the L-[methyl-^{3}H]methionine in the medium with assumption of a specific incorporation ratio of 1 (non-isotopic dilution). JH degradation by esterases was checked by incubating [^{3}H]JH in medium in the presence or absence of CA complexes and analyzing the recovery of labeled JH. 95–99% of the hormone was recovered intact after 4 h of incubation (results not shown). In some experiments, the JH esterase inhibitor OTFP (Hammock et al., 1984) was added to the incubation medium. Results of incubations in the presence or absence of OTFP were not significantly different.

Statistical analysis

Statistical analysis of the data was performed by *t*-test or one-way analysis of variance (ANOVA) with Tukey's post-test using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA, USA). The results were expressed as means ± S.E.M. and were considered significantly different at $P<0.05$.

Results

Effect of larval rearing conditions on teneral nutritional reserves

The two protocols used for rearing the larvae produced two distinct teneral adult phenotypes with significant differences in body size. Adult females emerged from larvae raised under a low diet protocol (small females) were approximately 25.7% smaller than females emerged from larvae reared under a high diet protocol (large females) [wing length: small, 2.54±0.05 mm (N=86) *vs* large, 3.42±0.06 mm (N=84)]. Teneral small and large females were analyzed within 2 h of eclosion to determine the total amount of reserves present as lipids, glycogen and protein (Fig. 1). All three groups of nutrients were significantly higher in teneral large mosquitoes.

Groups of small and large females after eclosion were kept on water or sugar, and the total amount of lipids, glycogen and protein was measured 24 and 48 h after eclosion (Fig. 2). Lipids and glycogen showed significant increases in both small and large sugar-fed females.

Effect of larval rearing conditions on adult ovarian development

Fig. 3 shows the length of the terminal follicle of ovaries dissected from water-fed and sugar-fed small and large females. Follicles from newly emerged small and large females were ~40 µm long (Fig. 3A). 24 h after emergence, the terminal follicles of large females raised on water or sugar had doubled their size. The follicles of small females raised in water had grown very little, while those of sugar-fed small females increased their size by ~30% (Fig. 3B). Similar results were observed at 48 h after emergence; while follicles of small females raised on water had grown to only 65 µm, those of sugar-fed small females increased their size to ~83 µm but were still significantly smaller than the follicles from large females (Fig. 3C).

Effect of topically applied methoprene on adult ovarian development

To confirm a role for JH in previtellogenic ovary

development, methoprene (a JH analogue) was topically applied to newly emerged small mosquitoes (500 ng per 0.5 µl of acetone). Acetone alone was applied to controls. After 24 h, the length of the terminal follicles from methoprene-treated small females was significantly larger than that from small females that were topically applied with acetone (Fig. 4). Topically applied methoprene induced the development of previtellogenic ovaries in small mosquitoes to levels comparable with those observed in large sugar-fed females.

JH synthesis in small and large females

The basal rate of JH biosynthesis by CA dissected from small and large mosquitoes was measured at different times after emergence using the *in vitro* radiochemical assay. The biosynthetic activity of the *Ae. aegypti* CA was significantly reduced in females emerged with low teneral reserves (Fig. 5A). Raising the small females on a high sugar diet (15%) resulted in a significant increase of JH synthesis compared with small females raised on water (Fig. 5B).

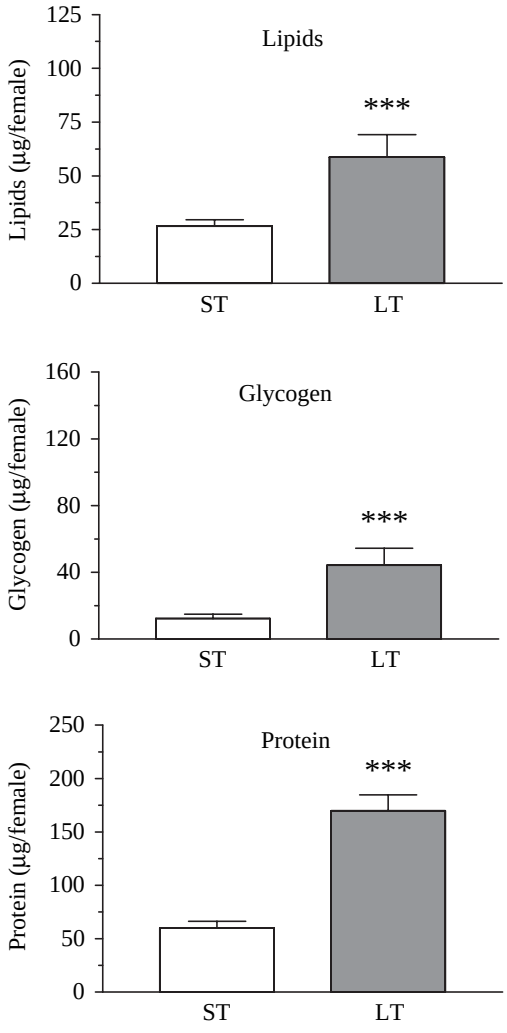

Fig. 1. Nutritional reserves in newly emerged females. Nutrients from teneral females were analyzed within 2 h of eclosion. ST, small teneral mosquito; LT, large teneral mosquito. Each bar represents the mean ± S.E.M. of three independent determinations of groups of five mosquitoes. Asterisks denote significant differences in each group (unpaired *t*-test; ***P<0.001).

Discussion

Differences in the amount of reserves accumulated during the larval life affect egg maturation in mosquitoes (Briegel, 1990). While mosquitoes reared in the laboratory can be provided with an optimal diet, those in the field often encounter suboptimal conditions that result in a high variability in teneral

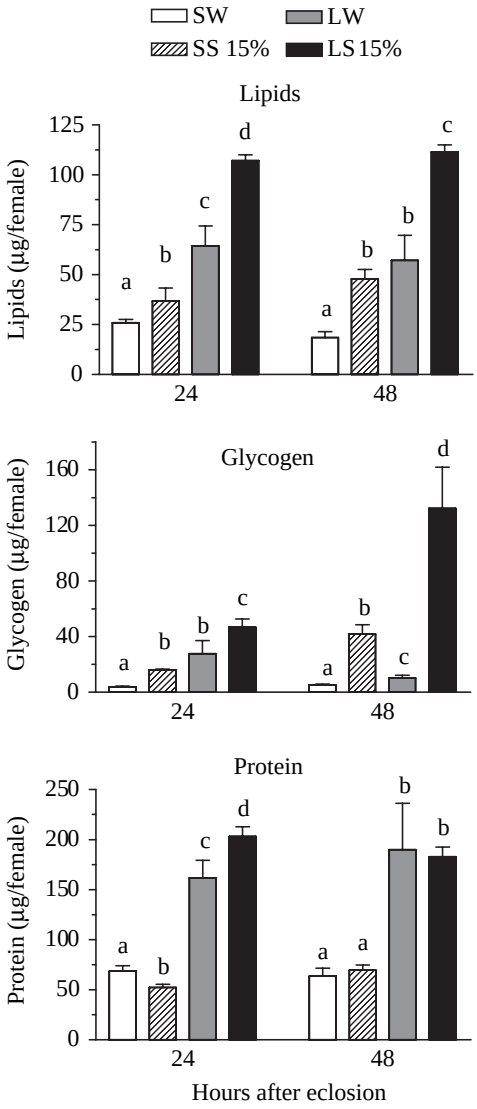

Fig. 2. Nutritional reserves in females fed on sugar. Adult females were offered a cotton wool pad soaked in either water or a 15% sucrose solution. Nutrients were analyzed at 24 or 48 h after eclosion. SW, small females raised on water; SS, small females raised on sugar; LW, large females raised on water; LS, large females raised on sugar. Each bar represents the mean ± S.E.M. of three independent determinations of groups of five mosquitoes. Values labeled with different letters are significantly different by Tukey's test after ANOVA at P<0.05.

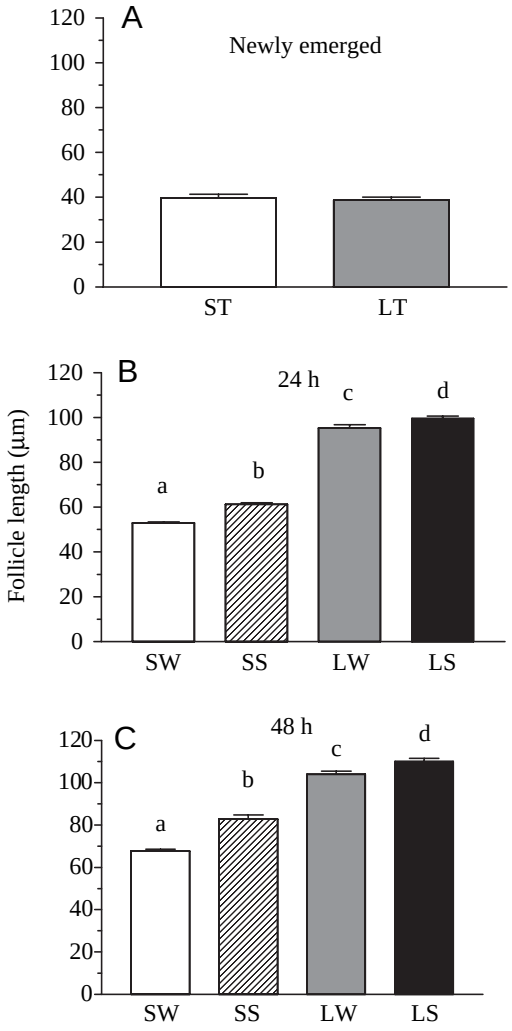

Fig. 3. Effect of nutrients on previtellogenic follicle development. We measured the length of the terminal follicle of ovaries dissected from water-fed and sugar-fed small and large females. (A) Follicles from newly emerged females. ST, small teneral mosquito; LT, large teneral mosquito. (B) Follicles from females 24 h after eclosion. (C) Follicles from females 48 h after eclosion. SW, small females raised on water; SS, small females raised on 15% sugar; LW, large females raised on water; LS, large females raised on 15% sugar. Each bar represents the mean ± S.E.M. of at least 20 independent determinations of individual females. Values labeled with different letters are significantly different by Tukey's test after ANOVA at $P<0.05$.

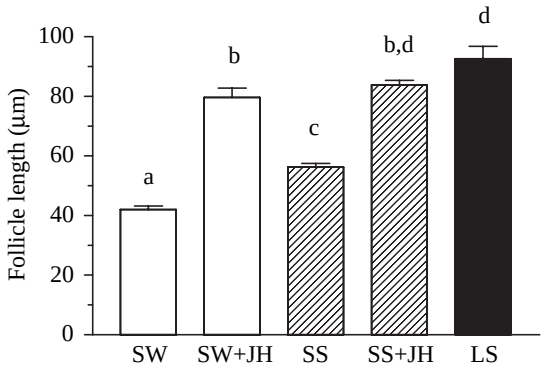

Fig. 4. Effect of juvenile hormone (JH) on previtellogenic follicle development. Follicle length was measured 24 h after eclosion. SW, small females raised on water; SS, small females raised on sugar; SS+JH, small females raised on sugar and treated with methoprene; SW+JH, small females raised on water and treated with methoprene; LS, large females raised on sugar; JH, females treated with methoprene (500 ng per 0.5 μl acetone) within 1 h of eclosion. Controls (SW, SS and LS) were treated with 0.5 μl of acetone. Each bar represents the mean ± S.E.M. of 10 independent determinations of individual females. Values labeled with different letters are significantly different by Tukey's test after ANOVA at $P<0.05$ or higher.

size and reproductive potential (Nasci, 1990; Feinsod and Spielman, 1980; Tun-Lin et al., 2000). Rearing conditions can be manipulated in the laboratory to elucidate the effects of suboptimal environmental conditions. We generated two very distinct adult phenotypes (large and small females) by raising larvae under different diets. Females that were significantly smaller in size at eclosion (measured by wing length) had significantly lower lipid, protein and glycogen teneral reserves than larger females. Our studies confirm similar conclusions from comprehensive reports previously published (Briegel, 1990; Briegel et al., 2001). Newly emerged females utilize reserves carried over from the pupal stage but soon seek out sugar meals as an important energy source (Clements, 1992). High sugar meals led to an increase in glycogen and lipid reserves.

Growth of the primary follicle to the resting stage in *Ae. aegypti* is linear and reaches maximum development about 60 h after adult eclosion (Hagedorn et al., 1977). Follicles of small mosquitoes did not develop much beyond the teneral stage when they were kept on water for 48 h. By contrast, sugar feeding permitted partial growth of ovarian follicles in small mosquitoes. Feinsod and Spielman (1980) also reported that a sucrose meal stimulates partial development of follicles from nutrient-deprived adult *Ae. aegypti*.

There are several reports describing correlations between teneral nutritional reserves, JH activity and previtellogenic egg maturation in anautogenous mosquitoes (Lea, 1963; Gwadz and Spielman, 1973; Hagedorn et al., 1977; Feinsod and Spielman, 1980). Decapitations, CA removal and abdominal ligations were used to prove that the growth of the previtellogenic follicles is under the control of factors from the brain and CA (Lea, 1963; Gwadz and Spielman, 1973; Hagedorn et al., 1977). Decapitation within 1 h of emergence or CA removal soon after eclosion prevents ovarian previtellogenic growth. Topical application of JH analogs stimulates normal growth of previtellogenic ovaries in decapitated or CA-ablated teneral females (Gwadz and Spielman, 1973; Hagedorn et al., 1977). We confirmed the role of JH on ovary development. A topically applied JH analogue stimulated previtellogenic ovarian development in nutrient-deprived mosquitoes. Follicles of methoprene-treated small females attained normal previtellogenic growth.

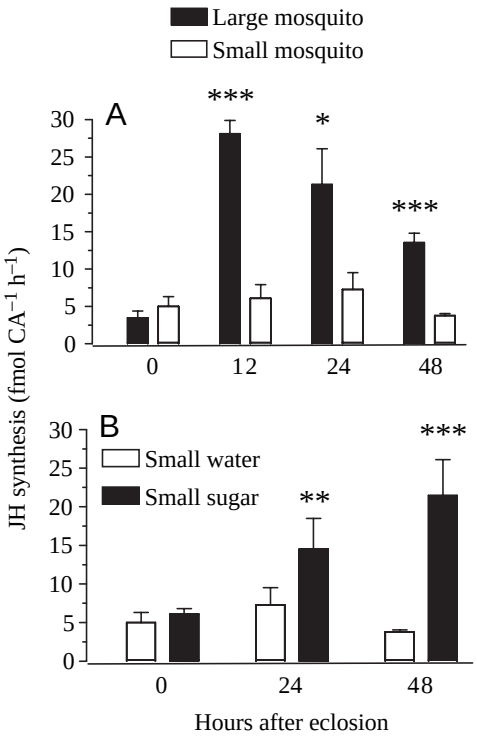

Fig. 5. Effect of nutrients on biosynthesis of juvenile hormone (JH) *in vitro*. JH synthesis was evaluated *in vitro* in CA complexes dissected at different times after emergence. (A) Synthesis in large and small mosquitoes raised on water. (B) Small mosquitoes with low teneral reserves were raised on water or sugar. Each bar represents the mean ± S.E.M. of five independent determinations of individual corpora allata (CA) complexes. Asterisks denote significant differences in each group (unpaired *t*-test; **P*<0.05, ***P*<0.05 and ****P*<0.001).

JH levels in *Ae. aegypti* are low at eclosion, increase during the first day after adult emergence and remain high in sugar-fed females (Shapiro et al., 1986). This initial rise in JH is essential for female's reproductive maturation (Klowden, 1997). Rates of JH biosynthesis by the CA *in vitro* closely reflect the levels of JH in the mosquito; biosynthesis of JH is very low in newly emerged females and increases dramatically during the first 24 h after adult eclosion (Li et al., 2003a). We have investigated for the first time the effect of larval diet on synthesis of JH by the CA of teneral females. We observed that biosynthesis of JH by the *Ae. aegypti* CA was significantly reduced in females emerged with low teneral reserves (small females). When small females were fed on a high sugar diet, JH synthesis was significantly increased.

How do nutrients activate JH synthesis after adult eclosion? Allatotropins (AT) are peptides that stimulate JH synthesis by the CA (Kataoka et al., 1989; Veenstra and Costes, 1999). We have previously described that the CA of a newly emerged mosquito needs to be exposed to *Ae. aegypti* AT before it is capable of synthesizing JH (Li et al., 2003b).

Studies using antibodies against *Ae. aegypti* AT showed that the peptide is present in cells of the brain of *Aedes* and *Anopheles* mosquitoes (S. Hernández-Martínez and F.G.N., unpublished observations). Removal of the medial neurosecretory cells (mnc) from *Ae. aegypti* immediately after adult emergence suppresses egg maturation; in older females, mnc ablation has little effect (Lea, 1967). It is reasonable to hypothesize that AT is one of the factors from the head that is essential for reproductive maturation. When the amount of nutrients is appropriate, the brain would release AT, and the CA would become capable of synthesizing enough JH to activate reproductive maturation. Therefore, the previtellogenic maturation of ovaries seems to depend exclusively on the capacity of the CA to produce high amounts of JH during the first day after the imaginal molt.

A similar mechanism controls diapause in mosquitoes. Readio et al. (1999) reported that diapause in the mosquito *Culex pipiens* is caused by inhibition of JH synthesis by the CA. Nondiapausing adult females synthesize four times more JH than do diapausing mosquitoes. Although small quantities of JH are produced during diapause, diapausing females lack sufficient JH to stimulate growth of ovarian follicles to the resting stage.

How are nutrients sensed? A recent paper by Colombani et al. (2003) describes a nutrient sensor mechanism that controls *Drosophila* growth. These authors propose a model where the amino-acid-responsive serine/threonine protein kinase target of rapamycin (TOR) signaling in the fat body modulates insulin signaling and nutrient-dependent responses in peripheral tissues. The brain mnc sense high circulating carbohydrate levels and secrete insulin-like peptides (ILPs), which interact with factors released by the fat body in response to amino acids and increase insulin signaling in the peripheral tissues. According to this model, the sensing of amino acids in the fat body and the sensing of carbohydrates in the brain are important for the regulation of nutrient-dependent responses.

One of the most difficult challenges in the future will be to discover whether one type of nutrient is more important than another in triggering JH secretion, as well as the mechanisms by which mosquitoes sense and respond to changes in their nutritional reserves.

In summary, CA activity is promoted by the presence of enough nutrient stores; inadequate larval nutrition prevented the early peak of JH biosynthesis that we detected in large females. There seems to be a mechanism involving a minimum threshold of nutrients that elicits the release of AT by the brain.

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