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Absence of Photoperiodism and Digestive Enzymes in Rats: The Role of Age and the Endogenous Melatonin Level

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Abstract—The effect of the prolonged absence of a photoperiodic signal (constant lighting and constant darkness) on changes in the activity of digestive enzymes in the late postnatal ontogenesis of rats is studied. Age-related alterations were characterized by a change in enzyme activity, as well as a redistribution of the functional activity between the amylo- and lipolytic links and the upper (pancreas) and lower (small intestine) divisions of the gastrointestinal tract. It was shown that, with age, the enzyme activity was affected both by an absence of photoperiodism and by a melatonin level associated with the light regime. Age-related changes characterizing "aging" of the digestive system were observed later under light deprivation conditions than with standard illumination and especially constant illumination.

Keywords: digestive enzymes, total proteolytic activity, amylase, lipase, pepsin, stomach, pancreas, thin intestine, light regimes, melatonin, ontogeny, rats

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INTRODUCTION

The majority of physiological processes in mammals are synchronized with external living conditions due to the photoperiod-controlled secretion of pineal melatonin [16]. Digestion in this regard differs from most physiological functions, since meals, which often do not coincide with the photoperiod, can play a significant role in its synchronization [24]. Approximately 400 times more melatonin is produced in the digestive tract than in the pineal gland [9, 19, 24, 31]. However, the amount of enteric melatonin is regulated not by light rhythms but by the consumption and composition of food. Occasional increases in the melatonin level are due to a change in the concentration of its precursor, tryptophan, which is produced by the digestion of proteins in the digestive tract [21, 23, 33].

The blood hormone level fluctuates significantly during the day. The majority of melatonin circulating in the bloodstream is secreted by the pineal gland, and the contribution of the digestive system is small, since about 90% of the hormone moving from the gastrointestinal tract into the portal vein is metabolized already upon entering the liver [50]. Melatonin produced by the pineal gland has endocrine action, while that synthesized by the enterochrochromaffin cells of the gastrointestinal tract has mainly paracrine action or acts via the intestinal lumen [19, 51]. G.A. Bubenik et al. [21, 23] demonstrated an increase in the formation of melatonin in the gastrointestinal tract in response to food intake, while its synthesis by the pineal gland remained unchanged. Thus, extrapineal melatonin, despite its high volume, cannot serve as a chemical signal of the light regime, and the synthesis of extrapineal melatonin in the tissues of organs, with the exception of the retina, does not have a circadian rhythm. Epiphyseal melatonin plays a significant role in the regulation of the digestive tract. The impairment of the daily cyclicity of its synthesis can provoke the development of peptic ulcer and acute pancreatitis [30, 32]. Regardless of the place of synthesis, the effect of melatonin on the digestive function is associated with the inhibition of peristaltic movements of the intestine [24, 26, 54], as well as with the regulation of the activity of digestive enzymes [22].

The prolonged absence of a photoperiod affects the secretion of melatonin by the pineal gland, impairing the operation of the biological clock. However, if constant illumination leads to a "physiological pinealectomy," there is suppression of the synthetic function of the pineal gland and a sharp decrease in the concentration of its hormone, melatonin, in plasma [14, 36, 49]. Constant darkness causes persistent hormone secretion and the total daily concentration of the hormone in the blood is even higher or, at least, not lower than that under conditions of regularly alternating illumination [52]. The melatonin concentration in the

body can be changed not only by the action of certain light regimes; tryptophan administered *per os* increased the level of melatonin by about six times in the pineal gland and ten times in the intestine and liver [21]. In another study, tryptophan also increased the level of circulating melatonin, especially in the portal vein, and even a previous epiphysisectomy did not affect the changes in its concentration in the gastrointestinal tract after tryptophan administration [31].

The presence of melatonin in the mucous membrane of the gastrointestinal tract, liver, and bile, as well as in the portal and peripheral venous blood, in addition to its modulating effects on the functions of the intestine and liver, indicates the role of this neurohormone as a mediator of intestine–liver interactions [37]. The hormone synthesized in the organs of the gastrointestinal tract, unlike that produced by the epiphysis, is necessary to maintain its concentration at a certain level in the circulating blood in the daytime. The liver is able to accumulate melatonin from portal blood [33]. After its metabolism, decay products, as well as an unchanged melatonin molecule, are excreted into the bile.

The goal of the study was the identification of age-related features of the functioning of the digestive tract enzymes in rats with reduced and increased synthetic pineal gland activity under constant lighting and light deprivation, respectively.

MATERIALS AND METHODS

The studies were carried out on the scientific equipment of the Center for Collective Use of the Karelian Scientific Center, a Federal Research Center of the Russian Academy of Sciences. The experiments were performed on males and females of outbred rats of the LIO line of the Petrov Research Institute of Oncology [15]. All animals received standard, prepared laboratory feed and water without restrictions. At the age of 25 days, the rats were randomly divided into three groups and kept under standard, regularly alternating lighting (12 h light/12 h darkness; *LD*, control group, $n = 97$), continuous lighting (*LL*, 750 lux, $n = 104$) and constant darkness (*DD*; 0–0.5 lux, $n = 112$).

The rats were weighed monthly throughout their lives, starting at 3 months and until natural death. At the age of 6, 12, and 18 months, five animals from each group were decapitated and tissue samples of the stomach, pancreas, and small intestine were taken for subsequent analysis. The activity of digestive enzymes was determined spectrophotometrically: the total proteolytic activity was determined according to the Nikolaevskaya method [3], amylase according to Drozdova and Fekson [2], lipase according to Ugolev [6], pepsin according to Helander [28]. The enzyme activity was expressed in micromoles of cleaved (for amylase, in mg of starch) or resulting substance per 1 min calculated per 1 g of tissue. The obtained data were processed with generally accepted methods of variation statistics. The study was carried out in accordance with the requirements of the WMA Declaration of Helsinki [8].

The obtained digital materials were processed with the Microsoft Excel and Statgraphics 2.0 statistical software packages by generally accepted methods of variation statistics and presented as mean ± SEM. The significance of differences between experimental groups and rats of different ages was determined by the nonparametric Wilcoxon–Mann–Whitney *U*-test. Correlation Analysis was used to identify the dependences and their nature between the studied indices in each age group for each light regime. Among the studied parameters, the identification of those that most fully characterized the separation of rats depending on the light regime was carried out with discriminant analysis. The influence of factors on the studied parameters was assessed with multifactor analysis of variance (MANOVA). The differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

The presence or absence of age-related changes in the activity of digestive enzymes in rats under conditions of regularly alternating illumination (*LD*) primarily depended on the food component lysed the enzyme, as well as the part of the gastrointestinal tract in which the enzyme functioned (Fig. 1). Age-related changes were noted for amylase and lipase in the pancreas and for general proteolytic activity, amylase, and lipase in the small intestine. An increase in the activity of the same enzymes in the pancreas was accompanied by the same increase in their activity in the small intestine, although with a certain temporal (age) shift. An age-related increase in proteolytic activity could be associated with the need to supply energy and to provide the body with amino acids, including tryptophan, which is necessary for melatonin synthesis. The absence of alternating light and dark (*LL* and *DD*) changed the age dynamics of the activity of most of the studied digestive enzymes (see Fig. 1). Moreover, while the maximum number of correlations of the studied parameters (evidence of functional load on the system) was noted in 12-month-old rats for the *LD* regime and in 6-month-old rats for the *LL* regime, the maximum number of correlations was detected much later with the *DD* regime—only in 18-month-old rats (Fig. 2).

Mutually compensatory relationships (the presence of positive and negative feedbacks) were detected in the observed correlation cohorts in the *LD* and *DD* regimes; in the *LL* regime, the were observed without such compensation. In this case, a correlation with cohort was noted only in *LL* 6-month-old rats, including, along with digestive enzymes, the animal's body weight, which suggested that the changes in enzyme activity observed at this age were associated precisely with the specificity of growth processes. Between the

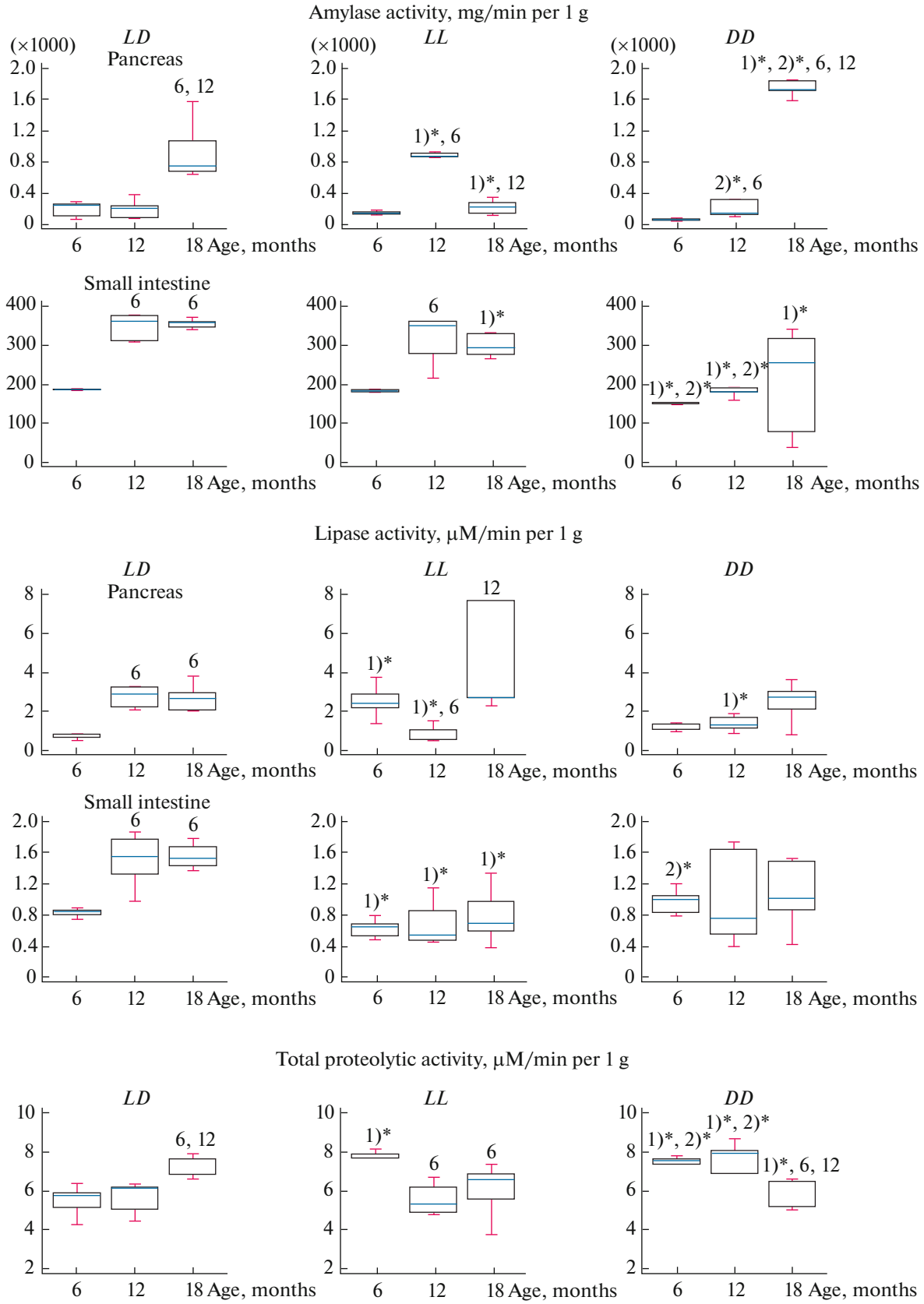

Fig. 1. Activity of digestive enzymes in the digestive tract of rats under different light conditions with respect to age. [1)]* Changes are significant as compared with animals of the same age in the *LD* group; [2)]* differences are significant as compared with animals of the same age in the *LL* group; [6, 12] differences are significant compared with 6- and 12-month-old rats under the same light conditions. Here and in Figs. 2, 3, 5: *LD*—standard regularly alternating lighting; *LL*—continuous lighting; *DD*—light deprivation.

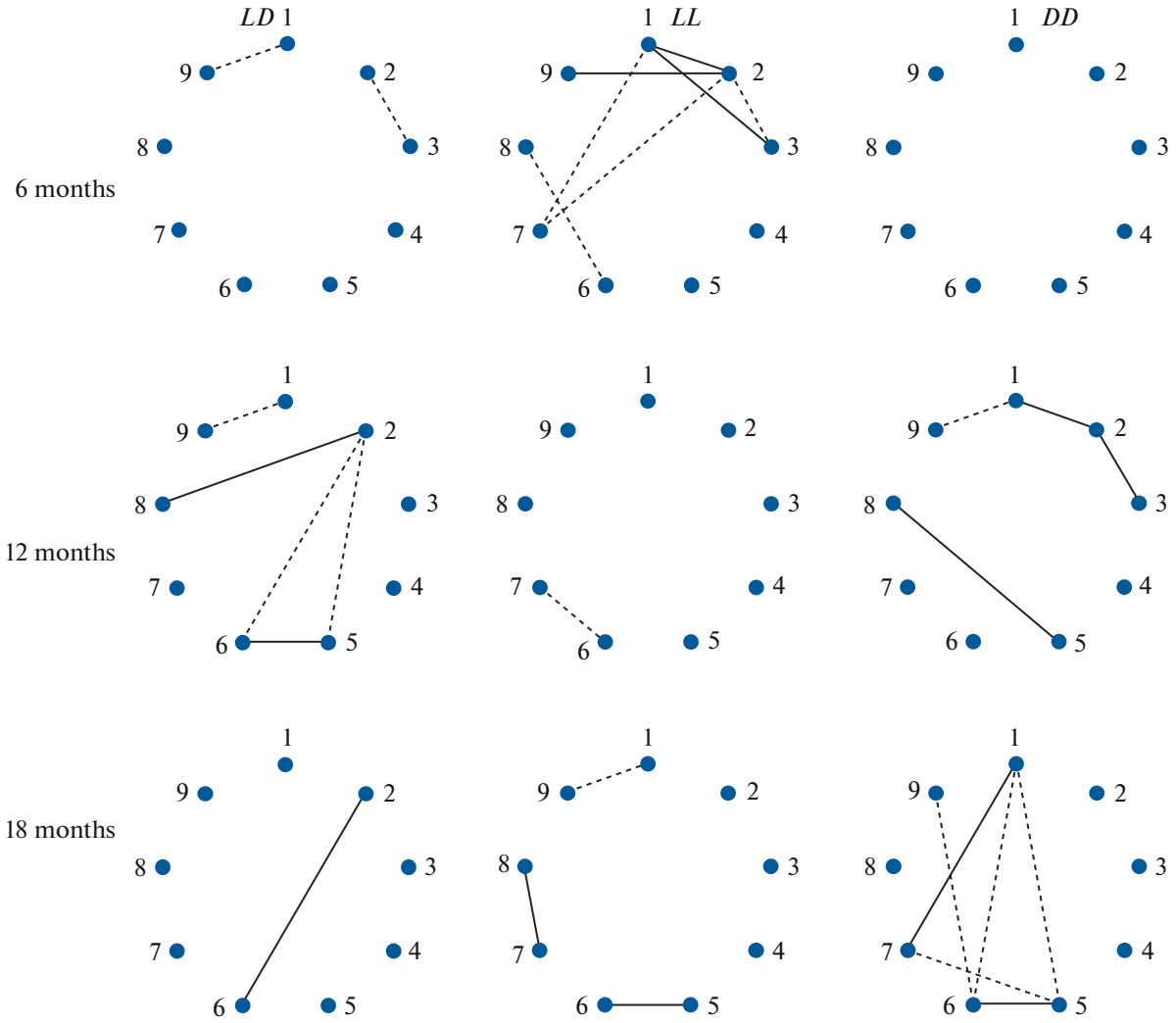

Fig. 2. Correlation of the studied parameters in rats under different light conditions. 1—sex of animals; 2—total proteolytic activity (TPA) in the pancreas; 3—amylase activity in the pancreas; 4—lipase activity in the pancreas; 5—TPA in the small intestine; 6—amylase activity in the small intestine; 7—lipase activity in the small intestine; 8—pepsin activity in the stomach; 9—animal body weight; solid line—positive correlation; dashed line—negative correlation. Only significant dependencies are shown ($p < 0.05$).

LD and *DD* light regimes, there were more similarities in the dynamics of age-related changes in the activity of the studied digestive enzymes than between each of these regimes and *LL*.

A similarity between the *LL* and *DD* regimes is the absence of photoperiodism, although the two states are fundamentally different at the level of melatonin synthesis by the pineal gland: if the first regime leads to "functional pinealectomy," then persistent secretion of melatonin is observed in the second case [52]. Discriminant analysis (Fig. 3) allowed the identification of three indices (amylolytic activity in the pancreas and small intestine and lipolytic activity in the small intestine) based on the three studied groups of rats that can be clearly differentiated (with probability of 72%). Moreover, the first discriminant function characterizes to a greater extent the presence/absence of photoperiodism (separates the *LD* group from *LL* and *DD*), and the second is obviously associated with the level of synthesis of epiphyseal melatonin (a clear separation of *LL* rats from *LD* and *DD*). The age trend of the first discriminant function in the *LD* group indicated a gradual synchronization of the activity of these three enzymes from the age of 6 to 12 months, followed by desynchronization by 18 months, which was also confirmed by the results of correlation analysis (Fig. 2). In the *LL* and *DD* groups, the age trend for this function has an opposite direction to the *LD* group. As for the second discriminant function, its values practically did not change in the *LL* group, and it increased equally in the *LD* and *DD* groups. This was obviously due to an age-related change in melatonin synthesis and the reaction of the described digestive enzymes to this.

MANOVA showed that the animal age did not have a significant effect only on the overall proteolytic

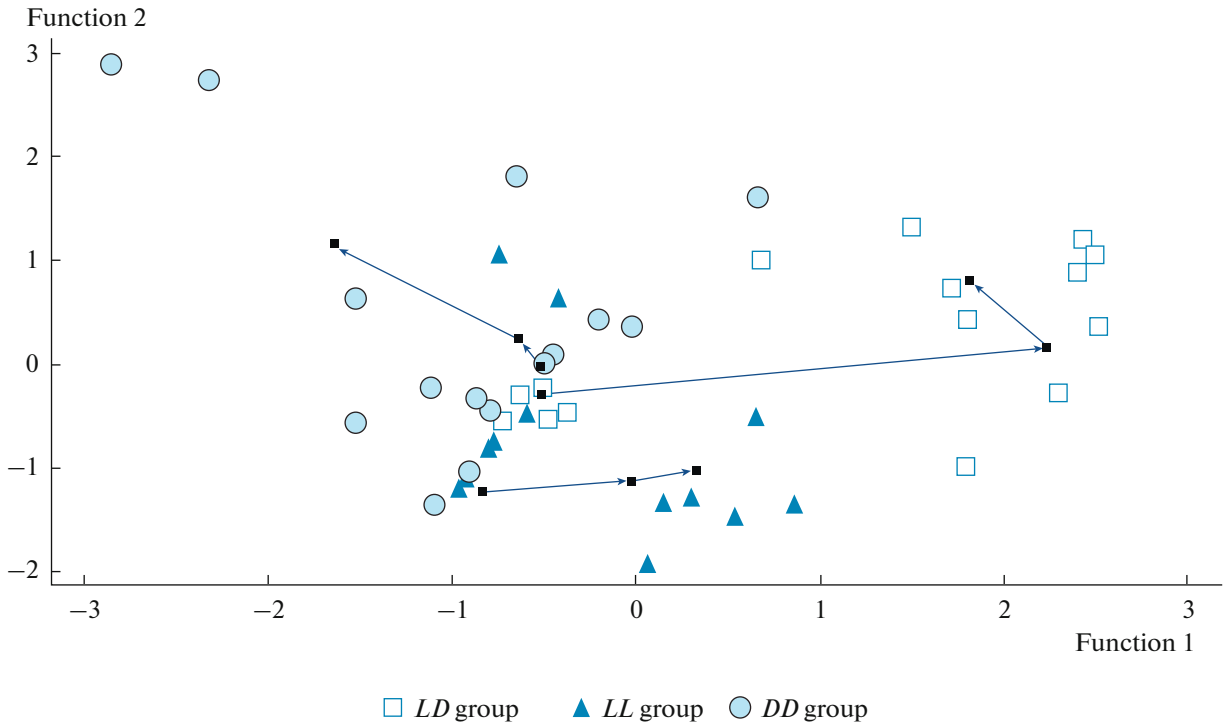

Fig. 3. Distribution of animals in the area of discriminant functions. Arrows show the dynamics of age trends for rats housed under each light regime.

activity in either the pancreas or small intestine (Fig. 4). The remaining enzymes were influenced by this factor to varying degrees, while the maximum influence (over 30% of the total dispersion) was detected for amylase. In the pancreas, "age" was the only factor affecting the activity of this enzyme of all studied enzymes. The influence of the "melatonin level" factor (which takes into account the fact that the maximum concentration of the hormone was observed with *LD*, and its synthesis was almost completely inhibited with *LL* [52]) was noted for lipase and amylase in the small intestine, as well as for lipase in the pancreas. The presence/absence of periodicity of the light regime, both separately and in interaction with age, was reflected in all enzymes, with the exception of pancreatic amylase. This effect was maximal for pepsin and total proteolytic activity both in the pancreas and in the small intestine; in total, it was more than 30% of the total dispersion of the index.

Changes in digestion (an increase in the contribution of lipolysis to the energy metabolism) and the use of more energetically beneficial substrates (carbohydrates and lipids) by animals [7] as the rats aged under standard light conditions influenced the age dynamics of body weight. Thus, the body weight of animals of both sexes in the *LD* group simultaneously increased in the first 15 months of postnatal life and did not change further in females, but it slightly decreased in males (Fig. 5). It should be noted separately that the body weight gain was 3.5 times higher in females and 7 times higher in males in the period of 3 to 6 months than for the next age period (6–9 months). The ontogenetic rearrangements of the enzyme profile of the gastrointestinal tract in rats of the *LD* group revealed by us can be cause metabolic shifts that lead to changes in body weight and also affect physical performance and endurance. A decrease in static endurance by the age of 18 months and dynamic performance by the age of 24 months of postnatal development was revealed [1]. Since the final result of the digestion function is aimed at providing plastic and energetic material to body cells [5], it can be stated based on our data that rats from *LD* group mainly use food carbohydrates for their metabolic needs during the period progressive and stable growth (up to 15 months). Rats then gradually switch to the joint use of carbohydrates with lipids. During the presenile period (18 months), they also use proteins that are necessary both for the provision of additional energy and the production of tryptophan, a precursor of melatonin, the synthesis of which by the pineal gland decreases with age.

The revealed age-related changes in the digestive function of rats in the *LL*-light regime had an effect on the growth of animals in postnatal ontogenesis. The age-related dynamics of rat body mass in the *LL* group was characterized by a shorter period of progressive growth as compared with animals in the *LD* and *DD* groups: it ended by the age of 9 and 12 months, respectively, in females and males (Fig. 5). Moreover, asynchronized growth was noted between the sexes. The

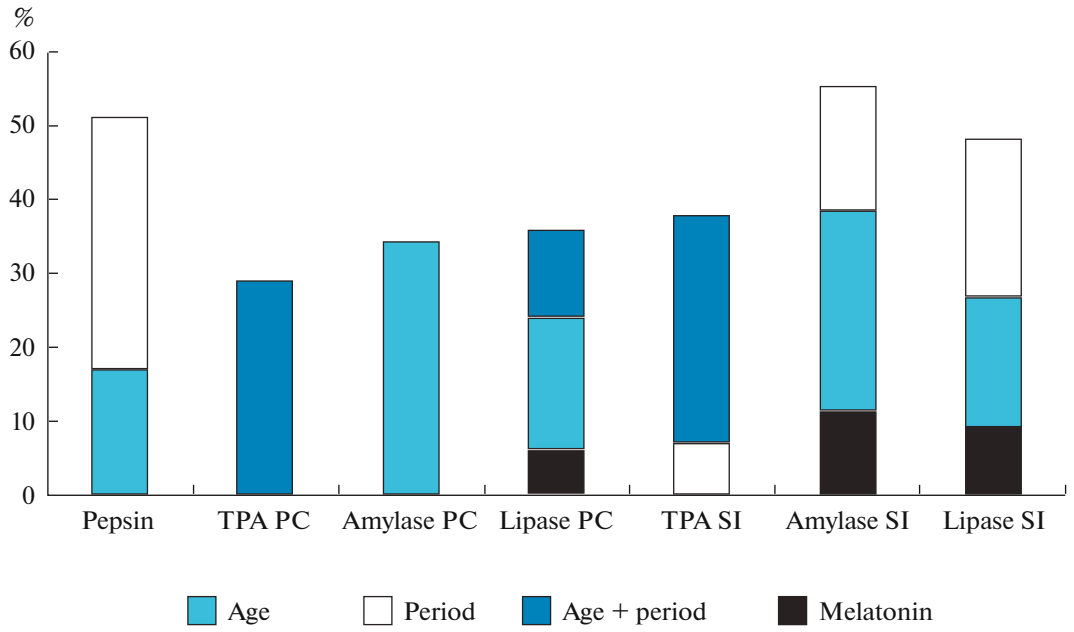

Fig. 4. Influence of various factors on the activity of digestive enzymes in the digestive tract in rats (only significant effects of factors according to analysis of variance are presented). TPA—total proteolytic activity; PC—pancreas; SI—small intestine; "period" factor—the presence/absence of periodicity of lighting in the experimental groups; "melatonin" factor—the melatonin level (see explanation in the text).

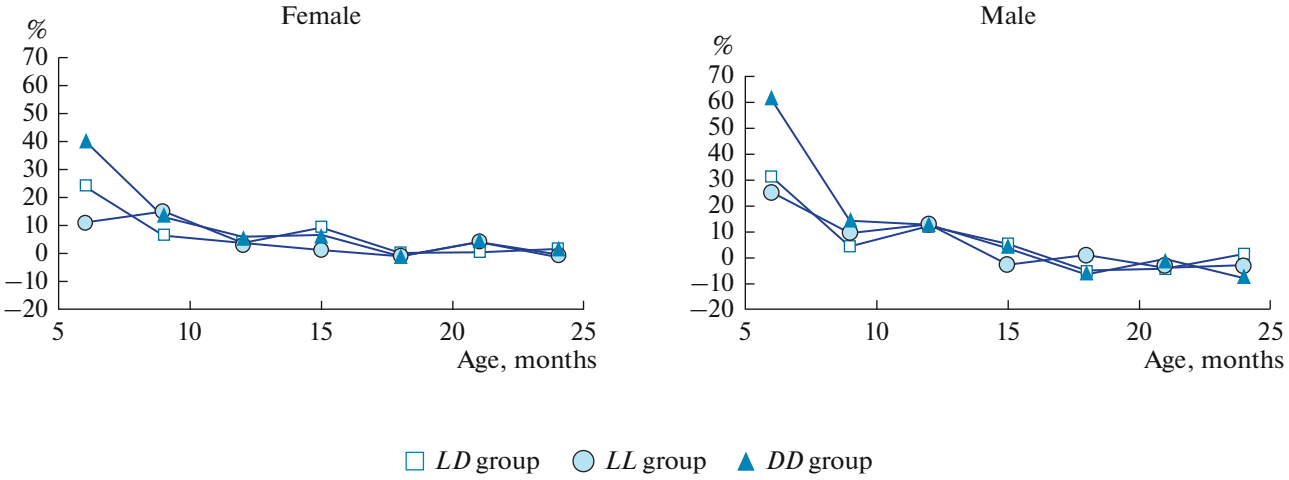

Fig. 5. Relative increase in body weight (% of the previous age period) of female and male rats under different light conditions.

increase in body weight in rats during the first 6 months of postnatal development under constant illumination conditions was minimal as compared to animals in the *LD* and *DD* groups. Negative growth dynamics and a gradual decrease in body weight were observed in males in *LL*-light regime, starting from the age of 15 months. The growth of females after 9 months of life also stopped and did not significantly change during aging. In addition, our previous study [1] found an earlier decrease in the physical endurance of *LL* rats, both static and dynamic, than that for rats of the control group. A decrease in the total retention time on the net was noted from the age of 12 months and in the duration of swimming from the age of 18th month.

Our study, like previously conducted studies, demonstrated a significant effect of the level of endogenous melatonin on digestive enzymes [4, 19]. We found further significant changes in the activity of amylase rather than lipase in the gastrointestinal tract of rats. These changes were related to the fact that the pineal hormone is the key regulator of a number of metabolic processes [42]. Even earlier studies showed that both a pinealectomy and the introduction of pineal-gland extracts significantly change the levels of glucose and its use in different tissues [25, 46]. Mela-

tonin deficiency can lead to glucose intolerance and insulin resistance [35, 39]. These changes can be stopped by the introduction of exogenous hormone. Melatonin, acting through signaling pathways associated with MT1 and MT2 receptors [13], regulates pancreatic function, affecting both glucagon and insulin biosynthesis [17, 41]; it also affects adipose tissue and consequently influences many aspects of the metabolism [10, 12, 18].

It was shown [38] that continuous lighting suppresses lipolysis and the breakdown of carbohydrates in the liver and stimulates the use of the carbohydrates as a preferred energy substrate in the kidneys. The opposite effect was observed during the implantation of melatonin in animals in this light regime. The authors also noted the presence of clear sexual dimorphism in the reaction to both exogenous melatonin and constant illumination. Recent studies have shown significant fluctuations in the concentration of extrapineal melatonin, as well as the expression level and activity of enzymes of the terminal stages of hormone synthesis, depending on the age of the animals [43, 48]. For example, a V-shaped age curve of the described indices was observed in the organs of the gastrointestinal tract (liver and intestines). This curve was described by a significant decrease in mature, 12-month-old rats and an increase in old 36-month-old animals as compared with young animals (3 months old). The increased expression of enzymes involved in melatonin synthesis in the liver of old (36-month-old) rats can be considered a compensatory response to an age-related decrease in the level of pineal melatonin. According to the authors of [43], the observed changes in the level of extrapineal melatonin were the result of additional protection from "harmful agents" that formed during aging rather than the insufficient activity of these enzymes in the early period.

Insulin, the main regulator of energy metabolism, is closely related to the level of epiphyseal melatonin. It was found that the removal of epiphysis causes a loss of sensitivity to glucose and reduces the daily secretion of insulin stimulated by glucose uptake [35]. In addition, melatonin enhances the expression of leptin and its release by rat adipocytes in the presence of insulin [11], and it also enhances the effect of insulin on leptin expression [10]. As a result, prolonged administration of melatonin at night leads to a decrease in the accumulation of abdominal fat [45, 55], regardless of food intake, in middle-aged males [55] and in obese rats [44]. This probably explains the various effects of light regimes on age-related changes in the activity of amylo- and lipolytic enzymes in the gastrointestinal tract of the rats in our study.

The enzyme activity was affected not only by the melatonin level but also by the presence of a periodic change in its blood concentration (Fig. 4). Until now, there is no consensus on how lighting conditions affect the level of melatonin secretion by the pineal gland and gastrointestinal tract. With standard light regimes, the authors found a 24-hour rhythm of NAT activity in the duodenal preparations, which coincided with the circadian rhythm of this enzyme in the pineal gland and retina [34]. However, even in early studies, the absence of a pronounced daily rhythm of melatonin in the digestive tract was noted, as well as the fact that the hormone level in the intestine did not decrease in pinealectomized animals [20]. The housing of rats under conditions of light deprivation (*DD*) does not significantly change the diurnal dynamics of melatonin synthesis (increases the daytime level and decreases the concentration of nocturnal melatonin) and does not violate the circadian rhythm of corticosterone synthesis [52]. The circadian rhythm in the central nervous system is very plastic, but the rhythms are usually preserved under *LD* and even under *DD* conditions when the main components of the mechanism of the biological clock are genetically impaired [27].

The change in the activity of proteolytic enzymes could be related both to age-related energy and plastic material requirements and to the tryptophan requirement, a kind of compensation for melatonin deficiency during the termination of growth processes. The melatonin synthesis in the intestine, but not in the pineal gland, depends on the presence of its main amino acid precursor in food [29]. This was also confirmed by the fact that the circadian fluctuation of residual melatonin in plasma in epiphysectomized rats was determined by the feeding schedule [40]. The administration of tryptophan stimulated the synthesis of melatonin, which led to an increase and earlier achievement of the peak concentration of this hormone in plasma [47].

Our study demonstrated an age-related increase in the activity of digestive enzymes in the small intestine in rats kept under *LD* light regime. This may be due to both the revealed increase in secretory activity [53] and the number of enzymes synthesized by the pancreas. The absence of a photoperiod in the form of constant illumination had a significant effect on age-related changes in the activity of gastrointestinal enzymes in rats. Negative age-related trends in the activity of digestive enzymes in animals with reduced synthetic activity of the pineal gland (under *LL* lighting conditions) led to a slowdown in growth processes and a decrease in physical endurance. Although age-related changes in the activity of digestive enzymes in rats under conditions of light deprivation (*DD*) were similar to those observed in animals of the *LD* group, their appearance was recorded at a later stage of ontogenesis in aging (18-month-old) rats.

CONCLUSIONS

In the standard light regime, the age-related trends in amylase activity indicate a deficiency of energy obtained from food in aging rats, which was most likely compensated for by lipids. The age-related increase in the activity of enteric proteolytic enzymes was probably due to the need to obtain more tryptophan to compensate for the melatonin level in aging

rats. Against the background of a simultaneous decrease in the synthesis of epiphyseal melatonin and impairment of the daily rhythm of its secretion under constant illumination conditions in rats, a mismatch were observed in the operation of digestive enzymes and premature aging of the system.

Animals under conditions of light deprivation when the diurnal rhythm of secretion of epiphyseal melatonin is maintained (in this case, its night peak was lower and the daily level was higher than with the *LD* regime) slowed age-related changes in the activity of digestive enzymes. An increase in amylo- and lipolytic activity as compared with rats of the control group was later observed. An age-related decrease in the total proteolytic activity in animals under the *DD* light regime indicates a rather high melatonin level in aging rats and the absence of the need for its precursor, tryptophan.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflict of interest.

Statement on the welfare of animals. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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