



## Effect of epitalon on the lifespan increase in *Drosophila melanogaster*

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### Abstract

The geroprotector activity of epitalon, a synthetic tetrapeptide Ala–Glu–Asp–Gly, was studied on the *Drosophila melanogaster* wild strain Canton-S. The substance was added to the culture medium only at the developmental stage (from egg to larva). Epitalon significantly increased the lifespan (LS) of imagoes by 11–16% when applied at unprecedented low concentrations — from  $0.001 \times 10^{-6}$  to  $5 \times 10^{-6}$  wt.% of culture medium for males and from  $0.01 \times 10^{-6}$  to  $0.1 \times 10^{-6}$  wt.% of culture medium for females. The increase in LS did not depend on the substance dose. Effective concentrations of epitalon were 16 000–80 000 000 times lower than those of melatonin. The possible mechanisms of the antioxidant and regulatory effects of epitalon are discussed. © 2000 Published by Elsevier Science Ireland Ltd.

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### 1. Introduction

Studying the biological activity of peptide regulators is one of the most promising fields of modern experimental gerontology. These studies started with a long-term

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investigation of the roles of epithalamin (a pineal gland preparation) and melatonin (a pineal gland hormone) in the structural and functional homeostasis of living systems (Anisimov et al., 1994).

The purpose of this study was to estimate the geroprotector effect of epitalon, a synthetic tetrapeptide Ala–Glu–Asp–Gly obtained by means of targeted construction based on amino acid analysis of epithalamin (Khavinson and Morozov, 1999). The data obtained indicated that the preparation increased the mean lifespan (MLS) of adult *Drosophila melanogaster* when added to the nutrient medium only at the developmental stage at concentrations as unprecedentedly low as  $0.001 \times 10^{-6}$  wt.%. In addition, we found that the geroprotector effect, namely, an 11–16% increase in MLS, was almost independent of the substance concentration within the range from  $0.001 \times 10^{-6}$  to  $5 \times 10^{-6}$  wt.%.

## 2. Materials and methods

In this study, *Drosophila melanogaster* of a wild strain, Canton-S, was used. All the experiments were conducted under constant conditions of 25°C, 75% relative humidity, and light/dark cycle of 0:24.

Two types of culture medium were prepared: one for the developmental stage and one for determination of adult LS. The culture medium of the first type contained 1000 ml water, 120 g yeast, 100 g sucrose, and 15 g agar. The culture medium of the second type contained 1000 ml water, 100 g cornmeal, 100 g sucrose, and 4.5 g agar. After 30 min cooking, the hot medium was poured into 100 × 25 mm glass vials whose surfaces were seeded with a suspension of living yeast and dried at room temperature for 24 h.

An outbreeding cultivation protocol was followed continuously and uniformly for all experiments. Randomly chosen 5-day virginal imagoes were pair mated for 1 day, and the next generation was observed. A total of 50–80 pairs were used to obtain each successive generation. After each eclosion, randomly chosen 5-day insects were crossed to obtain the next generation.

As a geroprotector, we applied the peptide Ala–Glu–Asp–Gly in solution form, produced by S. Petersburg Institute of Bioregulation and Gerontology (Khavinson and Morozov, 1999). The peptide as sterile (pure) solution (1 µg in 1 ml of isotonic solution) was introduced into the culture medium cooled down to 50–60°C, and the mixture was then thoroughly stirred. The concentration of peptide was calculated per unit mass of culture medium. For experimental investigation five concentrations of epitalon were used: from  $0.001 \times 10^{-6}$  to  $5 \times 10^{-6}$ % of culture medium weight.

The life span (LS) was registered as follows. A total of 90 virgin flies of both sexes were randomly chosen from each eclosion, and ten flies of each sex were placed separately in each vial. In the survival experiments, the culture medium was replaced three times a week.

As LS distribution parameters, we used mean values and standard errors. Standard *t*-test was used to compare the differences between mean values (Zar, 1984).

Table 1  
Mean life-span (MLS) and standard error (S.E.) in control and epitalon-treated groups

No.	Treatment	Concentration $\times 10^{-6}\%$	MLS $\pm$ S.D. days (relative effect, %)	
			Males	Females
1	Control		35.41 $\pm$ 1.31	32.07 $\pm$ 1.07
	Epitalon	0.001	41.30 $\pm$ 1.40 (+16.6) <sup>a</sup>	34.36 $\pm$ 0.98 (+7.1)
2	Control		32.55 $\pm$ 1.14	31.22 $\pm$ 0.91
	Epitalon	0.01	36.47 $\pm$ 1.09 (+12.0) <sup>a</sup>	35.41 $\pm$ 1.07 (+13.4) <sup>a</sup>
3	Control		31.44 $\pm$ 1.35	31.42 $\pm$ 1.10
	Epitalon	0.1	34.85 $\pm$ 1.18 (+10.8) <sup>a</sup>	35.13 $\pm$ 1.23 (+11.8) <sup>a</sup>
4	Control		29.99 $\pm$ 1.38 <sup>b</sup>	32.39 $\pm$ 1.17 <sup>b</sup>
	Epitalon	1.0	33.80 $\pm$ 1.28 (+12.8) <sup>a,b</sup>	33.43 $\pm$ 1.23 (+3.2) <sup>b</sup>
5	Control		33.90 $\pm$ 1.14	36.87 $\pm$ 1.40
	Epitalon	5.0	37.74 $\pm$ 1.16 (+11.3) <sup>a</sup>	38.04 $\pm$ 1.56 (+3.2)

<sup>a</sup> MLS difference is estimated as significant (*t*-test,  $P < 0.05$ ).

<sup>b</sup> Average data, original data are in Table 2.

### 3. Results

Table 1 shows the characteristics of LS distribution, including mean values, standard errors of the mean (Zar, 1984), and changes in MLS (expressed as a percentage) after epitalon treatment.

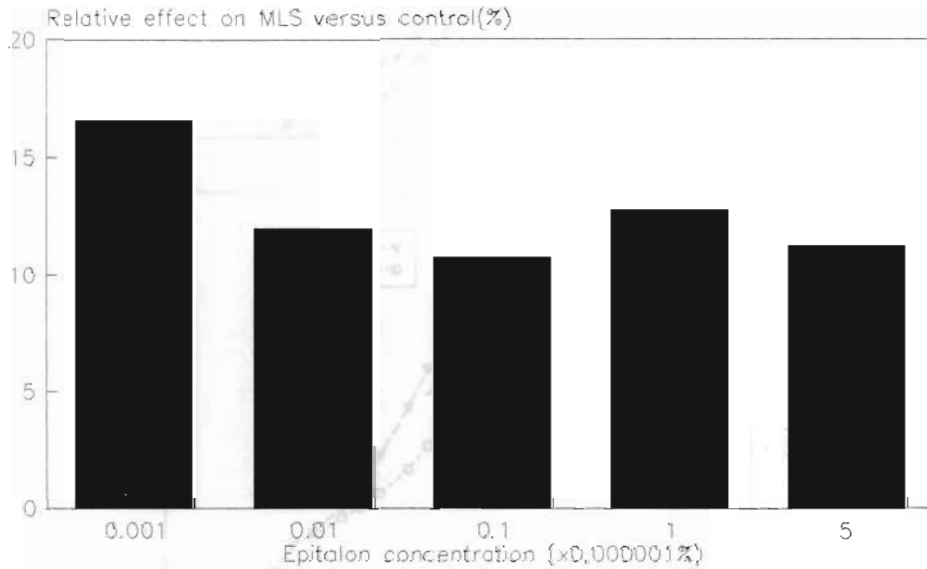


Fig. 1. Relative effect of epitalon treatment on mean life-span in *D. melanogaster* males.

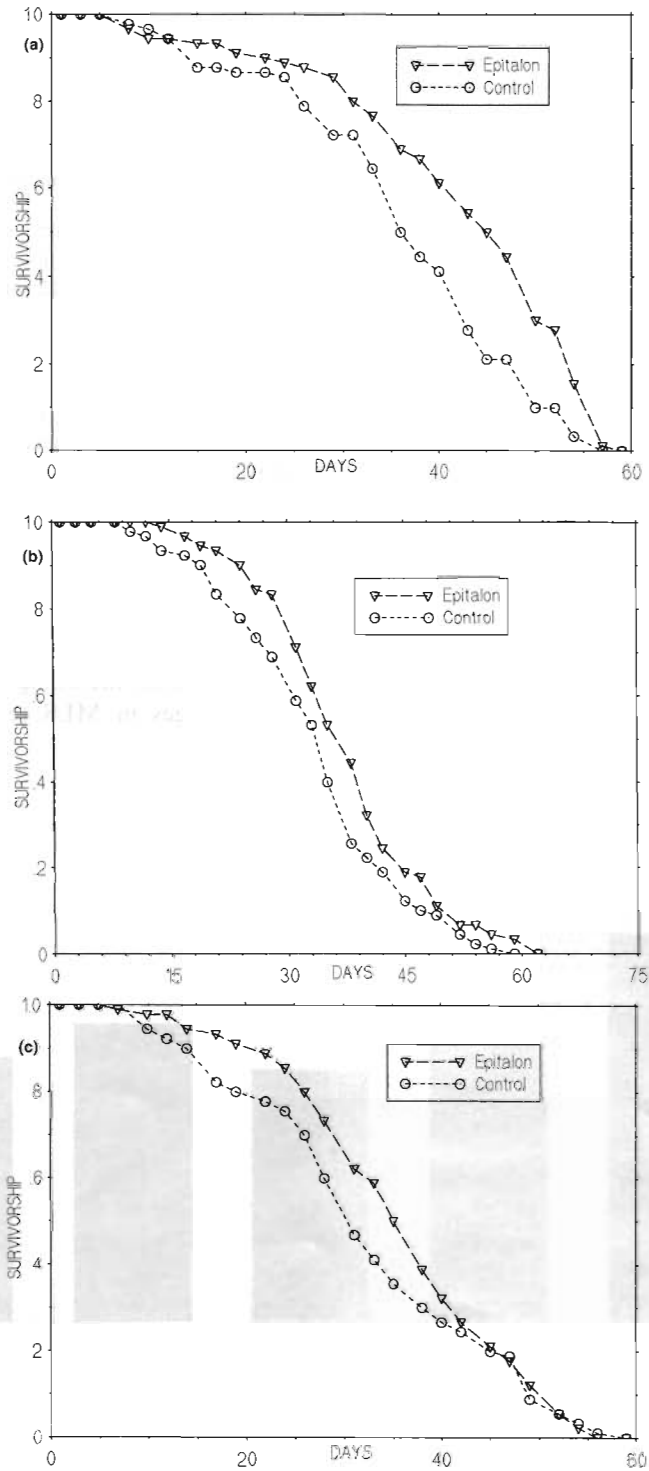


Fig. 2. Survival curves in *D. melanogaster* males (epitalon concentration): (a)  $0.001 \times 10^{-6}\%$ ; (b)  $0.01 \times 10^{-6}\%$ ; (c)  $0.1 \times 10^{-6}\%$ ; and (d)  $5 \times 10^{-6}\%$ .

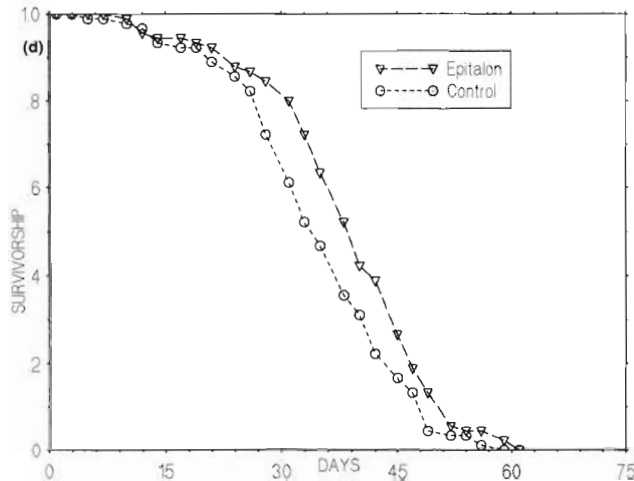


Fig. 2. (Continued)

Note that epitalon did not affect the duration of the *D. melanogaster* developmental stages from egg to imago, which generally suggests an absence of a genotoxic (mutagenic) effect of the substance.

Epitalon had a significant geroprotector effect on males at all concentrations studied: their MLSs significantly increased by 10.8–16.6% in different experimental groups. Fig. 1 shows the results obtained in the form of a diagram. The corresponding survival curves for the control and experimental groups are shown in Fig. 2(a–d). As is seen from the figure, the difference between the experimental and control survival curves in each experiment are mostly restricted to the segment of the mass mortality, with the initial plateau and the maximal LS being unaffected.

An experiment with an epitalon concentration of  $1.0 \times 10^{-6}\%$  was carried out in triplicate (Table 2). We found that, at the same concentration of the substance, the MLS may be considerably (by 21.8%), moderately (by 13.3%), or only slightly and nonsignificantly increased (by 3.3%) compared to the control value. The average MLS changes in the three experiments were +12.8 and +3.2% for males and females, respectively (Table 1, row 4). Fig. 3(a–c) show survival curves for experimental and control males after treatment with  $1.0 \times 10^{-6}\%$  epitalon. It is worth noting an obvious trend: the lower the control MLS, the higher the relative epitalon effect on MLS in the experimental group. Similar reverse relationships between the geroprotector effect and the varying viability in the control population were found earlier and described in detail for 4-hydroxy-TEMPO (Izmaylov and Obukhova, 1996) and melatonin (Izmaylov and Obukhova, 1999).

In females, epitalon was only effective at concentrations of  $0.01 \times 10^{-6}$ ,  $0.1 \times 10^{-6}$  and once at  $0.1 \times 10^{-6}\%$ , at which the MLS was significantly increased by 13.4 and 11.8% (Table 1) and 9.8% (Table 2), respectively. At other concentrations, differences between the experimental and control groups were not statistically significant.

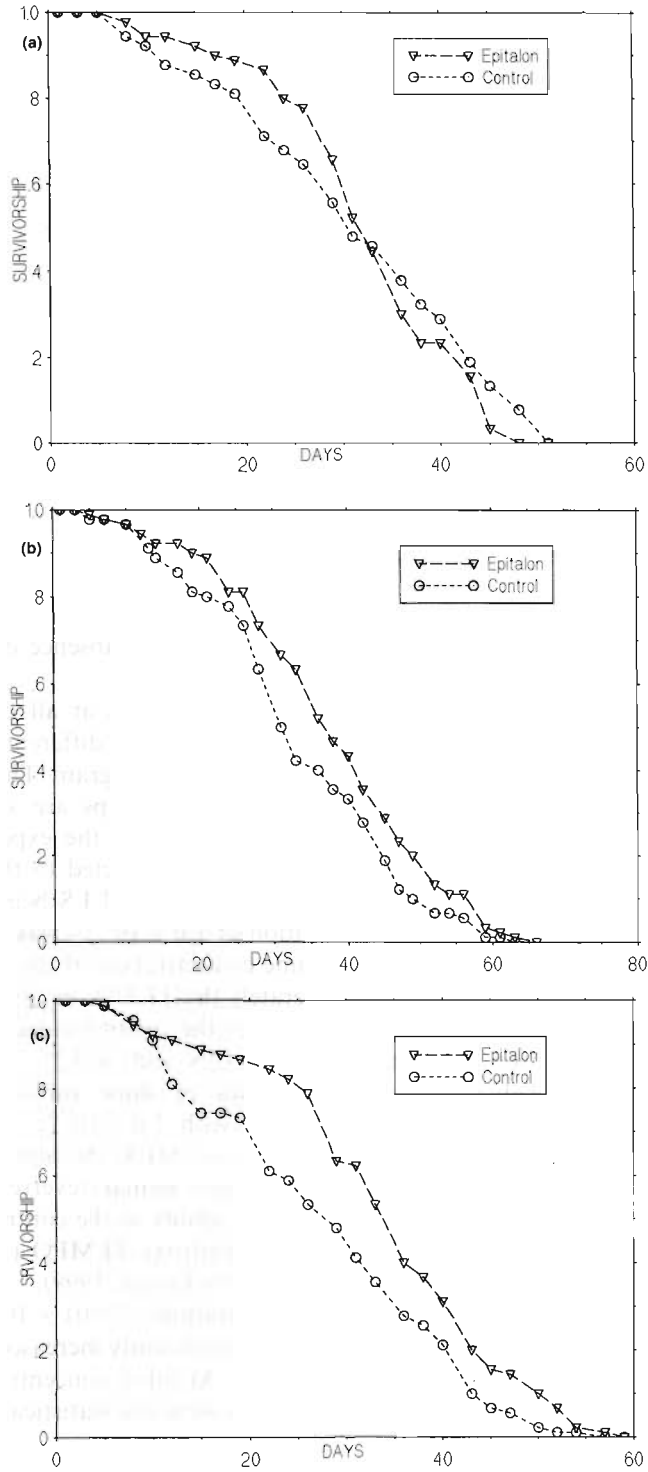


Fig. 3. Survival curves in *D. melanogaster* males. (a–c) epitalon concentration,  $1 \times 10^{-6}\%$  (three replays).

Table 2  
Mean life-span (MLS) and standard error (S.E.) in control and epitalon-treated groups (concentration  $1.0 \times 10^{-6}\%$ )

No.	Treatment	Concentration $\times 10^{-6}\%$	MLS $\pm$ S.D. days (relative effect, %)	
			Males	Females
4.1	Control	1.0	30.31 $\pm$ 1.33	32.32 $\pm$ 0.97
	Epitalon		31.30 $\pm$ 1.03 (+3.3)	32.59 $\pm$ 0.84 (+1.0)
4.2	Control	1.0	32.45 $\pm$ 1.41	36.17 $\pm$ 1.23
	Epitalon		36.77 $\pm$ 1.44 (+13.3) <sup>a</sup>	35.76 $\pm$ 1.35 (-1.1)
4.3	Control	1.0	27.20 $\pm$ 1.35	28.67 $\pm$ 0.93
	Epitalon		33.14 $\pm$ 1.32 (+21.8) <sup>a</sup>	31.49 $\pm$ 1.28 (+9.8) <sup>a</sup>

<sup>a</sup> MLS difference is estimated as significant (*t*-test,  $P < 0.05$ ).

#### 4. Discussion

The data obtained are fundamentally new and unique due to the ultralow concentrations of the peptide preparation.

The literature data indicate that concentrations of  $10^{-3}$ – $10^{-10}\%$  are the lower limit for the geroprotector effects of most substances on *D. melanogaster*. This concerns both synthetic and natural substances. Examples of the former are Gerovital, 3-hydroxypyridine, Dibunol, Centrophenoxin (Obukhova et al., 1979; Obukhova and Emanuel, 1984; Frolkis and Muradyan, 1988), and 4-hydroxy-TEMPO (Izmaylov and Obukhova, 1996). The latter are vitamins A, E, and C (Baker, 1993; Massie et al., 1993); hormones (Hochschild, 1971), lactic and gluconic acids, glutathione (Obukhova and Emanuel, 1984; Frolkis and Muradyan, 1988), melatonin, and epithalamin (Anisimov et al., 1994, 1997, 1998; Izmaylov and Obukhova, 1999; Khavinson and Morozov, 1999). For example, melatonin increased MLS to about the same extent as epitalon (by 13–16%) in similar experiments, with its optimum concentration being  $80\,000 \times 10^{-6}\%$  (Izmaylov and Obukhova, 1999). In other words, epitalon produced the same effect as melatonin at 16 000–80 000 000-fold lower concentrations.

Our data indicate that the effective concentration range of epitalon for *D. melanogaster* males is unprecedentedly wide: from  $0.001 \times 10^{-6}$  to  $5 \times 10^{-6}\%$  (the upper and lower limits differ by 5000 times). Note that the highest and the lowest effective concentrations yielded almost the same significant effect (on average, a 13% increase in MLS), i.e. the effect did not depend on the dose of the substance.

Analysis of the possible mechanism of epitalon action is of interest. For this purpose, it should first be noted that MLS was changed in adult populations, whereas only larvae were treated with epitalon. Between these stages, the insects undergo a complete metamorphosis, with intense lysis of larval and pupal tissues

and formation of adult tissues out of the small amounts of cells contained in imaginal disks (Ashburner, 1989). Only a structure capable of template duplication, i.e. nuclear DNA, may ensure a larva–imago informational succession. Therefore, epitalon affects the larval genetic material so that the adult MLS is changed. We observed earlier similar phenomena in experiments with 3-hydroxypyridine (Obukhova et al., 1979), melatonin (Izmaylov and Obukhova, 1999), and 4-hydroxy-TEMPO (Izmaylov and Obukhova, 1996).

Differences between males and females with respect to the dose–effect relationship apparently reflect the effect of epitalon on the female reproductive function, which masks the ‘pure’ effect of epitalon on LS. Results of other studies using melatonin (Anisimov et al., 1998; Izmaylov and Obukhova, 1999) also suggest differential sensitivity of males and females to the substance. As in the present work, female MLS in these experiments was changed to a lesser degree than male MLS. These effects of the pineal gland peptides call for further study.

Apparently, epitalon does not scavenge free radicals in the body of *D. melanogaster* as antioxidant geroprotectors do. In other words, the geroprotector effect of ultralow concentrations of this substance is not accounted for by an antioxidant mechanism. The effect obtained is most likely to be determined by optimization of vital functions due to the regulatory properties of short-lived peptides (Ashmarin and Kamenskaya, 1988), which are characterized by long-term action. Short-lived peptides are involved in an evolutionarily ancient regulatory mechanism and are widespread in lower organisms (Ashmarin and Kamenskaya, 1988).

The unusual pattern of the concentration–effect relationship with a large plateau (from  $0.001 \times 10^{-6}$  to  $5 \times 10^{-6}$ %) reflects the characteristic dynamics of interaction between a small number of molecules and their targets. There are other examples where the effect does not depend on concentration within a wide range (Burlakova, 1994). This is determined by fundamentally different kinetic patterns of relationships between biological structures and ultralow amounts of active substances (Burlakova, 1994), rather than specificity of the substances (antioxidants, herbicides, immune response modifiers, toxins, etc.) or the objects.

Consequences of epitalon metabolism after it enters the digestive tract of a *D. melanogaster* larva deserves special consideration. Is the geroprotector effect of the substance determined by the properties of the tetrapeptide itself or the products of its decomposition, i.e. dipeptides or amino acids? The tetrapeptide undoubtedly undergoes enzymatic hydrolysis (Ashburner, 1989). However, the products of the possible degradation of epitalon could not noticeably affect the general metabolic amino acid flow in the insect body, taking into account the low concentrations of epitalon in the medium. In addition, the Ala–Glu–Asp–Gly sequence is obviously not unique and may be found in native proteins; therefore, hydrolysis of food proteins might yield the same tetrapeptide. The geroprotector properties of epitalon found in this study apparently result from its interaction with specific receptors, the location of which in the body of *D. melanogaster* larva requires further investigation.



In order to estimate the geroprotector effectiveness of epitalon by means of extrapolation, let us make the following assumptions. A larva weighing  $\sim 1.5$  mg (Church and Robertson, 1966) eats approximately five times its own weight (Chiang and Hodson, 1950), i.e. 7.5 mg, while kept on the culture medium. This amount of the medium contains  $7.5 \times 10^{-11}$  mg of epitalon, given its concentration  $C = 0.001 \times 10^{-6}\%$ . An average human weighs 75 kg. If we set up a proportion, we will find that the effective dose of epitalon for humans is  $\sim 0.075$   $\mu\text{g}$  or 0.001  $\mu\text{g}/\text{kg}$  body weight.

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