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Original paper

The effect of low-dose spermidine supplementation on polyamine content and antioxidative defence mechanisms in honey bees

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Summary. The honey bee, a widespread pollinator, contributes to the conservation of biodiversity. In recent decades, a trend of declining colony numbers has emerged. The unsustainable exploitation of the environment may be the cause of this phenomenon. One protective strategy of organisms is to strengthen their antioxidative capacity. A class of positively charged molecules, polyamines, plays important roles in various cellular processes. They exert a regulatory effect on gene expression, have antioxidative properties, and promote longevity in model organisms. The three main representatives are putrescine, spermidine and spermine. The aim of this study was to determine whether supplementation of bees with low-dose spermidine leads to an increased level of the mentioned polyamines and whether this could strengthen the antioxidative defence system. Two experimental groups were established: C group (control), fed with a 50% (w/v) sucrose solution, and $S_{0.01}$ group, whose diet was supplemented with 0.01 mM spermidine. The experiment lasted for 10 and 17 days. A significant increase in putrescine, spermidine and spermine content was noted in the supplemented group after 17 days, compared to its control. These results show a positive impact of spermidine supplementation on maintaining polyamine levels throughout aging. FRAP and MDA biochemical assays were used for the assessment of oxidative status. FRAP assay showed increased antioxidative capacity in the $S_{0.01}$ group. These results are in accordance with the results obtained from the MDA assay, which showed a decreased level of lipid peroxidation in the supplemented group, in comparison to the control. The potential practical outcome of this study could be the use of spermidine in beekeeping practice to promote overall honey bee health.

Keywords: antioxidative defence mechanisms, health, honey bee, polyamines, putrescine, spermidine, spermine, supplementation.

INTRODUCTION

Wild and domesticated species that provide the ecosystem with the service of pollination greatly influence the maintenance of global wild plant biodiversity, as well as of agricultural crops (Potts et al. 2010). This service has become even more valuable in present times, as there is a global exponential human population increase that demands more food resources. In addition, the negative impact of anthropological activities can be seen through the pollinator population decline, as organisms become more susceptible to various diseases due to their compromised immune and antioxidative defence systems. As a result, this topic is of great interest and the focus of many studies, which have the aim of aiding in the promotion of pollinator health.

The honey bee (*Apis mellifer*a L.), is a globally distributed pollinator species, that increases the yield in 96% of crops pollinated by animals. Honey bees are the most prominent monoculture crop pollinators (Delaplane and Mayer 2000). Wild plants also depend on pollination by honey bees, although their exact contribution is poorly documented (Klein

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et al. 2007). What makes this species such a successful pollinator, particularly of large monocultural areas, is the fact that bee colonies possess a yearly large work force (Seeley 1985). Beekeepers can also aid in the population growth by feeding the honey bees with a wide array of supplements, which could elevate their overall health.

A promising contender for honey bee dietary supplementation is spermidine (Đorđievski et al. 2023). Spermidine belongs to a group of molecules, called polyamines. In addition to spermidine, putrescine and spermine are the most prominent polyamine members. Polyamines are positively charged molecules present in all living organisms, with heterogeneous cellular functions. To name a few, these biogenic amines are involved in gene expression, translation, cellular growth, differentiation, development, immunity, cell reprogramming and autophagy regulation (Minois et al. 2011; Choi and Park 2012). As polycations, polyamines interact with negatively charged molecules such as DNA, RNA, ATP and specific types of proteins and phospholipids (Minois 2014). The level of polyamines is determined by the cumulative effect of their biosynthesis and catabolism, and it is highly diverse in different organisms (Madeo et al. 2019). It is crucial that the level of polyamines remains within a narrow range, because an excess of these molecules leads to apoptosis and transformation of cells. In contrast, a significant decline of their level results in impaired cell proliferation and migration (Moinard et al. 2005). Nonetheless, one thing that both model organisms and humans have in common is that spermidine tissue concentrations decline with age and there is a possible link between reduced endogenous spermidine concentrations and age-related deterioration (Scalabrino and Ferioli 1984; Eisenberg et al. 2009; Pucciarelli et al. 2012; Gupta et al. 2013; Madeo et al. 2019). Moreover, exogenous spermidine supplementation reverses age-associated adverse changes and prolongs lifespan in different model organisms, including honey bees (Đorđievski et al. 2023) and humans (Ni and Liu 2021).

Spermidine influences aging through diverse mechanisms due to its interactions with numerous molecules. Autophagy is considered the primary mechanism of spermidine's action in delaying aging and prolonging the lifespan. In addition, spermidine exerts its effects through other mechanisms, including antioxidant activity (Minois et al. 2014; Ni and Liu 2021; Đorđievski et al. 2023; Jiang et al. 2023). Thus, increased oxidative stress, due to a higher concentration of reactive oxygen species (ROS), can be a result of naturally occurring polyamine depletion during aging, as these molecules also have antioxidative activity (Pegg 2013). In addition, ROS production can be stimulated by prolonged stress, including overuse of pesticides, malnutrition, habitat fragmentation and loss, changing environmental factors and disease (Goulson et al. 2015). Also, the innate antioxidative capacity weakens with age. Therefore, this study focuses on the effect of low-dose spermidine supplementation on individual polyamine content and antioxidative capacity of *A. mellifera*. More precisely, the aim of the experiment was to examine whether low-dose spermidine supplementation maintains polyamine levels and strengthens the response to oxidative stress.

MATERIAL AND METHODS

Experimental design and sampling of material

Three experimental bee hives, representing biological replicates, were maintained for 10 months in an apiary located in Susek, Fruška Gora mountain (45.2244°N, 19.5348°E) prior to the start of the experiment in laboratory conditions. These hives were left untreated for the whole duration of the mentioned period. In the spring of 2022, two brood frames were transferred from the experimental hives into glass hives, which were kept in an incubator with parameters set to simulate natural conditions in the hives. The temperature was set at 34 °C, relative humidity at 65%, in darkness. After a 24 h period, which was sufficient for the hatching of worker bees, the original brood frames were replaced with honey and bee bread frames to ensure food supply for the young bees. After five days bees were randomly transferred into 2 L plastic boxes, each containing on average 45 bees. Aeration was achieved by puncturing small holes in the boxes. In addition, 2 mL plastic syringes were used for feeding purposes, which were half-inserted through a larger hole made in the box. These boxes were kept under the same conditions in the incubator, except for the temperature which was lowered to 28 °C.

Two experimental groups were established: control group (C), which was fed with a basic 50% (w/v) sucrose solution and supplemented group ($S_{0.01}$), which was fed with 0.01 mM spermidine in a sucrose solution. Due to the unstable nature of spermidine, feeding solutions were prepared daily, by diluting 1 M spermidine stock with the sucrose solution. The mass of the consumed solution was measured daily. Sampling of bees from the described boxes was carried out after 10 and 17 days (10d and 17d, respectively). The sampled bees were frozen instantly in dry ice and kept in a freezer at –70 °C for subsequent analyses.

HPLC analysis

The content of all three polyamines (putrescine, spermidine and spermine) was determined in both the C and $S_{0.01}$ group, after 10 and 17 days. Each HPLC analysis was performed on a pool of two bees, in technical triplicates. The

sample preparation for this analysis included 24h lyophilisation, and polyamine extraction by the use of 4% perchloric acid in accordance with Scaramagli et al. (1999). After 1h incubation period on ice, the samples were centrifuged at 15,000 ×*g* for 30 min. The supernatants from the samples, as well as those from polyamine standards for each polyamine, were derivatized with dansyl chloride. The derivative was extracted by the addition of toluene, dried and resuspended in acetonitrile, after which the HPLC analysis was performed on a reverse phase C18 column (Spherisorb ODS2, 5-µm particle diameter, 4.6 × 250 mm, Waters, Wexford, Ireland). An acetonitrile-water step gradient was used. Eluted peaks were detected by spectrofluorometer (ex. 365 nm, em. 510 nm). Heptamethylenediamine was used as an internal standard.

FRAP and MDA

Each measurement was performed on a pool of five honey bees ground in liquid nitrogen using mortar and pestle and homogenized with ice cold 50 mM phosphate buffer pH 7,0 (20% w/v). These homogenates were centrifuged at 10,000 ×*g* at 4 °C for 10 min. Supernatants were aliquoted and frozen at –20 °C.

The Ferric Reducing Antioxidant Power (FRAP) was used to measure the antioxidant power of samples in both experimental groups, after 10 and 17 days, as described by Benzie and Strain (1996), in technical triplicates. The principle of FRAP analysis is the reduction of ferric-tripyridyltriazine (Fe^{III}-TPTZ), which is colourless, to an intensely blue ferrous (Fe^{II}) form with an absorption maximum at 593nm. This reduction is carried out by potential antioxidants present in the sample, at low pH. As a standard, ascorbic acid was used. Results were presented as equivalent of µg of vitamin C per mg of protein.

The level of malondialdehyde (MDA), as an indicator of lipid peroxidation due to oxidative stress, was determined according to the method of Rehncorna et al. (1980), in technical duplicates. The basis of this method is the formation of a coloured complex, with an absorption maximum at 532 nm, through a reaction between MDA and thiobarbituric acid (TBA). MDA content was expressed in nmols per mg of protein. Protein concentration in samples was obtained using the Bradford method, with γ-globulin as a standard (Bradford 1976).

Statistical data analysis

The results were analysed in the Statistica v13 statistical program using two-way ANOVA. In the case of a statistically significant difference between groups or over time (with age), the differences between groups and samplings were subsequently analysed using one-way ANOVA and post-hoc Tukey's test.

RESULTS

Polyamine content

The content of polyamines (putrescine, spermidine, and spermine) was analysed in the control group of bees fed with a 50% sucrose solution after 10 and 17 days (labelled as C 10d and C 17d, respectively), as well as in the group whose diet was supplemented with 0.01 mM spermidine, also after 10 and 17 days (labelled as $S_{0.01}$ 10d and $S_{0.01}$ 17d, respectively). The results of the two-way ANOVA are presented in Table 1 and show that differences in putrescine and spermidine content are influenced by age (10 or 17 days), treatment (spermidine supplementation), and the interaction of these two factors. Differences in spermine content are influenced

Table 1. Two-way ANOVA of polyamine content in whole honey bee body in control goup fed with sucrose (C) and spermidine supplemented group ($S_{0.01}$), after 10 days (10d) and after 17 days (17d).

	Effect	dF	MS	F	*P* value
Putrescine	Age	1	37,46	14,26	0,005422**
	Treatment	1	305,86	116,38	0,000005***
	Age x Treatment	1	313,15	119,16	0,000004***
	Error	8	2,63		
Spermidine	Age	1	0,10205	13,175	0,006687**
	Treatment	1	0,45215	58,378	0,000061***
	Age x Treatment	1	0,42667	55,089	0,000075***
	Error	8	0,00775		
Spermine	Age	1	0,00746	1,642	0,235960
	Treatment	1	0,01492	3,282	0,107640
	Age x Treatment	1	0,15003	33,007	0,000431***
	Error	8	0,00455		

Abbreviations: df – degree od freedom, MS – mean square, F – factor. $^{**}P < 0.01$, $^{***}P < 0.001$.

only by the combination of these two factors.

Further, the results of the one-way ANOVA (Fig. 1A, B, C) have shown that when comparing the control groups after 10 and 17 days (C 10d *vs.* C 17d), the content of putrescine and spermidine decreased with aging, while the content of spermine remained unchanged. On the other hand, when comparing bees whose diet was supplemented with spermidine to the control group fed only with sucrose, the results indicate that after 10 days (C 10d *vs.* $S_{0.01}$ 10d), there were no changes in the concentration of putrescine, spermidine or spermine, while after 17 days of supplementation (C 17d *vs.* $S_{0.01}$ 17d), their concentrations increased (Fig. 1A, B, C). The contribution of individual polyamines to the overall polyamine content is depicted in Figure 1D.

FRAP and MDA

FRAP assay and malondialdehyde (MDA) concentration were analysed, like polyamine content, in the control and spermidine-supplemented groups of bees after 10 and 17 days of the experiment (designated as C 10d, C 17d, $S_{0.01}$ 10d, and $S_{0.01}$ 17d, respectively). The results of the two-way ANOVA are presented in Table 2 and show that differences in the FRAP assay results are influenced by age (10 or 17 days) and the interaction of the two factors (age and spermidine treatment). Differences in MDA content are influenced by both of these factors, as well as their interactions.

The FRAP assay (Fig. 2A) revealed that antioxidant capacity decreased with aging in the group fed only sucrose (C 10d *vs.* C 17d), while it remained unchanged in the supple-

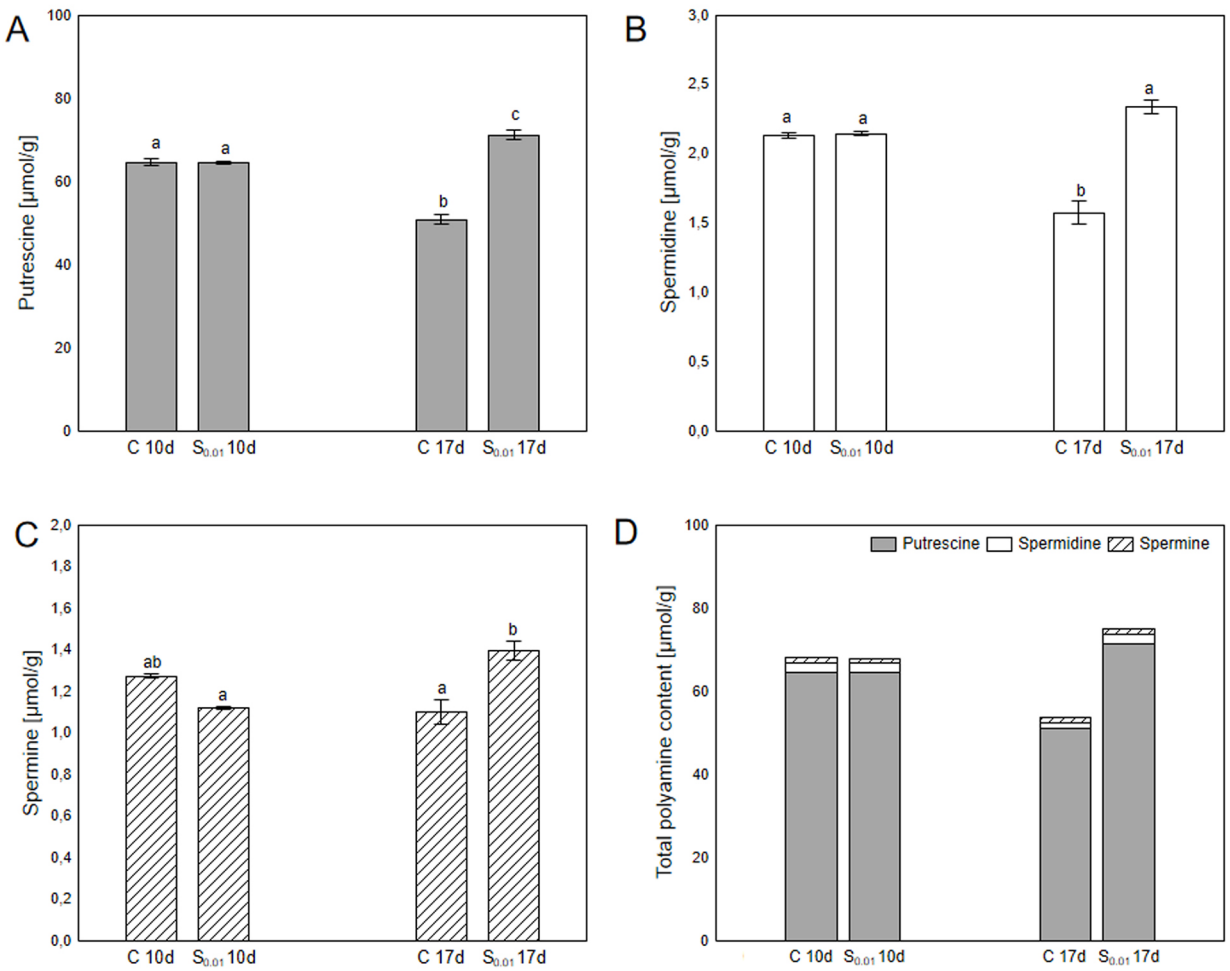

Fig. 1. Putrescine (A), spermidine (B) and spermine (C) concentration in whole honey bee body as µmol/g DW (dry weight) after 10 (10d) and 17 days (17d), and the contribution of individual polyamines in the overall polyamine content(D). Control C was fed with 50% sucrose solution, while the diet of experimental group $S_{0.01}$ was supplemented with 0.01 mM spermidine. Data is presented as mean ± standard error (SE). Differences between mean values were analysed with one-way ANOVA and post-hoc Tukey's test. Different letters indicate differences among all experimental groups shown on the graph for $P < 0.05$

Table 2. Two-way ANOVA of FRAP assay results and MDA level in whole honey bee body in control goup fed with sucrose (C) and spermidine supplemented group ($S_{0.01}$), after 10 days (10d) and after 17 days (17d).

	Effect	dF	MS	F	*P* value
FRAP	Age	1	90,961	20,0129	0,000125***
	Treatment	1	6,669	1,4672	0,236278
	Age x Treatment	1	73,485	16,1677	0,000419***
	Error	27	4,545		
MDA	Age	1	0,40698	15,718	0,000831***
	Treatment	1	0,44722	17,272	0,000537***
	Age x Treatment	1	0,50844	19,636	0,000287***
	Error	19	0,02589		

Abbreviations: df – degree od freedom, MS – mean square, F – factor. $^{**}P < 0.01$, $^{***}P < 0.001$.

mented group of bees ($S_{0.01}$ 10d *vs.* $S_{0.01}$ 17d). On the other hand, when comparing bees whose diet was supplemented with spermidine to the control group, the results indicate that after 17 days of supplementation, the antioxidant capacity increased in bees fed spermidine (C 17d *vs.* $S_{0.01}$ 17d) (Fig. 2A).

Results for MDA (Fig. 2B) are opposite to those of the FRAP assay, showing that MDA concentration increased with aging in the group fed only sucrose (C 10d *vs.* C 17d), while it remained unchanged in the supplemented group of bees ($S_{0.01}$ 10d *vs.* $S_{0.01}$ 17d). The spermidine treatment had an effect, as after 17 days of supplementation MDA concentration decreased in bees whose diet was supplemented with spermidine (C 17d *vs.* $S_{0.01}$ 17d) (Fig. 2B).

DISCUSSION

Honey bee health is an important topic due to the essential role that bees play in pollination, food production, and sustaining biodiversity (Potts et al. 2010). Threats to honey bee health include parasites and pests, pathogens, poor nutrition, and sublethal exposure to pesticides (López-Uribe and Simone-Finstrom 2019). Efforts to protect honey bee health include the strategy of supplementing their diet as a key factor for preserving their health. It provides bees with essential nutrients, strengthens their immune system and maintains an energy balance.

This study was aimed at investigating the effect of low-dose spermidine supplementation of honey bees on their individual polyamine levels and antioxidative capacity, during 10 and 17 days. Even though there is substantial evidence that polyamine content decreases during the aging process in other model organisms (Eisenberg et al. 2009; Soda et al. 2009; Minois et al. 2012), this phenomenon has not been widely explored in honey bees. Results from the present study demonstrate that the content of all three individual polyamines increased in the experimental group whose diet was supplemented with 0.01 mM spermidine, compared to control. In the control group, these levels did in fact decrease for putrescine and spermidine during the 17 days of experiment. These results are in accordance with the previously mentioned studies performed on different model organisms. More important, the results obtained by HPLC in the present study align with the results regarding individual polyamine content in the same model organism after higher-dose spermidine supplementation (Đorđievski et al. 2023). Also, a parallel between this and previously mentioned study can be drawn regarding the contribution of individual polyamines to overall polyamine content. The order of contribution is putrescine>spermidine>spermine. This order is different in mammalian tissues as demonstrated in Minois (2014), highlighting the fact that there is a diversity regarding polyamine levels in different organisms.

Maintaining the stability of polyamine levels in the body is a mode of preserving cellular redox homeostasis (Jiang et al. 2023). Natural polyamines themselves can act as ROS scavengers (Ha et al. 1998). Numerous other studies have pointed out the antioxidative activity of spermidine, either directly or indirectly, at the level of genes whose products are involved in defence against oxidative stress, or proteins, acting as antioxidant enzymes (Eisenberg et al. 2009; Guo et al. 2011; Minois et al. 2012; LaRocca et al. 2013; Đorđievski et al. 2023). To assess the antioxidative effects of spermidine supplementation, two assays, FRAP and MDA, were used in this study. FRAP assay is a method used to measure the antioxidant capacity of various biological samples in contrast to MDA assay, a commonly used biomarker of oxidative damage. FRAP and MDA assays showed that the spermidine-supplemented group increased its antioxidative capacity and decreased its lipid peroxidation level, compared to the non-supplemented group, after 17 days. The control group was characterised with a decreased antioxidative capacity and

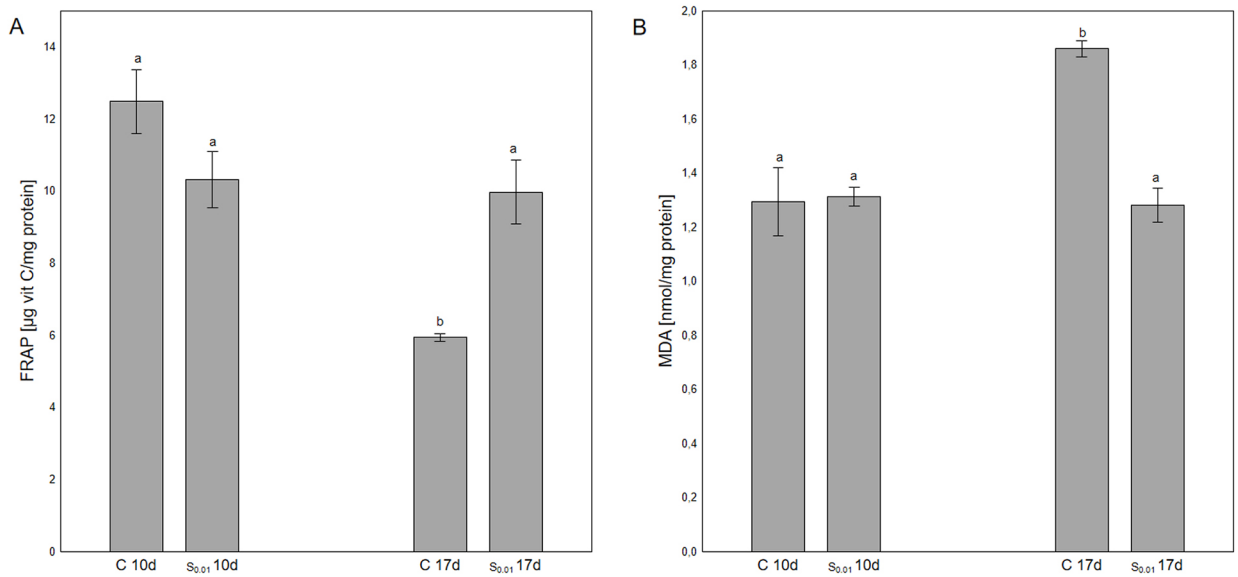

Fig. 2. FRAP (µg vit C/mg protein) (A) and MDA level (nmol/mg protein) (B) after 10 (10d) and 17 days (17d) in whole body of honey bees. Control C was fed with 50% sucrose solution, while the diet of experimental group $S_{0.01}$ was supplemented with 0.01 mM spermidine. Data is presented as mean ± standard error (SE). Differences between mean values were analysed with one-way ANOVA and post-hoc Tukey's test. Different letters indicate differences among all experimental groups shown on the graph for $P < 0.05$.

increased oxidative stress with age, thereby confirming the free radical theory of aging (Harman 1956).

We demonstrated that the addition of exogenous spermidine to honey bee feed has beneficial effects on maintaining the level of all three main polyamines, and that it increases resistance to oxidative stress. Although these results are promising in the context of understanding the effects of exogenous spermidine, they lack in providing the underlying mechanism by which this molecule exerts its impact. Also, a slightly different experimental design that includes the exposure of organisms to various stressors could yield information about the extent of the effects of spermidine supplementation. In addition, the previously mentioned study by Đorđievski et al. (2023) showed that the 0.01 mM spermidine concentration had no effect on the average lifespan of honey bees. Despite this fact, the spermidine concentration tested in the present study could easily be implemented in beekeeping practice, due to its health benefits and cost-efficiency.

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