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MELANINE PROTEIN-ENERGY ADDITIVE FROM *Hermetia illucens* LARVAE IN NUTRITION OF CALVES

R.V. NEKRASOV[1], A.A. ZELENCHENKOVA[1], M.G. CHABAEV[1], N.A. USHAKOVA[2]

[1]*Ernst Federal Science Center for Animal Husbandry,* Federal Agency of Scientific Organizations, 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail nek_roman@mail.ru (✉ corresponding author);
[2]*Severtsov Institute of Ecology and Evolution*, Federal Agency of Scientific Organizations, Moscow, Leninskii prosp., 33, 119071 Russia, e-mail naushakova@gmail.com
ORCID:
Nekrasov R.V. orcid.org/0000-0003-4242-2239
Zelenchenkova A.A. orcid.org/0000-0001-8862-3648
Chabaev M.G. orcid.org/0000-0003-1889-6063
Ushakova N.A. orcid.org/0000-0001-7914-1508
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Abstract

One of the natural sources of various biologically active substances, including melanin, are insects. In connection with the possibility of industrial breeding of some insect species for feed purposes and obtaining various products of processing their biomass, an assessment of the biological effectiveness of the substances obtained is necessary. The purpose of the present studies was to study the effectiveness of the use of the melanin protein-energy additive (MPEA) from the larva of the *Hermetia illucens* fly, in feeding black-and-white breed calves to enhance their safety and growth. Researches were carried out on 30 calves of black-and-white breed on the basis of experimental farm «Klenovo-Chegodaevo», Moscow. According to the principle of animal analogues, 3 groups of calves were formed, 10 animals in each. In the first period of the experiment (1-2 months old calves), the animals of the group 2 were fed individually with 5.0 ml of MPEA (6 mg melanin per head daily), and the animals of the group 3 were fed 7.5 ml of MPEA (9 mg melanin per head daily). Starting from 3 month age, a daily dose of the additive was increased to 7.5 and 10 ml per head, respectively, in the experimental groups. The duration of the experiment was 89 days. The study of the chemical composition of MPEA showed the absence of crude fiber (chitin), with a high content of protein, fat, minerals. In the experimental additive the concentration of melanin was 1.2 mg melanin/ml. MPEA practically did not contain pathogenic microorganisms and was recognized as non-toxic. During the experiment, the calves receiving the MPEA an average daily gain and gross gain were 4.13-2.43 kg and 46.44-27.34 g (or 4.23 and 2.49 %) higher, res[ectively, than those in the control group, with 4.1 and 2.4 % decrease in feed consumption per 1 kg of live weight gain. In the calves of the experimental groups, there was a tendency for lowering total blood protein level by 3.45 and 2.71 g/l compared to the control due to reducing the fractions of albumins by 1.86 and 1.29 g/l, and globulins by 1.60 and 1.43 g/l, respectively. Also, ALT activity decreases by 4.3 IU/l ($p < 0.05$ for group 3) and 2.38 IU/l ($p > 0.05$ for group 2). The content of lysozyme, per cent of lysis, the blood BA level of the calves from the experimental groups turned out to be practically the same, 0.47-0.49 μg/l, 27.27-28.28 %, and 80.39-82.35 %, respectively. However, the FA index of the calves from the group 2 and group 3 was 5.94 and 6.95 % higher, respectively, compared to the control. In the calves of the group 2 and group 3, the number of lacto- and bifidobacteria in the colonic intestine increased, by 2.23×10^5 and 10.3×10^5 CFU/g for *Lactobacillus*, and by 0.33×10^8 and 1.07×10^8 CFU/ml for *Bifidobacteria* compared to the control. There was a decrease in the amount of lactose-positive coliforms by 1.196×10^5 and 1.11×10^5 CFU/g, respectively, compared to the control animals. Calculation of the feeding efficiency of the MPEA during the experiment showed a profitability of (+)381.65 and (+)180.90 rubles, or (+)4.29 and(+) 2.03 rubles per head daily, in the experimental calves when compared to the control animals. MPEA dosage was established experimentally, and, possibly, is not definitively precise, since this is the first study of MPEA biological effectiveness for calves. Further research is needed to identify the biological effect of the *Hermetia illucens* larvae on various species of farm animals.

Keywords: calves, larvae, *Hermetia illucens,* melanin, immunity, microflora

Respiratory and gastrointestinal diseases of young animals result in low

viability, reduced growth rates and, often, death. These factors still remain serious challenges in livestock husbandry. Pre-weaning and the next transit growing period are most important in life of young animals since the need for nutritious substances due to the intensive animal growth is high, and development of fermentative systems of gastrointestinal tract has not been terminated yet. In pre-weaning period mother's milk plays important role in antibacterial protection of calves. In transit period the risk of diseases is especially high since milk in the diet gradually decreases in amount, while own immune system is still developing [1]. In new-born calves aged 1-30 days the core place takes digestion malfunction manifesting in diarrhea and, consequently, in sharp dehydration, anophthalmia, toxemia, and immune deficit [2]. Massive gastroenteritis in calves are mainly caused by infection agents, including viruses, microbes, protozoan, and fungi, virulence of which is increased under unfavorable breeding and keeping conditions [3].

Antimicrobial medicines are widely used for treatment and prevention of bacterial infections in animals but often do not provide the desired outcome, in particular, because of adaptive variability of microorganisms and immune deficit of animals under the effect of medicinal products [4]. More attention is drawn to bioactive feed additives able to stimulate non-specific immunity [5, 6] for prevention and treatment of mixed gastrointestinal infections and digestion disorders caused by malfunction of gut microbiocenosis [7, 8]. Natural herbal medicines and extracts of animal organs and tissues are perspective [9, 10]. Melanin is of significant interest [11]. Melanin, a condensed phenol compound, is irregular high-molecular biopolymer which contributes to dark color of insect cover, human hair, and cell wall of mushrooms, plants, and microorganisms [12]. Various functional groups, highly stable paramagnet centers, and conjugated double bonds in the molecule ensures diverse use of melanin as photo-, radio-protector and antioxidant [13].

Melanin refers to the most powerful natural antioxidants. Presence of melanin in feeds may promote their longstanding storage [14]. During digestion, melanin is partially used by intestinal microflora and also serves as enterosorbent and peristalsis regulator affecting intestinal microflora composition. Melanin is an active antidote at severe intoxications and effective removers of toxins from intestinal tract before their penetration into blood [15]. Melanin is used for treatment and prevention of hepatic diseases, stress, and tumors. Melanin is a powerful natural adaptogen and also has an anti-aging effect [16].

Insects are natural sources of various bioactive substances, including melanin [17]. Given possible farming of insects for various derivatives, the assessment of biological effectiveness of these substances is of interest [18].

Black soldier fly-larva (*Hermetia illucens*) deserves the attention. This species is considered the most perspective for industrial breeding and use of larva biomass as animal feed [19, 20] and in aquaculture [21, 22]. These insects do not accumulate pesticides or mycotoxins [23, 24] and are rich in protein and fat [25]. Chemical composition of larva partially depends on composition of growth medium [26]. Their lipid profile partially simulates the lipid profile of the substrate, at that, micronutrients such as minerals and vitamins are easily accumulated [27]. N.S. Liland et al. [28] confirm plasticity of the nutritional composition of larva allowing accumulation of lipids, as well as various water-soluble compounds.

Melanin and its effect on animal productivity and meat quality are not studied enough. A.I. Bastrakov et al. [29] had shown high anti-infection properties of melanin-chitosan complex from dead flies and empty covers of pupa cas-

es. Additive from larva of *Hermetia illucens* fly potentially accelerates the growth and development of young farm animals, increases their survivability and stress resistance. Feeding this additive to young animals allows them additional important nutrients, since fly larva contain full-value proteins and balanced ratio of mineral substances [30].

In this paper, melanin protein-energy additive (MPEA) from fly (*Hermetia illucens*) larvae was for the first time used in feeding farm animals. This results in improved composition of intestinal microbiocenosis, reduction of the diarrhea cases in calves during pre-weaning and transit periods, better feed conversion, higher daily growth rates, and increased survivability of young animals.

Our purpose was to study the effectiveness of use of melanin protein-energy additive from the larva of *Hermetia illucens* fly, in feeding Black-and-White calves to enhance their survivability and growth.

Techniques. Black-and-White pre-weaning calves (n = 30) aged 1-4 months were involved in study (experimental farming unit Klenovo-Chegodaevo, Moscow, 2017). Three groups, each of 10 analogue animals, were formed. Animals of group I (control) were fed basic ration (combined feed, whole and regenerated milk, cereal grass hay, maize silage and mineral additives) according to scheme accepted in the unit. Dietary Melanin Protein Energy Additive (MPEA) was used in groups II and III. Till 3-month age animals of group II were individually fed orally with 0.5 ml of MPEA (6 mg of melanin per animal daily), and animals of group III received 7.5 ml of MPEA (9 mg of melanin per animal daily). Starting from 3-month age, the dosage was increased up to 7.5 and 10 ml of melanin per animal daily. Studies lasted 89 days. Feed consumption and costs per living weight gain was estimated daily. Calves were weighted individually at beginning and at the end of test (in morning hours before feeding), as well as monthly during tests for determination of gross and average daily growth.

Feeds were sampled for chemical analysis subject to the State Standard GOST P ISO 6497-2011. Metabolic energy was expressed per raw nutritive substances [31], rations were calculated using software KormOptimaExpert (Version 2016.15.1.1, Kormoresurs LLC, Russia).

MPEA was produced from the mixture of larva and prepupa of *Hermetia illucens* flies (1:1) which were raised on cracked maize grain. Squeezed biomass was dissolved in distilled water (1:3) following by 2 hour sterilization of 250 ml suspension at 100 °C in vials. MPEA feeding dose was calculated based on content of water-soluble melanin in the additive. Melanin concentration was determined in 100 ml suspension aliquot after sedimentation with HCl and drying the residue. Suspension was preliminary centrifuged 15 minutes at 1200 g for separation of non-melanin component (centrifuge OPN-8, JSC DASTAN MNC, Russia). The obtained melanin residue was separated by centrifuging in the same mode, then neutralized, dried, and weighed. Melanin concentration in the test suspension was 1.2 mg/ml.

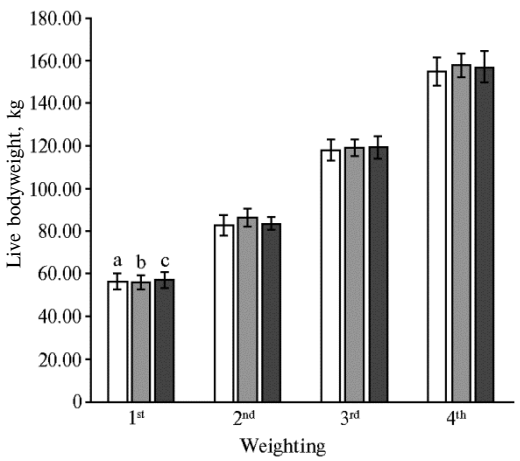
MPEA components were determined as follows: initial moisture content by GOST P 54951, air-dry substance by GOST 31640-2012, protein by GOST 32044.1-2012, fat by GOST 32905-2014, cellulose by GOST 31675-2012, nitrogen-free extractive substances by calculation, ash content by GOST 32933-2014, gross metabolic energy by calculation, calcium by GOST 32904-2014, and phosphorous by GOST P 51420-99). MPEA sample was also tested for *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella* spp., *Escherichia coli* (hemolytic), *Staphylococcus saprophyticus*, *Enterobacter* spp., *Citrobacter* spp., yeast-like fungi and mildew. Total toxic effect was assessed on 3-5-day culture of infusoria *Tetryhymena pyriformis*, toxicity degree was estimated as survivability

infusoria in the tested medium.

Excrements were individually collected from calves of each group (n = 3) at the end of the experiment. Microbiological profile was determined by serial dilution methods. Samples of gastrointestinal content were diluted 10-fold. Portions of 0.2 ml for spread-plating method and 1.0 ml for pour-plating method were used to isolate and identify microorganisms on selective and differential media with counting colony forming units (CFU/g or CFU/ml).

At isolation and determination of opportunistic pathogenic microorganisms, samples were incubated in liquid selective medium, and then plated on solid selective and diagnostic medium to confirm taxonomic affiliation of typical and atypical colonies. Identification criteria were morphology of colonies, microscopy, and biochemical properties on differential media (State Science Center of Applied Microbiology and Biotechnology, Russia) and with the use of test panels (BioMerieux, France). MRS and Bifidum media were used for identification of lactobacilli (lactobacteria and bifidobacteria), Endo-GRM agar for bacteria of *Escherichia* genus, meat peptone agar (MPA) with 5% sterile defibrinated sheep blood for hemolytic *Streptococcus* spp. and *E. coli* bacteria, and Sabouraud agar with 5 % potassium tellurite for yeast and yeast like fungi. Plates with 30-300 colonies were used for counting. Number of microorganisms (N) was calculated as $N = m \cdot V^{-1} \cdot d^{-1}$, where m stands for the average arithmetic number of colonies per two Petrie dishes, V stands for volume of inoculate in a dish (cm^3), and d stands for dilution coefficient.

At the end of test, blood was collected from animals of each group (n = 3). In samples of whole and stabilized blood, total protein, albumin, globulin, creatinine, urea, total bilirubin, total cholesterol, calcium, phosphorous, glucose, activity of alkaline phosphatase (AP), aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), hemoglobin, erythrocytes, lymphocytes, hematocrit value were determined by approved methods. Blood bactericide activity (BA) was measured photonephelometrically, blood lysozyme level was assayed by V.I. Mutovin (1974), phagocytic activity (PA) — by endocytosis and digestive ability of blood cells.



Living weight of Black-and-White pre-weaning calves fed dietary bioactive additive from larvae of *Hermetia illucens* fly: a — I group (control, n = 10), b — II group (n = 10), c — III group (n = 10). See description of groups in section "Methodology" ($M \pm m$, experimental farming unit "Klenovo-Chegodaevo", Moscow, 2017).

Expected economic effect of the additive used in calf preweaning period was calculated from the data on feed consumption, costs, and growth gain.

Results were processed by dispersion analysis method (ANOVA) with the use of Statistica 10 software (StatSoft, Inc., USA, 2011; http://www.statsoft.com). Average arithmetic mean values (M), error of mean square ($\pm m$) and significance level (p) were calculated. Statistically, results were deemed highly reliable at $p < 0.001$ and significant at $p < 0.01$ and $p < 0.05$. Trend towards reliability was recognized at $p < 0.1$, but $p > 0.05$. The difference was invalid at $p > 0.1$.

Results. According to some authors' opinion [32], MPEA mani-

fests biological effect at 0.1 mg/kg of living weight. This was the base value to calculated feed additive dosage in our tests.

The obtained biomass of black solder fly larva contains 36 % protein and 45 % fat. After defatting, the outcome of squeezed mass with 70 % moisture was 775 g from 1 kg of raw biomass.

Comparison of MPEA and protein concentrate compositions (Table 1) had shown high content of raw chitin in air-dry substance (ADS) of protein concentrate (PC) and its complete lack in MPEA ADS. MPEA ADS is high in protein, fat, and mineral elements, with melanin concentration of 1.2 mg/ml. The additive is also free from pathogenic microorganisms and non-toxic.

Initial weight of all calves was practically equal (from 56.0 to 56.90 kg) (Table 2, Fig.). Dietary MPEA accelerates growth of the animals, and the final indicators in groups II and III were 2.8 and 2.0 kg higher (or +1.81 and +1.29 %), respectively, compared to the control group.

1. Chemical composition of melanin protein energy additive (MPEA) and protein concentrate produced from larva of *Hermetia illucens* fly

Indicator	Feed specimen			
	MPEA		protein concentrate	
	NS	ADS	NS	ADS
Initial moisture, %	71.06		1.89	
Air-dry substance, %	28.94	100.00	98.11	100.00
Protein, g/kg	94.46	326.40	533.62	543.90
Fat, g/kg	126.84	438.29	50.82	51.80
Raw chitin, g/kg	–	–	183.07	186.60
NFES, g/kg	17.68	61.09	92.62	94.40
Ash, g/kg	38.20	132.00	76.13	77.60
Gross energy, MJ/kg	7.36	25.43	19.99	20.38
Metabolic energy, MJ/kg	5.45	18.83	14.55	14.83
Calcium, g/kg	4.68	16.17	15.00	15.29
Phosphorus, g/kg	3.58	12.37	5.10	5.20

N o t e. NS — natural substance, ADS — air-dry substance; NFES — nitrogen-free extractive substances. Dashes mean that no chitin was detected.

2. Growth, survival and feed consumption by Black-and-White pre-weaning calves fed dietary Melanin Protein Energy Additive (MPEA) from *Hermetia illucens* fly larvae ($M \pm m$, experimental farming unit "Klenovo-Chegodaevo", Moscow, 2017)

Indicator	Group		
	I (control, $n = 10$)	II ($n = 10$)	III ($n = 10$)
Living weight, kg:	56.30±4.02	56.00±3.45	56.90±3.79
at beginning of test			
at the end of test	155.00±6.67	157.80±5.76	157.00±7.42
To control, %	100.00	101.81	101.29
Gross growth rate, kg	97.67±5.18	101.80±5.25	100.10±7.64
Average daily growth rate, g	1097.38±58.23	1143.82±58.98	1124.72±85.82
To control, %	100.00	104.23	102.49
Survival, %	90.00	100.00	100.00
Gross consumption of ME, MJ per animal daily	2830.20	2830.20	2830.20
Consumption of EFU per animal daily	3.18	3.18	3.18
Feed consumption, EFU per kg weight gain	2.90	2.78	2.83
To control, %	100.00	95.90	97.60

N o t e. ME — metabolic energy, EFU — energy feeding unit. See description of groups in section "Methodology".

Average daily growth rate in calves of groups II and III was 46.44 g and 27.34 g, or by 4.23 % and 2.49 %, higher than in control group. The animals of group II showed the highest growth rates. Feed consumption per 1 kg gain in groups II and III decreased by 4.1 % and 2.4 % as compared to control due to increase of average daily gain. Animals were willingly consuming MPEA without any rejections. Calves from trial groups fell ill less frequently, no diarrhea and lethal cases occurred, while in group I one calf died.

Although studied blood values do not significantly differ between groups

remaining within the physiological limits, there are some specific differences (Table 3). Total blood protein in groups II and III decreases by 3.45 and 2.71 g/l as compared to control because of a decrease in albumin level by 1.86 and 1.29 g/l and in globulin level by 1.6 and 1.43 g/l. Total protein level is not enough to fully assess diet quality. Blood ALAT and ASAT activity additionally indicates on whether the diet is high-grade and allows characterization of protein metabolism intensity and hepatic function. We have confirmed reliable decrease of ALAT activity (by 4.3 IU/l, $p < 0.05$)) in calves of group III as compared to control. In group II this indicator was 2.38 IU/l less than in control. ASAT activity shows the same trend and is 7.63 and 7.93 IU/l less in groups II and III compared to control. Decrease in activity of these enzymes indirectly indicates a stabilizing effect of the additive on free amino acids which are less subjected to catabolism and more effectively involved in protein biosynthesis.

In this experiment dietary MPEA does not reliably affect alkaline phosphatase and blood Ca and P concentrations in calves, at slight increase in Ca/P ratio in animals of group III. Blood lysozyme level, lysis percentage, and bactericidal activity were practically similar, i.e. 0.47 and 0.49 μg/l, 27.27 and 28.28 %, 80.39 and 82.35 %. PA was 5.94 and 6.95 % higher in calves of groups II and III, respectively, at $p < 0.05$ for group III, as compared to control.

3. Hematological indicators and resistance parameters in Black-and-White preweaning calves fed dietary Melanin Protein Energy Additive (MPEA) from *Hermetia illucens* fly larvae ($M \pm m$, experimental farming unit Klenovo-Chegodaevo, Moscow, 2017)

Indicator	Group		
	I (control, $n = 3$)	II ($n = 3$)	III ($n = 3$)
Total protein, g/l	78.56±2.17	75.11±1.74	75.85±1.05
Albumin, g/l	33.08±1,16	31.22±0.64	31.79±1.36
Globulin, g/l	45,49±3.33	43.89±1.12	44.06±1.43
Albumin/globulin coefficient	0.74±0.08	0.71±0.01	0.72±0.05
Cholesterol, μmol/l	3.37±0.03	3.60±0.38	3.12±0.36
Creatinine, μmol/l	68.58±6.99	70.39±3.27	62.76±4.40
Urea, μmol/l	5.40±0.41	5.10±0.50	6.49±0.39
Total bilirubin, μmol/l	8.10±3.06	8.46±0.66	7.17±0.84
ALAT, IU/l	15.86±1.03	13.48±1.54	11.56±0.71*
ASAT, IU/l	65.52±5.69	57.89±5.67	57.59±3.04
Triglicerides, μmol/l	0.94±0.04	0.95±0.03	0.86±0.11
Alkali phosphatase, IU/l	361.33±24.35	435.48±56.05	244.71±65.05
Glucose, μmol/l	5.17±0.14	5.15±0.21	4.66±0.85
Calcium, μmol/l	2.84±0.08	2.87±0.07	2.83±0.08
Phosphorous, μmol/l	2.93±0.03	3.12±0.11	2.72±0.33
Ca/P ratio	1.25±0.02	1.19±0.02	1.40±0.21
Iron, μmol/l	35.64±2.03	34.55±2.39	31.64±2.27
Leucocytes, $\times 10^9$/l	12.92±0.25	13.26±0.88	12.30±0.51
Erythrocytes, $\times 10^{12}$/l	10.74±0.32	10.83±0.31	11.17±0.43
Hemoglobin, g/l	105.37±5.39	102.40±3.77	108.77±2.28
Hematocrit, %	41.21±2.18	41.92±0.81	41.98±0.83
Lysis, %	28.28±1.01	27.27±1.75	27.27±1.75
Lysozyme:			
mkg/ml blood	0.49±0.02	0.47±0.03	0.47±0.03
s.u.a, un.a/mg of protein	1.59±0.10	1.60±0.13	1.59±0.12
BSBA, %	82.35±0.00	80.39±1.30	80.39±1.30
PA, %	48.20±1.45	54.14±1.84	55.15±1.83*
PI	3.39±0.18	2.98±0.09	3.28±0.14
PN	1.63±0.10	1.61±0.06	1.81±0.09

N o t e. A/G — albumin/globulin, ALAT — alaninaminotransferase, ASAT — aspartaaminotransferase, Ca/P — calcium/phosphorous, BSBA — blood serum bactericidal activity, PA — phagocytic activity, PI — phagocytic index, PN — phagocyte number; s.u.a, un.a/mg — specific units of activity. See description of groups in section "Methodology".

* Differences from control are statistically significant at $p < 0.05$.

In large intestine of animals from groups II and III counts of lactobacteria increased by 2.23×10^5 and 10.3×10^5 CFU/g, of bifidobacterium — by 0.33×10^8

and 1.07×10^8 CFU/ml, respectively, as compared to control (Table 4). Abundance of lactose-positive *E. coli* forms decreases by 1.196×10^5 and 1.11×10^5 CFU/g ($p < 0.05$ for group III). No lactose-negative *E. coli* forms were found in animals of trial groups, whereas these forms were present in one calf of the control group (see Table 3).

4. Qualitative and quantitative composition of microbial flora of large intestine in Black-and-White pre-weaning calves fed dietary Melanin Protein Energy Additive (MPEA) from *Hermetia illucens* fly larvae ($M\pm m$, experimental farming unit Klenovo-Chegodaevo, Moscow, 2017)

Microorganisms	Group		
	I (control, $n = 3$)	II ($n = 3$)	III ($n = 3$)
Lactobacillus, $\times10^5$ CFU/g	3.10±1.35	5.33±0.99	13.4±3.68*
Bifidobacillus, $\times10^8$ CFU /ml	1.33±1.04	1.66±0.84	2.40±1.30
Hemolytic microorganisms, CFU g:			
streptococci, $\times10^3$	2.20±0.08	2.30±0.53	2.35±0.69
Escherichia coli	Not found	Not found	Not found
E.coli, CFU/g:			
lactose-positive, $\times10^5$	1.20±1.20	0.004±0.001	0.09±0.09
lactose-negative	Found in 1 specimen	Not found	Not found
Fungi of *Candida* genus, CFU/g	Not found	Not found	Not found

N o t e. CFU — coli form unit. See description of groups in section "Methodology".
* Differences from control were statistically significant at $p < 0.05$.

MPEA amounts per animal in groups II and III during the experiment were 520.0 ml and 742.5 ml. Cost of 1 l of the additive is 100.00 rubles, costs per animal in tests are 52.00 and 74.25 rubles. Revenue from conventional sale is (+)1355.35 rubles for control group I, (+)1737.00 rubles for group II, and (+)1536.25 rubles for group III. In the trial groups the indicator increases because of living weigh gain due to dietary MPEA. Additional revenue per each calf of groups II and III during tests is also in line with these findings. During the entire period total revenue, as compared to the control, is (+)381.65 and (+)180.90 rubles, or (+)4.29 and (+)2.03 rubles per animal dayly,.

Other authors report on anti-stress effect of melanin (0.1 mg/kg) derived from yeast *Nadsoniella nigra* X-1 [33], with no diseases and deaths of post-weaned pigs. Melanin in stressed animals promotes normal proteinase inhibition and prevents cytolytic syndrome of pancreatic gland [34, 35].

Thus, in this experiment differences in growth rates of calves fed dietary Melanin Protein Energy Additive (MPEA) from larva of *Hermetia illucens* fly were not statistically significant that allows for an increase of daily MPEA dosage. Interestingly, MPEA micro dose influences positively non-specific immunity and intestinal microbiocenosis. Biological effect of MPEA correlates with higher growth, animal survival rates, and better fed conversion. The MPEA doses established herein empirically are not ultimately recommended since biological effectiveness of the additive is studied for the first time. Further studies will clarify biological effect of both *Hermetia illucens* fly larva and larva-derived extracts of physiologically active substances on health and productivity of farm animals, poultry, and fish.

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