

Structure of the Midgut of the Queen Bee *Apis mellifera* L. Naturally Infected with *Sacbrood virus* (SBV)

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Abstract: Bees and their larvae are susceptible to various viral diseases, which leads to disruption of the bee family, and sometimes to its death. The *Sacbrood virus* is especially dangerous for the bee family. Sick families differ sharply in their condition and productivity from healthy ones, develop slowly in the spring, and grow little brood. Therefore, the aim of the study was to identify physiological changes in the intestines of mature uterus affected by *Sacbrood virus* or SBV disease. Histological examinations supplemented the data on the pathogenesis of honey bees affected by *Sacbrood virus*. Data on physiological and morphological changes in the middle part of the intestine of fertile queens are presented. Histological examinations were performed in different parts of the midgut. In patients of queens, in comparison with clinically healthy ones, changes in the function of the peritrophic membrane were revealed. An increase in the length of the midgut fold in the anterior part of the intestine by 23% ($p < 0.001$) was detected. Under the influence of pathogenicity of the virus there is an intensive proliferation of enterocytes in the middle part of the intestine. The defeat of the structural components of the mucosa is evidenced by the absence of a peritrophic membrane in the caudal direction of the midgut of diseased queens where necrotic lesions of the mucous membrane up to the basement membrane were detected.

Keywords: *Apis mellifera* L., *Sacbrood virus* (SBV), Fertile Queens, Midgut, Physiology Nutrition

1. Introduction

Honey bees are the main pollinators of entomophilous crops [1]. They, like all other insects, are exposed to infectious diseases [2-4].

The health of bees largely depends on immunity, which is affected by numerous abiotic and biotic factors [5]. The social way of life of large groups on the one hand helps them to counteract pathogenic factors [6]. On the other hand, such cohesion is an ideal place for the activity and spread of pathogens of many diseases [7, 8]. One of them is *Sacbrood virus*.

Sacbrood virus or SBV disease (*Sacculisato contagiosa larvae*) is a viral disease of honey bee larvae, which is characterized by the deposition of aqueous-granular fluid under the integumentary tissues of the body, which makes the larvae look like sacs [9-11].

The causative agent is the RNA-genomic virus *Morator aetatae* of spherical shape, with a diameter of 28-30 nm. [2,

12]. Affects the larvae of worker bees, queens and drones at 2-3 days of age during feeding infected food. The most sensitive are 4-7-day-old larvae, which die in the prepupa stage, less often - pupae. The incubation period lasts 5-6 days. Adult bees usually get sick without visible symptoms. However, SBV damage can also affect adult bees. At the same time, there is a reduction in their lives [13].

The disease occurs mainly in the first half of summer, more often after a sudden cold snap, in weakened families, in the absence of food or varroasis. The causative agent of the infection is adult bees, in whose body the virus can persist throughout the winter. The spread of the pathogen can be the carcasses of infected pupae, each of which contains so many virions that it can infect up to 3,000 healthy larvae. Within the bee family, the virus is spread by feeding bees, and between families by drones, wandering bees, as well as during the rearrangement of brood cells with brood and honey from sick families to healthy ones. The spread of the disease may also

depend on the hygiene of the apiary and the lack of disinfection of apiary equipment.

The entry of large amounts of nectar and pollen into the nests helps to reduce the incidence and even the disappearance of SBV disease, but in the autumn or spring of next year it reappears in the infected apiary. SBV is often associated with European or American rot or paragnylitis, which makes it very difficult to diagnose and organize control measures [14].

At SBV the mechanism of emergence and development of a disease, and also its display is characteristic. According to the literature, the saccular brood virus enters the midgut of 1–3 diurnal larvae together with food, and from there into the integumentary tissues and begins to multiply [13].

Reproduction of the virus in the cells of the integumentary tissues of the larvae is accompanied by the formation of specific rounded bodies-inclusions of black color. Together with the fat cells, the inclusion bodies give the destroyed tissues a granular appearance. Affected forebrains become brown, followed by sclerotization of their tissues. Affected larvae lose their pearly luster, become dull, increase significantly in volume and die at 8–9 days of age after being sealed with wax caps. The presence of variegated brood, which includes healthy, sick and dead larvae, is a characteristic clinical sign of pathology of family development. Covers over the dead larvae in many cells darkened, inflamed, perforated. Bees often open them completely or partially, which gives the impression of the death of open brood. In such cells are placed dead forelegs, the bodies of which are elongated along the lower wall. The development of the head of diseased larvae usually lags behind, the head area is darker than the rest of the body color and may lean towards the center of the cell. The dead forebrain is filled with watery-grained contents, has a flabby appearance, pale yellow color at the beginning of the lesion, then turns brown and becomes dark brown when dry. In contrast to putrefactive diseases, in SBV dead larvae do not decompose and have no odor. When removed from the cell, the recently died larva resembles a bag of fluid, the front end of which is raised to the exit of the cell, which is a characteristic feature of this disease. After drying, the corpses of the forebrains turn into dark brown crusts, which are easy to remove from the cell [15]. Scientists have developed methods for field research in the diagnosis of SBV [3].

However, there are insufficient data in the literature on the pathogenicity of the virus in the intestines of fertile uteruses. Therefore, the aim of the study was to identify physiological changes in the mature stage of individual development of affected SBV. Namely, to investigate the way in which morphological changes of midgut occur in the fertile queens of honey bees.

2. Material and Methods

The experimental part of the work was carried out in the conditions of Stepan Gzhytskyi National University of Veterinary Medicine and Biotechnologies Lviv (Ukraine). The research was conducted during 2018–2020 on a farm located

in the Stryj district of the Lviv region. The apiary of the farm had 230 bee families. The subject of the study were fertile queens aged 2 years. Control group (clinically healthy) 5 queens and experimental group (sick) 5 queens. Signs of SBV damage were found on the farm in 2018 and 2019. The diagnosis was confirmed on the basis of epidemiological data, clinical signs and laboratory tests. The control group included clinically healthy bee colonies. The study of interior measurements involved the study of morphometric parameters of midgut. It was dissected and histomorphologically examined. To do this, the fertile queens were dorsally immersed for 1/2 in Petri dishes with melted wax. After curing the wax, 30 ml of isotonic NaCl solution was poured into a Petri dish. In this medium, entomological scissors were used to dissect the pleural membranes that connect the sternites and tergites in the direction from the latter to the former. Each part of the sternite was removed to make the contents of the abdomen accessible. In some cases, the abdomen was fixed to the wax with entomological needles. Breast fixation was performed with a needle. Then the second needle was inserted into the sting chamber and the last abdominal sternitis of the abdomen was fixed. The abdomen was dissected by making two incisions with scissors. The midgut was then dissected. This drug was transferred to a glass slide and examined and measured length. Examination was performed using a stereoscopic microscope at a magnification of x 20. To study morphometric and histological studies, the midgut was fixed in a Buena fixator (24 hours). After using Buen's fixative, the material was washed in alternating portions of 70 ° alcohol until it was completely discolored. The recorded samples were poured into paraffin by the method of Horalskiy [16]. Also, according to classical methods, from paraffin blocks were made review histopreparations with a thickness of 5–7 µm, which were stained with hematoxylin and eosin. The study was performed histopreparations, which were obtained at a distance of 3, 7, 11 mm from the beginning of the midgut. Measurements of the length of the midgut folds were performed from the basement membrane to the apical edge, including microvilli. 10 folds arranged in order were chosen for measurement. Morphometric parameters of enterocytes were obtained by measuring their height, width and area. In the study of drugs paid attention to the state of histoarchitectonics of the mucous membrane, the preservation of structural and functional units, the integrity of epithelial cells, the presence of inflammatory processes. All digital research material was subjected to statistical processing [17] using standard software "StatPlus 2008". Differences between the average values of bees of the experimental group to the control were considered statistically significant at * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3. Results

The middle part of the intestine in the uterus is represented by the midgut. The segmental section gives an idea of the anatomical structure of the midgut. Figure 1 shows the histological structure of the anterior midgut of a clinically

healthy fetal uterus.

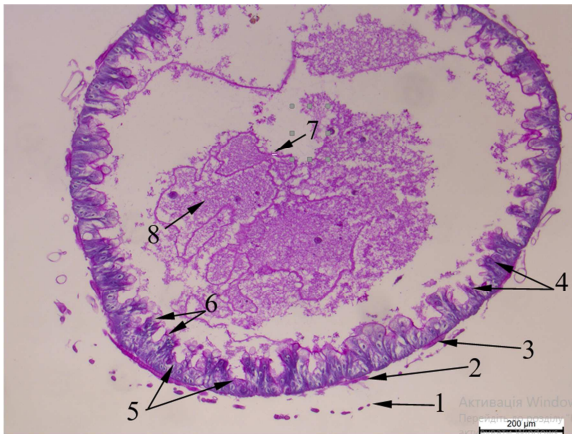


Figure 1. Segmental section of the anterior midgut clinically healthy fetal queen: 1 - longitudinal outer muscular layer, 2 - circular muscular layer, 3 - oblique muscle, 4 - intestinal folds, 5 - discrete crypts, 6 - proliferating small epithelium digestive cells, 7 - peritrophic membranes, 8 - containing intestines. (Magnification: ob. 8 x oc. 7, staining: hematoxylin-eosin).

The mucous membrane of the midgut of the queens of honey bees is represented by a single layer of epithelium, which is lined with many folds. This feature of the structure is due to the need to increase the surface area of the suction.

Food that gets into the midgut is contained in the peritrophic membranes. And each portion of food is enveloped by another membrane. Obviously, the layers of the peritrophic membrane, which are produced by epithelial cells, cover each part of the feed as it enters. Fertile queens consume royal jelly, so no pollen grains are found in the figure. When the SBV is affected, the membrane is disrupted due to motor, secretory and other functions of the midgut. This in turn leads to dystrophic and inflammatory changes of the superficial epithelium, as well as inhibits the regeneration process.

The function of the anterior part of the midgut is to produce digestive enzymes (mainly proteases and lipases). At research of this department midgut of uterus of the affected SBV some changes are shown (Figure 2).

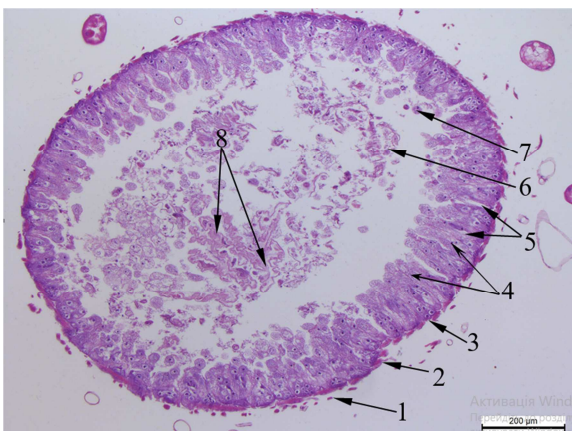


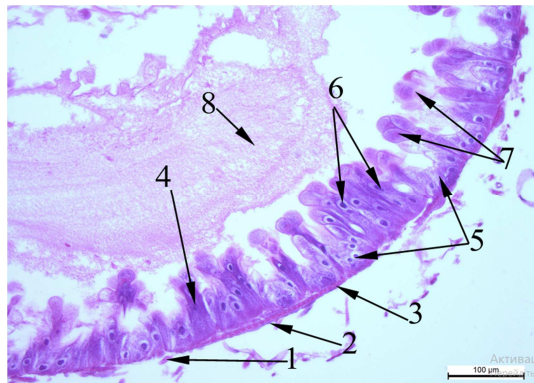
Figure 2. Segmental section of the anterior midgut of the fetal queen affected by SBV: 1 - longitudinal outer muscular layer, 2 - circular muscular layer, 3 - basement membrane, 4 - goblet cells, 5 - discrete crypts, 6 - intestinal contents, 7 - enterocyte, 8 - peritrophic membrane. (Magnification: ob. 8 x oc. 7, staining: hematoxylin-eosin).

The cytoplasm of enterocytes is filled with small and dense homogeneous inclusions. It is intact and has clear boundaries. On the segmental section of the midgut, in the cranial direction, there is an increase in the number of folds of the epithelium per unit area of the intestine. The distance between the enterocytes of the small folds is so small that in some sections it is visually impossible to see in the discrete crypts of the mucosa. Enterocytes are significantly elongated compared to midgut epithelial cells in healthy queens. Moreover, in some enterocytes the nucleus is located at the base of the basement membrane. The core is oval. The method of attaching mucosal cells deserves attention. The figure shows that the base of some enterocytes is attached to the basement membrane by a narrower end. At that time, the apical cells are expanded. The cytoplasm contains numerous secretory vacuoles. By shape, structure and functional purpose, such cells are called goblet cells. They are responsible for the production of protein-like substances of the peritrophic membrane. Goblet cells have almost no cilia and produce mucus, which is released on the surface of the epithelium. The placement of the nucleus in the cell is due to the fact that it is pushed to the base by the accumulated secretion. An increase in cell size due to an increase in the number of functionally active cells may indicate protective hyperplasia. As can be seen from the figure, the number of goblet cells increases in the distal direction of the intestine. If we pay attention to the histopreparation made from the midgut of a healthy individual, we can say that in pathology, due to *Sacbrood virus*, goblet cells are found in the midgut, in which they are normally either absent or present in much smaller quantities. Along with this, the defeat of the structural components of the mucosa indicates the absence of peritrophic membrane. In this case, in the lumen of the intestine there are exfoliated enterocytes. Most of them are discolored. This indicates intense secretion of the merocrine type.

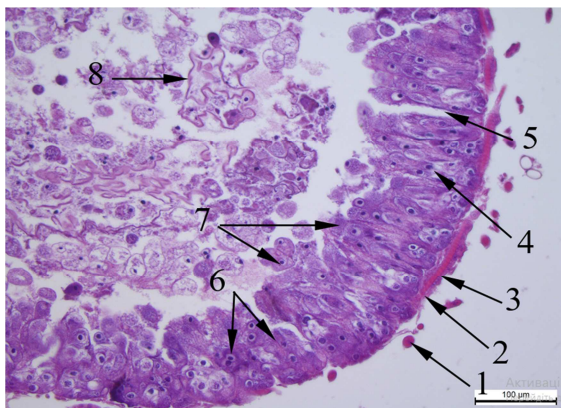
Figure 3 shows the changes that occur in the midgut of clinically healthy and virus-infected queens.

Studies of the midgut of the affected SBV suggest that in the middle department, which is responsible for the digestion of nutrients, there are changes that are not characteristic of the architecture of some cell structures of the medial direction. Examination of the segmental section of the midgut of the fetal uterus revealed that the number of intestinal folds is not clearly expressed. An increase in the number of regenerative cells was detected in the depth of diskette crypts. The nuclei of these enterocytes are clearly visible. However, the membrane membranes of older enterocytes do not have clear boundaries. Proliferative small epithelium of digestive cells was found on the apical surface of enterocytes. The cytoplasm of these cells, when stained with hematoxylin-eosin, is colored red, indicating that it is filled with substances of a proteinaceous nature, possibly proteases. In the lumen of the intestine you can find a significant number of nuclear cells with clear thin contours, which are completely discolored. The filling of these cells with enzymes is minimal because they are involved in digestive processes. The increase in the number of cells is balanced by their death and differentiation of cells that are

unable to divide. Examination revealed progressive cell proliferation. The proliferation index is increased in relation to indicators in healthy individuals.



A

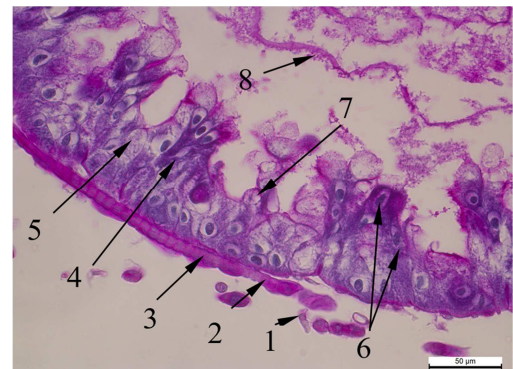


B

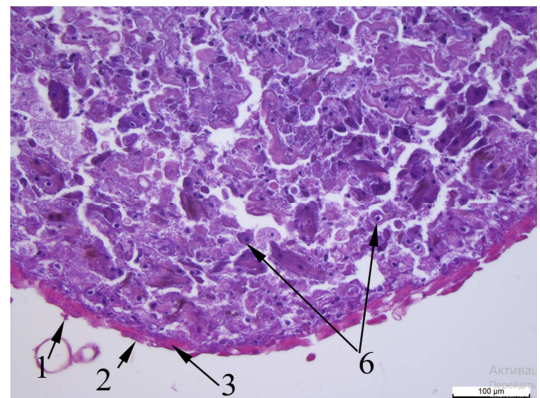
Figure 3. Segmental section of the middle midgut of the fetal queens clinically healthy (A) and affected SBV (B): 1 - longitudinal outer muscular layer, 2 - circular muscular layer, 3 - oblique muscle, 4 - intestinal fold, 5 - discrete crypts, 6 - enterocytes, 7 - proliferating small epithelium digestive cells, 8 - peritrophic membrane. (Magnification: ob. 10 x oc. 7, staining: hematoxylin-eosin)

In the sick body in most cases, the death of enterocytes does not occur due to aging, but under the influence of harmful factors (necrosis). Cells that have not performed their function die. The final destruction of cells is accompanied by an inflammatory process caused by the products of their decay. Figure 4 shows the differences in the posterior midgut of the uterus. In clinically healthy individuals, all structural components of the epithelium are clearly visible. The movement of food is carried out by means of a group of muscles. The following take part in this process: the longitudinal outer muscular layer, the circular muscular layer, the oblique muscle. In discrete crypts of folds clearly

distinguish regenerative epithelial cells. The distance between the folds ranges from 15 to 35 μm . Each fold contains 2-5 enterocytes of different degrees of maturity. On the apical surface of which proliferating small epithelium digestive cells are well visible. However, it is impossible to state unequivocally about the presence of rhabdiorium on the surface of enterocytes. Perhaps the membranes of enterocytes are covered with a thin layer of gelatinous mucus, which contains enzymes [18]. The width of the ecto-peritrophic space is smaller compared to the height of the intestinal wall.



A



B

Figure 4. Segmental section of the posterior midgut of the fetal queens clinically healthy (A) and affected SBV (B): 1 - longitudinal outer muscular layer, 2 - circular muscular layer, 3 - oblique muscle, 4 - intestinal fold, 5 - discrete crypt, 6 - enterocytes, 7 - proliferating small epithelium digestive cells, 8 - peritrophic membrane. (Magnification: ob. 10 x oc. 7, staining: hematoxylin-eosin)

Using morphometric research methods, we obtained an interesting pattern for intestinal folds of midgut in fertile uteruses (Table 1).

Table 1. The size of the intestinal folds midgut in fertile queens, μm ($M \pm m$).

Group of uteruses	Direction of midgut					
	cranial		medial		caudal	
	M \pm m	lim	M \pm m	lim	M \pm m	lim
Clinically healthy	152.12 \pm 5.46	138.19–199.33	161.23 \pm 4.45	146.16–201.04	169.93 \pm 4.45	155.22–218.65
Sick	170.58 \pm 1.8*	140.11–208.19	178.58 \pm 1.81**	125.91–226.14	12.11 \pm 0.11	0–14.76

Note: The difference with clinically healthy is probable at * $p < 0.05$; ** $p < 0.01$.

The table shows the length of the folds of the midgut which are obtained at a distance of 3, 7, 11 mm from the beginning of the intestine of the subjects. In clinically healthy queens, the length of the folds ranges from 138.19 to 218.65 μm . The smallest folds are observed in the front. In this part, the average length of the folds is $152.12 \pm 5.46 \mu\text{m}$. In the medial department, which is responsible for the secretion of digestive enzymes, there is a slight increase in the length of the intestinal folds. Positive dynamics in relation to the morphological parameters of the folds is manifested in the posterior part. In this part of the intestine, the average length is $169.93 \pm 4.45 \mu\text{m}$. The apparent increase in fold length is related to the physiological function of the midgut. The functional purpose of the distal part is the process of absorption of nutrients in the feed.

Analyzing the data in the table, we can see the morphological indicators of midgut development in the uterus affected by SBV. Depending on the location of the histo section, the length of the folds ranges from 0 to 14.76 μm . In particular, in the anterior part of the uterus of the affected SBV revealed a 12% increase in the length of the folds compared to healthy individuals. In the medial part of the midgut, a similar increase was found compared with healthy individuals: the length of the folds is probably increased by 11% ($p < 0.01$). A completely different situation was observed in the back of the midgut. The maximum fold length did not exceed the mark of 14.76 μm . Moreover, in most cases, necrobiotic foci of mucosal lesions were detected.

4. Discussion

The disease of SBV families is found everywhere where bees are bred [12, 19, 20]. Among viral diseases, SBV is considered the most common. The spread of the pathogen is facilitated by the migration of bees and especially Varoa mites. Bees infect larvae when fed infected food. In this case, the bees in the middle of the hive, in contact with dead larvae, become carriers. The virus enters the hemolymph through the midgut, which performs the function of digestion and absorption of food in honey bees.

There are insufficient data in the literature that due to the defeat of SBV the imaginary form of honey bees *Apis mellifera* L. does not show clinical signs. However, studies of the digestive system reveal new data on the pathogenesis of honey bees affected by SBV. It should be noted that the lesion of midgut cells in SBV has significant differences compared with the histological changes that are characteristic of the lesion of *N. cerana* [21-23]. The physiology of forage digestion in midgut in fetal queens affected by SBV is insufficiently described. Its length in experimental queens was 12-13 mm. The color of the midgut depends on the combination of muscle fibers and the outer shell of the connective tissue. In experimental queens dissected midgut, translucent and had a reddish-brown appearance. In worker bees, pollen and honey that has entered the midgut are exposed to enzymes of different spectrum of action [24, 25].

These chemicals are concentrated in the peritrophic membrane [26]. Similar processes are found in fertile queens. The presence of several layers of peritrophic membrane indicates a rather complex and gradual transformation of the feed. However, the peritrophic membrane does not protect the mucosa from damage by pollen because it is absent in the feed.

The conducted researches give the basis to consider that at sick individuals signs of defeat are found out in various sites of a midgut. Moreover, the relationship between the degree of damage and the distance from the beginning of the intestine. The proximal (anterior) part of the intestine, which is responsible for the secretion of digestive enzymes, began to perform the function of the distal, which is responsible for the absorption of nutrients. This is evidenced by the increase in the length of the folds of the anterior midgut. At first glance, the presence of all structural components of the intestine indicates that this part of the intestine physiologically copes with its functions. However, if we pay attention to the presence of the peritrophic membrane, we can see that compared to healthy individuals, its number is insufficient. In the caudal department there are foci of new destruction, which are morphologically characterized by necrobiotic changes of the newly formed epithelium. Of particular note is the fact that the virus penetrates the damaged peritrophic membrane, which serves as a factor of immune protection. During RNA replication, SBV virus, like other RNA-containing viruses, has a high mutation rate, which contributes to the huge diversity of SBV strains circulating in both *Apis cerana* and *Apis mellifera* populations worldwide [27]. There are currently 52 strains of SBV that can be found in the GenBank database [13].

A characteristic feature of SBV is the death of larvae in different parts of the cell. Moreover, under the influence of the virus affects all the internal organs of the larva, turning into a granular mass. The chitinous cover of larvae becomes dense and keeps putrefactive weight.

Insects have a variety of mechanisms to control infection with pathogens. Many of them are protected by a layer of antimicrobial secretions on their outside and the intestinal environment, hostile to pathogens. When pathogens go beyond these defenses, epithelium is often sufficient to stop further progress. If pathogens overcome the morphological defenses of insects, they often encounter factors of effective cellular and humoral immune defense. Insect immunity demonstrates many parallels with the innate immune response of humans and other vertebrates, involving a diverse set of actions, including antimicrobial peptide secretion, phagocytosis, melanization, and enzymatic degradation of pathogens [28, 29]. For example, significant violations of the epithelial lining of the trachea and peritrophic membrane caused by SBV infection [30] can lead to infection of hemolymph by bacteria in the tracheal or intestinal lining.

Different viruses affect the immunity of bees in different ways. In particular, infection with the DWV virus (deformed wing virus) leads to disruption of the intestinal epithelium. Therefore, the infection can significantly impair the digestive

process. DWV virus spreads throughout the body, including the ovaries and the fatty body of the uterus [31-34]. Acute bee paralysis virus (ABPV) revealed a significant degree of tissue specificity manifested by the accumulation of the pathogen almost exclusively in the salivary glands, with a slight accumulation in the midgut [19]. The slow paralysis virus (SPV) had a slightly wider distribution, also accumulating in the brain and adipose body [31]. Unlike several other viruses, some scientists found no evidence that *V. destructor* was able to transmit CWV during feeding, and concluded that if the virus was transmitted by a tick, the virus could not be infected as an overt infection in honey bees [35].

Bees belonging to *Apis mellifera* are more resistant to SBV infection compared to *Apis cerana* bees [13, 38].

With the onset of the main medical collection (first half of July), the signs of the disease usually disappear, there is a kind of temporary recovery. However, in the spring of next year there is a recurrence of the disease.

As with European rot, the degree of development of SBV is determined by the number of dead larvae, which the beekeeper detects when examining bee colonies affected by saccular brood [36].

One of the features of insect viruses is their ability to remain dormant in the host for many generations. The virus does not cause visible symptoms of disease. However, it can be activated by any internal or external factor. Spring climate variability and insufficient food supply are probably factors contributing to the intensive spread of the disease each spring.

At a time when the honey bee's body is developing new defenses against the pathogen, the latter is forced to adapt its own strategy of invasion and infection to counteract the host's immune defenses. Therefore, it has been suggested that the more stable the immunity of bees, the more often more pathogenic strains will be detected, and this in turn will accelerate the evolution of pathogens [27, 37-41].

The productivity of queens is determined by the number of eggs laid by her. Observations of the dynamics of egg laying indicate a decrease in egg production by 40-60% compared with healthy queens. During the day, the uterus affected SBV laid 800-1050 eggs. Such a decrease in the number of eggs laid by the uterus may be the cause of insufficient supply of nutrients during oogenesis due to damage to the epithelium of the midgut. The study of ways to strengthen the immunity of honey bees has an economic justification. There is a direct relationship between the productivity of the bee colony and the number of affected brood. In such families, the amount of honey obtained is reduced by 75-85%.

5. Conclusion

The information presented in this study expands the data on the pathogenesis of honey bees affected by *Sacbrood virus*. In the mild form of SBV, the symptoms of the disease usually remain asymptomatic in many infected bees. Most individuals remain functional. However, in fertile queens affected by the SBV virus, changes in the structure of the digestive system may occur. In patients of queens, in

comparison with clinically healthy ones, changes in the function of the peritrophic membrane were revealed. An increase in the length of the midgut fold in the anterior part of the intestine by 23% ($p < 0.001$) was detected. Under the influence of pathogenicity of the virus there is an intensive proliferation of enterocytes in the middle part of the intestine. The defeat of the structural components of the mucosa is evidenced by the absence of a peritrophic membrane in the caudal direction of the midgut of diseased queens where necrotic lesions of the mucous membrane up to the basement membrane were detected.

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