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THE EFFECTIVENESS OF VARIOUS METHODS FOR DETERMINING THE VIABILITY OF *A. SIMPLEX* LARVAE IN FISH PRODUCTS

Shevchuk T.V. *– Doctor of Agricultural Sciences, Associate Professor,*
Vinnitsa National Agrarian University,
Tatjana.Melnikova@ukr.net

The article presents the results of a comparative analysis of various methods for determining the viability of A. simplex larvae in fish products. The method of activating larvae in the physiological solution was the fastest, affordable and less expensive. The method of incubation in artificial gastric juice received more unsatisfactory estimates. Its execution is possible only in the presence of reagents and a thermostat.

During parasitological studies, the severity of the A. simplex invasion in fish products was 82,7%, the intensity of the invasion was 3-10 units, the invasion index was 7.8 units. All A. simplex larvae that have been detected in fish have been gradually tested by different viability methods.

It has been experimentally established that different methods for determining the viability of A. simplex larvae have different accuracy. The least accurate methods have been found to activate live larvae in a physiological solution with mechanical and electrical stimulation. More precise was the method for determining the viability of A.simplex larvae using methylene blue. During the painting live parasites retained their natural color, and the dead – acquired a blue color. The disadvantage of this method is the duration of the experiment and the ambiguity of the results of the study. Separate larvae after removal from capsules were painted differently. Therefore, the interpretation of research results was complicated. In this method, the largest number of live parasites were detected in a group of fish products. In addition, A. simplex live larvae were detected by this method, not only in the abdominal cavity, but also in the wall of the abdominal cavity and muscles.

The most accurate methods were electrical activation, color and digestion in artificial gastric juice. We were able to detect A. simplex live larvae that were not identified in saline solution, even after exposure to a thermostat and mechanical stimulation, by these methods. In this case, the accuracy of the method of electrical irritation was 4.35% lower than the color of the indicator and the use of artificial gastric juice.

In the future, it is planned to investigate the effectiveness of the method of availability and determination of the viability of A. simplex larvae by biological resonance method.

Keywords: invasion, A. simplex larvae, methods, viability, fishery products, efficiency.

Introduction. Anisakiasis is a parasitic gastrointestinal disorder caused by anisakis larvae. People become infected by eating raw or improperly cooked or canned sea fish. The surviving worms penetrate the intestinal wall and enter

the abdominal cavity. Symptoms are often non-specific, but more often it is abdominal pain, nausea and vomiting. Abdominal pain may persist for several weeks.

Anisakiasis is common in Europe (the Netherlands), Japan and the USA (and more and more cases of anisakis have been observed recently in Russia, because of the fashion for Japanese cuisine, sushi bars and restaurants in Russia). People become infected by eating fish containing viable third-stage larvae. However, humans are random hosts, since the transmission of parasites to humans cannot lead to the full life cycle of the parasite [4, 12, 20, 23, 26].

The types that cause anisakiasis in humans are *Anisakis simplex* and *Pseudoterranova decipiens*. The invasive larval stage of the parasite can be found in the internal organs and in the muscles of various fish [3, 6, 9, 8].

Non-infectious and invasive forms of anisakiasis are different. In a non-invasive form, the symptoms are rare, because the larvae do not damage the mucous membrane of the human digestive tract. In this form, a cough from pain and irritation of the throat may occur. The larvae can pass from the esophagus to the pharynx and stand out through the mouth. This occurs less than two weeks after eating infected fish. In the literature there are reports of the transition of larvae from the throat to the respiratory tract and lungs [22, 24, 25].

In the case of an invasive form of anisakiasis, the larvae penetrate the mucous membrane of the tract, especially into the stomach and small intestine. There have been cases of movement of larvae in the omentum, gallbladder, hepatic ducts, pancreas [16, 21].

Unfortunately, most cases of the disease remain unrecognized. For example, in Japan, out of about one hundred cases of anisakiasis, half the cases were incorrectly delivered. Therefore, methods for identifying viable larvae in fish products remain important.

Analysis of recent publications. Requirements for sampling methods for laboratory research, standards for assessing the nutritional suitability of fish products and the conditions for its use as a food product in the presence of parasites in fish belonging to one group or another are determined by sanitary norms and rules (1996). Practical recommendations for assessing the infection of marine commercial fish with parasites are given in the «Methodology of parasitological examination of marine fish and fish products (sea fish, chilled and frozen fish)», and in more detail in «Methods of sanitary and parasitological examination of fish, mollusks, crustaceans, amphibians, reptiles and their products» [2, 7].

For the detection of nematode larvae in the muscles of fish, the method of fluorescence of organisms under ultraviolet irradiation is used. Researchers note that dead worms are especially fluorescent after freezing meat. At the same time, the luminescent characteristics of nematodes of different species are

different: the *Anisakis simplex* larvae have bright bluish-white fluorescence, and the larvae and adults of *Hysterothylacium aduncum* vary from white to bright yellow [5, 15].

The method includes preparing a protein-antigenic extract from the third-stage larvae of *Anisakis simplex*, monitoring it for sterility, determining the protein content, preparing hyperimmune serum to the protein-antigenic extract from third-stage larvae *A Simplex* by immunizing rabbits with complete Freund's adjuvant; carrying out the immune diffusion reaction (RID) on an agar gel with the obtained hyperimmune serum and liquid formed during the defrosting of seafood [1, 10, 11, 18]. The manifestation of a precipitation strip between this fluid and hyperimmune serum to the protein-antigen extract of *A. simplex* indicates the presence of larvae antigens in seafood. The method is effective in detecting anisakids antigens in a liquid obtained by thawing the muscle tissue of fish [13, 14, 17, 19].

According to the rules of veterinary and sanitary examination, if individual parasites damage fish (up to 5 parasites per 1 kg of weight), it is sold without restrictions, and if the fish has more than 5 parasites per kilogram of weight and depletion, the fish is sent for industrial processing. At + 55°C the larvae die in a few minutes. If the fish has at least one parasite (or larvae) that can be detected with the naked eye, the fish is not for sale [2, 7]. Therefore, the effective detection of parasites in fish products and the determination of their viability is of great importance for the consumer. In this regard, the main goal of our study was the methods for determining the viability and degree of infection of herring (Atlantic and Black Sea herring) by the parasites of the *Anisacidae* family. The **object** of the study was methods for determining the viability of parasites, and the **subject** of research was the effectiveness of these methods and the degree of infestation of various types of herring treatment by parasites.

The aim of the work is to establish the species identity and viability of the larvae. *A. simplex* are found in the meat of sea fish.

Materials and methods. Material of our own research was obtained by us as a result of veterinary and sanitary examination 4 samples from industrial batch of products. Samples came to us from the trading Vinnitsa markets. For the examination were taken four species of fish arriving as food raw materials to the consumer market.

Laboratory studies of fish and fish products for compliance with safety requirements for human health from the point of view of parasitic purity are based on parasitological surveys.

Parasitological study of marine fish includes the identification of parasites that are dangerous to human health and alter the physicochemical properties of fish or spoil the appearance of commodity raw materials and products in parts of the body of fish (usually meat) that are sent for use as food. In some

cases, parasites of the liver, gonads (caviar) are taken into account, as well as parasites of the body cavity, if food products are made from small fish (capelin, Baltic herring, sprat) [2, 7].

We began the study with an external examination and weighing of fish. Then the left abdominal wall was cut after a short cut with medical scissors from the fish's anus, and the fish was cut in the midline of the abdomen to the angle of the lower jaw. The next cut was made from above, actuate, along the lateral line. Thus, the abdominal wall was separated. The fish turned on the right side. Conducted a survey of the body cavity and internal organs in order to identify the larvae of nematodes.

Next, the internal organs were removed, the sex glands (caviar or jelly) were cut out, which were placed in separate Petri dishes. Inspected the swim bladder. They cut out and examined the heart as well as the cavity of the heart. The body cavity was rubbed with a gauze napkin, and the peritoneum was scraped off.

We have prepared a set of digestive organs for external examination. Adipose tissue was cut into thin plates about 5 mm thick and examined for the presence of nematode larvae.

In the study of the digestive tract, freed from adipose tissue, the larvae were found in capsules on its surface or appeared through the serous membranes of the skin.

On external examination of the liver, larvae of *A. simplex* are visible on its surface with the naked eye. The liver was examined and cut into plates with a thickness of about 5 mm.

In the genital glands, we cut the shell, scraped the contents and examined them using the compressor method. This method is convenient for viewing only caviar. When examining large eggs, they must be disassembled with the help of dissecting needles in a Petri dish with a small amount of water.

After examining the internal organs, we removed the skin from the fish in the direction from the head to the tail, cut it with scissors and pulled it with surgical tweezers. Then they examined the inner side of the skin, and part of the muscles separated from the skin was cut into plates. We conducted a study of the muscles by the method of parallel incisions, but in some cases we used the method of examining muscle tissue for lumen.

The recommended technique for examining the muscles is to cut the muscles of the fish into pieces no thicker than 5 mm. The helminth larvae, localized in the musculature, are well detected in such areas with incident light. Thicker muscle sections should be viewed in transmitted light, for which each individual slice is placed on a transparent or milky-matte glass, and the light source (incandescent or fluorescent lamp) is placed below. These two methods are distinguished by their simplicity, and when they achieve a certain skill, they allow the detection of both living and dead parasites in the muscles of the fish.

The determination of the viability of helminth larvae that are hazardous to human health can be carried out using the following methods:

1. The nematode larvae are placed in a physiological solution. Nematode larvae are part of the cyst. At a temperature of 37 – 40°C, live helminth larvae show marked physical activity. The movement of worms can be stimulated by needle pricks. Mobility studies are best performed under a microscope or with a magnifying glass, especially when the larva is inactive or appears stationary. Then the larvae of *A. simplex* (in physiological solution) are placed for 2-3 hours in a thermostat with a temperature of + 37°C. At the same time, noticeable physical activity appears in living nematode larvae. Lack of motor activity, discoloration, detachment of the skin and other destructive changes in the body indicate that larvae of *A. simplex* are not viable.

2. Nematode larvae are placed on wet filter paper and exposed to direct electric current (battery with a voltage of 1.5 V). The manifestation of contractile movements is monitored under a microscope MBS or magnifying glass.

3. Nematode larvae are placed in a 1–2% solution of methylene blue dye. Dead larvae turn blue.

4. In the laboratory, the method of digesting infected marine fish in artificial gastric juice is widely used to identify live helminth larvae. The investigated parts of the fish are placed in a solution of pepsin (10 g of pepsin powder dissolved in 1 liter of 1% hydrochloric acid) and kept for 40 minutes at a temperature of + 52°C. After the specified time, the solution is dissolved. At the same time, the digested mass is filtered through a thin sieve. Motor activity of helminth larvae is well manifested in artificial gastric juice at a temperature of up to 40°C. After deformation and washing, the worms present are counted.

To identify free and larvae in helminth capsules in the body cavity of the fish, a thorough examination of the internal organs is carried out. In the study of the liver, gonad, it is advisable to apply the compressor method – viewing pieces of organs between two glasses in transmitted light.

The types of larvae of nematodes isolated from fish were determined using optical means, for example, a magnifying glass or a microscope.

To diagnose *A. simplex* larvae, we used the structure of the anterior part of the digestive tract as primary differential features, namely, the presence or absence of gastric or intestinal processes, the ratio of body length to esophagus length, body length to ventricle length, and the position of the excretory pore.

The digital material was processed statistically. The resulting digital data was processed using the MS EXEL 98 and Windows program, statistically processed by Student. The results were considered statistically significant at $p < 0.1$, $p < 0.01$, $p < 0.001$. In the table material of the work the following symbols are taken: * $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$.

Research results and discussion. From the tabular material (table 1) it becomes obvious that the method of activation of the larvae in saline is the fastest, most accessible and less expensive.

Table 1. **Characteristics of various methods of determining the viability of the larvae *A.simplex***

Index	Method:				
	Activation in saline with mechanical irritation	Heating in a thermostat to 40 degrees in a physiological solution with mechanical irritation	Electrical irritation	Coloring with methylene blue solution	Splitting in artificial gastric juice
The duration of the experiment	++++	+	+++	+	-
Availability	+++	-	+	+	-
Visibility (readability)	+	+	++	+++	++
Consumption of materials	+++	+	+	+	-

Note: in the table, the indicators were evaluated in relation to: + – satisfactory assessment of the indicator, ++ – rating "good", +++ – "excellent", if the indicator is unsatisfactory, then put "-",

It may even be recommended for home use for self-monitoring. The method of incubation in artificial gastric juice received more unsatisfactory ratings. Its execution is possible only in the presence of reagents and thermostat.

To study the effectiveness of various methods for determining the viability of *A.simplex*, a sample of fish (Atlantic fresh-frozen herring) was taken from the production batch. 25 samples were examined and evaluated organoleptically. The skin of the body of fish, eyes, gills, fin state corresponded to the norm. Samples of fish products were sent to the laboratory for parasitological studies. After opening the fish, we determined the presence and extent of damage to the fish by the larvae. *A.simplex*.

It was previously established that the extensiveness of the herring invasion in the frozen state was 84.5 ± 3.25%, the intensity – 1-12 units, and the invasion index – 6.0 ± 0.34. From each fish in which parasites were found, larvae were harvested. Their viability was determined by different methods. For this, the sample (25 copies) was divided into 5 groups according to the number of methods. The results of the experiment are presented in table 2.

The tabular material indicates that different methods for determining the viability of *A. simplex* larvae have different accuracy. Methods for the activation

of live larvae in physiological saline with mechanical and electrical stimulation were the least accurate. The larvae released from the capsules may have been alive, but did not respond to such weak stimuli (Fig. 1-3).

Therefore, in studies using the method of activation in a warm solution of 0.9% sodium chloride, it is difficult to determine the viability of this helminth. Inaccurate was the method of electrical irritation. The method for determining the viability of *A.simplex* larvae with methylene blue was more successful. When stained, live parasites retain their natural coloration, and the dead – painted (Fig. 4).

Table 2. **The effectiveness of various methods for determining the viability of larvae of *A.simplex*** *(M ± m, n = 5)*

Index	Method:				
	Activation in saline with mechanical irritation	Heating in a thermostat to 40 degrees in a physiological solution with mechanical irritation	Electrical irritation	Coloring with methylene blue solution	Splitting in artificial gastric juice
The intensity of infection with parasites, units	6,8 ± 3,96	8,2 ± 3,77	5,6 ± 4,22	6,4 ± 3,05	7,2 ± 5,22
Intensity of infection with parasites of the abdominal cavity, units	6,2 ± 3,63	7,6 ± 3,05	4,6 ± 3,91	5,6 ± 2,61	5,2 ± 3,42
% of total	91,18	92,68	82,14	87,5	72,22
including live parasites, units	0	0	0	0,6 ± 0,45*	1,67 ± 1,40***
% of total	0	0	0	9,38	23,19
The intensity of damage to the wall of the abdominal cavity, pcs.	0,5 ± 0,23	0,5 ± 0,20	0,9 ± 0,69	0,9 ± 0,69	1,4 ± 1,00
% of total	7,35	6,10	16,07	14,06	19,58
including live parasites, units	0	0	0	0,6 ± 0,45	0,6 ± 0,45
% of total	0	0	0	9,35	8,33
The intensity of muscle damage, pcs.	0,6 ± 0,45	0,6 ± 0,45	1,34 ± 0,6	0,6 ± 0,45	1,3 ± 0,8
% of total	8,82	7,32	23,93	9,38	18,06
including live parasites, units	0	0	0	0	0,6 ± 0,45
% of total	0	0	0	0	8,33

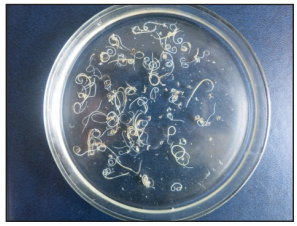


Fig. 1.
Method of activation in saline

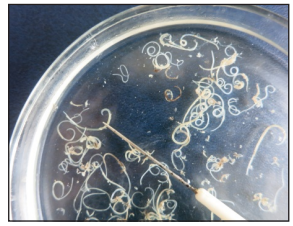
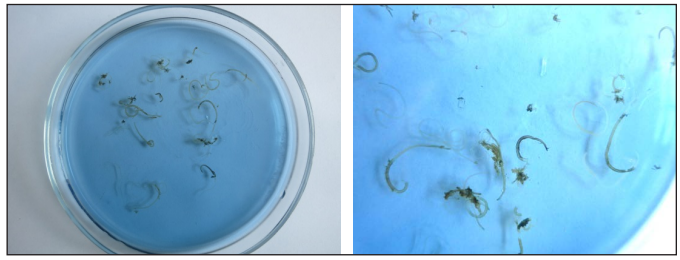


Fig. 2.
Method of activation in saline solution during storage in a thermostat and mechanical irritation

Fig. 3.
Electrostimulation method with current 1.5 W

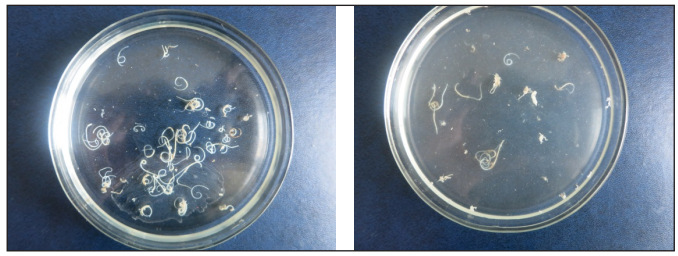


a b

Fig. 4. **Staining method: a – the beginning of staining, b – at the end of staining**

The disadvantage of this method is the duration of the experiment and the ambiguity of the research results. Individual larvae were stained differently after removal from the capsules. Therefore, the interpretation of the research results was difficult.

The most accurate was the method of digesting artificial gastric juice. It turned out that in the group of fish products in which this method was used, the largest number of live parasites was found. In addition, this method was used to detect live larvae of *A. simplex* not only in the abdominal cavity, but also in the wall of the abdominal cavity and muscles (Fig. 5).



a b

Fig. 5. **Splitting method in artificial gastric juice: a – the beginning of the experiment, b – the end of the experiment**

For the purity of the experiment, we selected 4 samples of frozen herring from a batch of fish products. The fish were assessed organoleptically. Conducted parasitological studies. The extensiveness of A. simplex invasion in the samples was 82.7 ± 2.33%, the intensity of invasion was 3-10, and the invasion index was 7.8 ± 0.61 units. All *A. simplex* larvae found in fish were successfully tested for viability by various methods. The results of the study are shown in table 3.

Table 3. **Phased determination of the effectiveness of methods for determining the viability of *A.simplex* larvae**

Index	Method:				
	Activation in saline with mechanical irritation	Heating in a thermostat to 40 degrees in a physiological solution with mechanical irritation	Electrical irritation	Coloring with methylene blue solution	Splitting in artificial gastric juice
The total number of studied larvae, pcs.	23	23	23	23	23
Found live larvae, pcs.	0	0	3	4	4
% of total	0	0	13,04	17,39	17,39

Experience has shown that methods of electro stimulation, indication of the dye and digestion in artificial gastric juice are the most accurate. Living larvae of *A. simplex*, which were not identified in the physiological solution, even after holding the thermostat and needle stimulation, we were able to determine using these methods. At the same time, the accuracy of the electro stimulation method was 4.35% less than when stained with methylene blue and when using gastric juice.

Conclusions. In the course of the research it was found that the method of determining the larvae of *A. simplex* in a physiological solution with mechanical stimulation is the most accessible and easy to use. However, this method was inaccurate.

The method of staining and splitting the larvae in artificial gastric juice was the most accurate and reliable. The latter of these methods was more than 4% more accurate than the electro stimulation method and 17.4% than the salt solution and mechanical stress method.

Prospects for further research. In subsequent experiments, the effectiveness of the method for determining the presence and viability of *A. simplex* larvae by the method of bioresonance will be investigated.

ЕФЕКТИВНІСТЬ РІЗНИХ МЕТОДІВ ВИЗНАЧЕННЯ ЖИТТЄЗДАТНОСТІ ЛИЧИНОК *A.SIMPLEX* У РИБНІЙ ПРОДУКЦІЇ

Шевчук Т.В. *– д.с.-г.н., доцент,*
Вінницький національний аграрний університет, Tatjana.Melnikova@ukr.net

У статті подані результати порівняльного аналізу різних методів визначення життєздатності личинок *A. Simplex* у рибній продукції. Найбільш швидким, доступним та менш затратним виявився метод активації личинок у фізіологічному розчині. Більше незадовільних оцінок отримав метод інкубації в штучному шлунковому соці. Його виконання можливе лише за наявності реагентів и термостату.

Під час паразитологічних досліджень установлено, що екстенсивність інвазії *A. simplex* в рибній продукції становила 82,7%, інтенсивність інвазії – 3-10 шт., а індекс інвазії – 7,8 одиниць. Всі личинки *A. simplex*, які були знайдені в рибі, поступово перевіряли різними методами на життєздатність.

Експериментально установлено, що різні методи визначення життєздатності личинок *A. simplex* мали різну точність. Найменш точним виявились методи активації живих личинок в фізіологічному розчині з механічним і електричним подразненням. Більш точним виявився метод визначення життєздатності личинок *A. simplex* за допомогою метиленового синього. Під час пофарбування живі паразити зберігали своє природне забарвлення, а мертві – набували блакитного забарвлення. Недоліком цього методу є тривалість експерименту и неоднозначність результатів дослідження. Окремі личинки після видалення з капсул зафарбовувалися по-різному. Тому трактовка результатів досліджень була ускладнення.

Самим точним виявився метод перетравлення в штучному шлунковому соці. За допомогою цього методу в групі рибної продукції було встановлено найбільша кількість живих паразитів. Причому, живі личинки *A. simplex* були виявлені цим методом не тільки в черевній порожнині, але і в стінці черевної порожнини та м'язах.

Методи електричного подразнення, пофарбування індикатором и перетравлення в штучному шлунковому соці були найбільш точними. Живі личинки *A. simplex*, які не ідентифікувалися у фізіологічному розчині, навіть після термостатування та подразнення механічно, ми змогли виявити вказаними методами. При цьому точність метода електричного подразнення була на 4,35% меншою, ніж пофарбування індикатором та використання штучного шлункового соку.

На перспективу нами планується дослідити ефективність методу наявності та визначення життєздатності личинок *A. simplex* методом біорезонансу.

Ключові слова: інвазія, личинки *A. simplex*, методи, життєздатність, рибна продукція, ефективність.

ЭФФЕКТИВНОСТЬ РАЗЛИЧНЫХ МЕТОДОВ ОПРЕДЕЛЕНИЯ ЖИЗНЕСПОСОБНОСТИ ЛИЧИНОК *A. SIMPLEX* В РЫБНОЙ ПРОДУКЦИИ

Шевчук Т.В. *– д.с.-х.н., доцент,*
Винницкий национальный агараный универсритет, Tatjana.Melnikova@ukr.net

В статье поданы результаты сравнительного анализа различных методов определения жизнедеятельности *A. Simplex* в рыбной продукции. Наиболее быстрым, доступным и менее затратным является метод активизации личинок в физиологическом растворе. Больше неудовлетворительных оценок получил метод инкубации в искусственном желудочном соке. Его исполнение возможно лишь при наличии реагентов и термостата.

При паразитологических исследованиях установлено, что экстенсивность инвазии *A. simplex* образцов составила 82,7%, интенсивность инвазии – 3-10 шт., а индекс инвазии – 7,8 единиц. Все личинки *A. simplex*, которые были найдены в рыбе, последовательно проверяли разными методами на жизнеспособность.

Экспериментально установлено, что разные методы определения жизнеспособности личинок *A. simplex* имеют различную точность. Наименее точным оказались методы активизации живых личинок в физиологическом растворе с механическим и электрическим раздражением. Более точным оказался метод определения жизнеспособности личинок *A. simplex* с помощью метиленового синего. При окрашивании живые паразиты сохраняли свою природную окраску, а мертвые – окрашивались. Недостатком этого метода является продолжительность эксперимента и неоднозначность результатов исследования. Отдельные личинки после извлечения из капсул окрашивались по-разному. Поэтому трактовка результатов исследований была затруднена.

Самым точным оказался метод переваривания в искусственном желудочном соке. Оказалось, что в группе рыбной продукции, в которой применяли этот метод, было выявлено самое большое количество живых паразитов. Причем, живые личинки *A. simplex* были обнаружены этим методом не только в брюшной полости, но и стенке брюшной полости и мышцах.

Методы электрического раздражения, покраски индикатором и переваривания в искусственном желудочном соке являются наиболее точными. Живые личинки *A.simplex*, которые не идентифицировались в физиологическом растворе, даже после термостатирования и раздражения иглой, мы смогли определить указанными методами. При этом точность метода электрического раздражения была на 4,35% меньше, чем покраска метиленовым синим и использование желудочного сока.

В последующих опытах будет исследована эффективность метода определения наличия и жизнеспособности личинок *A. simplex* методом биорезонанса.

Ключевые слова: инвазия, личинки *A. simplex,* методы, жизнеспособность, рыбная продукция, эффективность.

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