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THE EFFECT OF LOW TEMPERATURES ON THE MOTILITY OF DIPHYLLOBOTHRIUM LATUM PLEROCERCOIDS*)

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The main purpose of fish freezing is to prevent spoilage. However, freezing can also be used to make fish safe for human consumption with regard to the risk of infestation by fish tapeworm, Diphyllobothrium latum. In a previous series of experiments carried out on a size range of fish from 0.5 to 5 kg, the freezing time needed to kill the tapeworm larvae was determined (8). The temperature changes occurring in the fish during freezing were measured, and the treatment was considered to be sufficient when the temperature in the fish had decreased to —10°C. This is the lowest estimate of the lethal temperature of D. latum plerocercoids in the range of —8 to —10°C, as reported in earlier investigations (2, 5, 10, 11). Since for prophylaxis against diphyllobothriasis the lethal exposure of the larvae should be exactly known, and since the method by which the effect of freezing was studied differed from that of the earlier studies, the lethal low temperature exposure of the larvae was to be determined in the same experimental conditions as those in which the fish freezing tests were carried out. Motility was used as the criterion of the viability of plerocercoids.

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MATERIAL

The plerocercoids utilized in the experiments were obtained from pikes and burbots that were brought to the laboratory from the Lappeenranta region (fish from Lake Saimaa, Southeastern Finland). The total number of plerocercoids in the tests was 653. Fish flesh used to prepare the phantoms to simulate the environmental conditions in vivo in fish was acquired from pikes caught from coastal waters of the Gulf of Finland.

The fish had been caught approximately 1—2 days before arrival at the laboratory, and they were dissected within a few hours after arrival.

The medium used for the freezing tests on isolated larvae was normal horse serum without any addition.

METHODS

Isolation of the larvae

In the tests both isolated larvae and larvae enclosed in pieces of tissue were used. The plerocercoids in the fish were located by means of a systematic slicing of the musculature of the fish with a scalpel and by inspection of the viscera. Immediately after isolation the plerocercoids were transferred into horse serum. Larvae intended to be preserved in their natural environment were removed from the fish enclosed in pieces of tissue, the size of which was approximately 1 cm^3. The pieces of tissue were used within 3—4 hrs. to prepare the phantoms specially constructed for the tests. In the meantime the pieces were kept covered by several slices of fish musculature at a temperature of 4 °C, in order to maintain the environmental conditions of the larvae unchanged.

Low temperature exposures of the plerocercoids

a) *Isolated larvae.* The freezing tests on isolated larvae were carried out using horse serum as the medium. For the exposure the larvae were immersed in 3 ml serum in test tubes 12 cm in length and with a capacity of 10 ml. The motility and integrity of the plerocercoids were checked immediately before their immersion in the serum. The test tubes were placed in a freezing room at a temperature of —18 °C.

b) *Larvae in phantoms.* The phantoms were prepared by placing the pieces of tissue, each containing a plerocercoid, in

the central part of a plastic beaker, surrounded by densely packed minced fish musculature. The number of larvae frozen in this way at the same time varied between 10 and 20. The size of the beakers varied from 100 to 600 ml, with diameters of 4.5 to 8.5 cm and heights from 6.5 to 11.5 cm. In the central part of the phantom the decrease of temperature during freezing was considered to proceed in the same manner as in the internal parts of a whole fish. The freezing time needed to reach the temperatures tested varied between 5 and 10 hrs. in the environmental temperature of —18°C, corresponding to a size of fish in the range of 0.5 to 2.5 kg as compared with the earlier results (8).

Temperature measurement

The temperatures were measured by thermocouples made of copper-constantan wire 0.1 mm in diameter. The measuring joints were either free or permanently fixed in tips of thin injection needles. The former were used for the tests carried out on isolated larvae, whereas the latter were found more suitable for the tests with phantoms. In the latter case the measuring joint could be located close to the pieces of tissue with the enclosed larvae.

The recording apparatus was the same as that described before (8).

Recording of the temperature began immediately after the phantom or the test tubes had been placed in the freezing room, and was continued until the test temperature was reached. Usually the temperature continued to decline for a while in the phantoms after they had been taken out of the freezing room, but this decrease could be predicted and thus the minimum temperature controlled.

Determination of plerocercoid motility

After the exposure, the phantoms and the serum in the test tubes were allowed to thaw in a temperature of 4°C. The motility of the larvae was judged by microscopical inspection after a gentle warming to room temperature. The larvae were regarded as motile if distinct or even feeble continuous or intermittent contractions or other kind of movement could be observed. A number of plerocercoids were inspected twice; the first time

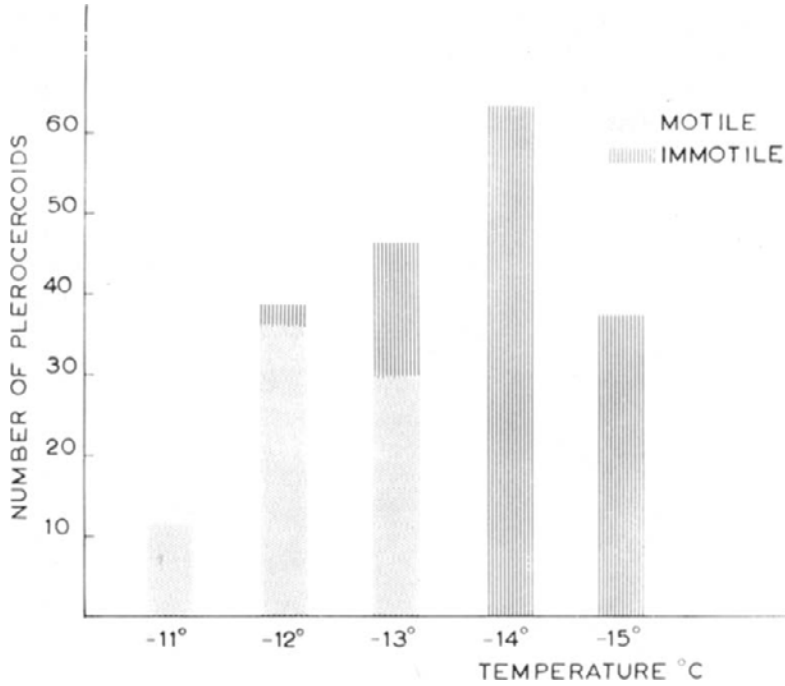

Figure 1. The effect of low temperature exposure on the motility of isolated plerocercoids immersed in serum.

immediately after thawing and the second time 24 hrs. later. However, in none of the second inspections could any deviation from the results of the first inspections be seen, and therefore they were omitted.

RESULTS

Freezing of the isolated larvae

The results of freezing tests carried out with isolated larvae are presented in Fig. 1. The total number of larvae studied was 200. The results are grouped according to the lowest temperature to which the larvae were exposed. A group consists of larvae exposed to temperatures between consecutive degrees centigrade, and the lower full degree stands for the entire group. Thus larvae that have been exposed to temperatures between < -7.0°C and -8.0°C have all been placed in one group, which is considered as having been exposed to -8.0°C. In most cases

the test was arranged so that the temperature of exposure was half-way between two consecutive degrees.

Fig. 1 shows that all the plerocercoids exposed to a temperature of —11°C survived, and most of those exposed to —12°C. As the temperature was lowered to —13°C, only about 64 % of the plerocercoids treated survived. None of the larvae exposed to —14°C and —15°C survived; consequently, in order to be certain that the exposure is lethal for Diphyllobothrium latum plerocercoids, the temperature must be lowered to —14°C as determined by the present method, and taking a possible fluctuation of ±0.3°C in the temperature measurement system into consideration.

The ocular microscopic inspection of the exposed larvae showed a great variety of noticeable changes. The intensity of the movements decreased as the temperature to which the larvae had been exposed became lower. In the range of —11°C to —12°C, obvious contractions could be seen. As the temperature declined further, a typical picture was a long larva, mainly relaxed in the caudal and middle part of the body, while the cranial part showed contractions moving to and fro. The relaxation seemed to be irreversible. The last movements before the permanent immobilization were bending and straightening that could be seen in larvae showing no more contractions. The appearance of the larvae varied from about normal to greatly changed. Because of the relaxation, the surface of the larvae became smooth and the length of the larvae increased considerably. Sometimes the cuticle was damaged and partly torn off, looking like a veil surrounding the larvae. Formation of air bubbles in the subcuticular tissue was seen in many plerocercoids. Occasionally the number of small air bubbles was so great that the larva was floating on the surface of the serum and its colour was almost white. Even though the macroscopic damages in the larvae were severe, they occasionally showed distinct movements.

Freezing of the larvae in phantoms

The results of the experiments with phantoms are shown in Fig. 2. The number of larvae studied was 453. The larvae have been ranged in groups according to the lowest temperature to which they have been exposed, analogous to the ranging described above. The results show that most of the larvae died at a tem-

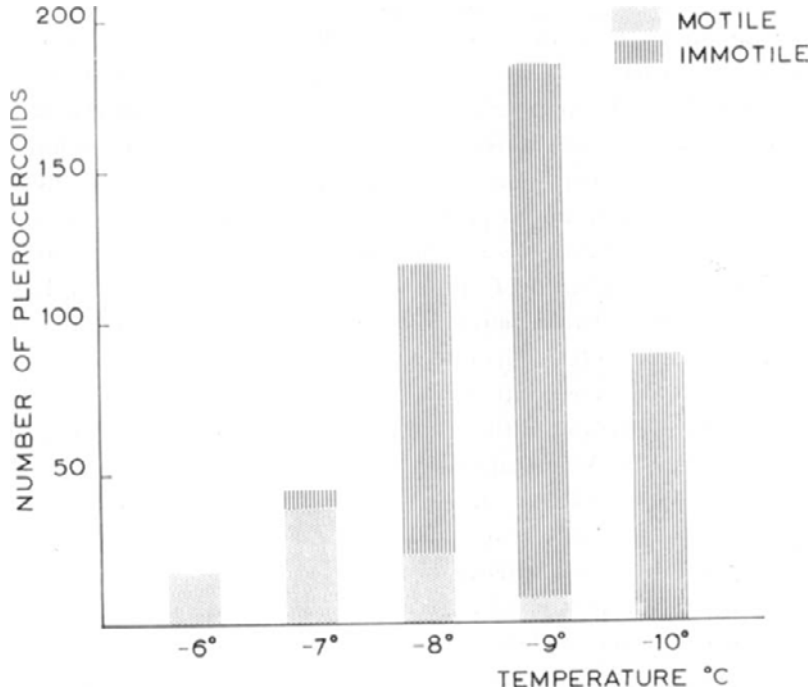

Figure 2. The effect of low temperature exposure on the motility of plerocercoids frozen in pieces of muscle tissue in fish phantoms.

perature of —8°C. Some larvae showed movements after having been exposed to a temperature of —9°C, but none after —10°C. Accordingly Diphyllobothrium latum plerocercoids in fish phantoms were found to be immobilized after having been exposed to a temperature of —10°C.

The observed changes in the treated larvae resembled those described above. The plerocercoids had become fragile as a result of the exposure, and thus while removing the larvae from the pieces of tissue a considerable number of them were broken. These larvae were excluded from the microscopic examination. Most of the permanently immobilized larvae were contracted in the cranial part of the body while the rest of the larva was relaxed. Air bubbles were frequently seen in the subcuticular layer. In comparative tests carried out with phantoms of varying size, no differences in plerocercoid survival were observed.

DISCUSSION

The purpose of the experiments was to study the effect of low temperatures on the motility of plerocercoids, and in particular to find the temperature which immobilizes all the larvae in fish freezing and consequently can be considered lethal for them. Therefore the main interest was focused upon a method by which the lethal temperature for the larvae in fish under in vivo circumstances could be determined. The observed differences in the results of the two methods described emphasize the need to have the experimental conditions resemble as closely as possible the actual circumstances to be studied.

The tests carried out with the phantoms were designed to give information about the inactivation of the larvae during the freezing of fish. As an alternative, the survival of plerocercoids in whole fish could have been studied. However, in this method the locations of the larvae in the fish are not known before the dissection, and consequently the measurement of the internal temperature in fish could give only approximate information about the temperature to which the larvae may have been exposed. This was overcome by using the phantoms. The facts that the freezing process could be strictly controlled, that the temperatures could be frequently registered and accurately measured, and that the larvae were kept in their natural environment made the method standardizable and the results indicative of the resistance of the larvae in vivo in fish.

The present problem has been studied previously by both methods described. *Wardle* (10), *Pesonen & Wikgren* (5) and *Wikgren & Nikander* (11) determined the lethal temperature of tapeworm larvae in various saline solutions. *Wardle* reports that the minimum lethal temperature of naked plerocercoids in 0.2 M-NaCl is —8°C. *Pesonen & Wikgren* found that plerocercoids regularly died in Ringer solution and 0.8 % NaCl after the temperature had been lowered to —10°C. In numerous tests, however, plerocercoids submitted to temperatures of —9°C and —9.5°C survived. The same results were obtained with Ringer solution with a double concentration of the solvents. *Wikgren & Nikander* used Hank's solution as medium. More than half of the plerocercoids cooled to —8°C survived the treatment, but none of those exposed to —9°C. *Kajava* (2) mentions that the tapeworm larvae in his experiments were enclosed in small pieces of fish muscle that were exposed for various time periods

to the temperature to be studied. He noted that plerocercoids exposed to a temperature of —3°C for 12 hrs. survived, but those exposed to —9°C for 12 hrs. died.

From the results of the above mentioned studies it can be concluded that the lethal temperature of isolated plerocercoids in saline solutions as determined by the described methods is between —8°C and —10°C. The results obtained in the present study are —14°C for isolated plerocercoids and —10°C for those in phantoms. The differences are due to many reasons, among which the methodological factors are of greatest importance. The principal factors differing between the methods described are the time of exposure and the character of the plerocercoid environment. In addition, the accuracy of temperature measurement and the difference in the definition of lethal temperature are to be considered.

When the larvae are studied by immersing the test tubes with saline solution or serum in a cold bath, the temperature decline is fairly rapid. In the present study the decline from 4°C to —14°C took place in approximately 50 min. The authors cited above do not indicate the time of exposure, but it can hardly deviate much from the time observed here when serum was used as the medium. It might be expected that the time of exposure would be even shorter, as the sub-zero temperatures were obtained by immersion in a cold bath of the tubes containing the solution. In the present study the cold exposures were carried out in a freezing room in which the decline of the temperature in the test tubes was probably slower, due to the lower conductivity of heat in air compared to liquid. On the other hand, the larvae in serum were exposed only momentaneously to the minimum temperature, whereas *Wardle,* for example, subjected the larvae to the selected temperature and then examined the viability of the larvae every consecutive hour. The time factor is obviously important when an instantaneous exposure and one of longer duration are compared. On the other hand, the differences in time of exposure in phantoms did not show any correlation with the inactivation of the larvae depending on the differences in size between the phantoms.

The environment of the larvae is important when lethal exposure in fish freezing is studied. The liquids apparently are to be considered unnatural media for the larvae, since their natural milieu is the tissue of fish. However, plerocercoids are

able to survive in liquids for a long time, and consequently they are capable of adapting to a liquid medium.

Saline solution and serum as media for plerocercoids show some apparent differences. Serum is known to have cryoprotective properties. The cryoprotective media are capable of protecting cells against freezing injury so that they may survive comparatively lower temperatures. According to their ability to penetrate into the cells they can be divided into penetrating (endo- or intracellular) and non-penetrating (extracellular) media or additives. The former include compounds such as glycerol, other hydrocyclic compounds, acetamide, dimethylsulphoxide (DMSO), pyridine N-oxide etc. (1, 6, 9). Penetration of the cell by these compounds appears to be necessary to give protection during slow freezing. Thus permeability of the cell membrane becomes an important factor in the susceptibility of tissues to protected freezing. Their cryoprotective efficacy for cells appears to be related to their potential hydrogen-binding capacity. Multiple hydrogen bonds formed by the protective solute with water may take place in the suspending medium as well as at sites within and at close proximity to cell membranes (4).

The group of non-penetrating media includes sugars such as lactose and glucose, polymers as polyvinylpyrrolidone (PVP) and more complex organic solutions like milk or serum. The mechanism of action of non-penetrating agents is not known. To some extent preliminary crenation may occur, perhaps preventing osmotic injury during the rapid removal of extracellular water. It has been proposed that the loss of lipoprotein from the cell membrane makes it permeable to cations and to osmotic lysis on thawing (3). A coating of polymer might either prevent denaturation or stabilize the membrane against the subsequent osmotic stress. It has also been suggested that the non-penetrating cryoprotective agents cause dehydration in the cells. The plerocercoids of a closely related species, Diphyllobothrium dendriticum, lack the ability of osmoregulation, but there is communication between the larval tissues and the medium through the excretory canals (7). Serum as a medium for larvae may give rise to partial dehydration of the cells of larval tissues, and thus increase their ability to survive lower temperatures.

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SUMMARY

The effect of low temperature exposure on the motility of Diphyllobothrium latum plerocercoids was studied, with the particular aim of finding the exposure that immobilizes all the larvae in fish freezing. Both isolated larvae immersed in normal horse serum and larvae enclosed in pieces of muscle tissue of the size of 1 cm^3 were tested. The pieces of tissue containing a larva were placed in the middle of a plastic beaker filled with densely packed minced fish flesh. In the

central part of this phantom, where the plerocercoids were situated, the temperature decline was considered to take place in the same way as in the interior of a whole fish. A total of 200 isolated larvae were tested, and a temperature of —14°C was found to have a fully immobilizing effect on them. The number of plerocercoids frozen enclosed in muscular tissue was 453, and —10°C was found to immobilize them. The observed difference seems to be mainly due to the cryoprotective properties of serum.

SAMMANFATTNING

Effekten av låga temperaturer på motiliteten av Diphyllobothrium latum plerocercoider.

Den effekt, som låga temperaturer har på motilitet av Diphyllobothrium latums plerocercoider, undersöktes speciellt med målet att finna den kylbehandling, som immobiliserar alla larver vid nedfrysning av fisk. Både isolerade larver nedsänkta i normalt hästserum samt larver inneslutna i fiskmuskelbitar av 1 cm^3 storlek blev testade. Vävnadsbitarna, innehållande en larv, placerades i mitten av en plastbägare fylld med tätt packat finmalet fiskkött. I den centrala delen av denna fantom, där plerocercoiderna var belägna, ansågs temperatursänkningen försiggå på samma sätt, som i de inre delarna av en hel fisk. Inalles 200 isolerade larver undersöktes, och man fann, att temperaturen —14°C immobiliserade dem alla. Antalet plerocercoider nedfrysta i fiskmuskelbitar var 453, och man kunde konstatera, att —10°C hade immobiliserande verkan på dem alla. Den observerade skillnaden beror sannolikt på serumets köldskyddande verkan.

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