Reuling

ACQUIRED IMMUNITY TO AN ANIMAL PARASITE

F. H. REULING

From the U. S. Biological Station, Fairport, Iowa, and the Pathological Laboratory, Northwestern University Medical School, Chicago

During the summer of 1917, while engaged in the experimental propagation of mussels, the attention of the author was directed to an acquired immunity in fish, acting as the host, to glochidia, the larval form of the fresh-water mussels, acting as the parasite. It was noticed that after two successive optimum infections of glochidia on the gills of a fish (this constitutes about 2,000 per individual with the species used) that the fish, which previously had carried the larval mussel through to maturity, did not permit the complete metamorphosis of a third or any subsequent infection. In every case the glochidia would attach normally, both as to time of attachment and number, but if the fish had had two previous infections or preferably three, the glochidia would drop off in 24-72 hours without any noticeable progress in their metamorphosis. The glochidia during the summer are normally parasitic for two or three weeks during which time their metamorphosis is completed.[1]

This artificial immunity to the metamorphosis of an animal parasite was strikingly apparent during the summer in 1917, in the short and long-nosed gar, Lepisosteus osseus, and Lepisosteus platostomus, which were being used as the specific host for the yellow sand shell, Lampsilis anodontoides. Because of the strong vitality of the gar, lots of 8 or 10 were used repeatedly for artificial infections. But with striking uniformity the metamorphosis was not completed on the third or subsequent infections.

The probability of an acquired immunity has been supported by Mr. Thaddeus Surber. Howard[2] calls attention to Surber's views and mentions that Surber observed sunfish "which received glochidia on the first infection, but not the second." Mr. Austin F. Shira has told me of cases he has observed where an acquired immunity has been produced after two or three infections.

Received for publication Nov. 26, 1918.

1 For a discussion of the length of the parasitic period taking up Schierholz and Harms work see: Lefevre and Curtis, Bull. U. S. Bureau of Fisheries, Document 756.

2 U. S. Bureau of Fisheries, Doc. 801, 1914, p. 37.

In speaking of natural immunity, for example, such as the large-mouth black bass, Micropterus salmoides, has for the yellow sand shell, Lampsilis anodontoides, Lefevre and Curtis[3] mention such mechanical factors as configuration of mouth parts, texture of gills, smallness of gill openings, and rapidity of fin movements. Howard in discussing the same condition very aptly says that "it seems not improbable that the tissues or blood of the nonhost possess reactions in the nature of antibodies, precipitins, and other immunizing agents, such as those discovered in the higher vertebrates, while the glochidium is especially adapted to the reactions of the appropriate host."

During the summer of 1918, while still engaged in the propagation of mussels, I planned on determining the more important facts in regard to the acquired immunity encountered during the preceding summer.

Unfortunately, the yellow sandshell, Lampsilis anodontoides, which is parasitic[4] on the gar, did not spawn until fall. Hence other species of host as well as parasite had to be used. Through the ever ready efforts of Mr. H. L. Canfield, I was able to secure an unlimited supply of the Lake Pepin mucket, Lampsilis luteola, and a limited number of large-mouth black bass, Micropterus salmoides. The fish were separated into lots of 4 or more each and placed in sheltered troughs 12 feet long, 1 foot wide and 10 inches deep, supplied with running water from the Mississippi. The bottom of the troughs was covered with sand and gravel. The fish were fed twice a week with minnows, crayfish, or grasshoppers. The fish remained in excellent condition throughout the entire summer, their vitality increasing rather than diminishing.

To simplify the report, I will give in detail the infections, species of mussels used, and the results obtained from one lot of fish.

The fish to be described are designated in my records as Lot 2-B, composed of 8 adult bass, Micropterus salmoides, varying in size from 7-14 inches long and all in excellent condition. Some of these were caught in the Mississippi, others were raised in the ponds at the Fairport Biological Station.

June 23, 1918, these fish were put in one of the troughs already described. On June 25, they were infected with L. luteola. The glochidia attached rapidly and it was estimated that about 2,000 had encysted on the gills of each fish. Specimens of the gills were taken every 2nd or 3rd day. The glochidia were well encysted and the metamorphosis proceeded normally. The mussels dropped off after a parasitic period of 18 days. On September 1, well-developed mussels averaging 15 mm. in length were recovered from this infection.

After a 6-day rest the fish were moved to another trough and reinfected with L. luteola on July 18. The glochidia attached well and completed their metamorphosis in 11 days, the fish being free of the infection on July 30. On September 5, well-developed mussels averaging 10 mm. in length were recovered from this second infection.

[3] Bull. U. S. Bureau of Fisheries, 1912, 30, p. 109.

[4] For a discussion of the restricted parasitism among the fresh-water mussels, see Surber, T. 1913. Notes on the natural host of fresh-water mussels, Bull. U. S. Bureau of Fisheries, 1913, 32, p. 101.

On August 2, after a 3-day interval, the fish were again moved to another trough and reinfected with L. luteola. A control infection, using 4 adult bass which had been raised at the station and had never been infected with glochidia, was made at the same time. On all control infections I have suspended the glochidia from 3 or 4 mussels in a bucket of water and have divided this mixture between the control and experimental fish, thus eliminating the possibility of applying "unripe" glochidia, or glochidia which were unusually active from one mussel to one set of fish and not to the other. In this infection both the control and the original fish (Lot 2-B) received the glochidia rapidly and well, to the extent of about 2,000 glochidia per fish. On examination after 24 hours the gills of the fish which had previously been infected twice showed marked necrosis and sloughing of the epithelial cyst around each glochidium. Under the microscope the glochidia had about them an unusually heavy cyst which, in certain instances, was in the process of being sloughed off along with the glochidia; the glochidia themselves showed a certain amount of disintegration. The shell was still intact and in fact remained so throughout, but between the valves there was considerable cellular debris which had broken off fr: n the glochidium itself. The control fish, examined at the same time, showed well-encysted, normal glochidia. After 48 hours the original lot of fish, except one individual which was the smallest fish of the lot and which will be discussed later, was entirely free from its infection and the gills were a normal, healthy red. The control fish held their infection throughout; the glochidia completed their metamorphosis normally and dropped off in 12 days. Well-developed, growing mussels averaging 5 mm. in length were recovered on Sept. 3 from this control infection.

This result was in accord with my previous observations that the immunity was acquired after two infections and that the glochidia would react the same way on any subsequent infection.

The question now arose whether fish which were the host for more than one species of mussel and had become immune to one of those species would likewise be immune to the other species. It may be stated here that we recognize the large-mouth black bass, Micropterus salmoides, as the host for the glochidia of 4 mussels: Lampsilis luteola, L. ventricosa, L. ligamentina, and Quadrula plicata.

To solve this problem I used the 8 bass (Lot 2-B) which had become immune to Lampsilis luteola. These fish were allowed to rest 10 days after killing and sloughing off the third infection of L. luteola. They were then infected on August 14, with L. ventricosa. A control infection was made with 5 previously uninfected bass from the ponds. Both the original 8 fish (Lot 2-B) and the 5 control fish took the infection well. In 36 hours the fish which were previously immune to L. luteola had sloughed the infection of L. ventricosa entirely off, with the same pathologic changes previously described. The control fish held the infection normally and carried the glochidia to complete metamorphosis in 12 days.

These 8 immune fish were then allowed to rest a week. Their condition was even better than when they were received in June. On August 21, they were infected with Lampsilis ligamentina. A control infection was made with three bass which had never carried glochidia. The glochidia attached rapidly and exceptionally well to both lots of fish. The immune fish sloughed off the glochidia just as they had done before. Considerable necrosis occurred in 24 hours and the gills were absolutely clean in 48 hours. The control fish held the glochidia to maturity and dropped them with metamorphosis complete in 17 days.

Unfortunately, it was impossible to obtain any gravid Quadrula plicata; hence no infection could be made with this species.

The foregoing account of the immunity induced in the 8 bass described, is typical of the immunity induced in all fish with which I have worked and which have had two or three maximum infections of glochidia during the same summer.

THE NATURE OF THE IMMUNITY

With these facts as presented it became necessary to determine, if possible, the factors which made a nonhost of a fish which had successfully carried at least two infections.

The possibility of a large increase of fibrous scar tissue from the repeated irritation, thus producing a mechanical immunity, had to be considered. Even a superficial consideration makes this seem unlikely, since an increase in fibrous tissue sufficient to interfere with the nourishment of the glochidia would certainly interfere with the respiration of the fish, and, as has been above stated, the fish were in excellent condition throughout. However, a number of sections of the gill filaments from the immune bass and from normal, uninfected bass were made and stained for fibrous tissue. These were examined carefully but in no instance was there any noticeable increase in the amount of fibrous tissue in the immune gills.

Another factor which presented itself was a possible increase in the number of mucous cells around the tip and periphery of each gill filament in the immune bass. The mucous cells on the gills are considered to have two functions: (1) That their secretion is toxic for bacteria and that they thus keep the delicate gills free of infection, and (2) that the mucous secreted washes away dirt and debris which may collect on the gills. Hence, it is conceivable that an enormous

increase in the number of mucous cells, and thus a greatly increased secretion, might make the gills untenable for glochidia. To test this possibility, filaments of the same size were cut from normal and immune bass of the same age. These were sectioned serially, stained for mucous cells, and the cells counted on both immune and normal filaments. Although the number varied considerably there was no constant increase or decrease on either the normal or immune filaments.

With these two possibilities eliminated it was thought probable that the blood of the immune fish had acquired some specific antibody for the glochidia. With this in view, experiments involving two blood reactions were determined on. The first of these was to observe the reaction of the glochidia in the immune serum. To accomplish this the blood of one of the immune bass and the blood of a bass which had never been infected were drawn into sterile test tubes and allowed to remain in the icebox 12 hours. The glochidia of Lampsilis luteola were then carefully removed from the marsupium with a sterile pipet and washed 4 times with distilled water. A drop of the immune and normal serum was then drawn from the clotted bloods, placed on cover glasses, and 6-8 gaping glochidia introduced into each. These were inverted over hollow ground slides and sealed with vaselin. In both cases the glochidia immediately snapped shut. The glochidia in the immune and control serums remained the same for about 2 hours. Then the glochidia of the immune serum began to show a striking reaction. The cells of the mantle layer and around the adductor muscle in the glochidia were slowly desquamated and were eventually broken up into cellular debris. In some cases the valves opened partially and the debris protruded to the outside. In other cases the valves remained in fairly close proximity to one another and the debris collected in irregular clumps along the inside margin of the valves. This proceeded until the entire internal structure of the glochidium was destroyed; no further reaction occurred and the valves remained intact throughout. The control remained alive and normal for 48 hours. In other words, the blood of the immune bass contained a cytolysin for the cells of the glochidia. It will be noticed that this reaction is in accord with the observation I had previously made of the way the glochidia reacted on the gills of the immune fish. The reaction was naturally slower on the gills because the glochidia did not come in contact with as much of the fish's blood at one time. It may be noted here that the glochidia actually do come in contact

with some of the fish's blood and lymph when they first attach. To quote Lefevre and Curtis:[3] "Since the hookless glochidia, which are essentially gill parasites and, when taken into the mouth of the fish lodge among the gill filaments, produce abrasions of the delicate epithelium covering the latter, a more or less extensive hemorrhage from the blood capillaries occurs, as may be readily seen from a microscopic examination. It is therefore evident that blood exuding from the gill filaments in the immediate neighborhood of the glochidia must have the same effect as in our experiments, and by exciting vigorous contractions of the adductor muscle furnish an efficient stimulus in bringing about a firm and permanent attachment to the filaments."

This hanging drop experiment has been repeated several times with the glochidia of L. luteola, L. ventricosa, and L. ligamentina. The results have been uniform except that some of the antiserums apparently contained a less active cytolysin than others, some requiring 6-12 hours to produce the results.

Since a few of the glochidia must die and be partially absorbed by the fish's blood and since all of them come in fairly close contact with the blood, it was thought possible that a precipitin was formed in the blood of the immune bass.

Uhlenhuth[5] after 24 days produced a precipitin in a rabbit fed on egg white and Metalnikoff[6] produced a hemolysin in the serum of rats fed on horse blood, hence it was deemed not unlikely that a precipitin would be formed in the fish from such repeated and close contact of the glochidia with the respiratory apparatus.

The amount of blood obtainable from a bass is very limited, hence capillary serologic tubes were used for this work. The blood was collected in sterile test tubes and put in an icebox for 12 hours. The blood from normal, uninfected bass was used as a control. Glochidia were removed from the marsupium of a gravid L. luteola, ground to a homogeneous mixture in a sterile mortar with normal salt solution, and centrifuged. The glochidia extract was used in dilutions of 1:1, 1:5, 1:20, 1:50, 1:100, and 1:200. One drop of the immune serum was added to each dilution. Controls of normal serum were run side by side with the serum being tested. The technic used by Nuttall[7] was followed fairly closely. The results were not striking enough to

[5] Ztschr. f. Hyg. u. Infektionskr., 1897, 26, p. 384.
[6] Centralbl. f. Bakteriol., 1901, 29, p. 531.
[7] Blood Immunity and Blood Relationship, 1904.

invite much confidence. T
with one drop of the immu
stant cloudiness and a ligh
room temperature. The c
was the only dilution in t
constant precipitate—and ir
dilutions would often show
points out, no conclusions
is especially true when wo
chidial extract. Because of
were not undertaken. Hen
sions as to the presence or
tests indicated that a very
serums, but it will require n

THE FACTORS INVOL

Several factors might b
we assume, with Lefevre a
about the glochidium is sti
as well as by the mechani
that these stimuli are appli
a single infection, it does n
stimulus and to the glochid
which Calmette[8] has pro
Metalnikoff[6] produced, or
which Bashford[9] has pro
There exists also the pos
cytolysin, are stimulated to
which have died on the
microscopic examination t
the glochidia do die on the
mum infection, as they d
number which die, altho
increase. Hence, if these a
a fairly plausible explanat
Schültze[10] produced an ant
for muscle albumen, and a

[8] Ann. de l'Inst. Pasteur, 189
[9] Jour. Pathol. and Bacteriol.,
[10] Deutsch. med. Wchnschr., 19

quite much confidence. The 1:5 dilution of the glochidia extract with one drop of the immune serum added to it showed a fairly constant cloudiness and a light flocculent precipitate after 24 hours at room temperature. The control remained clear throughout. This was the only dilution in the several sets of tests which showed a constant precipitate—and in one set even this dilution failed. Other dilutions would often show a cloudiness or opacity, but, as Nuttall points out, no conclusions can be drawn from such cloudiness. This is especially true when working with a substance such as the glochidial extract. Because of the shortness of time more extensive tests were not undertaken. Hence I do not care to draw any final conclusions as to the presence or absence of a precipitin. In general, the tests indicated that a very weak precipitin was present in the antiserums, but it will require more extensive work to determine this point.

THE FACTORS INVOLVED IN PRODUCING THE IMMUNITY

Several factors might be involved in producing the immunity. If we assume, with Lefevre and Curtis,[8] that the epithelial cyst formed about the glochidium is stimulated by a secretion of the glochidium as well as by the mechanical "bite," and when we further consider that these stimuli are applied to the extent of about 2,000 per fish at a single infection, it does not seem improbable that antibodies to this stimulus and to the glochidia might be formed similar to the antivenin which Calmette[8] has produced for snake toxins, the hemolysins Metalnikoff[6] produced, or the antibodies including the precipitin which Bashford[9] has produced in a rabbit treated with crotin. There exists also the possibility that the antibodies, especially the cytolysin, are stimulated to production by the absorption of glochidia which have died on the fish during their metamorphosis. From microscopic examination there is fairly good evidence that a few of the glochidia do die on the gills. When the fish carry nearly a maximum infection, as they do in the artificial propagation work, the number which die, although very limited, must proportionately increase. Hence, if these are absorbed while still in the cyst we have a fairly plausible explanation of the cause of the immunity. For, as Schültze[10] produced an antiserum for the vegetable protein "Roborat," for muscle albumen, and a generalized yeast precipitin for the yeasts,

[8] Ann. de l'Inst. Pasteur, 1895, 9, p. 225.
[9] Jour. Pathol. and Bacteriol., 1902, 8, p. 59.
[10] Deutsch. med. Wchnschr., 1902, 28, p. 804.

it seems likely that antibodies would be formed by the absorption of albumen of the glochidia.

Late in the summer of 1918 in an effort to induce a similar immunity in a normal bass I made 3 injections into the abdominal cavity of an adult bass of filtered, ground glochidia. The injections were made 4 days apart and consisted of 0.5 c c, 1 c c, and 2 c c. When this fish was infected along with a known immune and a known normal fish it held the glochidia equally as well as the normal fish, while the immune shed the infection within 36 hours as previously described. In other words, the immunity was not induced by the injections made. The failure of this may be explained in one of two ways: (1) That the injections were small and did not last over a long enough period of time, and (2) that the glochidia were filtered. It may be that the shells of the glochidia are involved in the immunity production or that the immunity producing sustance was filtered out. It will be recalled that Graham-Smith[11] found that Limulus serum unlike mammalian and avian serums, when passed through a porcelain filter no longer produced a precipitin when tested with anti-Limulus serum, while the unfiltered serum did.

The question is now naturally raised: How long will the immunity persist and what effect will it have on the commercial application of the artificial propagation of the fresh-water mussel?

These two questions are unanswered as yet, and only the surveillance of the immune fish from year to year will determine it. If the immunity does last for several years it will constitute an additional hazard in the artificial propagation of mussels. This is true because under the methods now in use the fish are seined from the river, infected in an hour or so, and turned back again. There is at present no way of telling whether these fish hold the infection or not. If with the extension of artificial propagation a great many fish become immune in a restricted area, the fish may be infected over and over again with the false idea that they are retaining the infection each time, while in reality they may be sloughing them off in a day or two. This suggestion is merely offered as one of the factors which must be considered in the event of more intensive artificial propagation work and applies especially to any attempt at trough, pond, or small lake propagation on a commercial or even practical basis.

[11] Blood relationship amongst the lower vertebrates and arthropoda, etc., as indicated by 2500 tests with precipitating antisera, 1904.

The past summer there w
size which from the first of
I tried to infect this fish re
them off in 24-48 hours. Th
of the station over winter, t
times it was infected during
out like those fish which ha
as previously described, an
acquired an immunity duri
tained it over the period of

Early in the paper the f
bass—apparently a 2- or 3-
2-B—did not become immu
a third infection before it
larger and older fish showe
seems to be a fairly constan
stance by pointing out tha
natural and hence small i
started toward an acquired i
reached complete immunity
fish are artificially infected
nity is completed very rapid
bass have become immune
the small bass will often re
the younger bass have not
hence require 3 heavy inf
immunity.

Prof. H. S. Davis has
conditions produced by myx
bubalus and I. cyprinella, i
sporidia were abundant, he
while on large and old buff
tion will be very light or t
encyst themselves and eve
stream it is possible that th
the parasite similar to the o

Furthermore, Wilson[12] c
of glochidia and copepods

[12] Bull. U. S. Bureau of Fisher

The past summer there was under consideration one bass of normal size which from the first of the season refused to hold the glochidia. I tried to infect this fish repeatedly, but it would invariably slough them off in 24-48 hours. This bass is one that remained in the ponds of the station over winter, but there is no record to show how many times it was infected during the preceding summer. It acted throughout like those fish which had the artificial immunity induced in them as previously described, and it would seem likely that this fish had acquired an immunity during the preceding summer and still maintained it over the period of a year.

Early in the paper the fact was mentioned that one of the small bass—apparently a 2- or 3-year old belonging to the described Lot 2-B—did not become immune after the second infection but required a third infection before it exhibited the same reactions which the larger and older fish showed after 2 infections. In my records this seems to be a fairly constant condition. I would explain this circumstance by pointing out that the older bass have had a number of natural and hence small infections, and that thus they have been started toward an acquired immunity although they might never have reached complete immunity under natural conditions. But when these fish are artificially infected with 2,000 or more glochidia their immunity is completed very rapidly—in my records some of the very large bass have become immune after one infection. On the other hand, the small bass will often require 3 infections. This is natural since the younger bass have not received any preliminary glochidia and hence require 3 heavy infections to produce the same degree of immunity.

Prof. H. S. Davis has called my attention to certain unreported conditions produced by myxosporidia. On young buffalo fish, Ictiobus bubalus and I. cyprinella, in ponds where gill species of the myxosporidia were abundant, he has found practically a 100% infection, while on large and old buffalo fish, even in the same pond, the infection will be very light or totally absent. Since these myxosporidia encyst themselves and eventually come in contact with the blood stream it is possible that the older fish have acquired an immunity to the parasite similar to the one described above.

Furthermore, Wilson[12] calls attention to the fact that the presence of glochidia and copepods are antagonistic to one another. That is,

[12] Bull. U. S. Bureau of Fisheries, 1914, 34, p. 333.

if a fish has a heavy infection of copepods it will receive only a limited number of glochidia and vice versa. It may be stated here that the experimental fish which have been described in the present report have been exceptionally free from copepods. He goes on to say that this incompatibility is probably chemical or physiologic in its action. The same author[13] introduced free-swimming copepodid larvae, gills of a fish heavily infected with glochidia, and gills free of glochidia into an aquarium. On the following morning the gills that had no glochidia were well covered with copepodid larvae. But none of these larvae had attached to the gills that were already occupied by glochidia. This may point to a delicate secretion which the glochidia have, which is instrumental in producing the immunity and at the same time is antagonistic for other forms such as the copepods

CONCLUSIONS

An immunity to the metamorphosis of glochidia is produced in fish after repeated heavy infections.

The fish becomes immune to all the species of glochidia for which it is a host.

The immunity is a blood immunity and is not concerned with mechanical factors.

[13] Bull. U. S. Bureau of Fisheries, Doc. 854, 1917, 35.

THE SPECIFICITY (
ENZYMES AN

HAR

From the Department of Bacteri

Ever since the recognitio
large number of vital proces
organisms, much work has b
properties. Through this we
great deal is still left unknow
to obtain them in a pure form
indirectly, that is, by investiga
action.

One of the first properties
cases was that of specificity.
several carbohydrates a certai
but the nature of this specific
appeared that these ferments w
They are what we now know
proteolytic enzymes, however,
are given off by the cells into t
are susceptible of isolation.

The action of these enzymes
unity for the study of specif
between the several types of p
then one would expect that no
all of the widely different type
really seems probable that a dif
this were actually the case, di
enzyme would have to be consid
or mixtures of many distinct fe
seems probable, nor is it borne o
others, who came to the conclu
the digestive juices are units and

Received for publication Nov. 30, 191
Supported by a grant from the Nat