A Method to Mark the Time in the Scale and other Hard Tissues of Fishes to see their Growth*

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Introduction

Observing scale and other hard tissues under microscope for age determination of fishes, it so frequently happens that the one would be embarrassed to find out the right year mark from the similar ring or discontinuity, so in some times the problem is brought into argument.

In that case, the method to find out the right year mark or the spawning mark we have now is that either to get the fact on seasonal change of the feature of scale or to get the detail frequency curve of body size by measuring a huge number of specimens enough to find out the peaks for corresponding ages.

The authors have been trying to find out the way to mark the time in living tissues of scale and others usually used for age determination so as to desolve this problem since long time. Meanwhile, Drs. OKADA and MIMURA, Profs. of Tokyo Medico-dental College, succeeded to make a dark lead mark in teeth of Rhodenta by injecting dilute lead-acetate solution into vein.

The authors getting their idea and the technical advice, made a serries of similar sort of experiments to apply it to hard tissues of fishes. At the first step, we studies the adquet method for fish by using the fish reared in the laboratory tank. And at the second step, applied the established method in chance of tagging experiment to see the natural growth until the fish recaptured. The results we obtained are quite satisfactory, so the authors intend to introduce the method, which may available for any sort of fish the growth of these tissues are still under argument, and also would describe the feature of growth of these tissues observed by our experiment.

From our experience of several years, the authors want to recommend the application of this method to tagging experiment, if anybody wants to know about the natural growth of these hard tissues. And, we believe there may be more way of application of this method in fisheries biology.

Method to Mark Lead-line

As OKADA and MIMURA's method is for teeth of Rhodenta, we should reexamine and improve it to be appliable to the hard tissues of fishes. Prior to the mass experiment, the following experiments were done to find out the best method, using gold fish about 5 to 20 grams weight in aquarium inside of the laboratory.

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* 本研究は文部省科學研究費によるものである。

1) The Method of Injection

a) As Okada & Mimura did, we tried to inject the solution to the artery or vein. However, as the blood vessels of the tiny fish of this size is so fine in the part near to the body surface, that we shoud give up to do it after so many fruitless trials. Moreover, by the injection to the thicker vessels near the heart, we sacrificed many fishes in every trial with the fruitless effort, as it is hard to find out the right location from outside of the body wall.

b). Then we turned to do it by abdominal injection. In so many cases of our trial, we injured the viscera and it sometimes brought the fish to die.

c). The next trial is to keep the fish alive in the tank where dilute solution of Pb-acetate is kept during certain duration of time, so as to the fish may absorb it through gill membrane or other external part of body directly contacting with the water.

0.001% solution (10 mg per litre) of Pb-acetate is prepared in the glass tank (140 mgr per litre is lethal concentration), where gold fish of about 10 grams weight were kept about one hour long.

But the result of examination of scale shows no clear lead mark is deposited in any part. We did not continue the same sort of experiment in more concentrated solution, as the following method, carried on simultaneously proved to be better and easier.

d). Finally, we found that the method to inject the solution to body muscle is the one most satisfactory from the stand point of effect of deposition and easiness of handling. The most adequet part of body muscle to inject is the middle part of dorsal side, where the muscle is most thick in many kind of fish.

And the injection should be done pricking the scaled skin with the injection needle obliquely from back to foward. The needle we used in most of our experiments has its diameter ¼ mm, as we found the finer the needle the result are more satisfactory, and its point should be sharpened by grinding it so frequently.

The injection should be done as slowly as possible so as to avoid the solution might leak out from the body. There was no particular technical need to have fish to be narcotised prior to the injection about the fish kinds as far as we tried.

2) The Proper Amount of Pb-acetate For Fish

To decide the amount and the concentration of the solution, we had to do some experiment, as the amount decided by Okada & Mimura for Rhodenta for vein injection might be different from that for our purpose. The material used for these series of experiments is a number of fish having body weights from 5 to 7 grams, and the results obtained are as follows.

To obtain the precise deposition of lead in scale, the more the amount of Pb-acetate is injected the mark is the more clear. However, to reduce the effect to the health of fish especially to apply it for tagging experiment, the amount of Pb-acetate should be limited in certain amount.

As obviously shown in Table 1, the proper amount enough to present the lead mark and less effect for fish health is about 0.1 to 0.5 mgr of Pb-acetate weight in the solution

Table 1.

	Concentration of Pb-acetate	Volume of solution per 10 grm of fish weight	Weight of Pb-acetate in it	Pb-mark in scale	Influence to fish health
1	1 %	0.1 c.c.	1 mgr	Clear	Decomposition of muscle in the part injected.
2	1 %	0.05 c.c.	0.5mgr	Clear	Healthy
3	0.5 %	0.1 c.c.	0.5mgr	Clear	Healthy
4	0.1 %	0.1 c.c.	0.1mgr	Clear	Healthy
5	0.04 %	0.05 c.c.	0.02mgr	Invisible	Healthy

for every 10 gr of body weight of fish. This amount of Pb-acetate for fish is about 10 to 25 times of that amount found adequet for Rhodenta by Okada & Mimura.

This discrepancy would be caused by the difference of the injection method, the nature of animal and that of tissue. The authors in the application of this method for all sorts of fish kind, as far as the authors tried, injected 0.1 c.c. of 0.1 to 0.5% solution of it for every 10 gr of fish body weight. And this standard amount is also effective to present lead mark in other calcified tissue. though In the case of observation of bones, 0.1 c.c. of 0.1% is enough to present a lead mark if it is examined as a cross section, but in the case of observing it from the surface, it is necessary to inject 0.1 c.c. of 0.5 % solution, unless the mark is too faint to see. Fin rays were always observed in cross section by microscope, so 0.1 c.c. of 0.1% solution is enough to produce lead mark.

3) How To Examine Lead Mark

After the injection of Pb-acetate, the scale and other calcified tissues taken off from the fish killed shows no remarkable signiture of deposition of lead under microscope without having the following treatment.

Scale

The scale taken off from the fish is kept in the small glass bottle, containing the water to which the scale is immerged. And the H_2S gas are lead into the bottom of the bottle by glass tube, being supplied by Kipp's gas generater (FeS+HCl), passing through the washing bottle having water in it. By this apparatus, the water in which the scale is, could be saturated by H_2S continuously. In this condition, the scale of usual size should be kept during about one hour, and then Pb deposited in the tissue would turn into PbS, which appear as dark line under optical observation. To make the line more clear and more stable, it is better to gild the lead granules by gold. So, after washing the scale treated by H_2S by water, it is immerged into 0.1 % solution of $AuCl_3$ during 10 to 20 minutes. And after then, put it into 5 % solution of Sodium thiosulphate, so as to remove

the excess of gold adhered to the surface of the scale out. Then the dark lead line would turn to blue under optical observation by transmitted light.

Fin-rays, Centrums of Vertebrae and Opercle

For these tissues, H_2S gas should be lead into acid (1 % solution of HCl or 1.8 % HNO_3) where the tissue is immerged, during about 24 hours. And after washing it by water, the tissue are treated by $AuCl_3$ during a half hour, as mentioned in foregoing chapter. To use the acid for these tissues is a sort of decalcification, which is done simultaneously during sulphuration. It is necessary for the thick tissue like the centrum of vertebrae to do this during long time, while for the thinner tissue like the scale, the weak acid like H_2S itself is enough for the purpose.

How Soon Lead is Deposited

How soon after Pb-acetate solution was injected to the muscle the lead would deposited in these tissues is the problem to be cleared, so as to know the exact growth of tissue by this method So, the two experiments were undertaken.

1). A gold fish (about 4 gram weight) was injected by 0.04 c.c. of 0.1 % Pb-acetate solution to muscle, and have kept in tank where the water temp. 10^0 C, and scales are taken off one by one after certain period, and examined. The results are as follows,

Table 2.

Time passed after injection	10 min.	30 〃	1 hr.	2 〃	3 〃	5 〃	2 days	3 〃	4 〃	5 〃	6 〃	7 〃	8 〃	9 〃	10 〃	11 〃	12 〃
Presence (P) or Absence (A) of lead mark in scale	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	P

As shown in Table 2, it needs 12 days since injection to get obvious lead deposition in scale by this experiment.

The result we obtained is quite different from the fact that Okada & Mimura (1934) described, *i. e.* 2 hours are enough to have lead mark in teeth of Rhodenta after the injection to vein.

This considerable discrepancy might be caused by the differences of tissue, animal and the method of injection.

However, in later experience the common gobby (*Acanthogobius flavimanus*) being injected in occaision of tagging liberation and examined one hour after it, had clear lead mark in scale. So, the authors have to think that the speed of secretion of lead on scale layer would be various according to the species of animal used.

2). About twenty individuals of gold fish, from 3 to 7 gr in weight, are injected by 0.1 mgr of Pb-acetate per 10 gr of fish body weight at May 27th 1952, and reared in tank where the water temperature is about 20^0 C being fed by usual food (powdered silk worm cocoon). And, after various duration of time, an individual is killed to find out the

lead deposition in each case. The duration of time since injection is as follows,

2 hours, 5 hours, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days.

The result of examination is that no trace of deposition was seen on the scale from the one up to the 9 days'. The scale from fish killed after 10 days shows slight mark, which is somewhat clearer in that of 11 days, and more clearly appeared in that of 12 days. Also operculum and fin rays are taken from this series of specimens for the examination. However, even though comparing the one taken from the 11 days' and 12 days' specimen with that of not injected, no remarkable difference was recognised between them. One of the reason of it might be the amount of medicine would not be enough to make lead mark clear in these tissue. About our numerous specimens experimented, the shortest case in which lead deposition taken place successfully in fin rays and bones is about one month after the injection about gold fish.

The Microscopic Feature of Growth of Calcified Tissue

As lead deposition is supposed to happen followed by calcium deposition in these tissues the more active the calcium secretion the more obvious and dark the lead mark in these tissues. And the dark line clearly shows the time when it is deposited. So, the feature of growth can be cleared experimentally as follows.

1) Cycloid Scale

The following was affirmed by scales of gold fish and carp. If the scale treated by this method is observed from the surface, it shows the cloud like spread dark area at the central part of it as usual. (Fig. 1) It is darker in its central part and thinner in its periferal part. And always there is a considerable broad marginal area being transparent as deposition of lead is slight.

And it is remarkable that the parts where radius runs are transparent also. These features can be considered, as Neave (1940 already mentioned that the scale is formed by secretion of collagenous substance at first stage, and then after certain duration of time calcium is added to it. So the lead deposition occured only in the part where calcium secretion have taken place, and the marginal area newly made is left to be transparent.

And also the part where radius runs would have no calcium secretion, as already Tayler (1914) stated. It might be considered that the border line of central dark area is the line where calcium was depositing with Pb after certain duration of time since Pb-acetate is injected. So if the marginal line where calcium is deposited at the time when fish was killed is found, by the method such as haematoxylin or $AgNo_3$ staining, the grown part with calcium deposition since Pb had deposited can be measured from the surface of the scale. However, the border of the both lead area and calcium is not so clear by surface observation to measure it accurately, and the cross section of scale made by paraffine-section method shows something more exact feature. Fig 3 and Photo I & II shows a cross section of scale cut in the direction along the body axis of fish, which was taken

from the fish killed after the injection. As it is clearly shown by the figure, the lead lies in the layer upper from the extreme bottom of scale. The line lies much deeper in exposed part of scale (right side in Fig. 3) than the part covered by neighboring scale (left side in Fig. 3). And, at the part crossing the radius, the lead line comes up near to the surface, having its peak not stained as shown in Figs. 3, 4, 5 & 6 and Photo II as point R.

These features are quite coincident with that can be supposed from the surface view. However, it is not recognized by the surface observation that in cross section even though the lead deposition is more faint than in the central area, the lead line extends to the peripheral part of the scale up to the extreme termination of both anterior (left in figure) and posterior (right in figure) of scale. The feature of front termination is enlarged in Photo. I, and that of the part near radius is also enlarged in Photo. II.

The authors have to consider by these fact that the new layer of scale is formed by the secretion of substance from the epithelium of scale sac, which is especially active at the part lining the scale bottom, not as the form of calcium compounds but as the matter without calcium compound, as Neave (1940) thought it is a sort of collagenous substance.

To make this sure, the calcium staining by $AgNO_3$ was tried to the same section of scale. And as it is shown in Fig. 4 and Photo. III, the dark staining by Ag, which proves the presense of calcium compounds, is found merely at the part of section upper from the lead line.

And at the exposed part (right side in Fig. 4) the calcification takes place more sooner after the scale layer is formed than the covered part (left side in Fig. 4), as it can be seen from the section that more thicker bony portion (calcified layer) and more thinner fibrillary portion (non-calcified collagenous layer) in the exposed part than in the covered part.

So as to get the calcium compounds deposited on the layer lies in the middle of the scale layer, the calcium compound, which is usually thought to be secreted from the epithelium of scale sac, should have come from either external covering epithelium or the internal lining epithelium surrounding the scale.

This origin of calcium secretion was cleared by the experiment to do the second injection succeedingly after certain period of time since the first injection, using different amount of Pb-acetate for same weight of fish body.

The first time, 1/50 mgr of Pb-acetate for 10 gram weight of gold fish was injected. And after four months since then 1/2 mgr of Pb acetate (25 times of the first injection) was injected to the same fish. This coupled injection makes the clear double line in the cross section of scale, as shown in Fig. 6, having the thicker lead line at deeper layer than that of the first one. This tells that the calcium secretion from the scale sac would be made by the epithelium lining the bottom of scale, depositing to inner layer, penetrating through the collageneous part. As this experiment was done using the gold fish reared in the narrow tank, no remarkadle growth of both scale and body was shown. However, the authers want to suggest the application of this coupled injection, so as to

see the exact growth, to any sort of aquatic living which might have convenient chance to do such as recapture of tagged fish or the fish easily reared in natural condition.

Moreover, there is another method to see the natural growth of scale by single injection at chance of tagging liberation. That is, as Fig. 3 shows the lead line extends to the both termination, the covered and the exposed part of scale, and there very thin collagenous layer exists. So if the scale grown since the time of injection at tagging liberation, the new layer would be formed beneath the lead mark extending to the border of scale. Therefore, if the part outer than the lead mark (arrow marked part in Fig. 7) is measured that is it which grown while the fish had been in nature since the time when tagged, injected, and liberated. (The dotted line in Fig. 7 is the line supposed to be the margin of scale when injected) This is a way to know about the amount and feature of growth of scale. However, the method requires the scale examination by cross section of it.

2) Ctenoid Scale

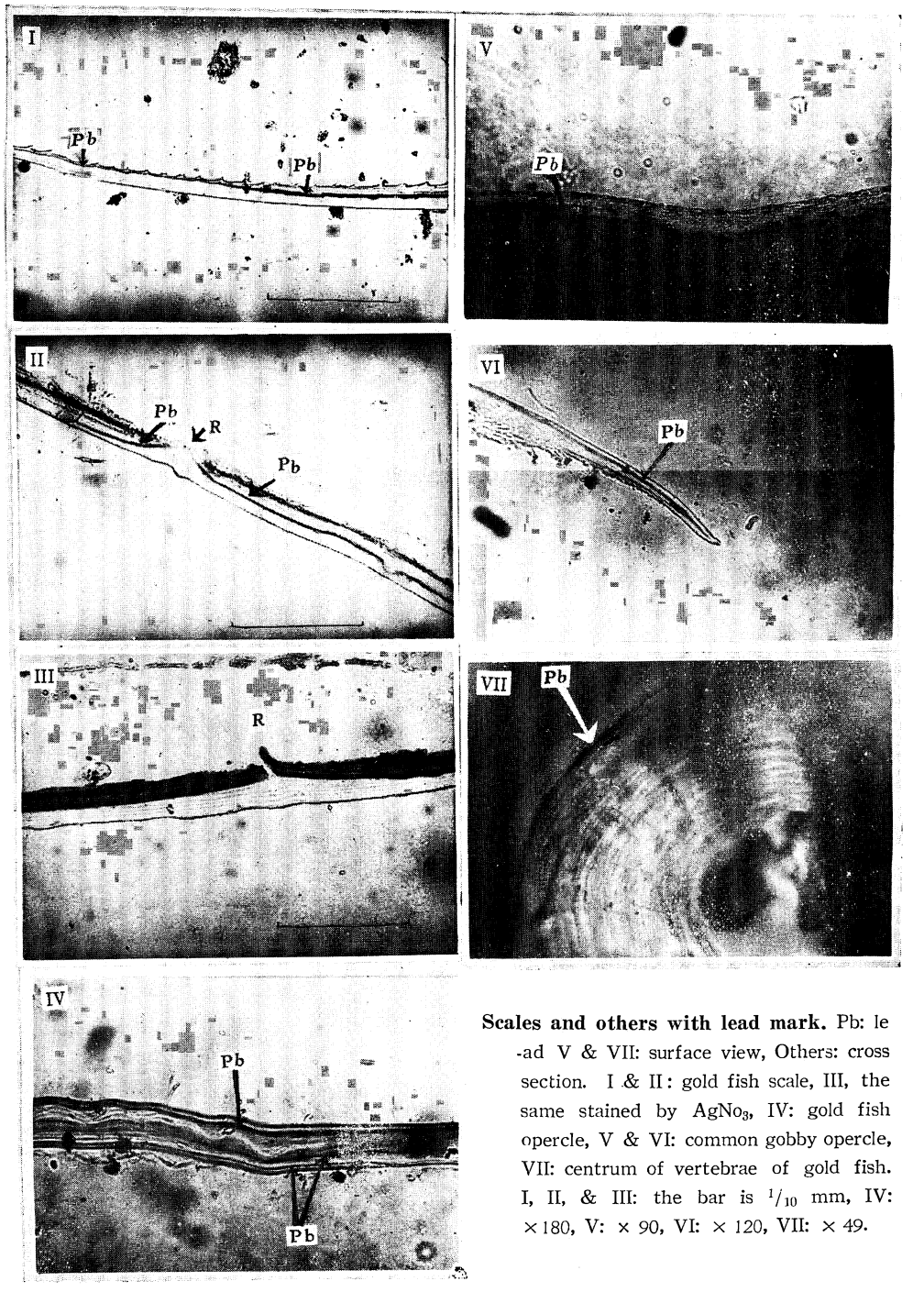
As the fish having ctenoid type of scale the Common gobby, *Acanthogobius flavimanus* was selected, as we had chance to do the tagging experiment by this. As most of features of lead mark of this caused by injection of Pb-actate are similar to those of cycloid scale already mentioned in foregoing chapter, such as the depth of lead line in cross section, and the feature around radii, so a few should be described here (Fig. 2) However, one peculiar feature was noticed by surface view of ctenoid scale which was not shown in cycloid scale.

That is a dark narrow lead line running along the margin of scale where fringed by the serration. (Pb in Fig. 2) The cross section of the scale (Fig. 5) tells the nature of this lead line clearly, *i. e.* it is caused by the vertical arrangement of the lead line at that point, while in most of other parts it runs horizontally in cross section. This vertical part is formed as the result of thick calcification at the part of serration (T in Fig. 5), which may add the strength to the construction of the serration. This peculiarity of ctenoid scales enables to know about the growth of scale by surface view. That is, if the newly grown part of scale is formed since the injection it should be added at the outer or marginal part from this lead line. Therefore, if the peripheral terminal of the lead line running along the serrated margin is distinct, the part outer from it should be considered as the newly made layer. So, the amount and the feature of growth since the fish was tagged, injected and liberated can be observed and measured by surface observation of scale.

3) Fin-rays

Though it is well supposed that the bony spines of fins should have the same lead mark if Pb-acetate is injected, but the soft rays is merely observed as it is more easy to make paraffine section for the examination. For this purpose, a gold fish of 3 gram weight is treated as follows.

The second soft ray of left pectoral fin was examined by section. (Fig. 9) The asymmetry shown in figure of coupled branch of a fin ray is caused by its nature, as



Scales and others with lead mark. Pb: le -ad V & VII: surface view, Others: cross section. I & II: gold fish scale, III, the same stained by $AgNo_3$, IV: gold fish opercle, V & VI: common gobby opercle, VII: centrum of vertebrae of gold fish. I, II, & III: the bar is $^1/_{10}$ mm, IV: ×180, V: × 90, VI: × 120, VII: × 49.

Fig. 1

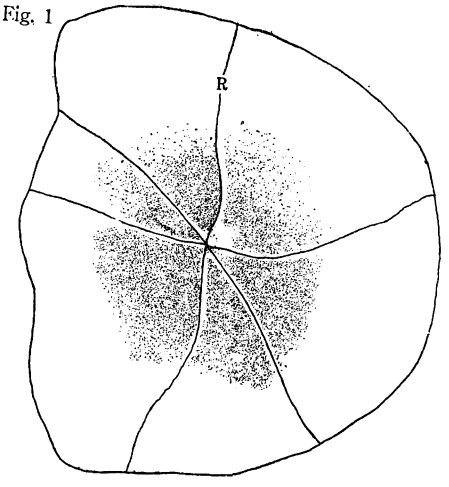


Fig. 2

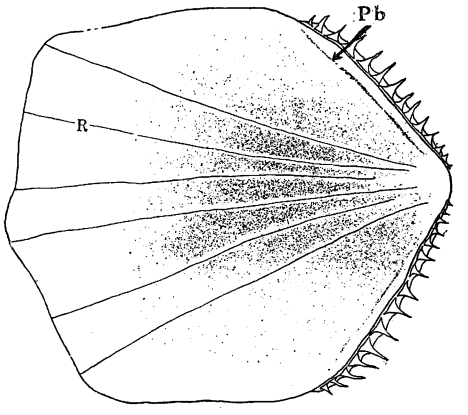


Fig. 3

Fig. 4

Fig. 5

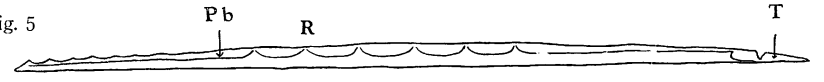


Fig. 6

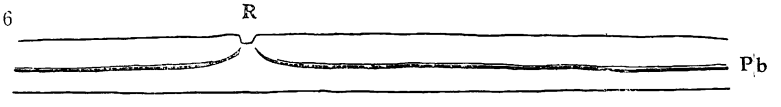
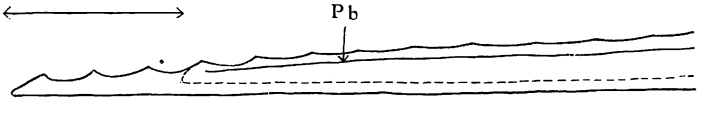


Fig. 7



Scales and others with lead mark. Pb: lead mark, R: radius, T: serration. Materials: Figs. 1, 3, 4, 6, 7, 8, 9, Gold fish, Figs 2 & 5 Commom goby. Figs. 1 & 2: surface view, ×25. Figs 3-7: cross section, Figs. 3, 4, 5, × 50: Figs 6 & 7, ×170. Right is exposed part in all figs. Fig. 1 shows dark central area. Fig 2 shows dark line beside it. Fig. 3 cycloid scale. Fig. 4. Calcium was stained by $AgNO_3$. Fig. 5. Ctenoid scale. Fig. 6. part of radius by coupled injection. Fig. 7. Newly grown part (arrow mark). Fig. 8. Surface view of opercle, Fig. 9. Fin ray by coupled injection.

Fig. 8

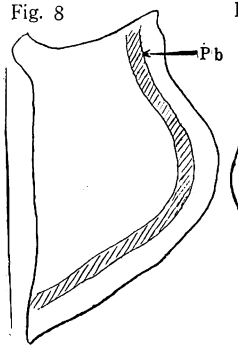


Fig. 9

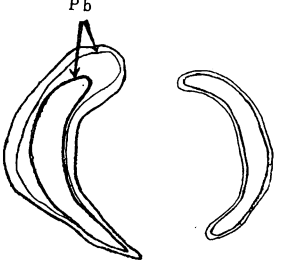


Table. 3

Date	Pb-acetate		Amount of Pb-acetate per 10 gram of body weight
	Concentration	Amount of Solution	
Aug. 14. 1951	0.5 %	0.03 c.c.	0.5 mgr
Sept. 19, 1951	0.1 %	0.03 c.c.	0.1 mgr
Nov. 7, 1951	Killed and examined		

the outer branch is usually bigger than the inner branch in pectoral fin rays. (the body located right side of Fig. 9) The feature of growth is shown in cross section by coupled injection, having the second mark thinner than the first one. That tells the secretion of calcium is made by the epithelium covering the ray, adding the new layer to the outer margine. And the activity of secretion is not homogeneous as the two lead line is not running equally parallel. It is more rapid and active by the outer branch, than the inner one, and more at external and upper part of the outer branch, as this degree of activity of secretion can be measured by the distance of two lead lines.

The bony spine is more common to be used as age determination than soft ray. The authors are quite certain the same would be observed in bony spine as well as soft ray.

4) Centrum of vertebrae

The centrum of vertebrae taken out from the same fish as used for the examination of fin rays described in foregoing chapter shows also the deposition of lead observing from the surface. (Photo. Ⅶ) Though we made the couple of injection, the lead line appeared was a single. Probably, the amount of Pb-acetate injected in the second time was too few to see it without getting the cross section of it. The grinding or paraffine section of this part is technically difficult, as its section should be cut by a surface near to cone. The feature of lead mark observed from surface is something near to the ring usually thought to be year-ring by majorities of fisheries biologist.

Though nothing new is added by us to the knowledge of growth of centrum, but this method tells the possibility to measure the grown part, which is the part from lead ring to the border, since Pb-acetate is injected.

5) Operculum

From the same fish as mentioned in the chapter of fin rays, the membrane bone of operculum was taken off and examined.

By the macroscopic surface view, a dark Pb-line came out along the posterior margin of opercle, nearly parallel to its marginal line. (Fig. 8) As the line is single, though the coupled injection was made, so the cross section was made to see it in detail. (Photo. Ⅳ)

As shown in photo. VI, the outer fainter lead line is found in the section, supposed to be the one made by the second injection. So, the one observed from the surface should be the one made by the first injection which is much thicker enough to be observed from the surface. The trace of both lead lines running parallel to its free margine tells the way of growth of this sort of membrane bone, which is not so far from the idea already thought by many biologists.

The operculum from the Common gobby recaptured after tagging with Pb-acetate injection is also examined (Photo. V & VI).

Although the specimen shown in Photo. V and VI was recaptured after twenty four days since tagging and injection, even if about the one examined immediately after injection, always the lead line apart from its free is posterior margine. This tells that, in this kind of fish, at the peripheral part of operculum the calcified layer is more thinner than gold fish described previosly, seeing it from the lead line in cross section (Photo. VI), and this one had few growth in twenty four days. So the dark broad area (Photo. V) observed from the surface is the part at where the two lines, outer and inner, are more thicker than other part. The part outer from the part surrounded by the two lead lines is not calcified being collagenous still.

This point should be considered to measure the grown part of opercle since injection in some sort of fish like this Common gobby. This is the difference between this species and the gold fish in which uncalcified part is very thin and negligible to measure the growth.

As the operculum is a part of body available to age determination, so to see the natural growth of it, our method is available. And if the amount of Pb-acetate is enough there is no neccessity to make cross section.

6) Otolith

As the otolith of fish is generally used for the age determination, so we had to work out the same method about this hard tissue. However, so many specimen we treated by various amount of solution have the otolith without any trace of lead deposition. This exception would be understood that calcium in the otolith is a form of calcium carbonate, which usually hard to deposit lead in secretion.

About tissues previously mentioned and got fine lead mark, calcium is supposed to be in the state of phosphate.

However, this is merely a supposition. So, about the reason and the method to make the time impression in this tissue would be studied further.

Effect of Injection to Survival Rate

Gold fish and carp injected by medicine and reared in the laboratory shows no significant physiological influence seeing from their behavior, and if the amount is adequet as mentioned in previous chapter no bad effect is found for their survival.

However, in the case injected when it is tagged, having the tag as a physiological burdon, some effect to the physiological condition of the fish could be considered.

So the differerence of survival rate between non-injected tagged fish and injected tagged fish are examined by the result of tagging experiment of the Common gobby.

977 individuals of the Common Gobby were tagged by silver band and celluloid disc at Tokyo Bay, in them 362 individuals were injected by lead-acetate and 615 were not injected. The number of fish recaptured and reported was 58 individuals in total, where 18 were injected and 40 were not, The rate of recovery is 4.9 % for injected fish and 6.5 % for non-injected fish.

As the difference between the figures of the rate of recapture is very small, so if we can keep the figure within this extent by other fish kinds, the application of this method would have so many hopes in future.

Conclusion

Here the authors introduced the method to make the time mark in hard tissues of fishes by lead-acetate injection to see the growth of these tissues, which is devised from OKADA & MIMURA's method. The method is combined with the tagging experiment to see the natural growth. The recapture of the tagged fish was too early to show the exact growth of tissues in this experiment, however, seeing the feature of growth of these tissues we could find out many points to be available to know the growth of these tissues beside the coupled injection method which is usually available for the fish in cultivation. We believe more other application of this method for fisheries biology would be found in future.

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