The Effects of Endocrine Disruptors on Fish Maturation and Reproduction - A Focus on Projects Underway at the Ministry of Agriculture, Forestry, and Fisheries -

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I am Kazunori Fujii of the National Research Institute of Fisheries and Environment of Inland Sea. I would like to thank Prof. Iguchi for inviting me here. Since my title, assignment and background were provided in the introduction, I should skip the first slide.

There have been reports that suggest various effects on fish in foreign countries. In Japan as well, the research groups of Prof. Iguchi, Prof. Nakamura of Teikyo University and Prof. Hara of Hokkaido University have reported abnormalities in carp in the Tama River. Arising from this report, abnormalities have been observed in the marbled sole in Tokyo Bay and gray mullet in Osaka Bay. All of the abnormalities had to do with feminization of males. There are still much we need to ravel about this, such as what substance or substances it is caused by, how the same type of fish in other areas are affected by the substance or substances, and what the effect is upon other types of fish.

The Ministry of Agriculture, Forestry, and Fisheries initiated a four-year plan for research projects concerning the effects of endocrine disrupters on the agriculture, forestry and fisheries and the mechanism by which endocrine disrupters work two years ago in 1999. The projects were divided among six teams. The projects involve research of livestock/poultry, waters, arable land, forests, food, food packaging/containers, preventing the effects and kinetics of dioxins. These six teams further consist of research groups called “sub-teams”. The waters team to which I belong consists of the state of influence sub-team, environmental kinetics sub-team and effects mechanism sub-team.

Today I would like to talk about the research results of the waters team over the past two years. Although my presentation is sort of like a mid-term report and kind of fragmented, I would like to focus on maturation and reproduction of fish.

Today I would specifically like to talk about vitellogenin and choriogenin, which we heard a lot about yesterday. I would also like to talk about these biomarkers and the effect of endocrine disrupters on sexual behavior, gametogenesis and sex differentiation of fish. Finally I would like to talk about the effects of endocrine disrupters in the waters of Japan. Although they are still fragmented, I would also like to give a brief description of the findings we have obtained so far.

First let’s talk about vitellogenin. We have already heard quite a lot about vitellogenin, so I’ll leave out the details. Vitellogenin is originally induced by endogenous estradiol-17β (hereinafter referred to as E2). It is a precursor of yolk protein produced by the liver due to the effect of female hormones. When a chemical substance that has estrogenic activity exists in the aquatic environment, it induces production of vitellogenin in the liver regardless of maturation period or male/female sex ratio. The protein is therefore noted as a biomarker for such estrogenic chemicals.

The rest of my presentation involves concrete data. The names of the principle researchers and institutions with which they are affiliated are given on the lower right. Two types of vitellogenin, 530 kDa (kilodaltons) and 320 kDa exist in the blood serum of Japanese goby. After being taken in by the oocytes in the vitellogenic stage, vitellogenin is processed into lipovitellin, the beta prime component, and the complex of lipovitellin and phosvitin.

Each vitellogenin was purified from blood serum. When the amino acid sequences were determined, we found an approximately 40% homology with the vitellogenin sequences of other fish reported so far in all cases. We prepared antibodies against each vitellogenin and developed a quantitative method using ELISA. The antibodies against Japanese goby vitellogenin however, mostly do not cross over with other fish. In order to study vitellogenin of various fish in various areas of Japan, therefore, each type of antibody must be prepared and each type of assay system must be established. In addition to the eight species of fish indicated here, the project has so far established vitellogenin assay systems for more than ten species of fish such as red sea bream and red tongue sole.

This graph shows how the concentration of vitellogenin in the blood varied in Japanese goby exposed to E2 for a period of two weeks. The horizontal axis indicates nominal concentrations of E2 in the water in ng/l. The order is in so-called ppt. The red bar shows the concentration of 530 kDa vitellogenin and the blue bar shows the concentration of 320 kDa vitellogenin.

As the graph clearly indicates, there exists a threshold at which both proteins manifest significantly at about 10 ng/l of E2.

The minimum concentration of E2 at which 530 kDa vitellogenin is induced in the order of 0.1 μ g/ml is 10 ng/l. We will talk about this again later, so please remember these figures.

Various species of fish will come up in the course of my presentation. One of these is the mudskipper, which is commonly seen in the Ariake Sea. Here are the results of a study of year-round variation in concentration of vitellogenin in the blood serum of mudskippers. The red graph at the top shows the year-round variation in females and the black graph at the bottom shows the year-round variation in males. The vitellogenin concentration was extremely high during the spawning period for females, as has already been reported in other fish. In some cases we found the concentration exceeded 10 mg/ml. We also found that the concentration becomes extremely low during the immature period.

For male mudskippers on the other hand, concentration was found to be much higher than for Japanese goby throughout the year, on the order of μg/ml. The highest concentration was detected during the spawning period, just as with females. Although I do not know what to make of it, as has been confirmed in several other fish, the year-round variation in vitellogenin concentration in the blood serum of males, in the case where the effect of vitellogenin as a biomarker was at least studied, the graph shows that the normal values need to be confirmed for each fish and each season.

Now I would like to talk about the choriogenin assay. Choriogenin testing was conducted on the marbled sole mentioned in the beginning of the presentation. Unlike vitellogenin, choriogenin is the precursor of vitellin envelope, the membrane in which the oocyte is enclosed. Just as with vitellogenin, because production by the liver is induced by endogenous E2, the protein is noted as a biomarker of estrogenic substances in the environment.

This shows the figure of SDS-PAGE and Western-blotting of choriogenin in the marbled sole. Indicated as Eg here, the vitellin envelope of the marbled sole is mainly composed by 61 and 37 kDa proteins.

We found that proteins resembling each molecular weight were induced in the blood serum by administering E2 to males. These types of protein from the blood serum are therefore purified and antibodies against each protein were prepared. Here we have established an assay system called “time-resolved fluoroimmunoassay.” Quantity from tens of pg/ml to tens of ng/ml is currently possible.

Here we have variation of concentration of 37 kDa choriogenin, 61 kDa choriogenin and vitellogenin in the blood of the marbled sole as a result of exposure to ethinylestradiol for a period of two weeks. The figures given at the bottom indicate concentration of ethinylestradiol in the water by ng/l dose.

We found that all proteins in the blood were increased according to increasing concentration of ethinylestradiol. We also detected a significant amount of low molecular weight choriogenin and vitellogenin in the blood even at the minimum concentration of ethinylestradiol of 0.3 ng/l.

As the result of a similar experiment using estrone occasionally detected in the sea, we found that low molecular weight choriogenin was already significantly induced at the minimum concentration of 10 ng/l. The concentration-dependent increases were also observed for vitellogenin and high molecular weight choriogenin.

Thus these results suggest that low molecular weight choriogenin of 37 kDa may be more sensitive to estrogen than vitellogenin. We are currently conducting a study of effects based on this.

Next I would like to talk about the effect of such substances on the sexual behavior of fish. This graph shows the effect on the sexual behavior of male masu salmon. The vertical axis shows the frequency of approaching behavior of males to females. The red bar shows frequency of quivering which is the act of the male to urge the female to lay eggs. Castration of the testis radically reduced this sexual behavior. When the castrated males were administered doses of steroid hormones such as testosterone, E2, 11-ketotestosterone (11KT) or DHP, the frequency of such sexual behavior that had decreased by castration tended to recover by androgens such as testosterone or 11KT.

These are the results of monitoring sexual behavior of males not castrated that were administered doses of E2, nonylphenol and bisphenol A. Sexual behavior did not decreased in those administered E2, but rather tended to increase at high doses. On the other hand, administering nonylphenol and bisphenol A tends to suppress such sexual behavior. These results suggest these chemical substances have an antagonistic effect on androgens.

Next I would like to talk about gametogenesis. These are the results of an experiment using red sea bream. The spawning period for red sea bream is in the spring from April through May. As for the results of experiments to see what happens when the specimens are administered steroid hormones at a much earlier stage, say November through December. The vertical axis indicates percentage of the testis weight accounted for by body weight.

The weight of the testes in the control group increased significantly during a one-month period. Just as with the control group, testis weight increased for the specimens administered 11-ketotestosterone. The weight of the testes of specimens administered estradiol and testosterone on the other hand suppressed whereas it should have increased, thus indicating that these steroid hormones inhibited increase in weight of the gonads.

Amount of GnRH and GTH expressed in the brain are currently being studied as possible causes, but no significant difference in amount due to the influence of steroids has been observed. Although I skipped this a little while ago, no significant change in GSI has been observed even when these steroids are administered during the period in which sperm formation is in full swing. We are currently studying the mechanism by which this happens and what the effects of other chemical substances are.

Next is the effect of such substances on sex differentiation. This shows the manifestation of steroid metabolizing enzymes during the sex differentiation stage in tilapia. The figures given at the bottom indicate the number of days since hatching. Genetically all-female and genetically all-male tilapia were used for the experiment.

In the females, these metabolizing enzymes for synthesizing estrogen were observed prior to the period in which the ovarian cavity was formed. In the males however, manifestation of the enzyme group for producing androgen was observed subsequent to sex differentiation, that is after formation of the testis and sperm duct.

If administered aromatase inhibitors during this period, genetic females have been observed to change into males. What this means is that estrogen strongly influences and is indispensable for sex

differentiation in females. There is still a lot we have not raveled about the mechanism of sex differentiation on males, but data suggests that androgen is unlikely to be necessary for sex differentiation on males.

These are the results of experiments conducted to see what happens when amago salmon, a fish of the salmon family thought to be more sensitive to steroid hormones, that is genetically all-male amago salmon, are exposed to various chemical substances during the sex differentiation period. The target group naturally consists of males. If exposed to E2 however – this shows the number of days since hatching – no males appeared at concentrations in excess of 50 ng/l, with more than 80% of the individuals becoming females. Others became intersex individuals. Even at the minimum concentration of 20 ng/l, intersex appeared in approximately 30%.

Even when exposed to the minimum concentration of 10 ng/l ethinylestradiol, which is even stronger, no males appeared, and more than 80% became females and the others became intersex. Although the dose is quite high, exposure to nonylphenol and bisphenol A produced females and intersex.

It will now be necessary to determine what will happen at lower doses, and we will need to study the androgenic effect of females turning into males.

Finally is the state of endocrine disrupter influence in the aquatic environment of Japan. Although we still do not have a lot of data, here are the results we have so far. These are the results using Japanese goby mentioned at the beginning of the presentation. This shows the amount of vitellogenin in the blood of males – this is 530 kDa vitellogenin – in waters thought to have a low level of pollution. From the data of the estrogen exposure experiment mentioned a little while ago ― here we drew a line at the blood concentration of 100 ng/ml for the sake of convenience – to show how many individuals whose blood concentration are in excess of this in each water. The denominator indicates the total number of individuals sampled and these are the results of testing in various waters of Japan.

As you can tell from the figures, almost none of the individuals exceeded the value on the coast of the Sea of Japan, but a high percentage of the males exceeded the value near large cities on the Pacific coast. The figures also vary according to the year sampled, for example. It will probably be necessary to continue this study in other waters over the years. We are currently involved in studying the influence of endocrine disrupters in various waters for other fish as well.

We are starting to run out of time, so I have to leave out a lot of things. These are concentrations of various chemical substances in the aquatic environment measured as part of a study conducted by the Ministry of the Environment last year. The top three is data concerning rivers and the bottom three data concerning seas. The number of locations where measurements were taken is given in parentheses. In the graph, the name of the chemical substance is given in the center. The names are ethinylestradiol, nonylphenol and octylphenol. This white number is the detection limit and the ratio of waters not reaching that figure. In the case of seas for example, ethinylestradiol did not reach the detection limit in any of the 17 locations sampled.

Here we have data of di(2-ethylhexyl) phthalate, bisphenol A and female hormone estradiol (E2). As is evident from sex differentiation and vitellogenin exposure test data, the locations where the concentration exceeds the threshold, are primarily in rivers. This therefore suggests that such waters may be affected by endocrine disrupters.

The concentration in the seas is much lower than in rivers, but several estrogenic chemical substances have already been detected. Although a low dose, considering complex contamination of each respective chemical substance, I think there is probably a need to continue to study the effect of endocrine disrupters in the seas as well.

This presentation does not venture to provide a conclusion. There is still two years remaining in the research period. We will offer a conclusion when all of the research has been concluded.

These are the joint researchers who have provided the data for this presentation. I would like to thank the fish that cooperated in the research as well as the researchers. This brings my presentation to a close. Thank you very much.

Q&A

Iguchi: Thank you very much. We heard an interim report on research involving various species of fish from various angles. If you have a question, please feel free. Just step up to the microphone. Any questions? Go ahead.

Guillette: Guillette, University of Florida. Quick question: have you tried to look at the effective mixtures on the choriogenin and the vitellogenin response? We know that androgens and progestins block the vitellogenesis in fish, and one of the questions I have is whether in the environment with complex mixtures of whether, in fact, positives tell us something, but negatives may be false-negatives because of complex mixtures.

Fujii: Compound effect is an extremely important theme. We must consider the putting together those showing positive reactions and those showing negative reactions. Furthermore the synergistic effect and the additive effect of positive on positive are interesting themes, but we still have no definitive data on that. If possible in the future we would like to study such effects, compound contamination and compound mechanisms.

Iguchi: Mr. Santo, go ahead.

Santo: My name is Santo from Towa Chemical. I have two questions. One concerns annual variation of concentration of vitellogenin in the blood of dusky mud hopper. Is that data from wild specimens? Also, do you know if there is any difference in data from wild fish and from cultured fish?

The other question concerns the national distribution chart of numbers of Japanese goby individuals exceeding the threshold value for vitellogenin concentration in the blood we saw at the end of the presentation. I think the proportion for example was about the same for Nagasaki and Osaka. I would like to know what sort of standard you are thinking of in place of comparison of large and medium-sized cities?

Fujii: Concerning the first question about mudskipper, if memory serves correctly, the data was from sampling in the field. If there was some misunderstanding, I would like to correct it. I at least do not think that value is a normal value that will serve as a future base. What I said, or rather what I wanted to say was, as we have observed carp and Japanese dace, there seemed to be an annual variation in concentration of vitellogenin in the blood in males as well. In order to see this as a biomarker, we must pay attention to annual variation and the season in which sampling is conducted.

As for the second question, all I can say now is the cause is not yet known. A large percentage of the individuals in which a high concentration of vitellogenin was detected came from comparatively [large] cities and the areas near those cities. As I said a little while ago however – although I don't want to speak in very specific terms – if you take Hyogo Prefecture for example, there was a certain percentage for a certain year. Thirteen individuals within 70 were detected in a study conducted in 1999. The following year however none were detected. It is therefore hard to make a conclusive statement.

One other thing, concerning certain effects, we are currently studying the substances that caused those effects. We fractionate water and see what activity is in what fraction by both *in vivo* and *in vitro* tests. Activity is mainly estrogenic activity. We are using this technique in an attempt to isolate the causal substances. These data and the analytic data of water in the environment by the Ministry of the Environment and our group, taking this into consideration, we would like to assess the various aquatic areas. Does that answer your question?

Iguchi: Thank you very much.