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The Relationship of Hepatoma in Rainbow Trout to Aflatoxin Contamination and Cottonseed Meal[1]

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SUMMARY

A test diet was formulated to approximate the commercial feed associated with the 1960 trout hepatoma epizootic in California. The same diet without the cottonseed meal portion served as the control. A third ration with food grade cottonseed flour substituted for the cottonseed meal was also tested. The trout on the 2 diets with the cottonseed products showed a high incidence of hepatoma. These results confirm previous observations associating the development of hepatoma in trout with the feeding of cottonseed meal. Chemical analyses relate the occurrence of hepatoma to aflatoxin B_1 in the cottonseed components of the test diets. These observations provide cumulative evidence that the hepatoma epizootic in California trout resulted from aflatoxin contamination of cottonseed meal ingredient.

Confirmation of the carcinogenicity of the cottonseed flour sample was obtained in a second experiment using a semipurified ration. Initial analysis of the cottonseed flour detected no aflatoxin. Subsequently an improved method detected 2 parts per billion of aflatoxin B_1; this is equivalent to 0.4 ppb in the experimental diet. Results from other studies indicate that the naturally occurring cyclopropene fatty acids and gossypol in the cottonseed components probably enhanced the carcinogenic action of the minute quantity of aflatoxin.

INTRODUCTION

In 1960 the widespread occurrence of trout hepatoma was recognized in many hatcheries in the United States (14). Epidemiologic study of the disease in California hatcheries showed that dietary factors were responsible and subsequent feeding experiments indicated that the etiologic agent was contained in the cottonseed component of the ration (20). Later Sinnhuber *et al.* (17) reported that aflatoxin, a mold metabolite and known liver carcinogen, was present in some samples of commercial cottonseed meal. This report presents the results of further work to determine the etiology of the epizootic of trout hepatoma in California hatcheries.

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MATERIALS AND METHODS

Experiment 1

The test diet for this work was the Santa Monica pelleted fish ration, which contains over 40 ingredients including vitamin and mineral supplements. As far as possible, the ingredients of the test rations, their sources, and percent composition were the same as those of the Santa Monica ration associated with a high hepatoma incidence in 1960 (20). The exceptions to this were the sources of the fish, meat, and blood meals. The major components of the diet were: wheat middlings, 25%; cottonseed meal, 20%; and fish meal, 19%.

The 3 diets fed in this experiment were based on the Santa Monica ration and its modifications as follows: (*a*) The DA diet, which tested the effect of cottonseed meal and consisted of the Santa Monica ration containing 20% prepressed solvent extracted cottonseed meal; (*b*) The DB diet was the control ration and consisted of the Santa Monica ration without cottonseed meal; and (*c*) The DC diet tested the effect of cottonseed flour and consisted of the Santa Monica ration with 20% food grade cottonseed flour (trade name Proflo) substituted for the cottonseed meal. The designations DA, DB, and DC were used for labeling diets and the respective trout pools.

The rainbow trout (*Salmo gairdneri*) used were the fall-spawning Shasta strain. These fish were fed and cared for according to standard hatchery management except for their not being periodically graded in size. All fish were hatched at the California Department of Fish and Game's Darrah Springs Fish Hatchery where the experiment was carried on for the first 16 months. The water temperature there ranges from 14.5° to 15.5°C.

The trout were sampled monthly for the first 10 months and again at 14 and 24 months. Approximately 25 fish were taken from each diet group at each examination. The fish were weighed and the livers were removed, examined grossly, and fixed in 10% formalin. The liver specimens were coded and sent for histologic examination to an independent laboratory. The diagnosis of hepatoma established by microscopic examination was the basis of the data presented here.

The 3 diets were exhausted after 14 months, and, subsequently, standard hatchery diets were fed. At 16 months of age, the remaining fish were moved to the Crystal Lake Fish Hatchery where holding tanks for larger fish were available and lower water temperatures would not adversely affect fertility and egg viability. Four pairs of fish from the DA group and 5 pairs each from the DB and DC diets were spawned

at 21 months of age. The eggs were handled in the routine hatchery manner with those of the individual pair held in separate hatching trays, as is done with select brood fish where hatching records are desired.

Biochemical analyses of the diets were carried out to determine the presence of agents which were tentatively implicated in the etiology of the disease. Aflatoxin analyses were made on samples of the total rations and on the cottonseed meal and flour components of the experimental diets. Gossypol determinations (2) were done on the 2 cottonseed ingredients. Analyses for insecticides, chlorinated hydrocarbons, and thiophosphates were performed on each of the 3 total rations.

Experiment 2

Because interpretation of the results obtained in the trout tested with 20% cottonseed flour was uncertain, a second experiment was conducted. Rainbow trout of the Shasta strain were held in test laboratory conditions at Oregon State University, Corvallis, Oregon, on a semipurified test ration described by Lee *et al.* (10). The control diet was composed of casein, gelatin, salmon and corn oils, and dextrin, with additives of salts and vitamins. This was modified to form the experimental ration by adding 20% cottonseed flour from the same lot used in the DC diet, keeping the diet isocaloric and iso-nitrogenous. The cottonseed flour was analyzed for aflatoxin by an improved method (13), and, in addition, the cyclopropene fatty acid determination was done using the method of Bailey *et al.* (3).

Four tanks containing 100 fish each were used. Two groups served as controls; the other 2 tanks of fish were fed the diet containing cottonseed flour. The water temperature was 11.5°C. At the start of the experiment, the fish were 1 month old and, prior to that, had been fed only the control ration. The trout were held 1 year; at 3-month intervals, a 10% sample of the trout was taken from each tank. The fish were explored for gross abnormalities and placed in Bouin's fixative. Samples of the livers were embedded in paraffin, skip-sectioned, and stained with hematoxylin and eosin.

RESULTS

Experiment 1

The rainbow trout fed the DA ration (20% cottonseed meal) first revealed hepatomas at 5 months of age; by the ninth month, 88% of the fish sampled had tumors (Table 1). Hepatomas were not observed in the DC group (20% cottonseed flour) until the ninth month, when 5 out of 25 were positive, and by 14 months of age, when 56% of the fish sampled showed hepatomas. Trout on the DB control diet showed neither gross nor microscopic hepatomas while on the test rations. However, a sampling of 2-year-old trout, after 14 months on the control diet and 10 months on the standard hatchery diet, revealed 3 out of the 26 examined with microscopic hepatomas.

Analysis of a sample of the cottonseed meal used in the DA diet revealed 36 ppb of aflatoxin B_1 (Table 2). As 20% of this ration was cottonseed meal, the aflatoxin level in the total ration would have been 7 ppb. No aflatoxin was detected in a sample of the cottonseed flour from the DC diet in the initial attempts, but the improved method described by Pons *et al.* (13) later revealed 2 ppb of aflatoxin B_1. Accordingly, the DC ration with 20% cottonseed flour would have contained 0.4 ppb aflatoxin B_1. Gossypol, both free and total, was slightly lower in the cottonseed meal than in the flour (Table 2). Attempts to detect aflatoxin in the total diets, DA, DB, and DC, were not successful due to the presence of interfering pigments. Insecticide determinations in each of the total diets (Table 2) show the total chlorinated hydrocarbons to be very similar in the 3 diets. However, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) and 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE) present in the DA diet were not detected in the DB and DC diets. Thiophosphates were remarkable only in that all fractions were lower in the DA ration which produced the highest incidence of hepatomas.

Table 1

Age of fish (mo.)	*DA* test diet[a] 20% cottonseed meal	*DB* control diet[b] no cottonseed meal	*DC* test diet[c] 20% cottonseed flour
1	0/24[d]	0/25	0/25
2	0/25	0/25	0/25
3	0/25	0/25	0/25
4	0/25	0/25	0/25
5	7/25	0/25	0/25
6	12/25	0/25	0/25
7	19/25	0/25	0/25
8	19/25	0/25	0/25
9	22/25	0/25	5/25
10	nd[e]	0/25	5/25
14	nd	0/25	14/24
24	4/4	3/26	17/26

Microscopic hepatoma diagnosis in rainbow trout by diet and age.

[a] Santa Monica Trout Feed, commercial ration containing 20% cottonseed meal, associated with high incidence of hepatomas in California.

[b] Santa Monica ration with no cottonseed meal.

[c] Santa Monica ration with 20% food grade cottonseed flour substituted for the conttonseed meal.

[d] Number with tumors/number examined.

[e] Not done.

In comparison with the DB control group, high egg mortality was observed in all 4 lots from the DA group, as well as in the eggs from 3 out of 5 DC parents (Table 3). High sac fry mortality was also observed in the progeny from the same 3 DC parent fish having high egg loss; however, eggs from the other 2 DC pairs showed no significant increase in either egg or sac fry mortality. All parent fish from the DA and DC groups showed gross hepatomas.

Because there were many deaths in the DA experimental group during shipment and spawning, only a few trout remained in this group at 24 months. Three of these 2 year olds were selected, and a complete dissection and histologic examination of the tissues was done. The enlarged nodular livers were sectioned, and the lesions were subsequently classified as hepatocellular carcinomas. Metastatic lesions similar to those described by other workers (1, 4, 9, 21) were found in stomach, ceca, pancreas, ovary, intercecal fascia, and lateral musculature.

Table 2

Analysis	*DA* test diet[a] 20% cottonseed meal	*DB* control diet[b] no cottonseed meal	*DC* test diet[c] 20% cottonseed flour[d]
Cottonseed component tested			
Aflatoxin (ppb) B_1	36	na[e]	2
Gossypol			
% total	0.71	na	0.99
% free	0.04	na	0.046
Total ration tested			
Thiophosphates (ppm)			
Ethion	0.07	0.11	0.13
Baytex	0.04	0.05	0.05
Parathione	0	0.07	0.05
Chlorinated hydrocarbons (ppm)			
Total	0.15	0.11	0.14
DDT	0.05	0	0
DDE	0.01	0	0
Unknown	0.04	0.06	0.04

Analyses of experimental diets and cottonseed meal and flour components. DDT, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane; DDE, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene.

[a] Santa Monica Trout Feed—commercial ration containing 20% cottonseed meal, associated with high incidence of hepatomas in California.

[b] Santa Monica ration with no cottonseed meal.

[c] Santa Monica ration with 20% food grade cottonseed flour substituted for the cottonseed meal.

[d] Also used in Experiment 2.

[e] Not applicable.

Experiment 2

Trout fed the semipurified control diet showed neither gross nor microscopic hepatomas during the 12-month experimental period, and the fish were considered normal. However, the trout on the diet containing cottonseed flour showed evidence of minor liver damage at 3 months; this consisted of parenchymal changes with accompanying lipid-filled cells with nuclei atypical in size and shape.

After 6 months on the cottonseed flour diet, liver damage was evidenced in all the fish examined; it consisted of lipid-filled parenchymal cells, bile duct hyperplasia, and the accumulation of appreciable gossypol-like material around the bile

Table 3

Diet group of parents		No. of eggs died/ No. taken	Percent eggs died	Mortality of sac fry	
				Number	Percent[a]
DA Diet	Total	2,288/3,835	59.6	164	10.6
Pair #1		670/670	100.0		
#2		272/931	29.2	86	13.0
#3		146/1,034	14.1	78	8.8
#4		1,200/1,200	100.0		
DB Control	Total	508/6,400	7.9	165	2.8
Pair #1		189/1,537	12.3	69	5.1
#2		87/1,304	6.6	27	2.2
#3		133/1,092	12.2	39	4.0
#4		53/1,273	4.5	18	1.5
#5		46/1,194	3.8	12	1.0
DC Diet	Total	2,821/6,006	46.9	663	20.8
Pair #1		113/1,465	7.7	110	8.1
#2		68/1,037	6.5	67	6.9
#3		1,030/1,233	83.5	171	84.2
#4		670/1,093	61.3	198	46.8
#5		940/1,178	79.7	117	49.1

Egg and sac fry mortality by diet group of parent rainbow trout.

[a] No. died × 100/no. of eggs hatched.

ducts. Livers from 3 of 20 fish sampled showed microscopic evidence of hepatomas to give a 15% incidence at this early stage.

The 9-month sample was similar in appearance, with 20% of the livers revealing hepatomas; gross tumors were also evident, varying in size from 1 to 4 mm in diameter. At 12 months the experiment was terminated with a 32% incidence of gross hepatomas; this was extended to 55% by microscopic examination.

Use of the improved analysis of the cottonseed flour (13) revealed 2 ppb aflatoxin B_1. In addition, cyclopropene fatty acid analyses of the cottonseed flour gave a value of 512 parts per million, implying a level of 102 ppm when fed at the 20% level in the semipurified ration. (The same level would also apply for the DC diet in Experiment 1.)

DISCUSSION

Experiments 1 and 2

Cottonseed Meal and Aflatoxin. It is clear that the cottonseed meal component of the DA diet is associated with a potent hepatoma-inducing effect. These results confirm earlier observations of trout hepatoma associated with the feeding of cottonseed meal (17, 20). This, along with the finding of aflatoxin in the cottonseed components in the present and an earlier study (17), provides cumulative evidence that the California trout hepatoma epizootic was caused by aflatoxin contamination of the cottonseed meal in the commercial rations. The invasive and metastatic lesions identified in 2-year-old DA fish leave no doubt as to the malignant nature of the neoplasm.

Control Diet. The DB control group in Experiment 1 showed no hepatomas while on that diet. However, 3 microscopic hepatomas were found out of 26 trout sampled at 2 years of age. This raises the possibility that a carcinogen was present either in the control diet which was fed during the first 14 months or in the standard hatchery rations fed the last 10 months. Particle analysis of the standard rations showed cottonseed meal to be present occasionally. Moreover, during the course of this work, a few gross hepatomas were observed in production trout reared in these same facilities, indicating that the standard rations have oncogenic potential. This is consistent with the view that the hepatomas in the DB control group resulted from an agent in the standard hatchery rations rather than in the control diet.

Earlier work showed a low level of hepatoma in trout tested on wheat middlings (20); Halver (7) reported a low hepatoma occurrence in some control groups. The low incidence of hepatoma in these instances is probably due to the occasional presence of aflatoxin or another carcinogen in the diet. Prior to the actual finding of aflatoxin in the cottonseed flour, the unexpected hepatomas in these and similar groups were called "spontaneous". The eventual detection of aflatoxin by improved procedures suggests the designation "spontaneous" should be viewed with mistrust.

Cottonseed Flour and Aflatoxin. The hepatomas associated with the feeding of cottonseed flour tested in the DC diet were not expected for two reasons. In earlier feeding experiments, cottonseed flour from the same producer did not result in hepatomas (17), and, further, the initial analysis of samples of cottonseed flour used in the DC diet failed to detect aflatoxins. Because of this, Experiment 2 was conducted to determine if the results could be reproduced when the same sample cottonseed flour was added to a semipurified diet. Hepatomas were associated with the feeding of 20% cottonseed flour in both the DC diet and in Experiment 2 using a semipurified ration. It was concluded from these results that the flour alone contained the necessary and sufficient agent(s) for oncogenesis.

It is worthy of note that a level of aflatoxin may be as low as 0.4 ppb in the semipurified ration. This corresponds to a dose level of approximately 0.4 μg/kg body weight. The total amount of aflatoxin consumed by a trout 6 months of age at this level and which produced an observable incidence would be about 0.018 μg. By 9 months a trout would have consumed 0.06 μg. Halver (7) has reported a 32% incidence of trout hepatomas after 1 year of feeding a semipurified ration containing only 0.5 ppb aflatoxin B_1. In Experiment 2, a 55% incidence of hepatomas was observed when only 0.4 ppb aflatoxin was present in the diet. This suggests that aflatoxin is an effective carcinogen in trout at levels not detectable by present analytical technics. It further implies that effective control of aflatoxin in experimental and commercial trout rations will prove to be a demanding and, in some instances, an empirical procedure.

Other Agents. It is likely that other agents identified in the experimental diets enhanced the carcinogenic effect of aflatoxin. One possibility is the cyclopropenoid fatty acids, which occur naturally in the cottonseed. Sinnhuber *et al.* (18) reported an 83% incidence of gross hepatomas in trout after 6 months on a purified ration containing 4 ppb aflatoxin B_1 and cyclopropenoid fatty acids (as 0.04% *S. foetida* oil equivalent to 200 ppm cyclopropene function), and no gross tumors were found in the 6-month-old control group fed the same level of aflatoxin without cyclopropenoids. It was concluded that the cyclopropene fatty acids are powerful cocarcinogens when fed with aflatoxin. The cottonseed flour revealed 102 ppm cyclopropene fatty acid functions, in addition to 0.4 ppb aflatoxin B_1. It is probable that the cyclopropenoids enhanced the carcinogenic effect of the minute quantities of aflatoxin in the flour accounting for the substantial incidence of hepatomas observed in both the DC group and the test group in Experiment 2.

Cottonseed also contains gossypol, a yellow phenolic pigment which is an acknowledged toxin (5, 12). A recent report by Lee *et al.* (11) indicated that gossypol, although not as active a promoting agent as cyclopropenes for aflatoxin-induced hepatoma in trout, more than doubled the tumor incidence. It is likely that gossypol in the cottonseed components (Table 2) also enhanced the oncogenic effect of aflatoxin.

The contribution of the thiophosphate and chlorinated hydrocarbon insecticides to the observed tumor incidences is less certain, and there is no pattern in their occurrence in this study to relate them to the differing incidences of hepatomas associated with the 3 diets (Table 2). Halver (8) has reported DDT to induce trout hepatomas. DDT was detected in the DA diet; however, none was found in the DC diet, indicating it was not a factor in the tumor induction associated with cot-

tonseed flour. In addition, the observations of Wales *et al.* (19) suggest that chlorinated insecticides are not highly suspect agents in diet-associated epizootics of trout hepatoma, as such occurrences were recorded prior to the commercial use of these materials.

Egg and Sac Fry Mortality. The observations following spawning (Table 3) demonstrate that eggs from parent fish fed the diets with cottonseed components experienced high egg and sac fry mortality. The adverse effects of the cyclopropenes on the reproductive system of chickens (15) and on chick embryo mortality (16), and the report of increased fetal deaths in mice due to aflatoxin (6), implicate these 2 agents in the high observed mortalities. However, it is possible that the diseased liver of the parent fish affected the quality of the eggs. Specific studies are needed to determine if there is a direct relationship between these agents and the lethal effects on trout eggs and sac fry.

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