

Differential effect of chronic aflatoxin B1 intoxication on the growth performance and incidence of hepatic lesions in triploid and diploid rainbow trout (*Oncorhynchus mykiss*)

Efecto diferencial de la intoxicación crónica por aflatoxina B1 en el crecimiento y en la incidencia de lesiones hepáticas en truchas diploides y triploides (*Oncorhynchus mykiss*)

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SUMMARY

Triploid trout has been considered to be more resistant than diploid trout to many diseases and to some adverse aquaculture conditions. Considering the common problems with animal food contamination by aflatoxins, the purpose of this research was to compare the incidence of liver lesions and growth performance in triploid and diploid trout (*O. mykiss*) exposed to chronic contamination with aflatoxin B1.

A total of 240 samples formed 4 groups, diploid and triploid control without aflatoxin in food and diploid and triploid treated with 80 ppb of aflatoxin/Kg of food. Five samples in each group were monthly sacrificed during one year. After the measurement of body weight and length, hepatic samples were obtained and fixed in 10% formalin-saline solution. The histopathological analyses were performed with liver slides stained with hematoxylin and eosin.

The comparative analyses of the growth performance showed significant difference between control and treated diploid, suggesting that aflatoxin B1 affects the growing of diploid trout. In triploid groups this aspect was not observed.

The histopathological analyses indicated that triploid trout is more resistant to aflatoxin B1, since this group did not present neoplastic lesions. Diploid fishes however, showed 4 samples with neoplastic lesion. The triploid treated group had preneoplastic lesion, but with minor incidence and slower progression than diploid trout.

Key words: Rainbow trout, triploidy, aflatoxin, hepatocarcinogenesis.

Palabras claves: Trucha arco iris, triploidia, aflatoxina, hepatocarcinogenesis.

INTRODUCTION

The triploidization has been shown to be a promising technique in fish farming because triploid fishes, being sterile animals, are spared

from the physiologic stress of the sexual maturation and reproductive behavior (Benfey, 1991). Also, they are not subjected to the immunodepressive state commonly associated to the reproductive process. Their mortality is reduced and they show better growing rates after the first year of life and show less aggressive behavior than diploid fishes. These features open

new possibilities in meat fish market or in their use in fish farms dedicated to sport fishing which is, an expanding economic activity in the last decade.

Several studies have been developed in order to increase the knowledge of the metabolic activity and behavior of triploid cells in many organs and tissues since the triploidization technique was first applied to aquaculture (Graham *et al.*, 1985; Fauconneau *et al.*, 1990; Tambets *et al.*, 1991; Greenlee *et al.*, 1995; Arzel *et al.*, 1998; Johnston *et al.*, 1999; Sadler *et al.*, 2000). The objective of this study was to compare the productivity of triploid fishes vs diploid fishes, taking into account parameters such as growth rate, weight gain and susceptibility to diseases (Kim *et al.*, 1986; Kobayashi, 1992; Krisfalusi & Cloud, 1996; Bonnet *et al.*, 1999; Tabata *et al.*, 1999; Benfey & Biron, 2000).

Although triploid fishes had been evaluated in several adverse conditions found in aquaculture and under different stress situations, their response to aflatoxin contaminated food has not been investigated.

The usual commercial fish food is composed by cereal grains (corn, soybean, wheat). There is a possibility that 25% of such grains used for animal nutrition may be contaminated by aflatoxins, which may have serious implications for the human and animal health (Fink-Gremmels, 1999).

The effect of the ingestion of aflatoxin B₁ contaminated fish food on the growth performance and on the incidence and evolution of hepatic lesions in triploid and diploid rainbow trout are compared in this study.

MATERIAL AND METHODS

One hundred and twenty triploid and 120 diploid *Oncorhynchus mykiss* (Teleostei, Salmonidae) from both sexes were obtained at Núcleo Experimental de Salmonicultura Dr. Ascânio de Faria, Instituto de Pesca/SAA, in the city of Campos do Jordão/SP. and where kept under trout farming conditions throughout the experiment.

Triploid trouts were produced by keeping fertilized eggs, from diploid parents, in warm water (28°C) for 20 minutes, starting 10 minutes after egg activation. After the fry reached 8 grams of average weight, both diploid and triploid specimens were separated in the following groups: TC (triploid) and DC (diploid) fed with trout commercial food without aflatoxin; TT (triploid) and DT (diploid) fed with trout commercial food plus Aflatoxin B₁ at 80 ppb (AFB₁) (Sigma).

All groups received their respective experimental food twice a day for 12 months. Samples of food were monthly analyzed by the Soares & Rodriguez-Amaya (1989) method to verify the absence of aflatoxin in the control groups and to confirm the degree of contamination in DT and TT groups. Thirty days after the beginning of the experiment 5 animals from each group were sacrificed by immersion in 1:10,000 benzocaine solution; the total weight and total size were recorded. The procedure was repeated monthly until the end of the experiment. Blood samples were taken from the caudal blood vessels and blood smears were prepared. The triploidy was confirmed by measuring the main axis of erythrocytes nuclei per glass slide without staining (Benfey *et al.*, 1984). The measurements were obtained in a light microscope with a graded eyepiece (Leitz).

The celomatic cavity was open by a ventro-longitudinal incision and the liver was exposed and studied *in locu* before being removed. Liver samples were fixed by a 10% saline-formalin solution for 24 hours at room temperature and paraffin embedded. Sections 6 µm thick were cut, stained by hematoxylin-eosin method for further histopathologic study.

The criteria established by Arana (1997) were applied to establish three basic situations describing the incidence of liver preneoplastic lesions in hepatic section (focal and nodular), characterized by the histopathologic inspection: one lesion per hepatic histologic section 1. two lesions per section, 2. or three or more lesions per section 3. A dichotomous classification was applied to neoplastic lesions: absent (0 or not) or present (Ok. or yes).

The comparison between the biometric data of the different groups took into account their temporal component and a regression analysis was applied to each group of monthly average values. A statistical test (analysis of variance) was applied to verify the difference between the regression lines ($p < 0.001$) (Beigelman, 1996).

The statistical analysis of the incidence of liver lesions was carried out using the computer programs SAS and Minitab, taking the number of animals showing lesions and their occurrence per quarter as parameters. In data analysis, the null hypothesis was the effect of treatment. The time was the same for each combination of quantity/type of lesion. The Wald test (Mood *et al.*, 1974) was applied in each case with $\alpha = 0,05$. It was observed if the probability of the test value analyzed was under a table value (α). In the case this probability was less than α , the null hypothesis was rejected and the difference between the effect of treatment was accepted as significant.

RESULTS

In our work, the comparative analysis of the biometric parameters revealed a significant difference between the DC group and the other groups. The null hypothesis of similarity of treatments was found to be true to DT, TC e TT (Graphs 1 and 2). The small difference observed in the lines did not result in a significant difference between TC and TT groups.

The histopathologic study of the liver of the control groups (DC and TC) showed the typical morphology described for the species throughout the experiment and no one kind of lesion was identified (Fig. 1).

Diploid and triploid fishes treated with aflatoxin, DT and TT groups, presented several kind of lesions that are characteristics of intoxication and carcinogenesis process in liver induced by aflatoxin, such as the occurrence of foci of basophilic cells-BF (Figs. 3 and 5), nodules of basophilic cells-BN (Figs. 2 and 4), foci of acidophilic cells -AF (Fig. 5) and foci of vacuolated cells-VF (Figs. 6 and 7). The foci of basophilic cells and nodule of basophilic cells

were considered preneoplastic lesions. Preneoplastic lesions were found in both diploid and triploid animals, but the lesions occurrence and progression were reduced in triploid specimens. It may then be observed in Graphs 3, 4 and 5 that basophilic cells foci (BF) and basophilic cells nodules (BN) appeared later in triploid than in diploid. It may be noted in these graphs that the hepatocarcinogenesis process is slow in triploid and while TT group had BF type lesions, DT group showed predominance of BN type lesion.

Neoplastic lesions were observed in DT group, occurring in the last quarter, when four diploid animals showed hepatocellular carcinoma (Figs. 8 and 9), while no neoplasm was identified in triploid fishes until the end of the experiment (Graph 6).

DISCUSSION

Aflatoxin B1 is an important member of the aflatoxin family due to its highly toxic and carcinogenic properties (Wogan, 1973). Such effects have stimulated a great number of scientific investigations, most of them dealing with rainbow trout on account of its high sensibility to this mycotoxin. A revision from Bailey *et al.* (1996) shows that this animal model is used for aflatoxicosis experiments and in epidemiological surveys.

Particularly, triploid trout was less used in carcinogenesis experiments (Thorgaard *et al.*, 1999), but their resistance to several diseases is a stimulating factor to check their behaviour to many kind of carcinogens such as aflatoxin B1.

Our results indicate that AFB1 effectively reduced the growth rate and weight gain of diploid animals. The triploid fishes however, were resistant to this effect. The gap between the growth performance of DC group and the triploid fishes as a whole may be ascribed to the usual slower growth of triploid fishes along the first year of life when compared to diploid fishes, a fact that has been experimentally demonstrated by other authors. This situation is usually reversed when diploid trouts reach the sexual maturity period (Thorgaard, 1986).

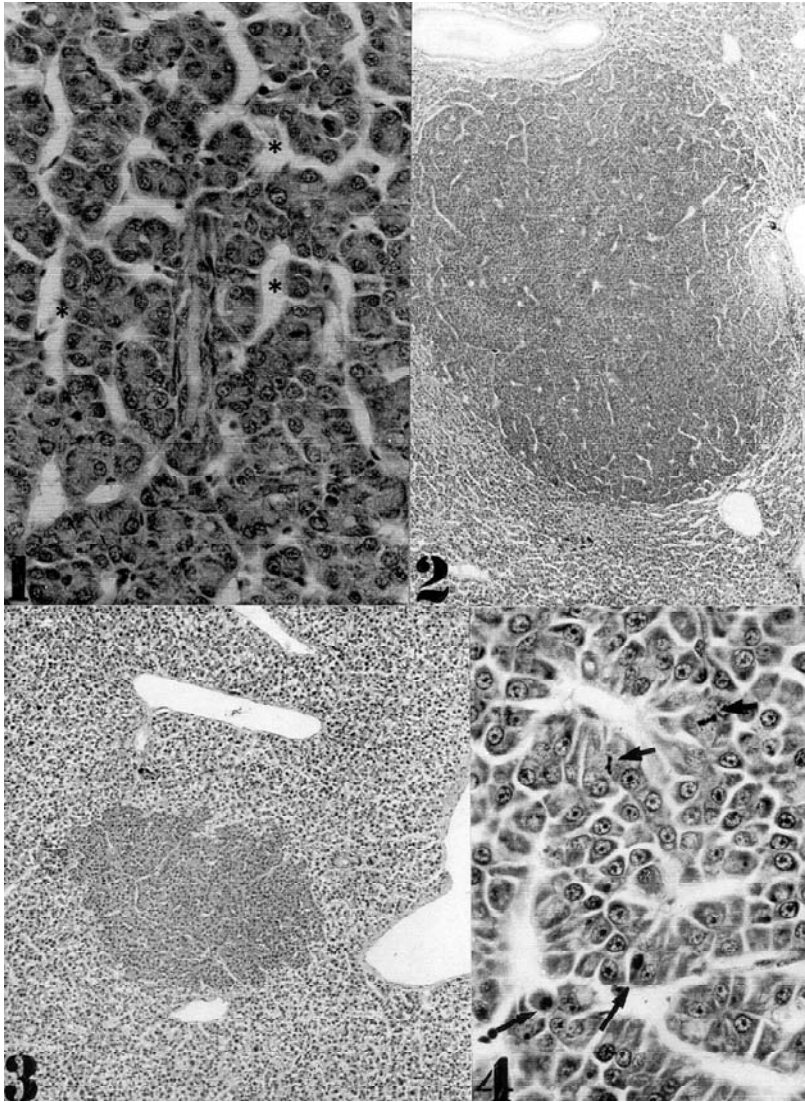


FIGURE 1: Liver section from a group control trout showing the typical tubular arrangement of hepatocytes surrounded by sinusoids (*) and a bile ductule in central area. H&E 780x.

Hígado de trucha del grupo control donde se nota la típica disposición tubular de los hepatocitos circundados por sinusoides (*). Se nota también, un túbulo biliar en el centro de la figura. H&E 780x.

FIGURE 2: Liver section from trout treated with AFB1 showing a basophilic cells nodule. H&E 90x.

Hígado de trucha tratada con AFB1 donde se observa un nódulo de células basófilas. H&E 90x.

FIGURE 3: A basophilic cells foci may be noted in liver section from trout treated with AFB1. H&E 96x.

Un foco de células basófilas puede ser observado en esta fotomicrografía de hígado de trucha tratada con AFB1. H&E 96x.

FIGURE 4: Detail of a basophilic cell nodule showing basophilic cells characteristics of this kind of lesion. Figures of mitosis (short arrows) and apoptosis (long arrows) are indicted. H&E 780x.

Detalle de un nódulo de células basófilas donde se observan las células características de ese tipo de lesión. H&E 780x.

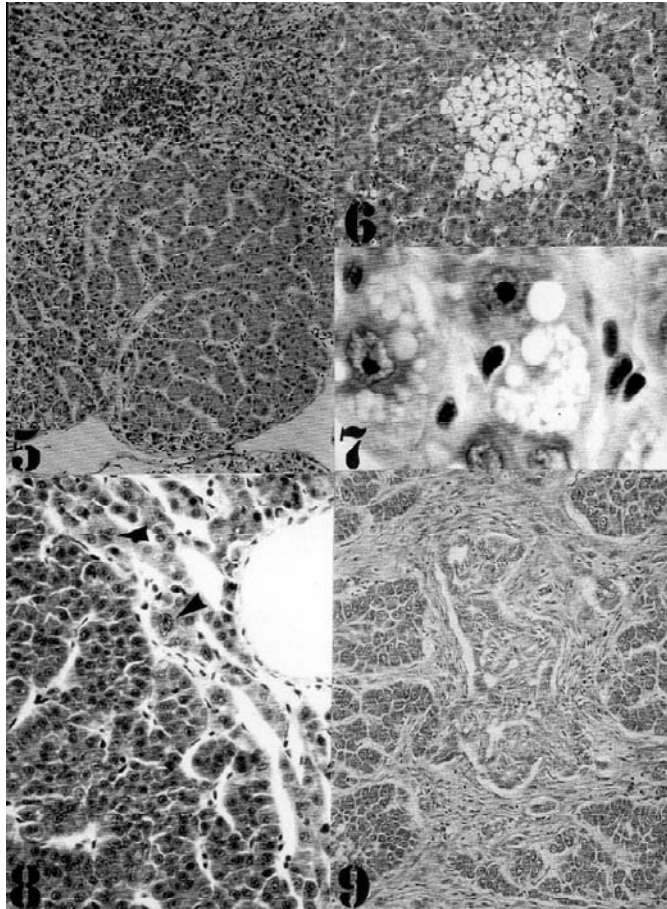


FIGURE 5: Liver section from a trout exposed to aflatoxin B1 showing acidophilic foci and a small basophilic foci. H&E 220x .

Hígado de trucha tratada con AFB1 donde se nota un foco de células acidófilas y un pequeño foco de células basófilas. H&E 220x.

FIGURE 6: A vacuolated foci composed by cells with macro and micro vacuolization in cytoplasm can be observed in this section of liver from a trout treated with AFB1. H&E 220x .

Hígado de trucha tratada con AFB1 donde se observa un foco de células vacuolizadas, con macro y micro vacuolización citoplasmática. H&E 220x.

FIGURE 7: Detail of vacuolated foci composed by cells with micro vacuolization in cytoplasm. H&E 2170x.

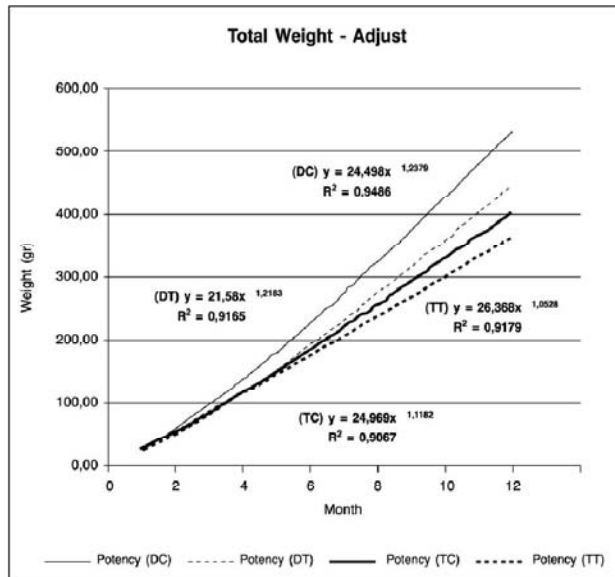
Detalle de un foco de células vacuolizadas compuesto por células con microvacuolización citoplasmática. H&E 2170x.

FIGURE 8: Part of a hepatocellular carcinoma surrounded by anaplastic cells (arrowheads) is showed in this liver section from diploid trout treated with AFB1. H&E 550x.

Hígado de trucha diplóide tratada con AFB1 donde se observa la región periférica de un carcinoma hepatocelular circundado por células anaplásicas (cabezas de flecha). H&E 550x.

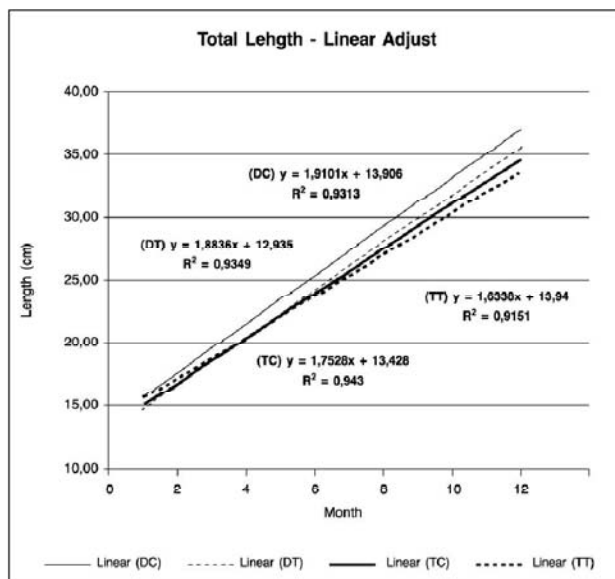
FIGURE 9: Detail of a section of hepatocellular carcinoma. A proliferating ductal area surrounded by desmoplastic tissue and micronodules composed by small basophilic cells may be observed in this field. H&E 490x.

Detalle de un carcinoma hepatocelular, donde se observa la región de proliferación tubular circundada por tejido desmoplásico y micronodulos compuestos por pequeñas células basófilas. H&E 490x.



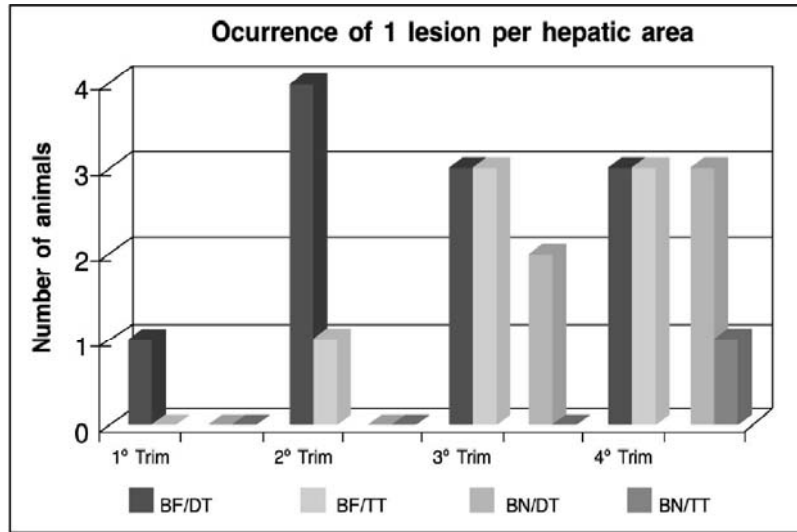
GRAPH 1. This graphic shows the adjusted lines to the weight measurement means obtained along the experiment and includes the mathematic expression by which they are represented, as well as the determination coefficient (R^2).

El gráfico muestra el ajuste linear para la media de peso obtenido durante el experimento e incluye expresiones matemáticas por las cuales son representadas, además del coeficiente de determinación (R^2).



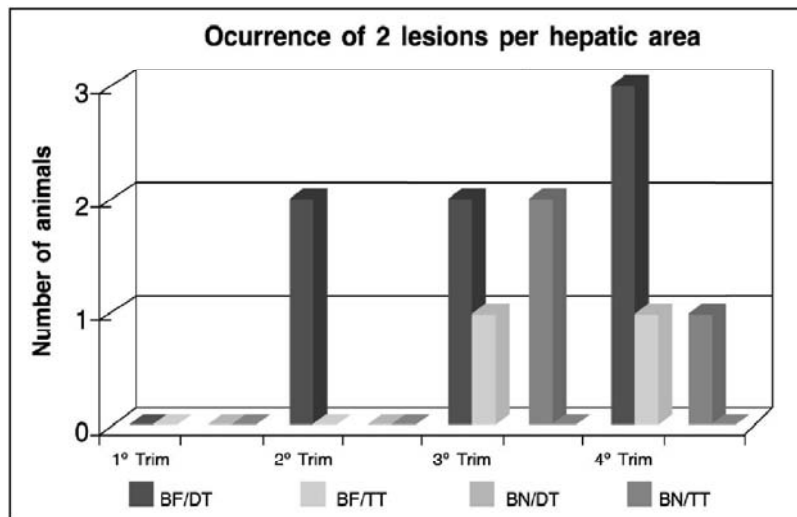
GRAPH 2. Representative graphic of the adjusted lines to the total length measurement means obtained along the experiment and includes the mathematic expression by which they are represented, as well as the determination coefficient (R^2).

El gráfico representa el ajuste linear de las medias del largo total obtenido durante todo el experimento e incluye las expresiones matemáticas por las cuales son representadas, además del coeficiente de determinación (R^2).



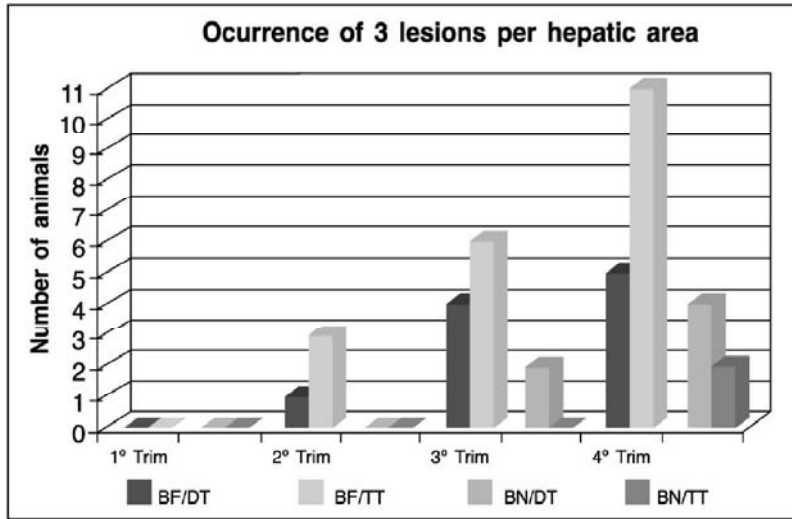
GRAPH 3. Quarterly evaluation of the incidence of basophilic cells foci (BF) and basophilic cells nodules (BN), taking in account the occurrence of 1 lesion by hepatic area studied in diploid trout (DT) and triploid (TT) treated with AFB1 contaminated food, considering the total number of animals with lesions in 15 samples analyzed during the quarter period.

Evaluación trimestral de la incidencia de focos y nódulos de células basófilas, considerando la presentación de 1 lesión por área hepática analizada en truchas diplóides (DT) y triplóides (TT) tratadas con AFB1. Se consideró el número total de animales con lesiones en 15 ejemplares analizados durante el trimestre.



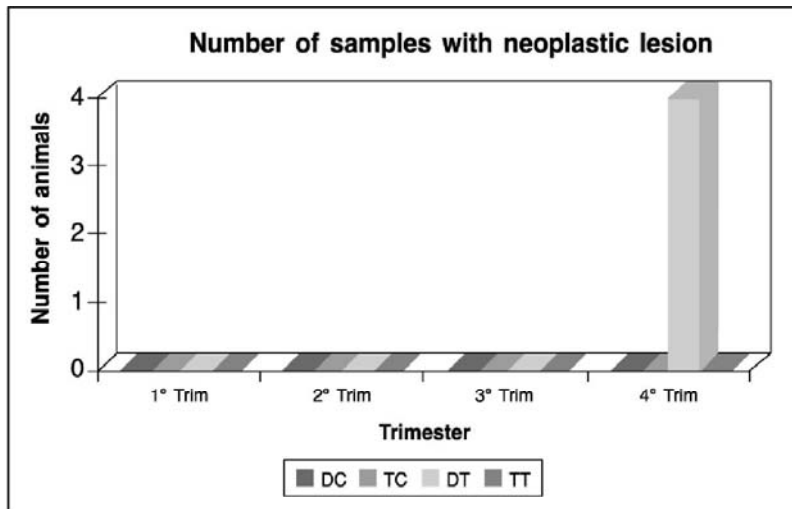
GRAPH 4. Quarterly evaluation of the incidence of basophilic cells foci (BF) and basophilic cells nodules (BN), taking in account the occurrence of 2 lesions by hepatic area studied in diploid trout (DT) and triploid (TT) treated with AFB1 contaminated food, considering the total number of animals with lesions in 15 samples analyzed during the quarter period.

Evaluación trimestral de la incidencia de focos y nódulos de células basófilas, considerando la presentación de 2 lesiones por área hepática analizada en truchas diplóides (DT) y triplóides (TT) tratadas con AFB1. Se consideró el número total de animales con lesiones en 15 ejemplares analizados durante el trimestre.



GRAPH 5. Quarterly evaluation of the incidence of basophilic cells foci (BF) and basophilic cells nodules (BN), taking in account the occurrence of 3 lesions by hepatic area studied in diploid trout (DT) and triploid (TT) treated with AFB1 contaminated food, considering the total number of animals with lesions in 15 samples analyzed during the quarter period.

Evaluación trimestral de la incidencia de focos de células basófilas y nódulos de células basófilas, considerando el caso de 3 lesiones por área hepática analizada en truchas diplóides (DT) y triplóides (TT) tratadas con AFB1. Fue considerado el número total de animales con lesiones en 15 ejemplares analizados durante el trimestre.



GRAPH 6. Quarterly evaluation of the occurrence of neoplastic lesion, hepatocellular carcinoma, in diploid trout diplóides (DC e TC) and triploid (TC e TT) treated with AFB1 contaminated food, considering the total number of animals with lesions in 15 samples analyzed during the quarter period.

Evaluación trimestral de la incidencia de lesiones neoplásicas, carcinoma hepatocelular en truchas diplóides (DT) y triplóides (TT) tratadas con AFB1. Fue considerado el número total de animales con lesiones en 15 ejemplares analizados durante el trimestre.

Presumptive preneoplastic lesions, characteristically described in the hepatocarcinogenesis process (Bannasch, 1986), were noted in AFB1 treated groups DT and TT: foci of vacuolated cells, foci of acidophilic cells, foci of basophilic cells and nodules of basophilic cells. However, as Hendricks *et al.* (1984) affirmed, only presumptive lesions formed by basophilic cells are really significative to the hepatocarcinogenesis process in trout, since other lesions do not contribute to the evolution of this disorder, a fact observed in previous works (Arana, 1997). We then emphasized the occurrence of the preneoplastic lesions formed by basophilic cells as a mean to evaluate the hepatocarcinogenesis process. The characterization of focal lesions and nodular lesions on the basis of lesion size adopted here is the same one used by Bannasch (1984, 1986), that is why we adopted the most common classification used in hepatocarcinogenesis experiments. However, Hendricks *et al.* (1984) did not use the term basophilic nodule and considered only basophilic foci and hepatocellular carcinoma as basophilic lesions, and they realize this distinction is arbitrary because the same type of basophilic cell comprises both lesions.

In fact, basophilic cells foci precede basophilic cells nodule, as it has been demonstrated by chemical carcinogenesis studies (Bannasch, 1986). Interesting data about the lesions evolution may still be inferred from Graph. 5, where it may be noted that TT group showed a greater number of animals with 3 or more BF by hepatic area after the second trimester than the diploid group, and this occurred without a significative increase of BN throughout the experimental period. These results suggest that some BF lesions may have regressed or that they have a very slow evolution thus, reducing the progression of the hepatocarcinogenesis process in triploid fishes. With respect to the neoplastic lesion itself observed only in diploid trout, the finding of neoplastic lesion after 9 or 12 months of AFB1 exposure has already been reported in the literature for rainbow trout (Bailey *et al.*, 1996).

Our data indicate that triploid animals are more resistant to the hepatotoxic effects of AFB1 in the food. Similar results were obtained by

Thorgaard *et al.* (1999) who tested several carcinogenic substances given by immersion in water to diploid and triploid rainbow trout fry. These authors observed a lesser incidence of neoplastic lesions in the different target organs of triploid trout, that was variable to the carcinogen applied.

The specific reasons for this resistance of triploid trout to the carcinogenic activity of AFB1 and other carcinogenic substances are not yet clear. Thorgaard *et al.* (1999) believe that tumor suppressor genes play a significant role in triploid trout carcinogenic process, since it would be more difficult to mutate or delete all three copies of tumor suppressor genes in these animals. However, to elucidate this hypothesis, or others that may be elaborated to explain the hepatocarcinogenesis in triploid trout, as greater expression of genes related to detoxifying enzymes, it will be necessary to carry out more experiments with a methodology that could evaluate the degree of involvement of these molecules or the role of the mechanisms associated to triploidy.

RESUMEN

El propósito del presente estudio fue comparar el crecimiento y la incidencia de lesiones hepáticas en truchas triploides y diploides tratadas con aflatoxina B1 (AFB1). 240 truchas fueron divididas en 4 grupos: DC: truchas diploides alimentadas con ración sin AFB1; TC: truchas triploides alimentadas con ración sin AFB1; DT: truchas tratadas con ración con 80 ppb de AFB1 y TT: truchas triploides alimentadas con ración con 80 ppb de AFB1. Durante doce meses, mensualmente, cinco ejemplares de cada grupo fueron anestesiados y sacrificados. Con posterioridad a la obtención del peso y medición del tamaño de los pescos, muestras hepáticas fueron fijadas en solución de formalina salina 10% y procesadas para análisis histopatológico. El análisis comparativo del rendimiento en crecimiento indicaron diferencias significativas entre truchas diploides del grupo control y del tratado, sugiriendo que AFB1 afecta el crecimiento de las truchas diploides. En

truchas triploides no se observaron diferencias entre peces del grupo control y los peces tratados. El análisis histopatológico señaló que truchas triploides son más resistentes a AFB₁, ya que ambos grupos tratados presentaron lesiones preneoplásicas, sin embargo, el grupo TT demostró menor incidencia de lesiones e igualmente un desarrollo más lento de las mismas. En cuanto a la ocurrencia de neoplasia, en el grupo DT 4 peces desarrollaron carcinoma hepatocelular en el último trimestre del experimento, mientras que ningún animal triploide desarrolló lesión neoplásica.

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