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The activity of several components of the innate immune system in diploid and triploid turbot

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Abstract

The use of triploid fish may be of interest in research, e.g. study of how this condition affects the size and activity of cells. In addition, triploid fish are sterile and production of triploids in fish species that are marketed after reaching sexual maturity may be of economic interest. In the present study, the effects of triploidy on the activity of several components of the innate immune system of turbot (*Psetta maxima* L) were determined. Triploid turbot had bigger cells (erythrocytes and neutrophils) but the number of blood erythrocytes, leucocytes and thrombocytes was lower than in diploid fish. The differential cell count was similar in both types of fish. The respiratory burst and the phagocytic activities were higher in neutrophils of triploid turbot. However, because the number of neutrophils was higher in diploids, the total respiratory burst activity and the phagocytosis per microliter of blood was similar in both types of fish. No differences were found in serum complement, lysozyme or bactericidal activities. The results indicate that the activities of the humoral components of the innate immune system tested are similar in diploid and triploid fish and that the lower leucocyte number found in triploids is compensated for by higher cell activity.
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1. Introduction

When fish reach sexual maturity, most of their energy is spent on reproduction and they show lower growth rates, increased mortality and inferior flesh quality (Donaldson and Devlin, 1996). Triploid fish, which contain three sets of chromosomes, are sterile and therefore all of their energy is placed on growth. For this reason, production of triploids may be desirable in those cases when fish become sexually mature before reaching marketable size.

One important aspect that should be considered before taking the decision to produce triploid fish commercially is their resistance to diseases in comparison with diploid fish. Depending on the experimental conditions and fish species used, survival was usually found to be similar or higher in diploids than in triploids (Johnson et al., 1986; Galbreath et al., 1994; Ojolick et al., 1995; Withler et al., 1995). Some studies suggest that diploid and triploid fish show similar resistance to infectious diseases. This was the case with ayu (*Plecoglossus altivelis*) exposed to *Vibrio anguillarum* (Inada et al., 1990) and Atlantic salmon (*Salmo salar*) experimentally infected with *Renibacterium salmoninarum* (Bruno and Johnstone, 1990). Mortalities in rainbow trout (*Oncorhynchus mykiss*) exposed to *Vibrio ordalii*, *Aeromonas salmonicida* or infectious hematopoietic necrosis virus were also similar in both diploid and triploid fish (Yamamoto and Iida, 1995a). However, according to other studies, triploid fish appear to be more susceptible to infections than diploids. The mortality of triploid rainbow trout due bacterial gill disease was higher than in diploids, although, in this case, the cause of death was mainly associated with a decrease in oxygen uptake due to the damage produced in gill epithelial cells (Yamamoto and Iida, 1994). Jhingan et al. (2003) found that triploid coho salmon (*Oncorhynchus kisutch*) were less resistant to vibriosis than their diploid counterparts. However,

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whether these differences are due to differences in the immune system is still unknown.

The innate immune system plays a decisive role in the defence of fish against pathogens and is of special importance in young fish in which acquired immune system is still not completely developed. There are very few studies that compare the activity of components of innate immune system in diploid and triploid fish. Triploids usually have larger cells but lower cell numbers. This is true for rainbow trout (*O. mykiss*) and brook trout (*Salvelinus fontinalis*) in which triploids possess lower leukocyte counts than diploids (Benfey and Biron, 2000). However, in other species such as tench (*Tinca tinca*), no significant differences in the total or differential blood leucocyte counts were found between diploids and triploids (Svobodová et al., 2001). In relation to other components of the immune system, the haemolytic and bactericidal activities of serum and the neutrophil activity did not differ significantly in diploid and triploid rainbow trout, suggesting that non-specific defence activities were similar in both types (Yamamoto and Iida, 1995b).

Turbot (*Psetta maxima* L., also known as *Scophthalmus maximus* L) is a cultured fish species of high economic importance in Europe. Under normal conditions, triploid turbot show similar survival rates to diploids. However, under certain conditions mortalities were found to be higher in triploid fish (Cal et al., 2006). To evaluate whether there are any differences in the innate immune systems of diploid and triploid turbot, the activity of several components of the innate immune system was determined and compared in both groups of fish.

2. Materials and methods

2.1. Fish

The fish used were 5 year-old diploid and triploid turbot (*P. maxima* L) (about 4 kg body mass); 23 diploids (14 males and 9 females) and 23 triploids (1 male 22 females), all obtained from the same lot of fertilized eggs. Triploids were produced by cold shock as described by Piferrer et al. (2000) and triploidy was verified by measuring the size of erythrocytes (Benfey et al., 1984; Cal et al., 2005).

2.2. Haematology

Blood was taken from the caudal vein of anaesthetized fish. A portion of the blood sample was heparinized and the rest was allowed to clot for 3 h and serum was obtained after centrifugation.

Red and white blood cell counts were determined with a haemocytometer, using blood diluted 1:50 in Kekic and Ivanc's (1982) fluid. Differential cell counts were estimated using blood films, prepared immediately after collection, then stained with Hemacolor (Merck®). A total of 200 cells (leucocytes and thrombocytes) were counted in each sample and the percentage number of different cell types was calculated. The neutrophil diameter and longitudinal and transverse erythrocyte diameter of 25 cells from each of the stained blood films were measured in both groups of fish.

2.3. Isolation of blood leucocytes

Suspensions of enriched blood neutrophils were obtained according to the method described by Lamas and Ellis (1994). To determine the proportion of neutrophils in the suspensions obtained, cells (200 μL) were fixed with 1% of formaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 10 min, washed twice with the same buffer and incubated with 3,3′-diaminobenzidine tetrahydrochloride for 10 min to demonstrate peroxidase activity (Kiernan, 1981). After centrifugation, the pellet was suspended in PBS and the number of neutrophils determined using a haemocytometer. After estimating the cell viability by the Trypan blue exclusion test, aliquots (100 μL) of the cell suspension containing the same amount of neutrophils (10^6 neutrophils mL^{-1}) were dispensed into each well of 96-well tissue culture plates.

2.4. Respiratory burst activity

Production of intracellular O_2^- by blood cells from diploid and triploid fish was determined by the nitroblue tetrazolium method (NBT). 100 μL of the cell suspension containing 10^6 neutrophils mL^{-1} were dispensed into each well of 96-well tissue culture plates. Cells were preincubated for 1 h at 18 °C with Macrogard® (a yeast β-glucan that stimulates the respiratory burst of turbot phagocytes (Castro et al., 1999), by adding 100 μL of a glucan suspension (0–100 μg mL^{-1}) in L-15 with 5% FCS to each well. After preincubation, the glucan was removed and 100 μL of L-15 containing NBT (1 mg mL^{-1}) were added to each well. Alternatively, cells were incubated for 1 h with NBT containing phorbol myristate acetate (PMA, Sigma) (1 μg mL^{-1}). After incubation for 1h, the NBT solution was removed, the cells were fixed with methanol and the reduced formazan was solubilized by adding 120 μL of 2 M KOH and 140 μL of DMSO. The NBT reduction was measured with a multiscan spectrophotometer, at a wavelength of 630 nm. Total respiratory burst activity in each fish was calculated by multiplying the NBT reduction per neutrophil by the number of neutrophils per microliter of blood. The results are expressed as the mean NBT reduction in all fish tested in each group.

2.5. Phagocytosis

The phagocytic activity in blood neutrophils was evaluated the day after obtaining the cells and the assays were carried out directly in the wells. As above, the same number of neutrophils was added to each well. After removing the medium, 100 μL of fixed yeast cells (1.5×10^6 cells mL^{-1} in L-15), opsonized for 1 h with foetal calf serum, were added to each well and incubated with the blood cells for 1 h. The wells were then washed twice with PBS and the blood cells were stained with Hemacolor. The percentage of phagocytosis (% of cells that had phagocytosed) and the phagocytic index (number of cells that had phagocytosed/number of yeast phagocytosed) were determined by counting 200 cells. Total phagocytic activity in each fish was determined by multiplying the particles phagocytosed per neutrophil by the number of neutrophils in 1 μL of blood.

Table 1
Haematological comparisons between 5-year-old diploid and triploid turbot (n=23) (NS, nonsignificant)

	Diploids (Mean±S.D.)	Triploids (Mean±S.D.)	
Erythrocytes (×10^6/μL)	1.90±0.073	1.18±0.055	$P<0.001$
Leucocytes and thrombocytes (×10^3/μL)	118±5.3	81±3.2	$P<0.001$
Thrombocytes (%)	60.40±8.14	65.08±5.83	NS
Lymphocytes (%)	36.47±7.50	31.85±6.45	NS
Neutrophils (%)	3.09±1.01	3.03±1.17	NS
Monocytes (%)	0.13±0.3	0.09±0.28	NS
Erythrocyte size (long axis, μm)	10.53±0.59	13.74±0.80	$P<0.001$
Neutrophil size (diameter, μm)	7.07±0.54	8.54±0.81	$P<0.001$

Results are expressed as mean activity for all fish tested in each group.

2.6. Determination of serum complement activity

The activity of the alternative complement pathway was determined using sheep red blood cells (SRBC, Biomedics) as targets. SRBC (6%) were washed three times with phenol red-free Hanks buffer (HBSS) containing Mg^{2+} (10 mM) and ethylene glycol tetraacetic acid-magnesium-gelatin veronal buffer (EGTA, 10 mM) and suspended in the same buffer. Test serum was serially diluted in the same buffer (from 1:4 to 1:128, v/v, serum/buffer). SRBC (100 μL) were mixed with the same amount of diluted serum, and incubated for 2 h at 18 °C. After centrifugation at 400×g for 5 min at 4 °C, 100 μL of supernatants were carefully removed and added to new wells. The relative haemoglobin content of the supernatants was assessed by measuring their optical density at 540 nm in a microplate reader (Bio-Rad). The values of maximum (100%) and minimum (spontaneous) haemolysis were obtained by adding 100 μL of distilled water or HBSS to 100 μL samples of SRBC, respectively. The dilution of serum producing 50% haemolysis (ACH50) was determined and the number of ACH50 units per milliliter was calculated for each experimental group as suggested by Yano (1992).

2.7. Determination of serum lysozyme activity

The method we used to determine the lysozyme activity in serum was based on the lysis of the lysozyme-sensitive Gram-positive bacterium *Micrococcus lysodeikticus*. Standard solutions (0–50 I.U. mL^{-1}) of hen egg white lysozyme (Sigma) were made up in 0.1 M phosphate citrate buffer, pH 5.8, and used to generate the standard curve. A suspension of bacteria (0.2 mg mL^{-1}) in buffer was also prepared. Dilutions of the standard and undiluted serum samples (2 μL) were placed into wells of a 96-well plate in triplicate. Twenty-three microliters of buffer and 175 μL of *M. lysodeikticus* suspension were then added to each well. After mixing, the change in turbidity was immediately measured every 15 s for 5 min, at 450 nm and at room temperature, using a microplate reader. The lysozyme activity was calculated from the standard curve generated from the hen egg white lysozyme activity and was expressed as units mL^{-1} serum.

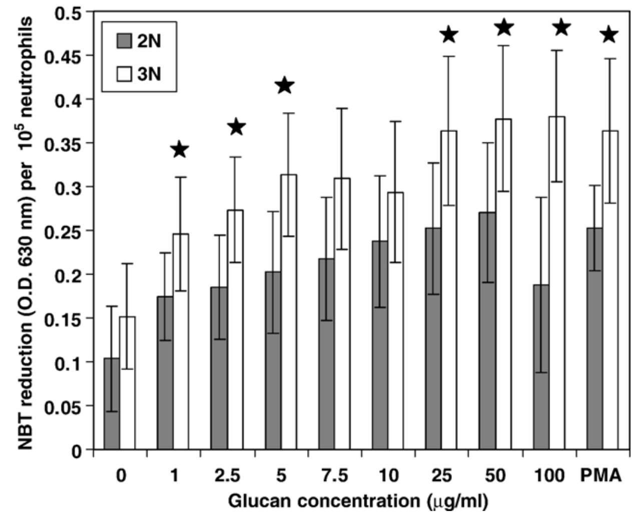

Fig. 1. Respiratory burst activity of diploid (2N) and triploid (3N) turbot neutrophils (per 10^5 cells) incubated with glucan or with PMA. Values represent the mean±S.D. (n=23). *$P<0.05$ denotes significant differences in the response between diploids and triploids for each glucan concentration or PMA.

2.8. Bactericidal activity

Bacteria, *V. anguillarum* strain R82, originally isolated from a vibriosis outbreak in turbot, were kindly provided by Dr. A.E. Toranzo and Dr. J.L. Barja (Department of Microbiology and Parasitology, University of Santiago de Compostela). Stock cultures were maintained frozen at −70 °C in Luria's broth with 1% NaCl (TSB-1, Cultimed) and 15% (vol/vol) glycerol until passage on tryptic soy agar containing 1% NaCl (TSA-1) for use in the assays. Colonies from agar were grown in TSB-1 for 8 h at 20 °C and with constant stirring to approximately 10^9 CFU/mL, as confirmed by counting the colonies on TSA-1 plates, washed twice in 0.9% saline and diluted to 10^7, 5×10^7 and 10^8 cells mL^{-1} in TSB-1. The bactericidal assay was conducted in flat-

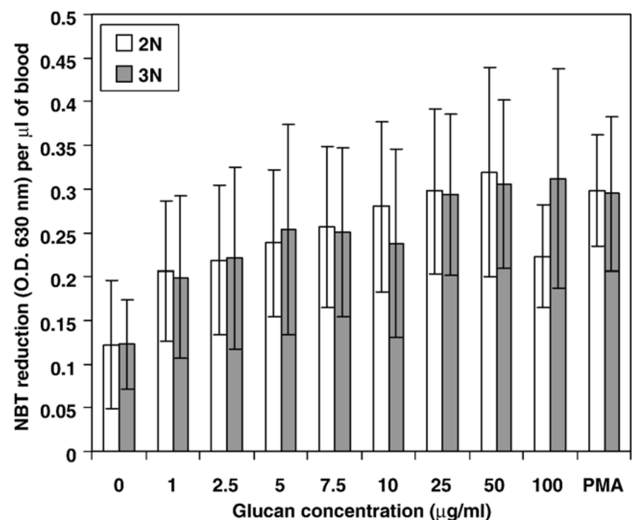

Fig. 2. Total respiratory burst activity of diploid (2N) and triploid (3N) turbot neutrophils per μl of blood. Cells were incubated with several concentrations of glucan or with PMA. Values represent the mean±S.D. (n=23).

bottomed 96-well microtiter plates and four replicate wells were used for each bacterial dilution described. The assay was carried out by adding 33 μL of fresh serum and 133 μL of each bacterial dilution to each well and mixing with a micropipette. Control wells contained PBS instead of serum. After incubation for 6.5 h at 20 °C, 86 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (2 mg mL^{-1}) were added to each well, mixed by pipetting and incubated for 15 min at 20 °C. The MTT reduction was measured with a multiscan spectrophotometer, at a wavelength of 630 nm. The bactericidal index was determined as absorbance of each sample/absorbance of control.

2.9. Cell counting

The mean cell number per well was estimated by counting the number of nuclei present after lysing the cells of several wells with 100 μL lysis buffer containing 0.1 M citric acid, 1% Tween 20 and 0.05% crystal violet (Secombes, 1990).

2.10. Statistical analysis

Data were analysed by analysis of variance followed by Duncan's test and by paired Student's *t*-test using SPSS for MS Windows.

3. Results

3.1. Haematology

The results of the blood cell counts and cell size for diploid and triploid turbot are shown in Table 1. The erythrocytes and neutrophils obtained from triploid fish were larger; however, the total number of blood erythrocytes, leucocytes and thrombocytes was lower in triploids than in diploids. As mentioned above, the ploidy level was verified by measuring the erythrocyte sizes. The mean length of erythrocyte long axis varied between 10.31 and 11.68 μm in diploids and between 13.43 and 14.81 μm in triploids. The differential cell counts (leucocytes and thrombocytes) were similar in diploid and triploid turbot.

3.2. Respiratory burst and phagocytosis

Both diploid and triploid blood neutrophils responded to glucan and PMA by increasing the respiratory burst activity. The activity per cell was significantly higher in triploids than in diploids ($P<0.01$) in both cells stimulated with glucan or with PMA (Fig. 1). However, because the number of neutrophils was higher in diploids, no differences between both groups of fish were found in the total respiratory burst activity per microliter of blood (Fig. 2).

The phagocytic activity was also estimated in blood neutrophils. The percentage of phagocytosis was similar in cells of diploid and triploid turbot. However, the phagocytic index was significantly higher in triploid neutrophils. Taking into account that the number of circulating neutrophils is higher in diploids, the total phagocytic activity per microliter of blood did not differ significantly different between diploid and triploid turbot (Table 2).

Table 2
Phagocytic activity in blood neutrophils (phagocytic rate and phagocytic index) of diploid and triploid turbot

	Diploids (Mean±S.D.)	Triploids (Mean±S.D.)	
Phagocytic rate (%)	54.04±10.99	53.53±9.68	NS
Phagocytic index	1.78±0.35	2.14±0.15	$P<0.05$
Total phagocytic activity per μl of blood	3504±680	2810±272	NS

Yeasts were opsonized with FCS ($n=23$). NS, nonsignificant.

Table 3
Complement and lysozyme activities in diploid and triploid turbot serum ($n=23$) (NS, nonsignificant)

	Diploids (Mean±S.D.)	Triploids (Mean±S.D.)	
Complement (ACH50, units/mL)	125.52±34.41	111.77±23.63	NS
Lysozyme (units/mL)	149.02±21.81	160.43±36.07	NS

3.3. Serum complement and lysozyme activities

No differences were found in serum complement (alternative pathway) or lysozyme activities of diploid and triploid turbot (Table 3).

3.4. Serum bactericidal activity

As shown in Fig. 3, both diploids and triploids had similar bactericidal index in serum. In relation to the control (absence of serum), a decrease of about 50%, 70% and 90% in MTT reduction was found when 10^8, 5×10^7, 10^7 bacteria mL^{-1} were used, respectively.

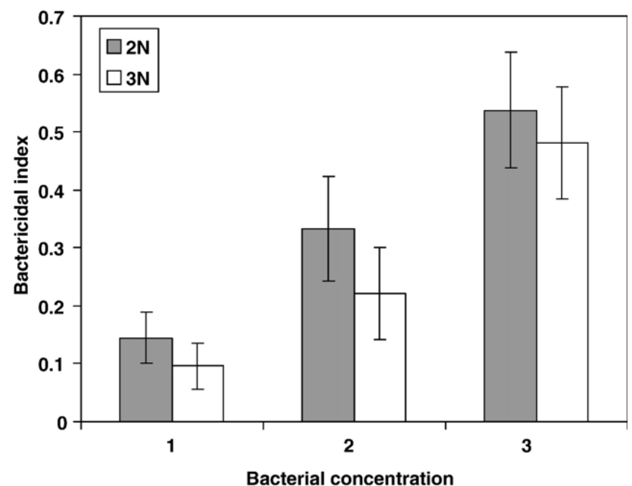

Fig. 3. Bactericidal index in serum of diploid (2N) and triploid (3N) turbot. Bacterial dilutions used were 10^7 cells mL^{-1} (1), 5×10^7 cells mL^{-1} (2) and 10^8 cells mL^{-1} (3). Values represent the mean±S.D. ($n=23$).

4. Discussion

The innate immune system, which is composed of cellular and humoral components, is well developed in fish and is fundamental in the protection against pathogens. Triploid fish have larger cell size, but these fish are not any larger than diploid fish because of lower cell numbers (Benfey, 1999). These differences in cell size and number may affect cell activity and produce differences in resistance to diseases in diploids and triploids. The purpose of this research was to determine whether there are any differences between diploid and triploid turbot in the activity of several important components of innate immunity that participate in humoral and cellular defences.

Triploid turbot had less but larger erythrocytes than diploids. These results are consistent with previous findings for turbot erythrocytes (Cal et al., 2005) and for erythrocytes in other fish species (Small and Benfey, 1987; Aliah et al., 1991; Parsons, 1993; Cogswell et al., 2002). The larger size and the lower number of erythrocytes in triploids may affect the oxygen exchange and the capacity for oxygen transport, causing stress under certain conditions and making fish more susceptible to diseases (Benfey, 1999). However, other studies indicate that triploids had similar oxygen carrying capacity and haematological responses to stress as diploid fish (Sadler et al., 2000).

The blood leucocyte number is probably very important in inflammatory reactions, as leucocytes migrate in response to inflammatory stimuli. The values for the total and differential leucocyte counts in diploid turbot were similar to those obtained by Burrows et al. (2001), although slightly higher than those reported by Quentel and Obach (1992). As also found in some fish species (Yamamoto and Iida, 1994; Ohtsu, 1993), triploid turbot had less blood leucocytes than diploid turbot. However, this is not always the case as no differences were found in other fish species (Svobodová et al., 2001). Differential cell counts were similar in triploid and diploid turbot, suggesting that triploidy affects the number of cells but not their proportion in blood.

Macrophages and neutrophils are efficient phagocytes and play a very important role in immunity. Fish phagocytes participate in the inflammatory responses, can phagocytose and kill microorganims and are involved in antigen presentation (Secombes, 1996). Phagocytosis is necessary to internalise pathogens and, in most cases, to kill them. The percentage of phagocytosis in blood neutrophils was similar in both groups of fish. However, the phagocytic index in phagocytes from triploids was higher than in those from diploid turbot, probably a consequence of the cell size. Large cells have higher membrane surface and volume than small cells, which increases their capacity to engulf particles. Our results are consistent with those obtained in diploid and triploid rainbow trout by Yamamoto and Iida (1995b). In the case of blood cells, and taking into account the number of neutrophils, the percentage and the phagocytic index, the total phagocytic activity per microliter of blood was similar in both groups of turbot.

During the respiratory burst activity, phagocytes produce oxygen free radicals that are important for killing microorganisms. This activity can be measured in several ways, one of which involves using the NBT method to determine O^{2-} production. Yeast glucans and PMA are good stimulators of the respiratory burst activity in turbot phagocytes (Castro et al., 1999; Couso et al., 2001). The respiratory burst activity of turbot monocytes/macrophages and neutrophils increased after stimulation with glucan or with PMA, although this activity was higher in neutrophils than in monocytes (Castro et al., 1999). As the percentage of monocytes in the blood of diploid and triploid turbot was very low, we believe that the NBT reduction measured in the present experiments was mainly related to neutrophils. When diploid and triploid turbot were compared, the activity per cell was higher in triploid fish, as also found in triploid rainbow trout (Yamamoto and Iida, 1995b). In turbot neutrophils, O^{2-} is produced in intracellular vesicles and tubular structures before being released extracellularly or into phagosomes (Couso et al., 2001). Large cells probably have more internal compartments than small ones and will thus be produce a respiratory burst activity after stimulation. However, despite the differences in cell activity, diploid turbot had more neutrophils in blood than triploids and the respiratory burst activity per microliter of blood was similar in both groups, indicating that the lower activity found in neutrophils from diploid fish is compensated by a higher number of cells.

Several serum activities (complement, lysozyme and bactericidal) were also evaluated. Complement not only forms membrane attack complexes that can kill pathogens but also promotes inflammatory responses and is recognized by receptors on phagocytes, which enhances their ability to phagocytose and kill pathogens. Classical and alternative complement pathways have been recognized in fish and the alternative pathway is more important in fish than in mammals (Yano, 1996). In fish, complement has virucidal, bactericidal, parasiticidal, opsonic and chemoattracting activities (Yano, 1996). Lysozyme is an enzyme that cuts linkages between monosaccharides in cell walls of bacteria. In fish, it is widely distributed through the body, usually at sites rich in leucocytes and also in eggs, in which plays an important role in the host defence mechanisms against pathogens (Yano, 1996). Serum lysozyme and complement activities and also bactericidal activity were similar in diploid and triploid turbot, indicating that there are no differences in the innate humoral defence systems in the two groups of fish.

In conclusion, diploid and triploid turbot showed similar innate cellular and humoral responses. The lower number of leucocytes found in the blood of triploid turbot appears to be compensated for by higher cell activity. These results suggest that diploid and triploid fish should have similar resistance to diseases. However, we cannot discard the possibility that having a lower concentration of leucocytes in tissues may be a disadvantage for finding and interacting with pathogens, making fish more susceptible to certain pathogens. On the other hand, having more potent cells could compensate for this or may even be an advantage in other cases. This may be the reason why in some studies triploid fish were found to be less resistant to certain diseases (Yamamoto and Iida, 1994; Inada et al., 1990; Bruno and Johnstone, 1990; Jhingan et al., 2003) but in others no differences were found between the two types of fish (Yamamoto and Iida, 1995b).

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