Haematological and physiological characteristics of diploid and triploid sea bass, *Dicentrarchus labrax* L.

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Abstract

The purpose of this study was to determine whether diploid and triploid sea bass differed in terms of main haematological and physiological characteristics. Diploid and triploid fish were produced by sub-optimal pressure treatments and held in communal environments under standard rearing conditions. Total red blood cell count (RBCC), haemoglobin concentration (Hb), hematocrit (Hct), mean cell volume (MCV), mean cellular haemoglobin content (MCH), mean cell haemoglobin concentration (MCHC), plasma metabolites, osmotic pressure, gill Na^+/K^+-ATPase activity, electrolytes, cortisol and 3,5,3'-triiodo-L-thyronine (T_3), were measured and compared. Triploidisation in sea bass led to an increase in erythrocyte size (32% in cytoplasm surface area, and 50% in nucleus) and a decrease in erythrocyte number (~34%).

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Haemoglobin and basal plasma cortisol levels were significantly lower in triploid sea bass than in diploids. There were also differences between ploidies in the plasma concentrations of some electrolytes, with triploids showing lower concentrations of K, Fe, Zn, S, and Cu than their diploid counterparts.

Keywords: Haematology; physiology; diploid; triploid; sea bass; *Dicentrarchus labrax*

1. Introduction

Efforts to induce triploidy in fish with the objective to generate sterility relate to potential applications in commercial farming and fisheries management. Triploid fish have impaired gametogenesis and investment in somatic growth may not be hindered by the metabolic costs of sexual maturation. Additionally, sterility in triploids may be a means to prevent the decline in flesh quality associated with sexual maturation, and also addresses concerns regarding the environmental impact of farmed escapees (Peruzzi et al., 2004). The methods used to induce triploidy, the performance of triploids, and the rationale for their practical use have been reviewed (Mair, 1993; Purdom, 1993; Pandian & Koteeswaran, 1998; Dunham, 1990; Arai, 2001; Felip et al., 2001a). Despite the interest, the performance of triploid fish remains variable, may be species-related and also seems to depend on rearing environment. Triploid fish may perform poorly under sub-optimal conditions, when cultured together with diploids, and they may be more sensitive to stress imposed by handling. Several studies are available on these and other aspects related to the physiology and behaviour of triploid fish compared to their diploid counterpart (see review by Benfey, 1999). This last author also put forward the potential advantage in the application of sub-optimal treatments that yield both triploid and diploid fish for use in comparative studies.

In triploid European sea bass (*Dicentrarchus labrax* L.), growth performance is comparable or lower than that of diploids, whereas some qualitative traits can be superior (Felip *et al*., 1999; Felip *et al*., 2001a; Peruzzi *et al*., 2004), but interest in the use of sterile triploid sea bass for aquaculture and stocking purposes exists. Blood constituents of diploid sea bass have been examined in relation to environmental factors and/or feeding (Carrillo et al., 1982; Gutierrez et al., 1984; Roche et al., 1989; Planas et al., 1990; Pavlidis et al., 1997; Cerdá-Reverter et al., 1998), handling and stress (Hadj Kacem et al., 1986; Hadj Kacem et al., 1987; Roche & Bogé, 1996; Marino et al., 2001), and for diagnostic purposes (Doimi, 1985). In

a comparative study on the reproductive physiology of diploid and triploid sea bass, Felip et al., (2001b) reported the plasma levels of sex hormones in both ploidies. Finally, erythrocyte and haematocrit measurements of diploid and triploid sea bass have been presented by Felip et al. (2001c).

The purpose of this work was to investigate the major blood and serum constituents in adult diploid and triploid sea bass with a view to provide information about possible adaptive physiological and behavioural interactions during rearing and husbandry procedures. For this purpose, the main haematological parameters, plasma metabolites, electrolytes, along with classical physiological indicators like cortisol, 3,5,3'-triiodo-L-thyronine (T_3), osmotic pressure, and gill Na^+/K^+-ATPase activity were examined in diploid and triploid fish produced by sub-optimal pressure treatments and held under communal environments.

2. Material and methods

2.1. Biological material and fish handling procedures

Diploid (n=16) and triploid (n=15) seabass were randomly selected from a mixed ploidy stock maintained under conditions of natural photoperiod and temperature at IFREMER Palavas-les-Flots. Fish were reared under communal rearing conditions and following previously published protocols (Peruzzi et al., 2004). These fish were produced by a sub-optimal pressure shock treatment (7,000 psi / 2 min duration) applied to newly fertilized eggs at 6 min post fertilization. Artificial fertilisation, pressure treatment procedures and flow cytometric analyses were performed following Peruzzi and Chatain (2000). All fish used in this study were individually PIT tagged and of certified ploidy.

2.2. Sampling

Sampling was conducted during May 2003, outside the normal spawning season of local sea bass stocks (January-February). Fish were left undisturbed and fasting for 2 days prior to experimentation. On the day of sampling, the water level was reduced by slow siphoning, fish were then lightly anaesthetized (Eugenol, Cooper, 15 ppm), sampled and their PIT-tag code and ploidy identified. Immediately after, two milliliters of blood were drawn by caudal puncture using syringes containing 2 mg of EDTA. Six operators performed the operation simultaneously and handling operations were completed within 15 minutes of first disturbing the fish. Two sub-samples of blood (approx. 1 ml) were then transferred to disposable test tubes and kept on ice. At the end of the operation, all fish were sacrificed by overdose of anesthetic, weighed (precision to the nearest 0,1 g) and their sex macroscopically determined (Chatain et al., 1999).

2.3. Haematology, plasma chemistry, metabolites and hormones

Blood smears were prepared and red blood cell counts were carried out using standard haematological techniques (Dacie and Lewis, 2001). Two 50 µl hematocrit tubes were filled with blood and kept refrigerated (4ºC) in an upright position until centrifugation (5 min at 12.000 g). One blood sample was refrigerated for later haemoglobin (Hb) determinations. The remaining sample was centrifuged (5 min at 9.000 g), plasma drawn off, divided into three "safe-lock" Eppendorf tubes which were immediately refrigerated or deep-frozen (-20ºC) to prevent any sample's evaporation. Total blood haemoglobin was determined a few hours after sampling, whereas plasma glucose, total proteins and osmotic pressure were determined on refrigerated plasma samples within two days. All other analyses were performed on deep-frozen samples within two months. Osmotic pressure (mOsm) was measured on 100 µl plasma samples using an automatic micro-osmometer (Autocal 13®Roebling). Red blood cell

measurements (cytoplasm and nuclear area, width and length) were made from blood smears using a Zeiss microscope fitted with a video camera module and computer-assisted image analysis (Visilog 5.2, ©Noesis Vision Inc., Canada). A minimum of 60 and a maximum of 90 erythrocytes/sample from 16 diploid and 15 triploid fish were analysed. Measurements of total red blood cell count (RBCC), haemoglobin concentration (Hb) and hematocrit (Hct) enabled the mean cell volume (MCV), mean cellular haemoglobin content (MCH), and mean cell haemoglobin concentration (MCHC) to be calculated according to the following formulas (Dacie and Lewis, 2001):

MCV (fl) = Hct / RBCC (10^6 μl^{-1})

MCH (pg)= [Hb (g dl^{-1}) x10]/ RBCC (10^6 μl^{-1})

and

MCHC (g l^{-1}) = [Hb (g dl^{-1}) x10] / Hct

Total blood haemoglobin concentration was measured by Drabkin's colorimetric assay, and plasma glucose by GOD-PAP enzymatic-colorimetric method using commercial kits and standards (Spinreact S.A., Spain). Plasma proteins were quantified according to Bradford (1976) with Comassie Brilliant Blue G-250 (Sigma) and using Bovine Serum Albumin (BSA) standards. All spectrophotometric measurements were performed using a Beckman DU®600 spectrophotometer.

Plasma concentrations of cortisol were determined in duplicate by radioimmunoassay (RIA) according to Balm et al. (1994). The cortisol antibody has marginal cross-reactivity with 11-deoxycortisol (5.9%), cortisone acetate (0.16%), cortisone (2.6%) and 17α-OH-progesterone (0.4%). The intra- and inter-assay coefficients of variation were 3 and 5% respectively.

Plasma Na, K and Cl concentrations were measured by flame photometry using an Auto Analyser. Ca, Mg, Mn, Fe, Si, Zn, Al, P, S, Cu, Cd and Pb were analysed with Inductively Coupled Plasma Atomic Emission Spectrometry (Plasma IL200, Thermo Electron, USA).

Plasma concentrations of lactate were determined with a pHOx Plus analyser (Nova Biomedical, The Netherlands), and triiodothyronine (T_3) concentrations were measured by RIA following the method of Boeuf and Prunet (1985).

2.4. Gill Na^+/K^+-ATPase

The first gill arch was immediately removed from the right side of the sacrificed fish, dissected tissue was rinsed in a solution (pH 7.4) containing 300 mM sucrose, 20 mM Na_2EDTA and 100 mM imidazol (Zaugg, 1982), placed in tubes containing the same solution, and stored at -20˚C until use. During preparation, the samples were kept on ice.

Stored samples were thawed at room temperature and briefly centrifuged. The medium was then removed, and 2 mL of isotonic isolation medium (IIM: 250 mM sucrose, 5 mM of $MgCl_2$ and 5 mM Hepes; pH 7.4) added to each tube. Samples were then homogenized and subsequently centrifuged at 4000 rpm for 5 min. The supernatant containing the plasma membrane fragments was transferred into new tubes. During the extraction procedure samples were maintained at 0-4˚C on ice.

Protein content was determined by a colorimetric method (Biorad) using BSA standards. Na^+/K^+-ATPase specific activity was assessed as the difference between total ATP hydrolysis (in presence of Na^+, K^+, Mg^{2+} and ATP) and that in absence of K^+ but in presence of an optimal concentration of ouabain (1 $mg.mL^{-1}$; Flik et al., 1983). The amount of phosphate released was assessed by comparison with commercial reference standards (Sigma). The enzyme specific activity was expressed in μmole $Pi.h^{-1}$ per mg protein. The total activity was calculated as the product of the specific activity and the total protein content of the sample.

2.5. Statistical analyses

Haematocrit proportion, total red blood cell count, osmolarity, glucose, haemoglobin and protein concentrations, electrolytes and T_3 were compared using single-factor ANOVA (ploidy). Erythrocyte numbers, area, width and length were compared using two-factor ANOVA (mix nested model with ploidy as fix factor and counting repetition or counted slide as random nested factor). The normality and the variance homogeneity were tested with tests of skewness, and kurtosis and Bartlett's test respectively (Dagnelie, 1975). Differences were accepted as significant when $P<0.05$. Values are expressed as means ± 95% Confidence Interval (CI).

3. Results

The analysed diploid fish were 8 males and 7 females (mean weight ± 95% CI = 990 ± 93 g), the triploids were 13 males and 2 females (mean weight ± 95% CI = 700 ± 65 g). Possible sex-related differences between the ploidy groups were not evaluated, due to low numbers of triploid females.

Haematological measurements, plasma glucose, proteins, cortisol, lactate and T_3 in diploid and triploid fish are given in Table 1. Haematocrit (~0.25) was similar in both ploidies. Triploid fish displayed significantly lower RBCC (~35%) and total [Hb] (~13%), but higher MCV (30%) than diploids. No differences in MCHC were found between ploidy groups. Total proteins, glucose, lactate and T_3 concentrations were also similar in both ploidies, whereas cortisol levels were significantly lower (~20%) in triploid fish.

Table 2 summarizes the results obtained from red blood cell measurements. Differences were observed in erythrocyte cellular and nuclear dimensions (area, width and length). Triploid *vs.* diploid ratios in erythrocyte cellular and nuclear areas were 1.32 and 1.50 respectively. Differences were also recorded between diploids and triploids in ratios of cytoplasmatic width to length, triploid ratios being significantly smaller than diploid, making triploid erythrocytes appear longer (~7%).

Plasma osmolarity and ion concentrations in diploid and triploid sea bass are given in Table 3. There were no significant differences in osmotic pressure between ploidies. Na, Cl, Ca, Mg, Mn, Al, P, Cd and Pb concentrations were also similar. The Na:Cl and (Na+K):Cl ratios were 0.98 and 0.99 respectively, and comparable in both groups. Triploid fish had lower K (~20%), Fe (~45%), Zn (~20%), S (~18%), and Cu (~34%) concentrations than did diploid fish. On the contrary, plasma concentrations of Si were significantly higher (~32%) in triploids.

Gill Na^+/K^+-ATPase activities (3.5 ± 1.08 μmol $Pi.h^{-1}$ mg $prot^{-1}$ in diploids and 3.85 ± 1.33 μmol $Pi.h^{-1}$ mg $prot^{-1}$ in triploids) were not statistically different between ploidies (F=2.570; P=0.126; n=1 and 18).

4. Discussion

The use of diploid and triploid fish exposed to the same shock treatment allowed elimination of confounding effects of treatments (Benfey, 1999). Under our experimental conditions, diploid and triploid fish produced by sub-optimal treatments did not differ in the majority of haematological parameters, but significant differences were found in red blood cell sizes and counts, Hb contents, plasma cortisol levels, and concentrations of some ions.

Triploidisation led to an increase of erythrocyte size (32% in cytoplasm surface area, and 50% in nucleus) and a decrease in erythrocyte number (~34%). This haematological profile of

fewer and larger erythrocytes is consistent with findings for other species (Benfey, 1999). This increase in cellular size offset by a decrease in cell number, explains the lack of difference in hematocrit observed between diploid and triploid sea bass, as reported in other fish species too (Benfey, 1999). Similar results were found in diploid and triploid sea bass by Felip and co-workers (2001c), who provided data on erythrocyte cellular/nuclear dimensions and accommodations in these two ploidies. They reported the erythrocyte number, as calculated from haematocrit and cell volume, to be ~37% lower in triploids whereas red blood cell volume was greater (54% in cell and 50% in nuclear volume, respectively). Haematocrit values were not significantly different between ploidies. For the triploid fish, the erythrocyte increase in length was significantly greater than the proportionate increase in width (25% and 12% respectively), resulting in a more elliptical shape to the erythrocytes. This is in agreement with previous findings for salmonids (Benfey and Sutterlin, 1984; Cogswell et al., 2002). According to Benfey (1999), the cause of the alteration of cell shape is likely to be a cytoplasmatic accommodation resulting from a significant increase in nuclear length. In the present study, the size of the erythrocytes of the diploid fish was consistent with that previously reported by Esteban et al. (2000), who measured sea bass blood cells using light and electron microscopy.

Total Hb concentration was reduced in the blood of triploids containing significantly less erythrocytes. The average volume of red blood cells (MCV) was greater in triploids, such that the mean cellular haemoglobin content (MCH) in erythrocytes of triploids was significant greater than in diploids and, the mean cellular haemoglobin concentrations (MCHC) were equivalent. Reported values for total blood haemoglobin (Hb) and MCHC concentrations in diploid and triploid fish are not consistent, whereas the mean cellular haemoglobin content (MCH) is commonly reported to be higher in polyploids (Benfey, 1999). Ballarin and co-workers (2004) reported no differences in total Hb but higher MCH values in triploid shi drum (*Umbrina cirrosa*) compared to their diploid counterparts. In salmonids, despite some

contrasting results on total blood haemoglobin levels and blood-oxygen carrying capacity, triploid fish were found to be similar to diploids in their overall oxygen-consumption rates and swimming performances under normal or stress conditions (Stillwell and Benfey, 1995).

Cortisol has both mineralo- and glucocorticoid actions (through expression of specialised transcription factors) in fish, being involved in both hydromineral balance and energy metabolism (Mommsen et al., 1999). It is the major corticosteroid produced during activation of the hypothalamo-pituitary–interrenal (HPI) axis, and is a major component of the stress response. In our study, both diploid and triploid sea bass showed relatively high levels of plasma cortisol although cortisol was significantly lower in triploids than in diploids. Cortisol levels for unstressed sea bass range from 15 to 133 ng ml^{-1} (Roche et al., 1989; Roche and Bogé, 1996; Cerdá-Reverter et al., 1998; Marino et al., 2001; Rotllant et al., 2003) suggesting that sampling and anaesthesia procedures can induce rapid changes in the hormone concentration in the blood. Eugenol in low concentration, as used in our study, is unable to block cortisol release in teleost fish (Iversen et al., 2003; Small, 2003) and this could explain the high cortisol levels that we measured. Also, cortisol might have been influenced by water temperature and photoperiod at the time of our sampling. In sea bass, significant annual variations in cortisol occur in relation to water temperature and photoperiod, with maxima during the warmest months (Planas et al., 1990). These last authors reported plasma cortisol levels ranging from 80 to 180 ng ml^{-1} for captive 2.5-year-old male sea bass measured during a period corresponding to our sampling. Few studies, mainly carried out on salmonids, have examined possible differences in the stress response between diploid and triploid fish; the general view is that there is no difference in plasma cortisol profiles following acute stress (Biron and Benfey, 1994; Sadler et al., 2000). Interestingly, in the present work the cortisol level was significantly lower in triploid than in diploid sea bass following a relatively short blood sampling procedure. The comparative physiology of the stress response in the two ploidies should be addressed in the

future but this would involve adopting particular and alternative blood sampling procedures for this species, which may be considered very stressor-sensitive.

Plasma concentrations of glucose, proteins, and lactate were similar for diploid and triploid sea bass under our experimental conditions. These blood metabolites are important indicators of fish health and basic metabolic activity. Increases in plasma glucose levels and hematocrit are secondary responses to stress, and arise following cortisol and catecholamine release (review in Wendelaar Bonga, 1997). Reduced capacity of haemoglobin to transport oxygen or reduced affinity for oxygen may result in anaerobic metabolism and accumulation of lactate in muscles, and possibly in blood. Ballarin and co-workers (2004) reported similar hematocrits, but higher plasma glucose, in triploid shi drum than in diploids. These authors considered that higher blood glucose concentrations in triploids might result from reduced aerobic metabolic capacities, to differences in glucose metabolic pathways and/or differences in insulin response between ploidies.

Reference values of serum glucose, total proteins, Na^+, K^+, Cl^-, Ca^{2+}, and osmolarity measured in captive diploid sea bass subjected to different handling and sampling procedures have been presented by Marino et al. (2001). Their results show that short capture and handling procedures, including light anaesthesia, did not elicit significant variations in the above blood parameters. Nevertheless, it remains difficult to compare studies that use different procedures, fish origins, and feeding regimes. Moreover, some authors (Planas et al., 1990; Pavlidis et al., 1997) report the presence of seasonal and daily variations in several blood parameters in sea bass, in accordance with observations in other fish species. The existence of such variations further complicates the possibility of performing comparisons. Nonetheless, the values that we measured for both diploid and triploid sea bass are within the range of reference values compatible with normal physiological and metabolic functions in this species (Marino et al., 2001). In the present work, K^+ differed between diploid and triploid sea bass, with lower levels

of this element being recorded in triploids. However, $(Na^{+}+K^{+})$/Cl- ratios were not different which suggests a strong homeostasis for monovalent ions; similar adjustments were seen in acid-base balance related ions between ploidies. Of the minor blood ions, triploid fish had lower Fe, Zn, S, and Cu concentrations than did diploid fish. In triploids, the lower (45%) Fe concentration can be partly explained by the reduced (13%) blood haemoglobin content compared to diploids, but the three-fold greater reduction in plasma iron suggests the possible involvement of other factors regulating their Fe status. Possibly, a comparative study on the activity of hepatic ferrireductase, the binding and storage capacity of serum transferrin and ferritin in both ploidy groups could provide some explanations to this. The other observations on minor elements suggest that additional studies should be carried out to clarify the role of these ions in sea bass physiology, and their possible relevance as metabolic indicators.

T_3 is involved in the intermediary metabolism of fish and its production is commonly associated with nutritional status and anabolism (Leatherland et al., 1977; Leatherland et al., 1984; Eales and Shostak, 1985; Eales, 1988; Eales and MacLatchy, 1989; Pérez-Sanchez and Le Bail, 1999). Thyroid hormones participate in growth promotion by stimulating appetite, by improving food conversion (Markert et al., 1977; Donaldson et al., 1979; Pérez-Sanchez et al., 1992; Le Bail and Boeuf, 1997; Rasmussen et al., 2001), and by stimulating protein synthesis (Narayansingh and Eales, 1975; Markert et al., 1977; Fauconneau et al., 1996). For these reasons, T_3 levels might differ in diploid and triploid fish if the latter are more sensitive to rearing conditions and have lower growth rates than diploids. Our results, showing no significant differences in T_3 levels between ploidies do not support this. Since the level of thyroid hormones is nutritionally-dependent, our results suggest that the inferior growth observed in triploid sea bass (Felip et al., 1999; Felip et al., 2001c; Peruzzi et al., 2004) may not be due to reduced access to food and depressed appetite. In the present study, the plasma T_3 levels were approximately 3.5-fold higher (60 *vs* 17 ng ml^{-1}) than those reported by

Cerdá-Reverter et al. (1996), two-fold higher (60 *vs* 25-35 ng ml^{-1}) than those of Pavlidis et al. (1997), but comparable to those found in male sea bass by Carrillo et al. (1991). We realize ourselves that T_3 is just one component of the thyroid hormone system, predominantly and locally produced from thyroxine (T_4) by tissue deiodase activity, and thus that plasma T_3 may not be the ultimate indicator of thyroid status in fish.

Gill Na^+/K^+-ATPase activities of diploid and triploid sea bass were not significantly different. Na^+/K^+-ATPase participates in ion transport either directly through movement of Na^+ and K^+ across the cell membrane, or indirectly through generation of ionic and electrical gradients that drive secondary active transports. The enzyme is considered to be the driving force for ionic exchanges in both fresh and seawater-adapted teleosts (review in McCormick, 1995). The strong euryhalinity of sea bass, which can live in salinities ranging from fresh water to hypersaline seawater, partially relies on adjustments of branchial Na^+/K^+-ATPase activity and intracellular distribution (Varsamos et al., 2002). The importance of branchial Na^+/K^+-ATPase in the maintenance of homeostasis through its involvement in osmoregulation and acid-base regulation allows this enzyme to be considered as a pertinent indicator of physiological status. The absence of difference in enzyme activity between diploid and triploid sea bass suggests that triploidy does not affect osmo- and ionoregulatory capacity in this species. However, experiments involving rigorous challenges are required to confirm this statement.

We have previously shown that the performance of triploid sea bass are equal to or lower than that of diploids (Peruzzi et al., 2004). In this work we confirm that similar levels of performance are reflected in similarities of several major haematological and physiological indicators. Further studies considering husbandry related experimental challenges should allow gaining a better understanding of the comparative adaptation capacities of diploid and triploid sea bass.

Acknowledgements

The authors are grateful to Iulia Grecu ("Dunărea de Jos" University of Galati, Romania) for her suggestions and assistance in some haematological procedures. They thank Malcom Jobling for reading the manuscript and revising the English. This study was partly supported by the European Community – Access to Research Infrastructures/Action of the Improving Human Potential Programme - ASEFAF Project (Contract NR. HPRI-CT-2001-00146).

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Table 1

Haematology and plasma metabolites in diploid and triploid sea bass. Values are means ± 95% CI.

	Diploid (n=16)	Triploid (n=15)	*F*	*P*
RBCC (10^6 mm^{-3})	2.17 ± 0.10	1.43 ± 0.07	18.51	<0.001
Hb (g l^{-1})	5.28±0.31	4.60±0.32	8.59	0.006
Hct	0.26±0.02	0.24±0.02	1.58	ns
Glucose (mg dl^{-1})	112.91±15.68	112.31±6.95	0.005	ns
Proteins (mg ml^{-1})	30.49±1.28	30.84±1.11	1.63	ns
MCV (fl)	120.85±7.41	172.20±15.78	34.747	<0.001
MCH (pg)	24.51±1.12	32.58±2.91	27.106	<0.001
MCHC (g l^{-1})	204.66±11.04	191.15±12.77	2.479	ns
Cortisol (ng ml^{-1})	152.00±17.44	122.27±17.72	5.320	0.029
Lactate (mM)	2.93±0.68	2.35±0.79	1.174	ns
T_3 (ng ml^{-1})	60.64±3.42	60.90±2.88	0.013	ns

Table 2

Red blood cells measurements of diploid and triploid sea bass. Values are means ± 95% CI.

	Diploid (n=16)	Triploid (n=15)	*F*	*P*
Nuclear area (μm^2)	10.89 ± 0.06	16.38 ± 0.17	43.67	<0.001
Nuclear width (μm)	4.04 ± 0.02	4.52 ± 0.03	13.06	<0.001
Nuclear length (μm)	5.15 ± 0.02	6.46 ± 0.03	22.63	<0.001
Cytoplasm area (μm^2)	60.42 ± 0.58	79.89 ± 1.04	48.16	<0.001
Cytoplasm width (μm)	8.34 ± 0.05	9.18 ± 0.81	15.48	<0.001
Cytoplasm length (μm)	11.38 ± 0.06	13.51 ± 0.08	161.91	<0.001
Cytoplasm width/length	0.73± 0.01	0.68± 0.01	24.56	<0.001

Table 3

Blood plasma osmolarity and ion concentrations in diploid and triploid sea bass. Values are means ± 95% CI.

	Diploid (n=16)	Triploid (n=15)	*F*	*P*
Osmolarity (mOsm)	367.31±2.93	372.27±6.98	0.16	ns
Na (mM)	175.56 ± 7.43	177.17 ± 2.61	0.152	ns
K (mM)	3.51 ± 0.25	2.83 ± 0.13	20.993	<0.001
Cl (mM)	180.12 ± 7.76	179.31 ± 3.37	0.034	ns
Na/Cl	0.97 ± 0.01	0.99 ± 0.01	3.729	ns
(Na+K)/Cl	0.99 ± 0.01	1.00 ± 0.01	2.147	ns
Ca (mM)	2.51 ± 0.27	2.23 ± 0.18	2.739	ns
Mg (mM)	1.07 ± 0.10	1.22 ± 0.17	2.412	ns
Mn (μM)	5.08 ± 2.39	4.16 ± 2.40	0.280	ns
Fe (μM)	34.18 ± 10.48	18.93 ± 2.78	7.135	0.013
Si (mM)	3.83 ± 0.34	5.65 ± 0.11	92.433	<0.001
Zn (mM)	0.26 ± 0.03	0.21 ± 0.02	8.479	0.007
Al (μM)	28.10 ± 5.68	29.28 ± 7.91	0.058	ns
P (mM)	9.00 ± 0.91	7.92 ± 0.53	3.912	ns
S (mM)	15.91 ± 1.56	13.06 ± 0.71	10.172	0.004
Cu (μM)	18.95 ± 4.58	12.50 ± 3.92	4.338	0.047
Cd (μM)	8.43 ± 3.84	7.25 ± 2.78	0.230	ns
Pb (μM)	40.78 ± 28.67	35.95 ± 18.93	0.074	ns