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Increased water current induces micro-architectural changes to the vertebral bone of juvenile rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

In terrestrial animals the link between exercise and bone physiology is well described, however this is not the case for fish. Abnormal bone physiology is a growing problem for intensive aquaculture, we therefore examined if water current affected bone quantity and quality in juvenile rainbow trout. Random groups of trout were assigned to one of two treatments, high current (two body lengths per second) or low current (zero body lengths per second), and fed a commercial diet for ten weeks. At the end of the trial no significant differences were elucidated for growth or body conformation. However the histomorphometry of the vertebrae from the trunco-caudal area of the spinal column was assessed and total bone area and trabecular thickness were found to be reduced ($p = 0.04$, $p = 0.01$), while the whole bone mineral content, and autocentrum width were observed to increase ($p = 0.01$). These changes however did not result in any significant differences in the mechanical properties of the vertebrae. This data suggests that exercise induces morphological changes to vertebrae which, over a longer production period than utilised in the present study, may influence the mechanical properties of the bone.

1. Introduction

The concept that bone form reflects the mechanical loading history during life is well recognised (Forwood, 2008). The phenomenon of bone adaptation to mechanical loading is often referred to as 'Wolff's law' (Wolff, 1892). Wolff's law states that bone in a healthy animal will adapt to the loads placed under, therefore if the loading on a particular bone increases, the bone will remodel itself over time to resist that load. Though the specifics of the 'law' are subject to considerable debate the general concept of bone functional adaptation is widely accepted (Ruff et al., 2006). This mechanical adaptation was summarised by Lanyon et al. (1982) who proposed a simple feedback loop where increased strain, through increased activity, leads to deposition of more bone tissue with decreased activity leading to a resorption of bone tissue. The ability of bone to adapt to mechanical loads is brought about by continuous bone resorption and bone formation. If these processes occur at different locations, the bone morphology is altered. Frost defined this as modelling (Frost, 1990a). In a homeostatic equilibrium resorption and formation are balanced. In that case old bone is continuously replaced by new tissue; this ensures that the mechanical integrity of the bone is maintained but it causes no global changes in morphology. Frost defined this as remodelling (Frost, 1990b).

Fish farms use water current to maintain water quality with an increase in the water current and velocity resulting in an increase in the speed at which fish must swim in order to maintain position (Kihara et al., 2002). The increased water velocity elevates the frequency of tail beats and therefore the physical stresses imposed on muscle ligaments and ultimately bone (Kranenbarg et al., 2005). However it is generally assumed that at favourable swimming speeds, exercise training, especially in salmonids leads to increased growth, increased feed conversion efficiency, increased protein turnover, higher plasma haematocrit, increased growth hormone and thyroxine, reduced circulating catecholamines and lower blood cortisol (Davison, 1997).

The vertebral body of the salmonid backbone consists of four layers or compartments, two of which are formed through mineralization of preformed collagenous tissue, (the notochordal sheath and the intervertebral ligament) and two of which are formed through ossification. The two ossified layers consist of both cortical (compact) bone which forms the bulk of the amphicoel and the trabecular bone. The three inner layers have ordered lamellar collagen matrixes, which alternate perpendicularly from layer to layer, whereas the outer layer of trabeculae has a woven matrix (Nordvik et al., 2005). Early reports considered most teleost bones incapable of remodelling and therefore unable to repair micro-fractures (Moss, 1962), however more recent studies have found that the trabeculae of the lower pharyngeal jaw of the cichlid *Astatoreochromis alluaudi* have been shown to adapt to diet, with fish fed on molluscs having more abundant

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trabeculae than those fed a soft diet (Huysseune et al., 1994). High current velocity has been linked to the development of lordosis in carp (Backiel et al., 1984), and has been implicated in the occurrence of deformities in sea bass and red sea bream (Divanach et al., 1997; Kihara et al., 2002). The mechanical integrity of cancellous bone is determined by its volume and microstructure, the composition of matrix and mineral and the balance between fatigue damage and repair (Compston 1994). For example short term resistance training increases bone turnover in rats, while long term exercise increases both bone strength and mass (Notomi et al., 2000).

Therefore this study was undertaken to examine if increased swimming activity induced by increased water current altered the microstructure of the cancellous bone of juvenile trout vertebrae and therefore the mechanical properties of whole vertebrae.

2. Methods

2.1. Fish and husbandry

One hundred and twenty six *O.mykiss* (68.71 ± 3.33 g) were randomly stocked into six replicate 130 L fibreglass tanks. After an acclimation period of one month the fish were fed a commercial diet (EWOS Sigma 50P) for a further 52 days (two meal portions/day, six hours apart) according to the digestible energy need (DEN) of the fish as described by Bailey and Alanara (2006) and Alanara et al. (2001). The fish were group weighed every two weeks, after 24 h starvation, and the model adjusted accordingly. The specific growth rate (SGR, %) was calculated from the biomass gain in each tank using the following equation:

$$SGR = Ln(W(ti)/W(t0))/(ti-t0) \times 100$$

where W(ti) and W(t0) were the biomass at the end (ti) and the start (t0) of the trial, and (ti − t0) was the duration of the trial in weeks. The feed conversion ratio was calculated based on the biomass gain in the tanks and the registered feed waste during the whole trial according to:

$$FCR = feedconsumed(ti-t0) : biomassgain(ti-t0)$$

The tanks were randomly assigned to one of two treatments; high current (25 L min^{-1}, directional/laminar current, at pressure, representing 2 BL s^{-1}) and low current (25 L min^{-1},uni-directional current, low pressure, 0 BL s^{-1}) (Fig. 1). These two treatments were experimentally induced by using a PVC end cap drilled with an 8 mm hole pointing at 45°, 15 cm above the water surface; and by directing the 'low' current vertically into the tank from 32 mm inlet diameter and using 6" PVC pipes as baffles. In-tank current rates were established from a mean of three readings, measured with a current meter (Ott type V C2) and represented swimming speeds of two body lengths per second (BL s^{-1}) and zero current rate at the start of the trial with current rates adjusted according to the length of the experimental animals determined at each weigh point. A random sampling of initial point fish was removed for carcass composition ($N = 6$). After 52 days of dietary conditioning a sample of fish ($N = 3$) was removed, anaesthetised and a sample of blood (1 mL) was removed by caudal veinipuncture. The blood was allowed to clot for 18 h (4 °C) prior to being centrifuged (13,000 rpm, 5 min) and the serum removed and snap frozen in liquid nitrogen before storage at −80 °C until analysis. A further three fish were also removed and the tenth to thirteenth vertebrae in reverse order from the urohyale were removed and placed into neutral buffered formalin prior to being decalcified for seven days (2 M ethylenediaminetetraacetic acid: Sigma Aldrich, UK), and processed by standard techniques for paraffin embedding. Serial samples of three vertebrae were also removed for bone ash (V13–16 in reverse order from urohyale) and bone strength determinations (V16–19 in reverse order from the urohyale). Vertebrae from this area of the spinal column were studied as this is the region commonly showing malformation in farmed salmonids (Berg et al., 2006, Hansen et al., 2010). The feeding regimen was carried out under license granted by the UK Animal (Scientific Procedures) Act, 1986. Fish were killed in accordance with UK Home Office Schedule One regulations.

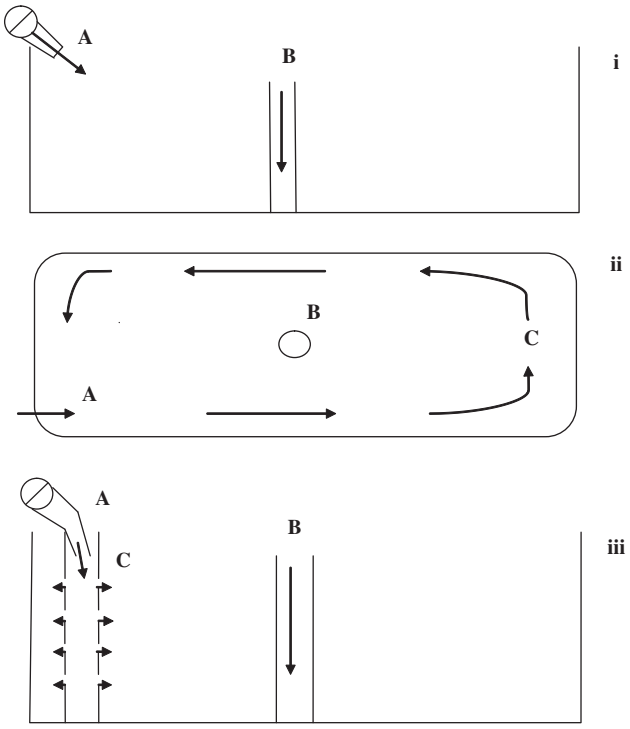

Fig. 1. (i-iii): Diagrammatic representation of experimental layout. i = cross section of 2 BL s^{-1} tank where A = water inlet, B = water outlet. ii = aerial view of tank depicted in i showing approximate water flow throughout the tank. iii = cross section of 0 BL s^{-1} aquaria, C = baffle to diffuse incoming water.

2.2. Vertebral mechanical properties

The frozen vertebrae were defrosted and allowed to equilibrate to room temperature, the diameter and height recorded and then the sample compressed, in the anterior posterior direction, using an Instron universal testing machine with a 500 N load cell with parallel stainless steel plates. The Young's modulus (N/mm^2), compressive extension (mm), and the load to initial peak (Kg F) (that is where a 10% plastic deformation occurred) were determined and recorded using the Bluehill 2 (Instron, High Wycombe, Bucks, UK) materials testing software. A 5 N preload was used on all vertebrae to negate the effects of soft tissue on the plane of compression. Compressive force application was performed at a rate of 2 mm min^{-1} and was terminated after the initial elastic deformation had reached a plateau.

2.3. Bone mineral content

Three vertebrae of the trunco-caudal region of the spine as defined by Kacem and Meunier (2003) were pooled, dried for 12 h at 110 °C, and weighed (W_{dry}) to the nearest milligram prior to incineration for 12 h at 550 °C. The ashes were then weighed (W_{ash}) to the

nearest milligram. The vertebral bone mineralization (BM, %) was calculated from the following equation:

$$Bone\text{mineral content}(\%) = \left(W_{ash}/W_{dry}\right) \times 100$$

where W_{ash} = weight of ash after incineration and W_{dry} = weight of dried sample.

2.4. Alkaline phosphatase and serum protein

Blood (1 mL) was removed by caudal puncture, allowed to clot overnight at 4 °C and the serum removed and stored at −80 °C until serum alkaline phosphatase and protein levels were determined following the protocol of Deschamps et al. (2009). Briefly 100 μl of plasma sample, in triplicate was incubated at 37 °C for 1 h with *p*-nitrophenyl phosphate (pNPP: 1 mg mL^{-1}, Sigma N9389) in an alkaline buffer (1.5 M, pH 10.3, [Sigma Aldrich, A9226]). The reaction was stopped by adding 50 μl of 3 N NaOH. Absorbance was measured at 405 nm against a blank and converted into the amount of produced *p*-nitrophenyl (pNP) using a standard dilution-curve (pNP: 10 mM, Sigma N7660). Serum protein concentration was determined using a commercially available test kit (Bio-Rad, Hemel Hempstead, UK) which uses a modification of the Bradford method (Bradford, 1976).

2.5. Histological sections

Histological samples were dissected and stored in 10% neutral-buffered formalin until processing via standard histological protocols into wax blocks. The wax blocks were cut into 8 μm sections using a Leica microtome (Reichert Jung) and mounted on slides prior to staining. Vertebral samples were decalcified in 10% EDTA [w/v] (ethylenediaminetetraacetic acid: Sigma Aldrich, UK) with the decalcification time dependant on the size of sample ranging from 7 to 10 days. Prior to staining the paraffin embedded samples were sectioned using a Leica microtome (Reichert Yung) at 6–8 μ, dried, cleared with xylene, and hydrated through a series of graded alcohols (100, 90, 70%) before being rinsed in distilled water for 5 min. The slides were stained with Mallory's trichrome stain following the protocol of Handy et al. (1999). This stain results in erythrocytes staining orange, nuclei stain red, collagen stains blue and muscle staining red.

2.6. Vertebral trabecular bone connectivity

A binary image of the stained sections was created using Image J and from this a histogram was used to quantify the ratio of white: black pixels and thus measure trabecular bone area (B.Ar) and tissue area (T.Ar) as well as bone perimeter (B.Pm) (Fig. 2i, ii). From these measurements the following indices, as defined by the American Society of Bone and Mineral Research in Parfitt, 1988, were derived:

$$\text{Trabecular thickness}(\text{Tb.Th}) = 4/\pi \times \text{B.Ar}/2/\text{B.Pm}$$
$$\text{Trabecular number}(\text{Tb.N}) = \text{Tb.Ar} \times 10/\text{Tb.Th}$$
$$\text{Trabecular separation}(\text{Tb.Sp}) = 1000/\text{Tb.N} - \text{Tb.Th}.$$
$$\text{Euler Poinacre number}(\text{E}) = \text{n} - \text{m}$$

where n = the total number of (disconnected) trabecular profiles and m = the number of marrow cavities with the more negative the Euler number the more connected the compartments are.

In total five sections per animal were measured and all analyses were performed blind. It should also be noted that the equations defined by Parfitt, 1988 are predicated upon the fact that histomorphometric assessment of bone connectivity is independent of the plane of quantification.

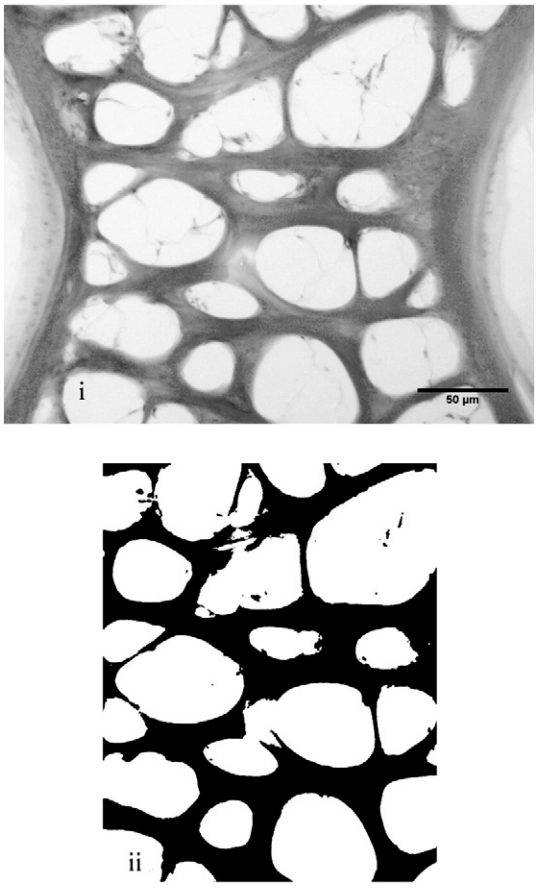

Fig. 2. (i-ii): Image processing in Image J for quantitative analysis. i = 8-bit greyscale image, ii = cropped B/W picture used for histogram/connectivity analysis. Note due to the decreasing connectivity of the trabeculae at the innermost layers of the vertebrae, connectivity was assessed using sections where a minimum of 100 μm between cortical surfaces were used.

2.7. Statistical analysis

All data are shown as mean ± standard deviation. Where appropriate data was analysed by one way analysis of variance (ANOVA) and *post hoc* Tukeys test, after Anderson–Darling normality test, using Sigmaplot 11.0 (Systat software Inc. 2008). Percentage data were arcsine transformed prior to subsequent analysis. A difference of $p<0.05$ was considered significant.

3. Results

A summary of the growth results can be seen in Table 1. Current rate did not have a significant effect on growth with no difference in % body weight gain, specific growth rate, or thermal growth coefficient. Nor did current rate significantly affect the body shape or feed conversion, though the feed conversion ratio was marginally reduced in the 2 BL s^{-1} group. No significant differences were found between the proximate analysis (moisture, protein, lipid, ash) of fish under either treatment.

Significant effects were seen in total trabecular area (Tb.Ar), trabecular thickness (Tb.Th), autocentrum width and bone ash content. Tb.Ar was seen to decrease with a higher current rate as did the Tb.Th however the autocentrum bone width was seen to increase (Table 2/Fig. 3). No discernable effect of current rate was found on the number of trabeculae, the trabecular separation or the Euler number with both treatments recording a highly negative Euler number indicating a highly connected trabecular network (Table 2).

Table 1
Summary of experimental results of trout fed a commercial diet for 10 weeks and subject to either a high (2 BL s^{-1}) or low current (0 BL s^{-1}) rate.

Parameter		0 BL s^{-1}	2 BL s^{-1}
Initial body weight (g)		62.84 ± 2.51	64.57 ± 4.38
Final body weight (g)		136.76 ± 6.81	140.82 ± 11.61
% BWG		122.10 ± 7.45	123.33 ± 12.41
Specific growth rate (% BW day^{-1})		1.64 ± 0.11	1.63 ± 0.06
Thermal growth coefficient		0.17 ± 0.01	0.18 ± 0.01
Feed conversion ratio		0.80 ± 0.04	0.78 ± 0.04
Condition factor		1.26 ± 0.05	1.26 ± 0.02
Proximate analysis	Initial fish		
Moisture (%)	74.18 ± 1.87	66.71 ± 1.90	68.94 ± 2.64
Protein (%)	14.92 ± 0.77	17.43 ± 0.91	17.04 ± 0.82
Lipid (%)	5.42 ± 1.06	13.32 ± 1.10	11.61 ± 0.79
Ash (%)	1.92 ± 0.11	2.25 ± 0.07	2.20 ± 0.18
NFE[a]	3.56	0.29	0.21

Data is shown as mean ± standard deviation. Different superscripts denote significant differences ($p<0.05$).
[a] Nitrogen free extract (by calculation).

The Young's modulus was approximately 15% higher in the 2 BL s^{-1} treatment however this increase was not significant. This trend was repeated for the compressive load, while the compressive extension was reduced by 14%, but again this was not significant at the $\alpha = 0.05$ level. No differences were observed in serum alkaline phosphatase (ALP) activity however the total mineral content of the vertebrae was seen to significantly increase in the 2 BL s^{-1} treatment compared to the 0 BL s^{-1} group.

4. Discussion

Resistance training induced no negative effects on growth, proximate composition or body condition of the fish. The FCR achieved in the current study (approximately 0.8) is much lower than the 1.3 salmonid economic FCR (total feed fed/total species group biomass increase) reported by Naylor et al. (2009). The FCR of the current study is comparable to the best achievable modern aquaculture, however it is acknowledged that in practical terms feeding models are balance between achieving the low FCR required to reduce negative environmental inputs and the economic need to maintain a high growth rate.

Similar to this study Deschamps et al. (2009) found that sustained exercise increased bone density (through a reduction in the bone cross sectional area and an increase in bone mineralisation) and hypothesised that the increased mineralisation would result in stronger bones that are more able to resist the higher forces arising from the increased exercise. In the current study the trabecular bone area was observed to decrease by approximately 15% with exercise while the autocentrum width increased by 10%. This suggests that in our experiment, the increased strain from exercise acts to decrease trabecular bone in favour of compact autocentrum bone and therefore increase the bone density. Nevertheless this finding disagrees with that of Krossoy et al., 2009 who found no significant differences between the trabecular and compact bone mineral content in adult salmon. It is therefore possible that differences between the mineral content of trabeculae and the autocentrum exist in younger and faster growing animals but these differences are diminished in adult animals.

It should also be noted that while no significant differences in the mechanical properties of the vertebrae were found in the current study the Young's modulus mean value was 15% greater in the exercised fish. The Young's modulus, determined from the stress/strain curve of a material, is a measure of stiffness, which is a material's ability to withstand plastic deformation, with brittle materials therefore having higher Young's modulus than elastic materials. Thus the increased autocentrum bone may have served to increase stiffness but the experimental design was unable to resolve these differences due to the variability between treatments. These findings suggest that exercise induces modelling changes that serve to increase the stiffness of the bone without affecting overall strength in the anterior posterior direction; however the strength of the bone in this plane is unlikely to be representative of the strain patterns *in vivo*. The subcarangiform locomotion of the trout is defined by the movement

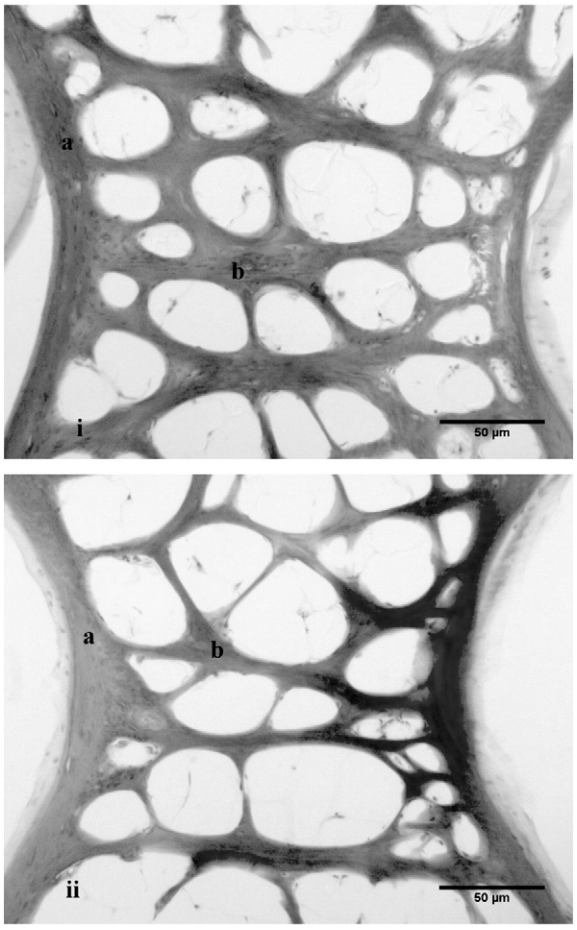

Fig. 3. (i-ii): Histology sections of trout vertebrae from fish trained at either 0 BL s^{-1} i or 2 BL s^{-1} ii for ten weeks. a = autocentrum, b = trabeculae. Scale bar represents 50 µm.

Table 2
Vertebral histomorphometric and mechanical properties, bone mineralisation and serum alkaline phosphatase of trout fed a commercial diet for 10 weeks and subject to either a high (2 BL s^{-1}) or low current (0 BL s^{-1}) rate.

Parameter	0 BL s^{-1}	2 BL s^{-1}
Trabecular bone area [Tb.Ar] (%)	43.80 ± 1.14[a]	38.30 ± 3.63[b]
Trabecular thickness [Tb.Th] (µm)	10.28 ± 0.37[a]	9.17 ± 0.30[b]
Trabecular number [Tb.N] (mm^{-2})	42.90 ± 1.23	42.23 ± 3.23
Trabecular separation [Tb.Sp] (µm)	13.18 ± 0.62	14.88 ± 2.04
Autocentrum width (µm)	15.30 ± 0.65[b]	17.00 ± 0.24[b]
Euler number	−8.53 ± 2.99	−4.56 ± 2.80
Serum ALP (µmol L^{-1} min^{-1})	83.44 ± 19.98	82.72 ± 22.16
Bone mineralisation (Ash %)	27.72 ± 1.35[a]	30.50 ± 0.45[b]
Young's modulus (N mm^{-2})	158.39 ± 25.76	181.38 ± 23.74
Compressive extension (mm)	0.57 ± 0.21	0.54 ± 0.02
Compressive load (Kg F)	6.88 ± 1.06	6.91 ± 0.30

Histomorphometric data values represent a mean of a minimum of five slides from three fish pre replicate. Data is shown as mean ± standard deviation. Different superscripts denote significant differences (ANOVA, $p<0.05$).

between one half and two thirds of the muscle mass (Sfakiotakis et al., 1999) resulting in sinusoidal movement, thus the strain patterns are unlikely to be running parallel in the anterior posterior direction. Therefore this represents a limitation of the methodology currently employed as the mechanical assessment will not be generating loads akin to those engendered by muscular action, which the modelling response has tried to normalise, thus greater changes may be noted if similar loads were applied experimentally.

The data of the current study does not support the hypothesis that sustained exercise improves vertebral histomorphology as no difference in the mechanical properties of the bone was found. Thus more evidence is required to determine the practical implications of the increased bone density. It could be argued that the loss of elasticity in the bones as a result of exercise may increase the risk of deformities due to the inability of the bone to resist plastic deformation, alternatively the stiffer bones may transmit forces more readily to the inter-vertebral tissue which has been implicated in the aetiology of deformities in Atlantic Salmon (Witten et al., 2005).

Deschamps et al. (2009) stated that the reduced bone area in exercised fish, was due to bone resorption rather than bone deposition but experimentally the author found no variation in plasma TRAP between treatments, the data of Deschamps et al. (2009) and that of this study, support the theory that the changes have occurred due to bone modelling, rather than remodelling as defined by Frost (1990b,a) as in both cases the animals grew significantly over the experimental period.

Furthermore some authors have implied a relationship between the incidence of deformities and the rapid growth rates required in modern aquaculture, citing the soft tissue growth may be at the expense of mineralised tissue (Lall, 2002). In the current study high SGR's and low FCR's were induced but no increase in the number of obvious deformities was observed, indeed no differences in the number of deformities in exercised and control fish were found by Deschamps et al. (2009) either. Thus there appears to be little current evidence for this hypothesis in trout past larval stages. Similarly some authors have stated that during rapid growth in active conditions dietary uptake and absorption of minerals from the surrounding water may not fulfil the entire physiological requirement and lead to a reduction of bone area (Deschamps et al., 2009). However scales have been shown to be a significant reservoir of minerals in fish with some authors regarding them as the most labile source (Witten and Huysseune, 2009). Also for more minerals to be available for soft tissue biological processes a loss of bone tissue would coincide with either a maintenance or loss of bone mineralisation, however in both this study and that of Deschamps et al. (2009) the bone mineralisation increased in exercised fish. Thus there is a need to identify where minerals are resorbed from; bone, scales, or both, under mineral deficient conditions.

In both the study of Deschamps et al. (2009) and this, the bone area was reduced in exercised fish however the plane of quantification was cross sectional in the former and sagittal in the latter. While neither can be confirmed to be correct without the determination of the plane of force, bone mechanical adaptation theory stipulates that the trabeculae will be aligned in the direction of the major force. Thus as the trabeculae occur as inter-connected plates in the anterior posterior direction plane (Nordvik et al., 2005) for standardisation of future teleost bone histomorphometric studies we suggest that bone area, trabecular thickness and trabecular separation be determined in the sagittal plane at spatially defined points. In addition quantification of vertebral bone area in sagittal sections removes the requirement for vertebrae size normalisation of cross sectional bone areas.

In conclusion the effect of exercise in rapidly growing farmed fish fed commercial diets appears to be an increased autocentrum bone width and mineralisation coupled with a reduction in the trabecular thickness. It is probable that this adaptation of mineral tissue serves to maintain the mechanical integrity of the bone while resisting the increased forces imposed upon it from the increased tail beat frequency needed to maintain position in the water column.

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