Changes in vertebral structure during growth of reared rainbow trout, *Oncorhynchus mykiss* (Walbaum): a new approach using modelling of vertebral bone profiles

M-H Deschamps¹, M Girondot², L Labbé³ and J-Y Sire¹

¹ Équipe Évolution et développement du squelette, UMR 7138, Université Pierre & Marie Curie-Paris, France
² Université Paris Sud, UMR 8079, Orsay, France
³ Pisciculture Expérimentale INRA des Monts d’Arrée, Barrage du Drennec, Sizun, France

Abstract

Severe bone resorption of the vertebral body in reared rainbow trout was thought to be a dysfunction in mineral balance induced by increased growth rate in unfavourable rearing conditions. To verify this assumption, we sampled market-sized trout (c. 250 g) from 20 fish farms with different rearing conditions. Growth rate was also studied by sampling trout reared in three different water temperatures from fry to market-size. Transverse sections of vertebrae were microradiographed, then digitized. Total bone area (Tt-B.Ar.) and bone profiles were obtained using BONE PROFILER 3.23 software and a mathematical model was developed to statistically compare bone profiles using 12 parameters in four vertebra regions. Tt-B.Ar. and bone profiles were found to vary with rearing conditions and growing temperatures, indicating obvious influences of these factors on bone remodelling. However, vertebral resorption was found to be a general phenomenon. In trout from 190 to 235 mm in length, vertebrae underwent important remodelling resulting in large resorption of the middle area, while the transition and peripheral areas showed an increase in bone deposition. Changes in vertebra architecture seem to be a good compromise between the need to mobilize stored minerals during growth while maintaining vertebral biomechanical properties.

Keywords: bone area, growth, modelling, *Oncorhynchus mykiss*, resorption, vertebrae.

Introduction

A recent histological study of normally shaped vertebrae of reared rainbow trout (market-size, c. 250 g) revealed the presence of extended ‘holes’ in the large, middle and cancellous bone region (Kacem, Meunier, Aubin & Haffray 2004). In salmonids, during vertebra growth this region extends by successive bone deposition at the trabecula surface, i.e. elongation and branching from the amphicoel compact bone (reviewed in Nordvik, Kryvi, Totland & Grotmol 2005). The presence of such holes can only be interpreted as the result of a destruction of the bone matrix through osteoclast activity, a process that is also revealed by the presence of numerous Howship lacunae at the trabecula surface. Histological observations usually show that these holes are mainly filled by fat and neither cartilage nor unmineralized bone matrix are present. Cartilage has been previously reported in this vertebra region, but only in vertebrae that showed obvious pathological changes, as for instance fused vertebral bodies (Kacem et al. 2004).

In salmonids living in fresh water, resorption of bone (mainly vertebrae) and scales is known as a means of mobilizing mineral ions when mineral uptake from the environment is insufficient to fulfil physiological processes such as ovarian maturation, spawning migration and starvation (Weiss & Watabe 1979; Carragher & Sumpter 1991;
Persson, Sundell & Björnsson 1994; Persson, Sundell, Lundqvist, Shiramick & Björnsson 1997; Skonberg, Yoge, Hardy & Dong 1997; Kacem, Meunier & Baglinière 1998; Kacem, Gustafsson & Meunier 2000; Kacem & Meunier 2000, 2003; Witten & Hall 2003). In market-sized reared rainbow trout, vertebral resorptions can hardly be related to these physiological factors because, (i) the trout do not reach maturity, as the time required to obtain this commercial length is generally too short in most fish farms (Davies & Bromage 2002) and producers frequently prevent sexual maturation using triploidization (Benley 1999); (ii) in tanks, water flow is often low and sustained exercise is limited when compared with the wild (Egna & Boyd 1997) and (iii) there is no starvation, as trout are usually fed to satiation to enhance rapid growth (Talbot, Corneillie & Kørsoen 1999). Therefore, given the lack of physiological constraints, vertebral bone resorption in reared trout was thought to be the consequence of a dysfunction in phosphorus and calcium metabolism induced by increasing growth rates in unfavourable rearing conditions (Kacem et al. 2004). Dietary uptake and absorption of minerals may not be able to fulfil body requirements (Lall 2002; Helland, Refstie, Espmark, Hjelde & Bae 2005; Kaushik 2005; Lall & Lewis-McCrea 2007). It is important to understand how such large areas of resorption take place during growth, and whether or not the various regions of the vertebral body are all involved in this process. However, one difficulty remains: how to quantify accurately vertebral bone resorption?

Thus far, in salmonids, 2D-measurements of the relative amount of vertebral mineralized area on the total bone area (Tt-B.Ar.) 1 have generally been used to estimate indirectly the amount of bone resorption (Kacem et al. 2004). The higher the Tt-B.Ar., the less the vertebrae were resorbed and vice versa. However, we have observed that bony areas are not distributed homogeneously. In addition, vertebrae showing weak resorption, but narrow trabeculae, can have a Tt-B.Ar. similar to that of vertebrae with extended resorption but thick trabeculae. Therefore, changes in the vertebra structure resulting from bone resorption (and bone deposition) cannot be accurately described by using only Tt-B.Ar. values. A similar difficulty in the evaluation of Tt-B.Ar. was deduced from studies of long bone sections in tetrapods. To solve this problem, Girondot & Laurin (2003) have developed a software, called BONE PROFILER (www.ese.u-psud.fr/epc/conservation/Bone-profiler), to quantify, model and statistically compare bone area (B.Ar.) profiles (also specified as ‘bone compactness profiles’) in long bone cross-sections. In measuring Tt-B.Ar and B.Ar. in several concentric regions of a vertebral section (i.e. vertebral bone profiles), BONE PROFILER appeared a suitable tool to quantify accurately vertebral B.Ar. in reared trout. However, because vertebral sections are more complex than long bone sections, it was necessary to develop an appropriate model adapted to the vertebral structure to analyse vertebral bone profiles.

In the present study, we have developed a mathematical model and characterized parameters that accurately describe the complexity of vertebral bone profiles obtained using BONE PROFILER. Furthermore, to evaluate the influence of rearing conditions, we have compared vertebral Tt-B.Ar and vertebral bone profiles of trout of the same length (market-sized) but reared in different fish farms. Also, to evaluate the influence of growth rate, we have compared the vertebral features of trout, of the same age or length, reared experimentally at different temperatures from fry to market-size.

Materials and methods

Trout

A total of 350 rainbow trout, Oncorhynchus mykiss (Walbaum), were randomly sampled from 20 fish farms. These trout were market sized and ranged from 207 to 320 mm in total length (TL), and showed no external deformities. They were either diploid (n = 180) or triploid (n = 170).

Growth rate experiment

Fertilization and hatchery procedures were carried out at the experimental fish farm of INRA (PEIMA, Sizun, France). The rainbow trout broodstock used in our experiments, five females
and five neomales (i.e. XX females hormonally masculinized) were from INRA’s Autumn strain (i.e. broodstock selected to spawn in autumn). Each spawning was in December 2004 and eggs were incubated separately until the ‘eyed’ stage. Then, the eggs were evenly divided into three batches of 1200 eggs. Diploid fry were reared in tanks supplied with spring water at 11.0 ± 0.5 °C. First feeding started on 17 January 2005 and fry were fed to satiety with commercial diets (Bio Optimal Start, Biomar, Nersac, France).

One and a half months after first feeding (1 March 2005), 500 trout from each batch were transferred into tanks at the following locations with different rearing conditions:

- **Batch 1**, designated ‘NOR’, remained at the PEIMA experimental farm. The tanks were supplied from a river source with seasonal temperature variations from 5.0 to 18.9 °C.
- **Batch 2**, designated ‘SLOW’, was transferred to Lées-Athas fish farm (Pyrénées-Atlantiques). The tanks were supplied with mountain spring water at a constant temperature of 7–8 °C.
- **Batch 3**, designated ‘FAST’, was transferred to Donzacq (Landes) fish farm. The tanks were supplied with spring water at a constant temperature of 17 ± 1 °C.

Trout of batches 2 and 3 were transported in oxygenated containers (30 L) at a controlled temperature of 10 °C, and acclimatized by successive water mixings allowing a maximum temperature variation of 2 °C h⁻¹. During the three experiments (SLOW, NOR and FAST), trout were fed to satiety with the same commercial diet (ECOLIFE 66–67, Biomar).

The aim was to compare the vertebrae of individuals with different growth rates, (i) at the same length (i.e. market-size trout of ≈250 g) and (ii) at the same age. Thus, three samples of 20 trout were taken from each batch at 7.5, 9 and 13 months after first feeding, i.e. when FAST, NOR and SLOW trout reached the appropriate size, respectively (Fig. 4). A total of 180 trout were analysed. All experiments were in conformity with French animal welfare laws, guidelines and policies.

**Vertebra preparation and vertebral measurements**

According to the methods of Kacem *et al.* (2004), trout were cooked in boiling water for 10 min and soft tissues were removed from spines and bones with dissecting tweezers. Three ‘normally shaped’, adjacent vertebrae (i.e. showing no pathological changes) from the middle region of the vertebral column (V34–V36) were selected from each trout. The vertebrae were dehydrated in a graded series of ethanol (70, 95 and 100%; 24 h each), and lipids were removed in acetone (2 × 24 h), and then in trichloroethylene (2 × 24 h).

Vertebrae were embedded in resin (98% stratyl, 2% Luperox catalysor) and 125 ± 10 µm-thick, transverse ground sections were obtained with a Leitz 1600 Saw Microtome (Leitz Company, Wetzlar, Germany). The single section through the mid-region of the vertebrae, in which the notochord canal is the narrowest and bone tissue area the largest, was retained for B.Ar. analysis (Kacem *et al.* 2004).

Ground sections were radiographed using an X-ray tube CGR Sigma 2060 (CGR-GE, Buc, France), adjusted to 8 kV and 6 mA, on a Kodak Industrex film Ready Pack (Eastman Kodak, Rochester, NY, USA) set at 30 cm from the source. The enlarged radiographs (35X) were digitized using a Olympus Camedia digital camera (Olympus Corporation, Tokyo, Japan) mounted on an Olympus SZX12 binocular microscope (Olympus Corporation, Tokyo, Japan). The pictures were then transformed into binary images (black and white images; TIFF format) with ADOBE PHOTOSHOP 7.0 software (Adobe Systems Inc., San Jose, CA, USA), with a threshold value chosen to visualize space between bone trabeculae. Vertebral bone profiles and Tt-B.Ar. were obtained from these images using BONE PROFILER 3.23, a modified version of a software developed to study B.Ar. profiles of long bone sections (Girondot & Laurin 2003).

**Theory of the model**

Transverse sections through the mid-region of vertebrae can be divided into four distinct regions (Fig. 1a): (i) a central ring of compact bone surrounding the notochord, hereafter called the ‘notochord area’; (ii) a ‘transition area’, immediately adjacent to the notochord area, that represents the proximal region of bone trabeculae; (iii) a ‘middle area’, that represents a region in which bone trabeculae are most often subjected to resorption and (iv) a ‘peripheral area’, i.e. the distal region of bone trabeculae to which muscles are attached in.
teleost fish (Westneat & Wainwright 2001; Alami-Durante & Rescan 2003).

As the notochord canal does not contain mineralized tissues, this central region of the vertebral section was not included in the analysis of vertebral bone profiles. The diameter of the notochord canal being variable between cross-sections, we took into consideration the relative distance (d, proportion of the radius of cross-sectioned vertebra) from the periphery of the notochord canal (value: 0) to the periphery of the vertebra (value: 1) to ensure accurate comparison between vertebral sections with various total bone areas (Fig. 1b).

The main pattern of B.Ar. from the notochord canal to the vertebral periphery always shows a sigmoid signature (Fig. 1). Therefore, B.Ar. can be adequately modelled by scaled logistic functions, which have the advantage of being between 0 and 1, as observed for B.Ar. The general formula for a scaled logistic function is:

\[
1/(1 + e^{-P(d-d)}) (1 - \min) + \min
\]

where \( P \) is the distance to the centre where the most abrupt change in B.Ar. is observed, \( S \) is the slope of B.Ar. change at point \( P \), \( d \) is the distance to the centre and \( \min \) is the lower asymptote.

This main pattern, called hereafter \( Q_0 \), is the ‘unresorbed’ model, modelling the rapid decrease of B.Ar. to an asymptotic B.Ar. value observed at the vertebral periphery (\( \min_2 \); Fig. 1a,b).

Superimposed on this ‘unresorbed’ model, two zones of B.Ar. decline are observed within transition and middle areas (Fig. 1a,b). Each decrease and increase of B.Ar. is modelled using the product of two scaled logistic equations (1). \( Q_{1,2} \) and \( Q_{3,4} \) are the products used to model B.Ar. changes within the transition and the middle area, respectively.

Therefore, the overall model that best explained B.Ar. as a function of the distance from the notochord canal (d) of a vertebra cross-section is:

\[
B.A.(d) = Q_0 * Q_{1,2} * Q_{3,4}
\]

The parameters of the model are described in Table 1 and Fig. 1b.

**Parameter estimation**

Bone area was measured in 50 concentric zones, each representing 2% along the radius length from the periphery of the notochord canal to the periphery of the vertebra. The parameter values describing a vertebra cross-section were fitted using maximum likelihood with a binomial link function (Burnham & Anderson 2002). Such a binomial model was chosen because the data from radiographs were composed of bone (B) and...
Table 1 Parameters of the model describing bone area profiles of trout vertebrae (see also Fig. 1)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>( Q_0 )</td>
<td>Maximal ( B.Ar. ) value in the periphery area</td>
</tr>
<tr>
<td>( P_0 )</td>
<td>Relative distance from the border of the chordal area where a 50% decrease in ( B.Ar. ) is observed between ( B.Ar. ) of the chordal area and the ( Q_0 ) value</td>
</tr>
<tr>
<td>( S_0 )</td>
<td>Reciprocal of the slope at point ( P_0 )</td>
</tr>
<tr>
<td>Model for the transition area ( Q_{1,2} = [B.Ar.(d) \times B.Ar.(d)] )</td>
<td>% of ( B.Ar. ) lost compared with ( Q_0 )</td>
</tr>
<tr>
<td>( P_1 )</td>
<td>Relative distance from the border of the chordal area where a 50% decrease in ( B.Ar. ) is observed between ( Q_{1,2} ) and ( Q_0 )</td>
</tr>
<tr>
<td>( S_1 )</td>
<td>Reciprocal of the slope at point ( P_1 )</td>
</tr>
<tr>
<td>( S_2 )</td>
<td>Reciprocal of the slope at point ( P_2 )</td>
</tr>
<tr>
<td>Model for the middle area ( Q_{3,4} = [B.Ar.(d) \times B.Ar.(d)] )</td>
<td>% of ( B.Ar. ) lost compared with ( Q_0 )</td>
</tr>
<tr>
<td>( P_3 )</td>
<td>Relative distance from the border of the chordal area where a 50% decrease in ( B.Ar. ) is observed between ( Q_{3,4} ) and ( Q_0 )</td>
</tr>
<tr>
<td>( S_3 )</td>
<td>Reciprocal of the slope at point ( P_3 )</td>
</tr>
<tr>
<td>( S_4 )</td>
<td>Reciprocal of the slope at point ( P_4 )</td>
</tr>
</tbody>
</table>

The search for parameters maximizing likelihood was performed using GCR2 algorithm (Fylstra, Lasdon, Watson & Waren 1998). The parameters estimated using maximum likelihood are normally distributed (Burnham & Anderson 2002). Results are expressed in mean values with standard error of the mean (SEM).

Data analysis

Bone area values are proportions; therefore, prior to statistical analysis an arcsine transformation was performed to ensure normality, using the formula: 
\[
p' = \arcsin \sqrt{p} \quad \text{(Zar 1999).}
\]

For comparisons of trout from various fish farms and for the experiment on growth rate, vertebral Tt-B.Ar. and vertebral bone profiles (assessed by the parameters of the model: \( Q_0, P_0, S_0, Q_{1,2}, P_1, S_1, P_2, S_2, Q_{3,4}, P_3, S_3, P_4 \) and \( S_4 \)) were tested using MANOVAs. Trout growth (assessed by TL) as a function of lots and samplings was tested using an ANCOVA. Tukey’s honestly significant difference tests were used a posteriori to detect differences among means. For the comparison of trout from various fish farms, partial correlation coefficients were also used to express the correlation between each parameter of the model and Tt-B.Ar., assuming that the other parameters were kept at constant values. All statistics were performed using JMP™ Statistical Software version 5.1 (SAS Institute, Cary, NC, USA) with a significance level of 5%.

Results

Trout from different fish farms

A similar value of Tt-B.Ar. was obtained for some cross-sections of vertebrae with different organization and structure (i.e., different bone profiles) and,

![Figure 2 Inverse radiographs of transverse sections of vertebrae in market-size trout, with their associated total bone area values (Tt-B.Ar.). a–c: Slightly resorbed vertebrae; d–f: resorbed vertebrae. Some vertebrae with weak resorption but with numerous trabeculae had similar Tt-B.Ar. as vertebrae with extended resorption in the middle area but with thick trabeculae in the notochord and peripheral areas, e.g. compare (a) with (f), and (b) with (e). The sections shown in (a) and (d) represent the least and greatest resorption, respectively, in the trout sample studied (> 500 vertebral sections).](image-url)
conversely, a similar organization resulted in different vertebral $Tt$-$B$.Ar. values (Fig. 2).

Most market-sized trout sampled from different fish farms showed ‘resorbed’ vertebrae (Fig. 3), i.e. similar to that described in Fig. 1b. This means that the resorption of the central region (=middle area) is a general phenomenon, which is not related to particular rearing conditions.

However, when comparing fish farms, we found significant differences in $Tt$-$B$.Ar. and vertebral bone profiles (Pillai’s trace value $= 1.6596$, $P < 0.0001$ followed by Tukey tests, $P < 0.05$) (Fig. 3). Indeed, the comparisons showed differences between lots for $Tt$-$B$.Ar., $min_{1,2}$, $P_1$, $S_1$ and $min_{3,4}$, although no difference was observed for $P_0$, $S_0$, $P_3$, $S_2$ and $S_3$. Therefore, even if the presence of large resorptions is not related to particular rearing conditions, the latter still significantly affect vertebral bone remodelling in trout.

Taking all the trout together ($n = 373$), partial correlations (partial $r^2$ are given in brackets) between $Tt$-$B$.Ar. and the parameters of the mathematical model showed that when $Tt$-$B$.Ar. increases, this resulted in (i) no structural change in the notochord area, (ii) an increase of bone deposition in the transition area ($min_{1,2}$: $-0.0851$), (iii) a decrease of bone resorption in the middle area ($P_1$: $0.1127$; $P_4$: $-0.3729$; $S_4$: $-0.1536$ and $min_{3,4}$: $-0.1626$) and (iv) an increase of bone deposition in the peripheral area ($min_3$: $0.5786$). In other words, trout having a high vertebral $Tt$-$B$.Ar. showed reduced resorption in the middle area, and, more interestingly, an increase of bone deposition in the transition and peripheral areas.

**Growth rate experiments**

**Growth of rainbow trout at different temperatures**

As expected, in the three temperature experiments trout showed different growth rates (ANCOVA, $P < 0.0001$; Fig. 4): FAST, NOR and SLOW trout reached market-size length 7.5 (01 September 2005), 9 (10 October 2005) and 13 (7 February 2006) months after first feeding, respectively. At first sampling, i.e. when FAST trout reached an average of 250 g (238 ± 14 mm TL), the TL of SLOW trout was only 160 ± 17 mm. At the end of the experiment (third sampling), i.e. when SLOW trout reached 250 g (263 ± 16 mm TL), the TL of FAST trout was 376 ± 20 mm. In SLOW and FAST trout, growth speed was relatively regular as they were reared at a constant temperature (7 and 17 °C, respectively). In contrast, in NOR trout, growth speed was influenced by the variation of seasonal temperatures: rapid growth during summer and at the beginning of autumn, followed by slow growth in winter and spring. This explains why NOR trout roughly reached a similar mean TL as FAST trout at the second sampling (9 months), but had a smaller length at the end of the experiment (13 months).
Vertebral total bone area

Growth rates in trout were found to induce changes in vertebral Tt-B.Ar. (Pillai’s trace value = 0.9506, \( P < 0.0001 \) followed by Tukey tests, \( P < 0.05 \); Fig. 5). Until market-size, SLOW trout had a constant and higher Tt-B.Ar. than NOR and FAST trout. More precisely, at the first sampling (7.5 months), SLOW trout had a vertebral Tt-B.Ar. (0.38 ± 0.03) significantly higher than in NOR (0.33 ± 0.02) and FAST (0.33 ± 0.01) trout (Fig. 5). No significant difference was observed in Tt-B.Ar. between NOR and FAST trout.

At the second sampling (9 months), SLOW trout had a similar vertebral Tt-B.Ar. (0.39 ± 0.02) compared with that calculated at 7.5 months. It was still higher than in NOR (0.34 ± 0.02) and FAST (0.36 ± 0.03) trout (Fig. 5). Similarly, no change in Tt-B.Ar. was observed between NOR trout sampled at 7.5 and 9 months. In contrast, Tt-B.Ar. had increased significantly between 7.5 and 9 months in FAST trout.

At the third sampling (13 months), SLOW trout had still a similar vertebral Tt-B.Ar. (0.39 ± 0.02) as in fish sampled at 7.5 and 9 months. It was still higher than in NOR (0.33 ± 0.02) and FAST (0.36 ± 0.03) trout (Fig. 5). Similarly, no change in Tt-B.Ar. was observed in NOR trout sampled at 9 and 13 months. In contrast, FAST trout showed an increase of vertebral Tt-B.Ar. (0.44 ± 0.03), exceeding the value obtained in SLOW trout and NOR trout.

At market-size, Tt-B.Ar. was higher in SLOW trout (0.39 ± 0.02) than in FAST (0.33 ± 0.01) and NOR (0.34 ± 0.02) trout, which showed similar values (Fig. 5).

Vertebral bone profiles

Growth rates were observed to significantly influence vertebral bone profiles (Pillai’s trace value = 0.9506, \( P < 0.0001 \); Fig. 6, Table). Indeed, the comparison of the parameters (Tukey tests, \( P < 0.05 \)) showed differences between lots for \( \min_0, \ P_0, \ S_0, \ min_3,4, \ P_3, \ S_3, \ P_4 \) and \( S_4 \). No difference was found for \( \min_1,2, \ P_1, \ S_1, \ P_2 \) and \( S_2 \).

The vertebral structure of SLOW trout changed during the experiment from an ‘unresorbed’ structure, as observed at 7.5 and 9 months, to a ‘resorbed’ structure at 13 months (Fig. 6, Table 2). No structural change of the vertebrae was observed between trout at 7.5 and 9 months. However, at 13 months, the amount of bone increased in the notochord, transition and peripheral areas (\( S_0, \ P_0 \) and \( \min_0 \) increased significantly). Also, significant resorption occurred in the middle area (\( \min_3,4; \% \) of bone lost increased significantly). The balance of
both processes led to a neutral result, i.e. a similar vertebral Tt-B.Ar. over the whole experiment (Fig. 5).

The vertebrae of NOR trout always showed a ‘resorbed’ structure, a pattern which did not change significantly during the experiment (Fig. 6, Table 2). No difference was observed between the parameters of the model at 7.5, 9 and 13 months. This explains why vertebral Tt-B.Ar. was similar over the whole experiment (Fig. 5).

In FAST trout, the vertebral structure changed significantly during the experiment (Fig. 6, Table 2). At each sampling, the vertebrae of FAST trout showed extended resorption in the middle area (i.e. ‘resorbed’ structure). However, the amount of bone in the peripheral area (mino) increased significantly with age. Bone deposition in this area explains why Tt-B.Ar. increased in 13-month-old FAST trout (Fig. 5).

Vertebral structure comparison at the age when trout had reached market-size in each experiment also showed significant differences (Fig. 6, Table 2). Compared with NOR and FAST trout, the vertebral structure of SLOW trout showed: (i) a much less abrupt B.Ar. decline within the notochord and transition areas and (ii) a resorption surface in the middle area that begins far away from the notochord canal (P0, S0, mino and P3 of SLOW...
### Discussion

This study is the first to propose the use of vertebral bone profiles in addition to total vertebral bone area (Tt-B.Ar.) to determine the amount of bone resorption in trout vertebrae. Previously, this type of study was carried out by calculating Tt-B.Ar. on transverse ground sections of vertebrae (Kacem et al. 2004). Tt-B.Ar. was used to estimate indirectly vertebral bone resorption resulting from bone remodeling processes. Indeed, the heterogeneous distribution of vertebral bone resorption in relation to genetic and environmental factors has been clearly demonstrated that the present study has focused on the tubular structure (resorbed fraction) to calculate Tt-B.Ar. on the intermediate area (Tt-I-Ar.). However, it was possible to compare indirectly vertebral bone resorption in relation to physiological demands and/or biomechanical constraints. Therefore, the Tt-B.Ar. alone could not accurately describe the degree of remodeling processes. Indeed, the significant bone deposition in the notochord, peripheral area, and transition areas relative to the resorption in the middle area.

In contrast, FAST trout had a lower amount of bone in the peripheral area (Tt-B.Ar. calculated for FAST trout (Fig. 6) is more related to the significant bone deposition in the notochord and transition areas than to the resorption in the middle area).

Furthermore, compared with NOR trout, SLOW trout had a greater amount of bone in the peripheral area (Tt-B.Ar. calculated for SLOW trout (Fig. 6) is more related to the significant bone deposition in the notochord and transition areas than to the resorption in the middle area). Differences in the degree of bone deposition in the notochord and transition areas were of little importance compared with NOR trout (Fig. 6). Therefore, it appears that any change from the normal fluctuations in temperature affects the vertebral structure.

### Table 2

Parameters (mean values ± SEM) of the model (unresorbed and middle area)* describing bone area profiles of transverse sections in vertebrae of reared rainbow trout with different growth rates (SLOW, NOR, FAST) sampled at 7.5, 9, and 13 months after first feeding.

<table>
<thead>
<tr>
<th>Growth speed</th>
<th>Age (months)</th>
<th>Unresorbed model (Q_0)</th>
<th>Model for the middle area (Q_{3,4})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( \text{min}_0 (%) )</td>
<td>( P_0 )</td>
</tr>
<tr>
<td>SLOW</td>
<td>7.5</td>
<td>42.3 ± 2.4</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>36.6 ± 5.2</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>47.8 ± 1.6</td>
<td>0.28 ± 0.03</td>
</tr>
<tr>
<td>NOR</td>
<td>7.5</td>
<td>39.5 ± 1.4</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>46.4 ± 1.8</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>52.2 ± 1.8</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>FAST</td>
<td>7.5</td>
<td>37.8 ± 1.3</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>41.8 ± 1.6</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>63.3 ± 1.3</td>
<td>0.09 ± 0.02</td>
</tr>
</tbody>
</table>

*Parameters of the model for the transition area (min 1,2,3,4) are not presented.

Bold characters indicate parameter values at the age when trout reached market-size in each experiment.
methodological approach allows discrimination of different vertebral structures in modelling vertebral bone profiles from the notochord canal to the periphery of the vertebrae and provides precise information about the location and extent of the resorption. Modelling of vertebral bone profiles can now be a complementary tool for future studies concerning trout vertebral histomorphometry and this method can be easily applied to other fish species that exhibit similar vertebral organization and structure.

Modelling of vertebral bone profiles enables us to characterize the distribution of mineralized bone in transverse sections of vertebrae by distinguishing four regions. In teleosts, a transverse section through the vertebral body (i.e. excluding neural and haemal arches) is generally described as composed of an amphicoel compact bone (notochord area), which results from the mineralization of pre-existing concentric collagen fibres of the notochord sheath, and of a cancellous bone deposited by sclerotomal osteoblasts (direct ossification), which gives the initial ‘trabecular’ structure (Nordvik et al. 2005). The amphicoel compact bone constitutes the structural base of the vertebra, which enables growth in length through extension at the margins, whereas cancellous bone allows growth in volume by elongation and branching of trabeculae (Nordvik et al. 2005). Our analysis of hundreds of vertebral sections in reared rainbow trout clearly showed that the cancellous B.Ar. can be divided into three structural sub-regions (transition, middle and peripheral areas) with regard to their susceptibility to resorption. Indeed, the more centrally located region, the middle area, is the only region subject to large resorption, while the notochord, transition and peripheral areas are well conserved. The notochord area represents about 10% of the total vertebral surface, while the transition, middle and peripheral areas represent 15%, 60% and 15%, respectively. Therefore, in contrast to classical histological approaches, which distinguish only two main vertebral regions according to structural features (François 1966; Arratia, Schulze & Cascio-otta 2001; Nordvik et al. 2005), we suggest that the vertebra may be divided into four ‘histophysiological’ regions, which take into account bone distribution and remodelling processes.

In this study, we have shown that trout reared in different conditions and with different growth rates do not have the same amount of bone in their vertebrae, indicating a clear influence of external conditions, including growth rate, on bone remodelling. In fish, bone quality is related to calcium and phosphorus metabolism, as these ions are directly involved in the development and maintenance of skeleton mineralization through the formation of hydroxyapatite crystals (for reviews, see Francillon-Vieillot, De Buffrénil, Castanet, Géraudie, Meunier, Sire, Zylberberg & de Ricqlès 1990; Lall & Lewis-McCrea 2007). Tt-B.Ar. depends on the balance between bone formation (osteoblast) and resorption (osteoclast) (Sire, Huysseune & Meunier 1990). The resorption process leads to mobilization of mineral ions from the skeleton to fulfil various demands, principally from physiological processes, e.g. osmo-regulation, muscular activity and reproduction (Kacem et al. 2000; Witten & Hall 2003; Helland et al. 2005; Gil Martens, Witten, Fivelstad, Huysseune, Sævareid, Vikesæ & Obach 2006). In fish farms, several environmental and/or physiological factors (e.g. genetic strain, spawning period, ploidy, water temperature and acidity) could affect vertebral Tt-B.Ar. (Kacem et al. 1998, 2000; Helland et al. 2005; Gil Martens et al. 2006; Helland, Denstadli, Witten, Hjelde, Storebakken, Skrede, Åsgård & Baeverfjord 2006). However, discussion of the respective effects of these factors is beyond the scope of this study.

The growth rate experiment showed that an increase in temperature increased growth rate in trout, as expected (Jensen 1985; Austreng, Storebakken & Åsgård 1987; Cho 1992). We also found that slow growth until market-size favoured bone deposition (high Tt-B.Ar.). Indeed, it appeared that the speed of bone deposition in NOR and FAST trout below 350 mm TL did not follow the rapid growth induced by increased water temperature (seasonal or constant, see below). This resulted in a more fragile vertebral structure (low Tt-B.Ar.) throughout growth to market size. Development and growth of a healthy vertebral skeleton takes time, as it requires the deposition of an organic matrix and its subsequent mineralization (Meunier & François 1992; Meunier 2002; Fjeldal, Lock, Grotmol, Totland, Nordgarden, Flik & Hansen 2006). Although the highest water temperature that occurred during this experiment (18.9 °C in the seasonal variation experiment) is within the range of optimal temperatures for rainbow trout growth (10–22 °C; Barton 1996; Sauter, McMillan & Dunham 2001), in trout fry it appears that the optimal temperature necessary for appropriate bone deposition is lower than the optimal temperature.
for growth. Indeed, in salmonids, fast growth induced by increased temperature is known to decrease bone quality by reducing vertebral mineral content, yield–load and stiffness (Fjelldal, Nordgarden, Berg, Grotmol, Totland, Wargelius & Hansen 2005; Fjelldal et al. 2006). However, to our knowledge, our results are the first clear evidence of low Tt-B.Ar. in market-sized trout experiencing normal and rapid growth.

In trout subjected to successive slow and rapid growth periods over the year (i.e. NOR trout with seasonal temperature variations from 5.0 to 18.9 °C), the vertebrae had a similar Tt-B.Ar. as in trout reared at a constant high temperature (FAST trout), at least for individuals smaller than 350 mm TL. Thus, in trout subjected to seasonal temperature, the low Tt-B.Ar. that could result from the periods of rapid growth (i.e. summer and beginning of autumn) was not counterbalanced by increased bone deposition during the slow growth periods (winter and spring). Moreover, in large individuals reared at 17 °C (> 350 mm TL), Tt-B.Ar. increased significantly even exceeding the Tt-B.Ar. in SLOW and NOR trout. Unfortunately, these results do not allow us to determine whether the increase in Tt-B.Ar. is only related to length or to the combined effect of length and temperature, because the only trout exceeding 350 mm were FAST trout. However, in trout, the specific growth rate (Jensen 1985; Austreng et al. 1987; Cho 1992) and the preference/tolerance to temperature changes with age and/or size of the animal (for review see Sauter et al. 2001). Moreover, in some circumstances, environmental factors such as light and temperature are known to induce heterochronic influences (Fjelldal et al. 2005, 2006; Wargelius, Fjelldal, Benedet, Hansen, Björnsson & Nordgarden 2005). Therefore, increased vertebral Tt-B.Ar. in large trout could be the result of the combined effect of a decrease in specific growth rate and an increase of bone deposition. Studies assessing specific growth rates (TL and vertebral bone deposition) in trout according to temperature, but using a large size range, must be carried out to understand the influence of each factor.

In teleost fish, as vertebrae are one of the main sites (along with scales) in which mineral ions are stored, vertebral Trt-B.Ar. is usually interpreted as the resulting balance of bone remodelling processes fulfilling various physiological requirements, e.g. maturation, spawning migration, muscular activity and starvation (Kacem et al. 1998, 2000; Kacem & Meunier 2000, 2003; Witten & Hall 2003). However, given the lack of such physiological constraints in reared trout, vertebral bone resorption was thought to be the consequence of a dysfunction in mineral metabolism induced by increasing growth speed in unfavourable rearing conditions (Kacem et al. 2004). In fact, we have seen that bone resorption of the vertebral middle area of market-size trout was a general phenomenon that can be neither related to growth rate (various temperatures) nor to particular rearing conditions (different fish farms). However, we cannot reject the hypothesis of a clinical pathology, as nutritional state in relation to mineral balance was not explored during this study. Furthermore, genetics has to be considered as reared trout were selected for high growth rate through generations. Further work should be conducted, for example, in comparing wild and reared specimens. Appropriate biological material is needed, i.e. fish of appropriate length, with no genetic influence from reared strains and which have not started sexual maturation.

Vertebrae play important biomechanical roles such as ensuring flexibility and elasticity of skeleton during swimming, and supporting muscle attachment (Webb 1975; Meunier & François 1992; Westneat & Wainwright 2001; Meunier & Ramzu 2006; Paxton, Bonser & Winwood 2006). The growth rate experiment indicated that extensive resorptions are the consequence of significant remodelling of vertebral architecture during rapid growth. The vertebral structure in young (small) trout (< 190 mm TL) is initially ‘trabecular’ (i.e. unresorbed). Bone resorption starts late in trout ontogeny and is only obvious in the vertebral middle area in trout between 190 and 235 mm TL. The ‘trabecular’ structure is progressively replaced by a ‘tubular’ structure (resorbed), mostly present in vertebrae from market-size fish onwards. Such a structure was found in the vertebrae of the largest trout analysed in our study (380 mm TL).

At first glance, these results are in contrast with those reported in Atlantic salmon, in which the ‘open’ trabecular architecture of the vertebrae was claimed to allow bone to grow without being remodelled, i.e. only by trabecular elongation and branching (Nordvik et al. 2005). This hypothesis was supported by the lack of osteoclast detection, although it seems necessary to clarify bone remodelling processes in parr and smolts (Nordvik et al. 2005; Fjelldal et al. 2006). In our study, histological examination was not needed to assess the
presence of osteoclasts because the numerous and wide Howship’s lacunae located along bone trabeculae was evidence of an obvious osteoclast activity (Francillon-Vieillot et al. 1990; Kacem et al. 1998, 2000, 2004). The largest salmon studied by Nordvik et al. (2005) were smolts (~100 g), i.e. a weight slightly larger than our smallest trout (~85 g; 9-month-old SLOW trout), in which we did not observe vertebral bone resorption. The lack of osteoclasts in smolts as in young trout means that vertebral growth is ensured by trabecular elongation and branching in young fish. Such an initial growth process seems suitable for small individuals in which biomechanical constraints are not important, while a transition towards a tubular architecture must take place to fulfil vertebral function in larger specimens. Here, we show that in ‘tubular’ vertebrae, bone deposition occurs in the transition area, which is strengthened, and in the peripheral area, which offers a greater surface for muscle attachment. Indeed, although there is no specific report for salmonids, horizontal septa are known to be attached to the peripheral area of the vertebrae in various teleost fish (Westneat & Wainwright 2001; Alami-Durante & Rescan 2003). This occurs at the expense of the middle area, which is resorbed with variable intensity depending on the individual. This resorption is not followed by an increase of bone deposition, which means that the balance of Tt-B.Ar. is displaced in favour of the transition and peripheral areas. The analysis of numerous sections of market-size trout vertebrae indicates that the bone thickness is variable in these two areas, suggesting that bone remodelling is acting on these surfaces, allowing mobilization of mineral ions. Therefore, structural changes are interpreted as being a compromise between the need to mobilize mineral ions stored in the vertebral bone and that to maintain the biomechanical properties of the vertebrae during growth. To test this hypothesis, further studies to compare the biomechanical properties (stiffness, yield-load, strength) of vertebrae according to the different structures (trabecular vs. tubular) should be carried out. It would also be of interest to determine whether such structural changes are also observed in salmonids with different mineral balances, i.e. comparing individuals transferred to salt water with those kept in fresh water.

Interestingly, we have seen that until fish reach market-size, any fluctuation of the temperature alters vertebral structure. A low growth rate (low temperature) seems to favour the reinforcement of the structural base of the vertebrae (i.e. notochord and transition areas). In contrast, a fast growth rate (high temperature) seems to prevent bone deposition in the peripheral area, reducing the relative surface for muscle attachment. Further studies (e.g. related to genetics, nutrition and mechanics) are needed to understand the consequences of such changes in vertebral bone remodelling during growth. Eventually, the high plasticity of vertebral bone remodelling could contribute to the appearance of vertebral abnormalities in reared fish. Vertebral abnormalities are a major pathological problem frequently reported in reared salmonids (Kvellestad, Høie, Thorud, Tørud & Lyngøy 2000; Kacem et al. 2004; Wargelius et al. 2005; Witten, Gil-Martens, Hall, Huysseune & Obach 2005; Helland et al. 2006; Witten, Obach, Huysseune & Bæverfjord 2006; Deschamps, Kacem, Ventura, Courty, Haffray, Meunier & Sire 2008). Abnormalities in vertebral structure, (e.g. compressed and/or fused vertebrae) give rise to several economic problems as well as welfare concerns. Therefore, the approach used in this study should be a useful tool for further studies on vertebral abnormalities in trout and other reared salmonids.

In conclusion, Tt-B.Ar. appears to be a good indicator to determine the balance of remodelling processes in a given bony element. However, in trout vertebrae, Tt-B.Ar. shows obvious limits in accurately determining bone remodelling. Modelling of vertebral bone profiles provides a new methodological tool, which allows comparison of bone deposition and resorption in different areas of a bone section. In trout, our method has allowed the description of a new vertebral regionalization, different from the classical histological approach, and taking into account bone remodelling processes. Also, during growth of reared trout, our analysis demonstrated that the vertebral body undergoes several morphological changes, which reinforce the vertebral structure and increase the bone surface available for muscle attachment without increasing the total bone volume. These findings have not been described in other teleost fish. The hypothesis of a pathology related to genetics and/or nutritional state cannot be excluded. In the future, this approach could be used to answer several questions dealing with normal and pathologic bone remodelling processes in reared trout as well as in other species having similar vertebral organization and structure.
Acknowledgements

We thank F. J. Meunier (MNHN, Paris, France), P. E. Witten (AKVAFORSK, Norway) and A. Huysseune (Gent University, Belgium) for their helpful advice and discussions. We gratefully acknowledge the expertise of INRA’s agents, V. Gayet (PEIMA), P. Maunas (Lées Athas fish farm) and F. Terrier (Donzac fish farm), and overseer F. Vallée (Saint-Pée-sur-Nivelle) in rearing trout for the growth rate experiments. We are grateful to producers for providing trout used for fish farm comparisons. This research was funded by the Office national interprofessionnel des produits de la mer et de l’aquaculture (OFIMER, contract No. 050/04/C), the European Union (IFOP/DPMA, contract No. 2005/010) and the Comité Interprofessionnel des Produits de l’Aquaculture (CIPA).

References


