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Effects of Starvation and Subsequent Refeeding on Formation and Resorption of Acellular Bone in Tilapia, *Oreochromis niloticus*

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ABSTRACT—Fish undergo a brief growth spurt known as "compensatory growth" when they resume adequate feeding after a restricted food intake. In the present study, changes in formation and resorption of acellular bone during the compensatory growth period were quantified using bone histomorphometry. Juvenile tilapia (*Oreochromis niloticus*) were starved for 15 days and subsequently refed for 15 days. In the starvation period, osteoblastic activity decreased linearly with time and almost reached zero on day 15, whereas the significant reduction in osteoclastic activity first became evident on day 15. This resulted in an increased percentage of eroded bone surface, which is the bone surface affected by osteoclasts. In the refeeding period, osteoblastic activity recovered to the levels of fed fish on day 3, briefly increased above the levels of fed fish on day 7, then returned to those of fed fish on day 15. Osteoclastic activity gradually increased and returned to the levels of fed fish from 7 days onwards. The eroded surface rapidly decreased to the levels of fed fish on day 3, became significantly lower than those of fed fish on day 7, and then returned to those of fed fish on day 15. It is concluded that starvation leads to a decreased turnover rate in tilapia acellular bone, which can be reversed by subsequent refeeding. A brief increase in bone-forming activity above the levels of fed fish, "the compensatory bone-formation", is a characteristic feature of the recovery period.

INTRODUCTION

Starvation is a potential threat to the survival of animals. In teleosts, many species often undergo starvation or restricted food intake in particular phases of their life cycles. For example, many salmonid species stop or reduce feeding during sexual maturation (Pentegoff *et al*., 1928). During a starvation period, fish have to direct energy reserves from growth to the support of vital processes, which triggers metabolic changes in many tissues. The metabolism of bone is no exception. For example, experimentally induced starvation greatly decreases bone formation through inactivation of osteoblasts in rainbow trout, *Oncorhynchus mykiss* (Takagi *et al*., 1992; Persson *et al*., 1997). After recommencing food intake, fish undergo a brief growth spurt known as "compensatory growth" or "catch up growth" (Weatherley and Gill, 1981; Dobson and Holmes, 1984; Blasco *et al*., 1992). During a period of compensatory growth, bone is assumed to also undergo a growth spurt. However, most studies on the compensatory growth have dealt with body weight changes; what happens to bone growth and metabolism during compensatory growth period is totally unknown.

Generally, three main cellular components are contained in bone: osteoblasts, osteoclasts and osteocytes. Through the combined actions of these cells, bone growth and turnover are performed. In contrast, a lot of evolutionally "higher" fish species have a characteristic type of bone, the acellular bone, which completely lacks osteocytes (Moss, 1961a, b, 1963). Since acellular-boned species are dominant in teleosts, the functional and adaptive significance of such a bone is of considerable interest. The lack of osteocytes had first led many scientists to question the metabolic activity of the tissue. In fact, the early studies had concluded that the acellular bone was metabolically "dead" bone (Moss, 1962; Simmons *et al*., 1970; Simmons, 1971). Recent studies, however, have shown that the acellular bone is a metabolically active tissue. For example, it actively participates in calcium turnover in the body through the combined actions of osteoblasts and osteoclasts. A calcium deficiency in environmental water induces calcium mobilization from the acellular bone and results in reduced bone mass in tilapia, *Oreochromis niloticus* (Takagi and Yamada, 1991, 1992), through increased osteoclastic activity and decreased osteoblastic activity. Addition of calcium to both the environmental water and diet restores the changes in bone

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metabolism induced by the calcium deficiency (Takagi and Yamada, 1993). However, the metabolic response of bone to a restricted food intake has been studied only in cellular-boned fish, rainbow trout (Takagi *et al.*, 1992; Persson *et al.*, 1997), and that of acellular bone is unclear. Moreover, the bone metabolism during the compensatory-growth period is unclear both in cellular and acellular bone.

The present study aimed to examine metabolic changes in the acellular bone during the periods of restricted food intake and subsequent compensatory growth. Changes in activities of bone formation and resorption in a starvation period and a subsequent refeeding period were quantified with histomorphometry of the pharyngeal bone in the acellular-boned fish, tilapia.

MATERIALS AND METHODS

Fish

Laboratory-hatched and -reared juvenile tilapia (*Oreochromis niloticus*), weighing about 3 g, were used. Before the experiments, fish were acclimated for 2 weeks in tap water at 23±1°C under light cycle of 16 hours light - 8 hr dark. During the acclimation period, fish were fed commercial carp pellets (No. 2C, Nippon Formula Feed, Co.) at 3% body wt/day.

Experimental protocol

In the first experiment, effects of starvation on bone metabolism were examined. At the start of the experiment (day 0), fish in the stock tank were divided into two groups. Fish in the first group were fed (3% body wt/day) and the fish in the second group were starved. Ten fish from the stock tank on day 0 and 10-11 fish from each group on days 3, 7 and 15 were sampled as described below.

In the second experiment, effects of refeeding after starvation were examined. Fifteen days before the experiment, fish were divided into two groups. Fish in one group (fed group) were fed at 3% body wt/day, while fish in the second group were starved. After 15 days (day 0), eight fish from each group were sampled as described below. The starved fish were then further divided into two groups. Fish in one group were kept starved (starved group) whereas fish in the other group were fed at 3% body wt/day (refed group). Fish in the fed group were fed throughout the experiment. Eight fish from each group were sampled 3, 7 and 15 days after the initiation of refeeding.

Sampling

After anesthetizing the fish deeply in a 0.2% solution of 2-phenoxyethanol, body weight and standard length were measured. Fish were then decapitated. The pharyngeal bone was dissected and processed for histomorphometric analysis.

Histomorphometric analysis

Pharyngeal bones were fixed in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) for one day at room temperature, and post-fixed with 1% osmium tetraoxide in the same buffer (pH 7.4) for 2 hr at room temperature. They were dehydrated through graded ethyl alcohol and embedded in a methacrylate resin as described previously (Takagi and Yamada, 1992). From each group, five bone samples were randomly selected and used for the analysis. Transverse sections (thickness 1 μm) were cut from a selected portion of each bone (Takagi and Yamada, 1992) without any trimming using an ultramicrotome (Ivan Sorvall, MT-1) using glass knives. The sections were stained with silver nitrate-toluidine blue and examined with a light microscope.

Histomorphometric analysis was performed largely following the methods of Takagi and Yamada (1992) and Persson *et al.* (1997). In

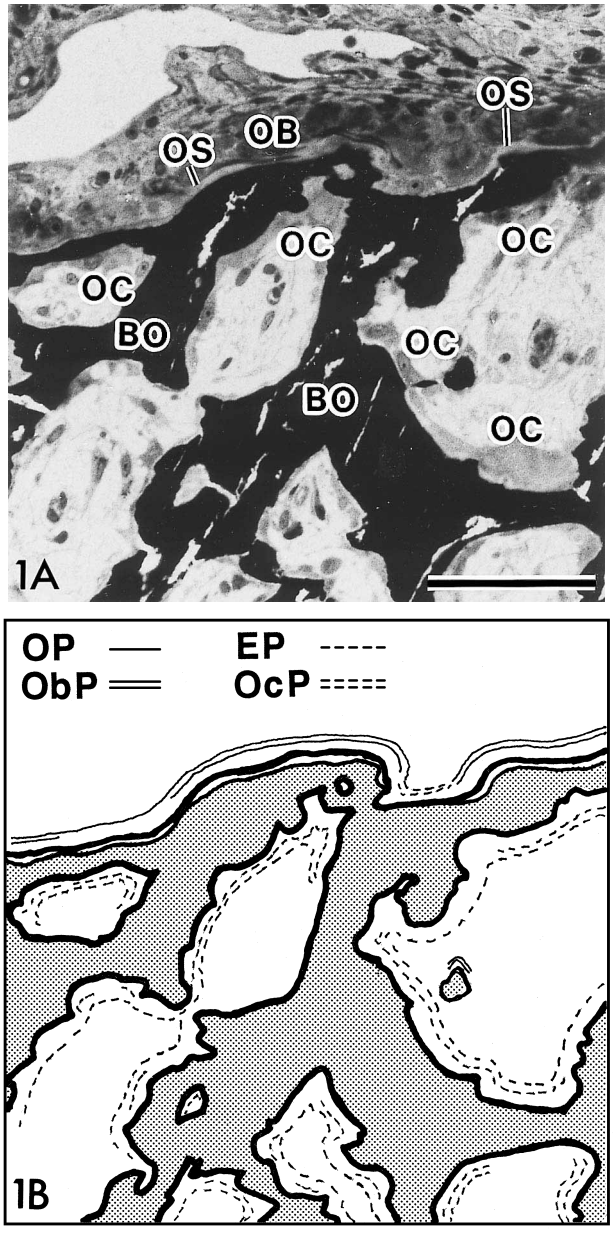

Fig. 1. Photomicrograph and schematic representation of the section of a tilapia pharyngeal bone. (A) Photomicrograph of a pharyngeal bone of fed fish stained with silver nitrate-toluidine blue. Bar = 50 μm. The calcified bone matrix (BO) is stained black with a silver nitrate solution. The bone surface where formation is in progress is covered with the osteoid seam (OS), which is a just-produced bone matrix and is not calcified yet. A part of osteoid seam is lined with osteoblasts (OB). The bone surface where resorption is undergoing shows irregularly eroded outline. A part of the eroded perimeter is covered with osteoclasts (Oc). (B) Schematic representation of (A), showing histomorphometric parameters. The osteoid surface perimeter (OP) is the bone perimeter covered with osteoid. The osteoblast surface perimeter (ObP) is the OP lined with osteoblasts. The eroded surface perimeter (EP) is the bone perimeter showing an irregular or scalloped outline. The osteoclast surface perimeter (OcP) is the EP lined with osteoclasts. Other parts of the bone surface perimeter are categorized into the resting surface perimeter.

brief, one section of each bone was randomly selected, and the entire bone surface in the section was subjected to the measurement. Figs. 1A and B show a part of a bone section used for the measurement and a schematic representation of histomorphometric parameters, respectively. Lengths of osteoid surface perimeter (OP), osteoblast surface perimeter (ObP), eroded surface perimeter (EP), osteoclast surface perimeter (OcP), and total bone surface perimeter (BP) were measured. Resting surface perimeter (RP) was calculated as RP = BP – (OP + EP). OP/BP and ObP/BP ratios were then calculated and expressed as percentages to evaluate the activities of bone formation and osteoblasts, respectively. Similarly, EP/BP and OcP/BP ratios were calculated and expressed as percentages to evaluate the activities of bone resorption and osteoclasts, respectively. The RP/BP ratio was also calculated and expressed as a percentage to evaluate the percentage of bone surface where no formation or resorption was taking place. In OP/BP, ObP/BP, EP/BP and RP/BP ratios, variations of the calculated values from the different sections of the same bone were about 10%. In RcP/BP ratio, the variation was about 20%.

Statistical analyses

For each experiment, histomorphometric data were analyzed, after arcsine transformation, by a two-way ANOVA with treatment and time as factors. If the interaction between treatment and time was significant ($P<0.05$), differences between each group at each time point were further analyzed by Student's *t*-test. In Exp. 1, differences between fed and starved fish were compared. In Exp. 2, a closed testing procedure was employed. At first, differences between fed and starved fish were analyzed in order to examine the sensitivity of the fish to starvation. Differences between starved and refed fish were compared to examine the efficacy of refeeding only when differences between fed and starved fish were significant. Then, only when the refeeding was significantly effective, differences between refed and fed fish were further analyzed in order to compare the effects of refeeding to the continuously fed fish. Significance was set at $P<0.05$.

RESULTS

Exp. 1 Effects of starvation on acellular-bone metabolism

During the experimental period of 15 days, fed fish gained in both weight [3.7±0.4 g (mean±SEM) to 6.3±0.9 g] and length (4.9±0.2 cm to 5.7±0.3 cm) (Fig. 2). Starved fish completely stopped growing. On day 15, their weight and length were 3.4±0.2 g and 4.8±0.1 cm, respectively.

Starvation significantly reduced OP/BP and ObP/BP, and the reduction increased as starvation lasted longer ($P<0.05$, two-way ANOVA, Fig. 3). OP/BP and ObP/BP in starved fish were significantly smaller than those in fed fish at days 3, 7 and 15 ($P<0.05$, Student's *t*-test). After 15 days of starvation, ObP/BP almost reached zero.

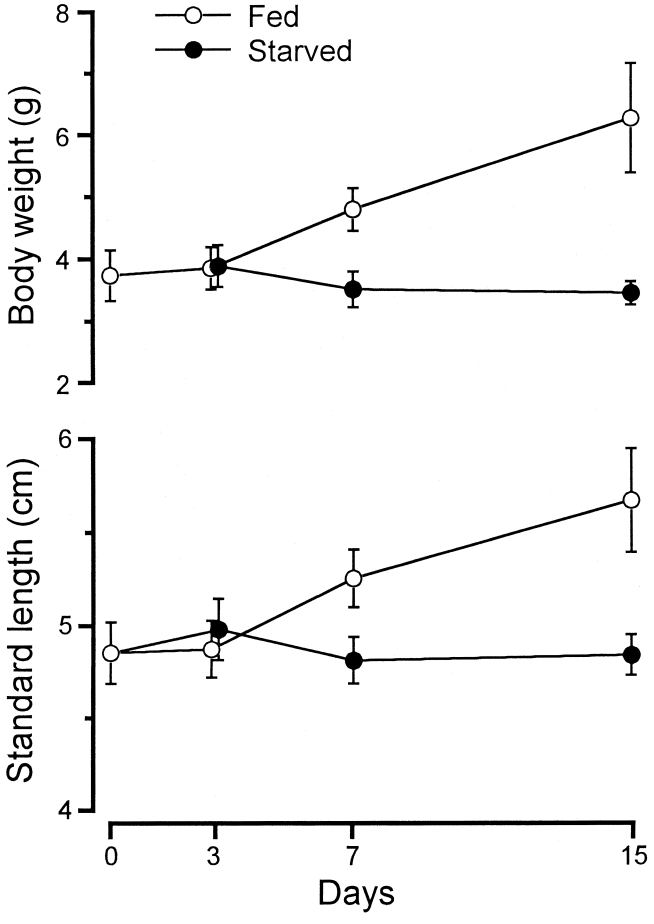

Fig. 2. Changes in body weight and standard length of juvenile tilapia in Exp. 1. Each point and bar represent a mean and SEM, respectively (n=10 or 11).

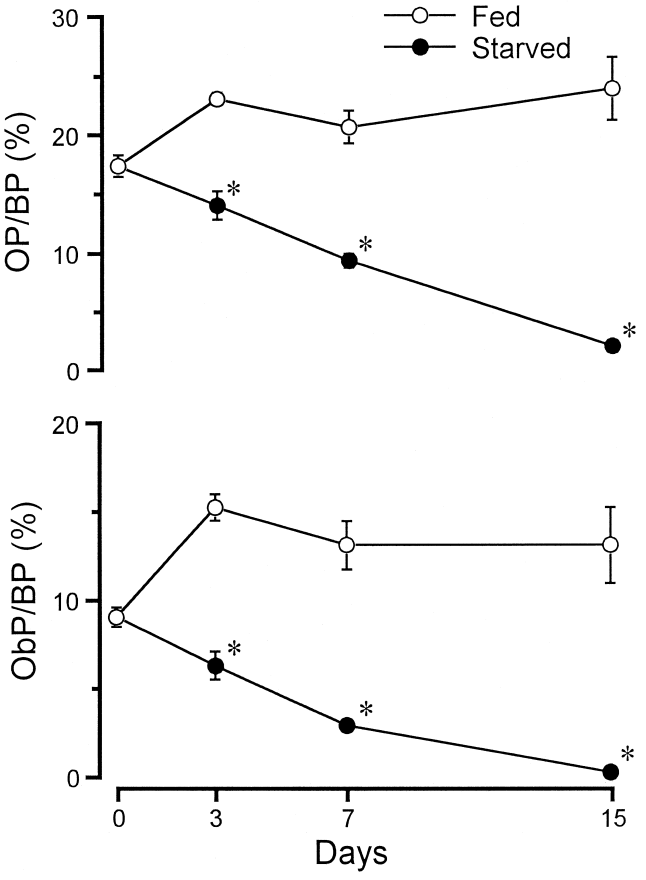

Fig. 3. Histomorphometric changes in activities of bone formation in Exp. 1. Each point and bar represent a mean and SEM, respectively (n=5). *Significantly different from the value of fed fish at the same day by Student's *t*-test.

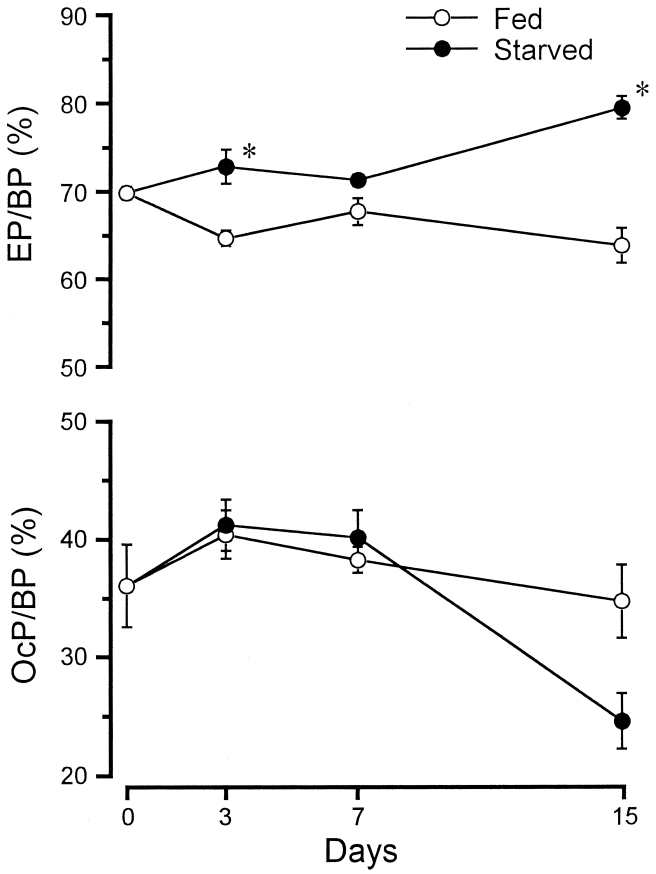

Fig. 4. Histomorphometric changes in activities of bone resorption in Exp. 1. Each point and bar represent a mean and SEM, respectively (n=5). *Significantly different from the value of fed fish at the same day by Student's *t*-test.

In contrast, starvation significantly increased EP/BP ($P<0.05$, two-way ANOVA), but the increase was not time-dependent ($P>0.05$, two-way ANOVA, Fig. 4). Compared with the EP/BP in fed fish, that in starved fish was significantly higher on days 3 and 15 ($P<0.05$, Student's *t*-test). The two-way ANOVA did not detect any significant effect of starvation on OcP/BP, but detected significant changes with time ($P<0.05$). This is probably due to the relatively low OcP/BP in starved fish at day 15 (Fig. 4).

RP/BP time-dependently increased with starvation ($P<0.05$, two-way ANOVA, Fig. 5). RP/BP in starved fish was similar to that of fed fish at day 3, but had increased significantly by day 7 ($P<0.05$, Student's *t*-test), and remained at similar levels on day 15.

Exp. 2 Effects of refeeding on acellular-bone metabolism

In this experiment, effects of refeeding after the 15-day starvation were examined. During the 15-day refeeding, refed fish gained in both weight [3.3±0.3 g (mean±SEM) to 4.4±0.3 g] and length (4.8±0.1 cm to 5.0±0.1 cm) (Fig. 6). During this period, fed fish gained in weight (5.2±0.6 g to 6.9±0.5 g) and length (5.2±0.2 cm to 5.8±0.1 cm). The compensatory growth

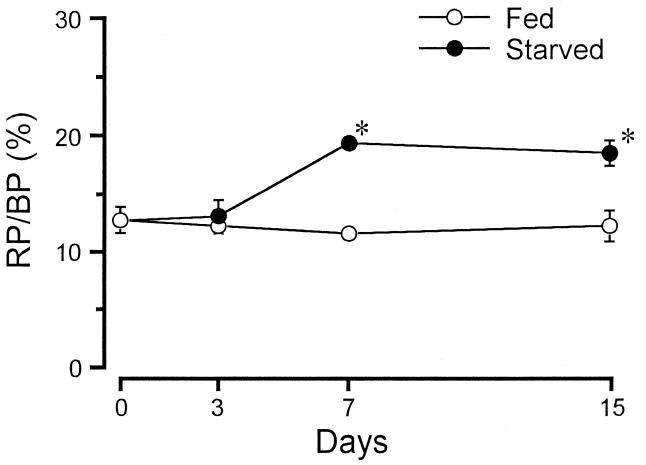

Fig. 5. Histomorphometric changes in the percentage ratio of the resting surface perimeter (RP) to total bone surface perimeter (BP) in Exp. 1. Each point and bar represent a mean and SEM, respectively (n=5). *Significantly different from the value of fed fish at the same day by Student's *t*-test.

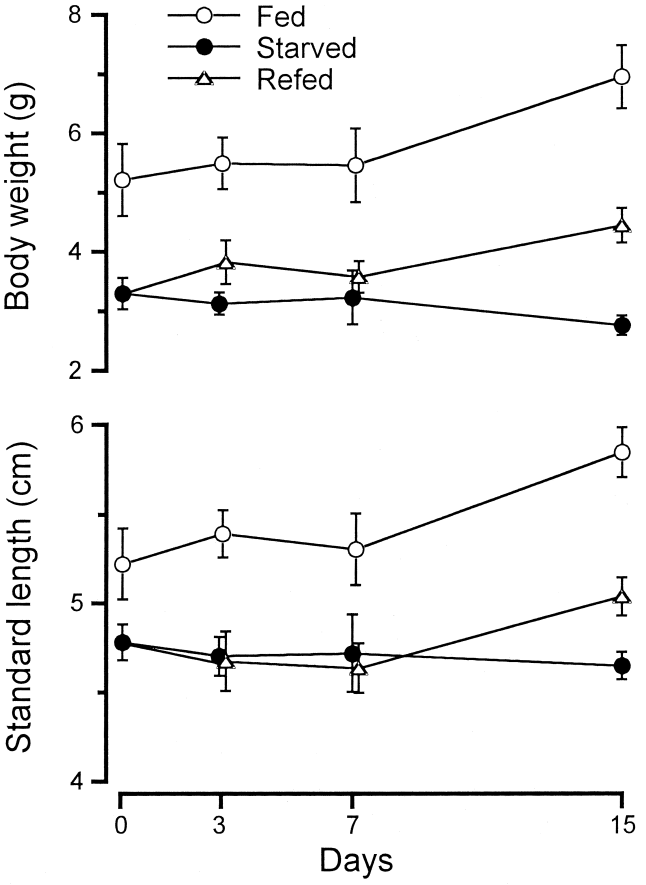

Fig. 6. Changes in body weight and standard length of juvenile tilapia in Exp. 2. Details of the experimental protocol are given in the Materials and Methods. Each point and bar represent a mean and SEM, respectively (n=8).

spurt in refed fish was not evident. The weight of starved fish decreased from 3.3±0.3 g to 2.7±0.2 g during the experiment. The length of starved fish did not change.

OP/BP and ObP/BP levels in starved fish were significantly lower than those in fed fish ($P<0.05$, Student's *t*-test) and remained relatively constant during the experiment (Fig. 7). Refeeding significantly increased OP/BP and ObP/BP time-dependently ($P<0.05$, two-way ANOVA). In refed fish, OP/BP and ObP/BP increased 3 days after the initiation of refeeding to levels comparable to those in fed fish, which were significantly higher than those in starved fish ($P<0.05$, Student's *t*-test). Both parameters in refed fish had further increased by day 7, with levels significantly higher than those in fed fish ($P<0.05$, Student's *t*-test). OP/BP and ObP/BP then decreased to levels similar to those in fed fish at day 15.

In starved fish, EP/BP remained relatively constant at levels significantly higher ($P<0.05$, Student's *t*-test) than those in fed fish during the experiment (Fig. 8). Refeeding significantly reduced EP/BP time-dependently ($P<0.05$, two-way ANOVA). After 3 days of refeeding, EP/BP levels in refed fish decreased to those of fed fish and were significantly lower than those in starved fish ($P<0.05$, Student's *t*-test). EP/BP decreased further to levels significantly lower than those in

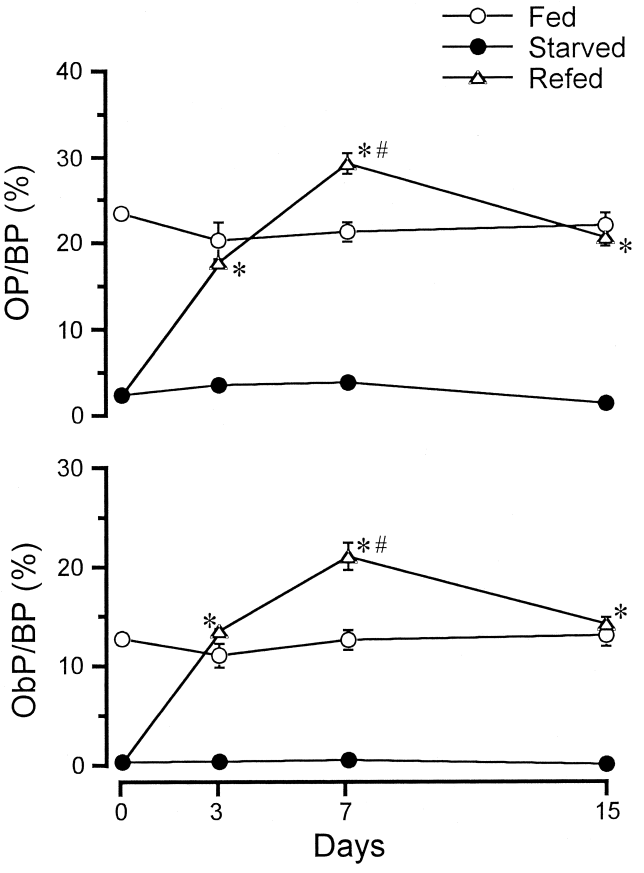

Fig. 7. Histomorphometric changes in activities of bone formation in Exp. 2. Each point and bar represent a mean and SEM, respectively (n=5). *Significantly different from the value of starved fish at the same day by Student's *t*-test. #Significantly different from the value of fed fish at the same day by Student's *t*-test.

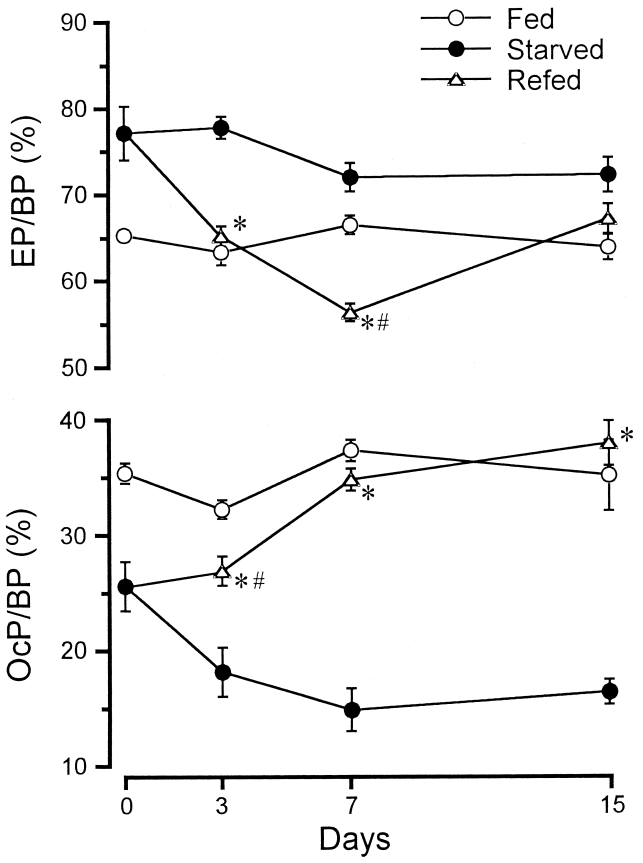

Fig. 8. Histomorphometric changes in activities of bone resorption in Exp. 2. Each point and bar represent a mean and SEM, respectively (n=5). *Significantly different from the value of starved fish at the same day by Student's *t*-test. #Significantly different from the value of fed fish at the same day by Student's *t*-test.

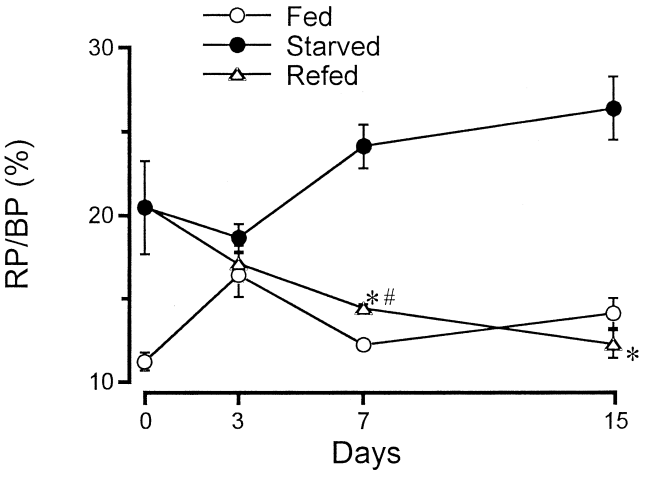

Fig. 9. Histomorphometric changes in the percentage ratio of the resting surface perimeter (RP) to total bone surface perimeter (BP) in Exp. 2. Each point and bar represent a mean and SEM, respectively (n=5). *Significantly different from the value of starved fish at the same day by Student's *t*-test. #Significantly different from the value of fed fish at the same day by Student's *t*-test.

fed fish after 7 days and then recovered to the levels in fed fish after 15 days.

OcP/BP in starved fish was significantly lower than that in fed fish at day 0 ($P<0.05$, Student's *t*-test, Fig. 8). Levels decreased until day 7 and thereafter remained constant until day 15. In contrast, refeeding significantly increased OcP/BP time-dependently ($P<0.05$, two-way ANOVA). OcP/BP levels in refed fish on day 3 were similar to those on day 0, thus they were significantly lower than those in fed fish and significantly higher than those in starved fish ($P<0.05$, Student's *t*-test). From 7 days onwards, levels recovered and were similar to those of fed fish, which were significantly higher than those in starved fish.

RP/BP was significantly higher in starved fish than in fed fish except on day 3 ($P<0.05$, Student's *t*-test, Fig. 9). Refeeding significantly reduced RP/BP ($P<0.05$, two-way ANOVA) to levels similar to those of fed fish but the reduction was time-independent ($P>0.05$, two-way ANOVA). RP/BP in refed fish at day 7 was significantly lower than in starved fish but still significantly higher than in fed fish ($P<0.05$, Student's *t*-test). On day 15, RP/BP decreased further to levels similar to those in fed fish.

DISCUSSION

Although compensatory growth after growth retardation is a well-known physiological phenomenon in fish (Weatherley and Gill, 1981; Dobson and Holmes, 1984; Blasco *et al*., 1992), most studies have dealt with body weight changes; what happens to bone growth and metabolism during compensatory-growth period has not been clarified. The compensatory growth is also evident in mammals (Osborne and Mendel, 1915; Prader *et al*., 1963; Wilson and Osbourn, 1960; Hermanussen *et al*., 1996). However, bone metabolism during the compensatory-growth period is unclear also in mammals. Hermanussen *et al.* (1996) measured the longitudinal bone length in rats to quantify bone growth rate during starvation and subsequent refeeding periods, but compensatory growth spurts in the bone were not evident. In the present study, activities of formation and resorption of acellular bone in tilapia were precisely quantified with histomorphometric analysis of the pharyngeal bone during starvation and subsequent refeeding periods. The characteristic feature of bone metabolism in the refeeding period was a brief increase in bone-forming activity above the levels of fed fish. From now on, I call such a reaction the "compensatory bone-formation".

When fish were starved, osteoblastic activity decreased, as indicated by a significant reduction of ObP/BP. The reduction of osteoblastic activity resulted in decreased synthesis of a new bone matrix, the osteoid, as shown by the decrease of OP/BP. Similarly, decreased activity of osteoclasts was indicated in starved fish from 15 days onwards by the reduction of OcP/BP. These results indicate that starvation significantly decreases bone-turnover rate. Supporting reduced bone-turnover in starved fish is the significant increase in RP/BP, which is the percentage of bone surface where no bone formation or resorption is in progress. Reduced bone-turnover rate during starvation has been reported for rainbow trout which has cellular bone (Takagi *et al*., 1992; Persson *et al*., 1997), and rats (Shires *et al*., 1980).

The osteoblastic activity significantly decreased when fish were starved for 3 days, and almost reached zero from 15 days onwards, whereas the significant reduction in osteoclastic activity first became evident after 15 days and it never reached zero during the experiment. The time lag in the reduction of osteoblastic and osteoclastic activities led to a significant increase in EP/BP, the percentage of bone surface affected by osteoclasts. This imbalance between bone formation and resorption may result in reduced bone mass in starved fish. Similar decreased bone-turnover rate resulted in significant reduction in bone mass when food was restricted for seven weeks in rats (Shires *et al*., 1980).

The refeeding experiment indicated that the starvation-induced reduction in osteoblastic and osteoclastic activity was restorable; OP/BP, ObP/BP, EP/BP and OcP/BP in refed fish recovered to the levels of fed fish after the 15-day refeeding. The reduced bone-turnover rate was also restored, as indicated by the recovered RP/BP in refed fish. Moreover, OP/BP and ObP/BP in refed fish rapidly increased above the levels of fed fish, whereas EP/BP in refed fish decreased below the levels of fed fish. Furthermore, there was a time lag in the recoveries of osteoblastic and osteoclastic activities. ObP/BP in refed fish restored to the levels of fed fish on day 3, whereas OcP/BP in refed fish was still significantly lower than the levels of fed fish at the same time. These data indicate greater bone formation in refed fish than in fed fish, showing that the "compensatory bone-formation" occurred in the refeeding period. A compensatory reaction of bone metabolism was also observed in tilapia when calcium was added to the environmental water after it had been withdrawn (Takagi and Yamada, 1993). Bone formation decreased and bone resorption increased in the Ca-deficient condition. When calcium was restored, bone formation increased above the levels of control fish and bone resorption decreased under the levels of control fish.

Although the compensatory reaction of bone cells after starvation was evident in the refeeding period, the compensatory growth spurt for body weight was unclear. The starvation period in the present experiment (15 days) may have been insufficient to show clear compensatory growth spurts in body weight, since starvation caused no severe weight loss.

In conclusion, starvation leads to a decreased turnover rate in tilapia acellular bone, which can be reversed by subsequent refeeding. A brief increase in bone-forming activity above the levels of fed fish, "the compensatory bone-formation", is a characteristic feature of the recovery period.

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