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Phosphorus in Haddock (*Melanogrammus aeglefinus* L.) Nutrition and Bone Metabolism

by

Prabir K. Roy

Submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
September 2002

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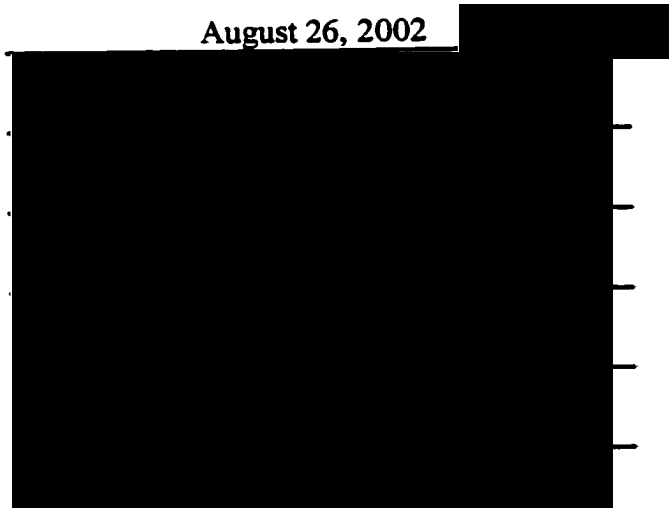

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Dedicated

To My Daughter
Prama Roy
&
My Mother
Sadana Roy

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ABSTRACT

The amount of phosphorus (P) in fish feeds must be carefully balanced to prevent deficiency signs as well as to minimize the urinary and fecal P excretions. A comprehensive study was undertaken to measure the quantitative dietary P requirement, bone mineralization, and the urinary and fecal P excretions, as well as to characterize bone deformities associated with dietary P deficiency or excess levels in haddock (*Melanogrammus aeglefinus* L.). The nutritional status of P was assessed in terms of growth, vertebrae ash and P concentration in plasma and tissue. According to the dose – response study using growth and bone mineralization as the requirement indicator, juvenile haddock require 0.96 % P or 0.34 g digestible P per MJ energy of diet. Growth, feed conversion ratio, vertebrae and opercula ash content and urinary P excretion were all positively correlated with dietary P levels. Approximately 43.2 % of organic P in feed was digestible for haddock, whereas the digestibility of inorganic P was 99 %. Haddock fed a commercial diet had a higher P concentration in the urine than in Atlantic salmon, however, a higher volume of urine was excreted by Atlantic salmon.

Bone deformities associated with P deficiency or an excess amount of dietary P was characterized using radiography, histology, histomorphometry, or enzyme histochemistry. Juvenile haddock were fed experimental diets containing low (0.42 %), optimum (1.02 %) and excess (1.42 %) levels of P for 20 weeks. Ash (total minerals) content of vertebrae, opercula, preopercula and ventral pharyngeal bone have indicated that the excess dietary P affects only vertebrae, though deficient P affects all bone equally. Tartrate resistant acid phosphatase (TRAP) staining employed for osteoclasts detection in vertebrae revealed that bone resorption occurs at the endosteal surfaces of the neural arches. Histomorphometrical examinations showed that the dietary P affects in bone formation, mineralization and resorption. Haddock juvenile diet deficient in P causes for the delay in bone mineralization. In contrast, excess amount of P in diet increased matrix deposition and an accelerated mineralization. Osteoclasts could be involved in P homeostatic balance. Observations of the number of osteoblasts and osteoclasts, and the amounts of mineralized bone and osteoid suggest that P deficiency in haddock bone initially increases bone resorption and subsequently decreases bone mineralization followed by a decrease in bone formation. It appears that skeletal tissue metabolism of haddock like other vertebrates is directly affected by the dietary phosphorus (P) intake. These findings will be useful in characterizing P deficiency and toxicity as well as to improve the health of farmed haddock.

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CHAPTER 1

1. Introduction

Haddock (*Melanogrammus aeglefinus* L.) is a marine cold water gadoid fish. Present investigation indicated that haddock bone is an acellular type. Its high growth rate, low fat and high protein content in muscle and high market demand makes it an ideal commercial aquaculture species in Canada, Scotland and other countries in Europe. Among several new marine aquaculture species, haddock is considered a good prospect for commercial farming in Atlantic Canada. Haddock are carnivorous and are sited at the top of the marine food chain stemming from phytoplankton through zooplankton to fish. Diets in free ranging haddock include a wide variety of invertebrates and fishes. The distribution and the nutrition of haddock correspond quite well with the distribution of the bottom animals. Data from stomach contents in haddock caught from the Nova Scotian Shelf of Atlantic Canada (Homans and Needler, 1944) show their diet content to be fish (53 %), Annelida (11.2 %), Echinoidea (7.0 %), Amphipoda (4.3 %), Decapoda (4.4 %), Gastropoda (3.1 %) and miscellaneous (17 %). The natural diet of haddock is rich in protein. Krill and other crustaceans consumed at early stages of development provide a low level of lipid, a moderate amount of chitin and carbohydrate as a minor component of their natural diet. Large sexually mature haddock have a lower optimal temperature for growth than smaller haddock (50-1000 g). The optimum temperature for growth of large haddock may lie within the range 9-12 °C.

Fish require amino acids and essential fatty acids, vitamin and minerals in diets for their growth and maintenance (NRC, 1993). Haddock feeds formulated from feedstuffs of fish or plant origin contain optimum levels of these components and energy for growth, reproduction and health. Body musculature in haddock as in the other Gadoidae is the main protein depot, while lipid reserves are primarily stored in the liver. The protein requirement for juvenile haddock has been estimated to be 49.9 % of their diet (Kim et al., 2001). Haddock is considered to be lean fish. In contrast with salmonids, the main energy storage organ in haddock is the liver and the lipid content of the muscle rarely exceeds 1.0 % of the wet weight (Nanton et al., 2001). The basic information on the nutrient requirements of haddock to develop a well-balanced and cost effective commercial feed is lacking.

In addition to macromolecules (protein, lipid and carbohydrates), fish require inorganic elements for its normal life processes. Of the 90 naturally occurring elements, 29 are known to be essential for normal health. The primary elements are carbon, hydrogen, nitrogen and oxygen distributed in protein, lipid and carbohydrates. The remaining elements are broadly classified into two groups, namely macro and trace elements. The macro-elements, which include the structural elements calcium (Ca), phosphorus (P), magnesium (Mg), potassium (K), manganese (Mn), sodium (Na), chloride (Cl) and sulfur (S), occur in fish body at a concentrations ranging from 0.1 to 2 % of fish weight and their dietary requirement is generally higher than 100 mg/Kg dry diet. In nature, marine fish accumulate and retain most of the elements from their water and plankton. However, in commercial aquaculture the minerals existing in water are not sufficient for normal health and body growth and therefore, must be supplemented in diet.

Phosphorus is considered one of the most important minerals. It is essential for growth (Åsgård and Shearer, 1997), bone mineralization (Baverfjord et al., 1998) and energy metabolism (Sugiura et al., 2000). Phosphorus as phosphate (HPO_4^{2-}) plays a major role in the function of all cells. The most important role of phosphates in life processes is as the central building block in nucleic acids. Since the di-ester bond in DNA carries multiple negative charges the phosphate di-ester bond is stable. Within cells, phosphate is an integral component of phospholipids, DNA and RNA. Phosphates are also involved in ATP and in other compounds for the storage of chemical energy. Further, phosphate residues, attached to several coenzyme and phosphate residues, combined with the hydroxyl groups of the serine and threonine and tyrosine residues of enzymes, control the action of these catalysts. Inorganic phosphate is also found in the cell and is important in osmoregulation, acid base and other types of physicochemical balance in the body. The electrostatic effect of phosphate (phosphorus carries a positive charge and each oxygen carries a negative charge) retards hydrolysis of the phosphate bond and makes it capable of reacting with any electrolyte to neutralize the body plasma, thus serving as a buffer in maintaining acid-base homeostasis. In extracellular space,

phosphate is present as hydroxyapatite in bone tissues. Approximately 80 % of vertebrate body phosphate is found in the skeleton.

Phosphorus content in both fresh and marine water is low in relation to the requirement of fishes. Therefore, P must be supplied in their feed. Imbalanced P is considered one of the important causes for bone deformities observed in farmed commercial salmonids in Norway, Chile and Canada. Deformed bones retard normal growth rate and render fishes more susceptible to stress and disease, causing considerable economic loss. However, much of the P in commercial fish diets may be released into the environment because of its low digestibility and or excess amount (Lall, 1991; Wiesmann et al., 1988). The amount and quality of P in feeds must, therefore, be formulated in a way that fish can digest, absorb, and retain as much of the feed nutrients as possible. This requires determination of optimum dietary P requirement, selection of highly digestible feed ingredients with higher retention of P and sensitive histological and histochemical techniques to detect P deficiency in farm and laboratory conditions.

Most studies on P deficiency in fish are limited to gross examination of fish and the change in either body or vertebrae ash content. The subsequent sections provide a brief review of P in fish nutrition and examine the role of dietary P in fish bone metabolism and deficiency. The factors that limit and control the P metabolism in dynamic bone mineralization in fish are also discussed.

1.1 Phosphorus metabolism of fish

1.1.1. Phosphorus balance in fish

Intestinal absorption, glomerular filtration in the kidney and storage of P in the bone control phosphate homeostasis in terrestrial vertebrates (Cross et al., 1990) and in fish (Vielma and Lall, 1998a). Mechanisms of intestinal phosphate transport in fish are similar to those of mammalian systems in that the active transport of inorganic phosphate is sodium-dependent, occurring against a concentration gradient and not coupled to calcium (Ca) transport (Avila et al., 2000). Phosphate is transported as orthophosphates and the absorption capacity is largest in the posterior part of the intestine (Nakamura,

1985a and 1985b). Fish demineralize bone and scales to supply phosphate for essential physiological functions when dietary P intake is limited (Lall, 1991).

1.1.2. Bioavailability and retention of dietary phosphorus in practical diets

In order for dietary nutrients to be utilized for various biological functions, including growth, they first need to be absorbed from the gastrointestinal tract. Knowing the content of digestible or available nutrients in ingredients is one of the pieces of critical information required for formulation of environmentally friendly feeds. Bioavailability is the degree to which an ingested nutrient is absorbed in a particular form that can be utilized by the animal in metabolism (Ammerman et al., 1995). It is a function of the quality of P in dietary sources and ability of particular species. To formulate a sustainable diet for commercial aquaculture, the choice of nutrients for selecting of feed ingredients is limited. Phosphorus in the diet must be in the correct form for its absorption and utilization by fish. Many factors including chemical form, digestibility, particle size, the technology of feed processing, and interaction with other nutrients in the gastrointestinal tract, influence P bioavailability (Lall and Vielma, 2001). The more soluble the P source, the higher the availability. About two-thirds of P in various plant feedstuffs is in the form of phytates (inositol hexaphosphate). Intestinal mucosa of monogastric animals, including many fish does not secrete phytase (myo-inositol hexaphosphate phosphohydrolase), an acidic phosphatase that catalyzes and liberates phosphate from phytate (Gibson and Ullah, 1990). Therefore, availability of P from plant feedstuffs is greatly reduced for these fish. Phytates also function as chelating agents, forming insoluble complexes with other dietary elements such as zinc and iron, calcium and thus reducing protein digestibility (Adeyeye et al., 2000; Singh and Krikorian, 1982, Spinelli et al., 1983). There are large differences in the availability of P from a variety of organic and inorganic sources (reviewed by Lall, 1991). Salmonids utilize P from fishmeal more efficiently than do carp. Low phytate content of practical ingredients are reported to be reduced to 50.2 % phytate P in rainbow trout feces (Sugiura et al., 1999).

Inorganic compounds such as, monobasic phosphates are utilized effectively by carp and rainbow trout, whereas dibasic and tribasic phosphates are less available (Ogino

et al., 1979). The P present in fishmeals has been shown to be highly available to rainbow trout and tilapia, but less available to carp, which do not secrete acid in their stomach (Lovell, 1978; Ogino et al., 1979; Yone and Toshima, 1979; Watanabe et al., 1980b). In general, fish species with acidic gastric secretions are better able to utilize the P in fish meals. However, among the feed stuffs used for fish feed formulation, fish meals contain 1.5 to 3.2 % P (Lall, 1991) which provides excessive amount of P in fish feeds. In order to minimize the P content without compromising growth, production and water quality, partial or complete replacement of fish meal P with alternative ingredients low in P content is essential. Bioavailability of P in feed ingredients may be affected by several factors including physiological differences among fish species, phytic acid content, particle size of feed etc. Thus, P bioavailability values for one species may not be directly applicable to all other species.

1.1.3. Role of calcium in phosphorus metabolism

In bones of terrestrial vertebrates and fish, P is complexed with Ca as $Ca_{10}(PO_4)_6(OH)_2$ (calcium hydroxyapatite). It is well known that an optimum Ca to-P ratio is important but increasing the Ca: P ratio of diet beyond optimum level interferes with the absorption of P. Fish deposit Ca and phosphates in bone and this reserve is readily mobilized for metabolism. The hormones of endocrine regulatory loops simultaneously control the metabolism of Ca and P. Marine fish consume large amounts of seawater thus enabling intestinal uptake of Ca whereas fresh-water fish extract Ca through active uptake by ion-transporting chloride cells (Flick et al., 1995). Fish can tolerate larger changes in plasma Ca levels than other vertebrates, possibly reflecting the open system of Ca regulation (Bern and Madsen, 1992). The role of dietary Ca in P metabolism is well documented in Atlantic salmon (Vielma and Lall, 1998b), redlip mullets (Hossain and Furuichi, 1999), tiger puffer (Hossain and Furuichi, 2000) and in an American cichlids (Chavez-Sanchez et al., 2000), although their essentiality from dietary sources still remains questioned.

1.1.4. Phosphorus excretion in fish

Fish excrete a significant quantity of P via feces and urine. A study conducted in rainbow trout cage culture located in a Scottish lake showed that 4.472 t of P was annually produced at this site (Gavine et al., 1995). Total P discharge ranging from 11.0 to 40.2 kg per ton of fish has also been reported (Lall, 1991) in salmon commercial farms in many countries. In order to minimize the impact of P discharged from fish farms to the environment, basic information on the role of P in bone metabolism and the relation of this element in fish body is required.

1.2 Phosphorus requirement and deficiency in fish

A great deal of information on the dietary P requirement of freshwater and marine fish species for optimum growth, feed utilization and bone mineralization has been reported (Lall, 1991). This indicated that the requirement ranged from 0.5 to 0.8 % of diet for freshwater fish, for example, rainbow trout, Atlantic salmon, chum salmon, carp and marine species, red sea bream. Estimated dietary P requirements for certain marine and freshwater fish are presented in Table 1.1.

The signs of P deficiency include poor growth, reduced feed efficiency and poor bone mineralization in channel cat-fish (Andrews et al., 1973), carp (Yone and Toshima, 1979) and Atlantic salmon (Baeverfjord et al., 1998). In carp, other clinical signs of deficiency include increased fat with decreased water content in the body carcass, and reduced blood phosphate levels (Ogino and Takeda, 1976; Onishi et al., 1981; Takeuchi and Nakazoe, 1981). In haddock, a decrease in serum phosphate levels and an increase in body lipid levels also have been recorded as a result of deficient dietary P. A low dietary P intake by red sea bream caused curved bones, enlarged vertebrae, increased serum alkaline phosphatase activity, higher lipid deposition in muscle, liver and vertebrae, as well as a reduction in liver glycogen content (Sakamoto and Yone, 1980). Deformed vertebrae also have been observed in our present investigation on haddock. A significant reduction in operculum and scale P concentration has been reported in salmon and trout (Vielma and Lall, 1998b; Vielma et al., 1998). In recent years, several cases of bone abnormalities in farmed Atlantic salmon have been reported in Europe and Chile and are

Table 1.1. Dietary phosphorus in various fish species: Requirement, deficiency signs and effects on growth

Species	Fish (g)	Effect on growth	Requirement (%)	Deficiency signs	Weeks	References
[1C]Striped bass, *Morone saxatilis*	321	no	0.58	Scoliosis characters and decreased bone minerals	14	Dougall et al., 1996
[1C]Striped bass	7.9	no	0.58	Scoliosis characters and decreased bone minerals	6	Dougall et al., 1996
[1C]Striped bass	48	no	0.58	Scoliosis characters and decreased bone minerals	10	Dougall et al., 1996
[1C]Rainbow trout, *O. mykiss*	4.6	yes ; P=2.51 %	1.50	Decreased bone minerals	18	Satoh et al., 1996
[1D]Rainbow trout	15	no	0.72	Deformed ribs and spines	18	Shearer and Hardy, 1987
[1B]Rainbow trout	9	yes; P>0.61 %	0.61	Poor growth, feed efficiency, bone ash and P	18	Ketola and Richmond, 1994
[1C]Rainbow trout	53	yes; P<0.28 %	0.56	Poor growth and feed efficiency and P deposition	8	Rodehutscord, 1996
[1C]Atlantic salmon, *Salmo salar*	4.7	yes	Not measured	Scoliosis, soft bone and wrinkly ribs	12	Baeverfjord et al, 1998
[3C]Atlantic salmon (SW)	113	yes	not measured	Decreased P, Ca. Soft bone and scoliotic changes.	15	Baeverfjord et al, 1998
[1C]Atlantic salmon	1.4	no	1.10	Decreased body P	9	Åsgård and Shearer, 1997
[1A]Atlantic salmon	15	no	1.0	Deformed ribs, neural and hemal spines	16	Vilema and Lall, 1998
[1B]Chum Salmon, *Onchorhynchus keta*	1.5	yes; P=0.5 %	0.60	Poor growth, decreased bone ash, P, feed efficiency	7	Watanabe et al., 1980
[1C]Channel catfish, *Ictalurus punctatus*	1.8	yes; P=0.18 %	0.45	Decreased bone ash and P	16	Lovel, 1978
[1A]Channel catfish	25	yes; P=0.22 %	0.40	Poor growth , decreased bone ash, P, feed efficiency	11	Wilson et al., 1982
[1D]Carp, *Cyprinus carpio*	18	yes; 0.24 % AVP	0.70	Low feed efficiency	8	Kim et al., 1998
[1D]Carp	4.6	yes	Not measured	Decreased vertebrae ash, P, Ca	12	Jahan et al., 2000
[1C]Carp	2.4	yes; P=0.69 %	Not measured	Increased lipid, decreased ash ca, P		Ogino et al., 1979
[1D]Carp	4.5	yes; P= 0.5 %	0.6-0.7	Poor growth and deformed head	6	Ogino and Takeda, 1976
[1B]Carp	1.2	yes; P =0.3 %	0.80	Poor feed efficiency, decreased bone ash and P	6	Ogino and Takeda, 1978
[1A]Sunshine bass, *Morone chrysops* ♀ *X M. Saxatilis* ♂	2.7	yes; P=0.34 %	0.50	Poor growth and decreased minerals	9	Brown et al., 1993
[1A]Tilapia, *oreochromis aureus*	1.5	not clear	0.50	Decreased bone ash, calcium and phosphorus	12	Robinson et al., 1987
[2D]Milk fish, *Chanos, chanos*	2.5	yes; P<8.5 %	0.85	Decreased vertebrae ash, P and Ca	16	Borlongan and Satoh, 2001
[3B]Red sea bream, *Chrysophrys major*		Yes	0.68	Increased lipid in muscle, liver and vertebrae	11	Sakamoto and Yone, 1978
[3B]Red lip mullet, *Liza hematochiela*	3.8	yes ; P<0.6 %	0.70	Increased lipid, decreased ash Ca and P	14	Elzibdeh et al., 1995
[3B]Red lip mullet	26.5	No	0.6	No effect	12	Elzibdeh et al., 1995
[3B]Gilthead sea bream, *Sparus aurata*	5	yes; P = 0.37 %	0.75	Low feed efficiency	6	Pimentel -Ridrigves and Oliva-Tales, 2001
[3D]Sea bass, *Lates calcarifer*	1.28	No		No effect	12	Chaimongkol and Boonyaratpalin, 2001
[3A]American cichlid, *Cichlasoma urophthalmus*	403	yes; P=0.5 %	1.50	Poor growth, low feed efficiency, high fat and decreased bone minerals	9	Chavez-Shnchez et al., 2000

[1]Fresh water culture; [2]Brackish water culture; [3]Marine water; [A]Basal diet; [B]Purified diet; [C]Semipurified diet; [D]Practical diet

closely correlated with P deficiency. The role of P in bone mineralization will be discussed in details section 1.4.

1.3 Bone metabolism of fish

Bones are the highly organized main component of the supportive tissues in the body of vertebrates (Stiassny, 2000). Bone as a tissue characterized by bone matrix and inorganic minerals. The bone matrix is an organic part, mainly composed of collagen. The inorganic minerals are mainly hydroxylated polymers of calcium phosphate $\{Ca_{10}(PO_4)_6(OH)_2\}$ (Clark, 1955; Simkiss and Wilbur, 1989; Moss and Posner, 1961). In addition, trace amounts of other organic compounds such as mucopolysaccharides, proteoglycans, phospholipids and other organic compounds are present and are considered as basic substances necessary for encrusting the inorganic minerals into the organic matrix (Fourman and Royer, 1963). Bone requires continuous remodelling for growth and metabolism (Witten et al., 2000). The processes of bone formation and remodelling are controlled by growth hormones and physiological conditions (Huysseune, 2000). The bone mineralization is controlled by mineral metabolism (Lall, 2002).

Mainly, three types of cells are involved in the production and resorption of vertebrate bone, osteoblasts, osteocytes and osteoclasts (Figure 1.1). Through the combined actions of these cells, bone growth and turnover are performed. Osteoblasts form the organic matrix and convert it into mineralized form. Osteocytes are derived from osteoblasts and remain entrapped inside the mineralized bone matrix (Simkiss and Wilbur, 1989; Ekanayake and Hall, 1988). They retain contact with each other via cellular processes and are considered to be largely involved in maintaining the bone mineral and controlling its exchange with the plasma. Osteoclasts are responsible for bone resorption. In terrestrial vertebrates as well as in many lower vertebrates, the osteoclast cells are multinucleated with ruffled border (Athanasou, 1996). Bone resorption is probably achieved by creating an acid extracellular environment that promotes bone-mineral dissolution and maintains the function of certain enzymes such as, β-glycerophosphatase, cathe pepsines and acid phosphatase (Baron et al., 1993; Lee et al., 1996; Stevens and Forgac, 1997). The acid phosphatase secreted by osteoclast cells is

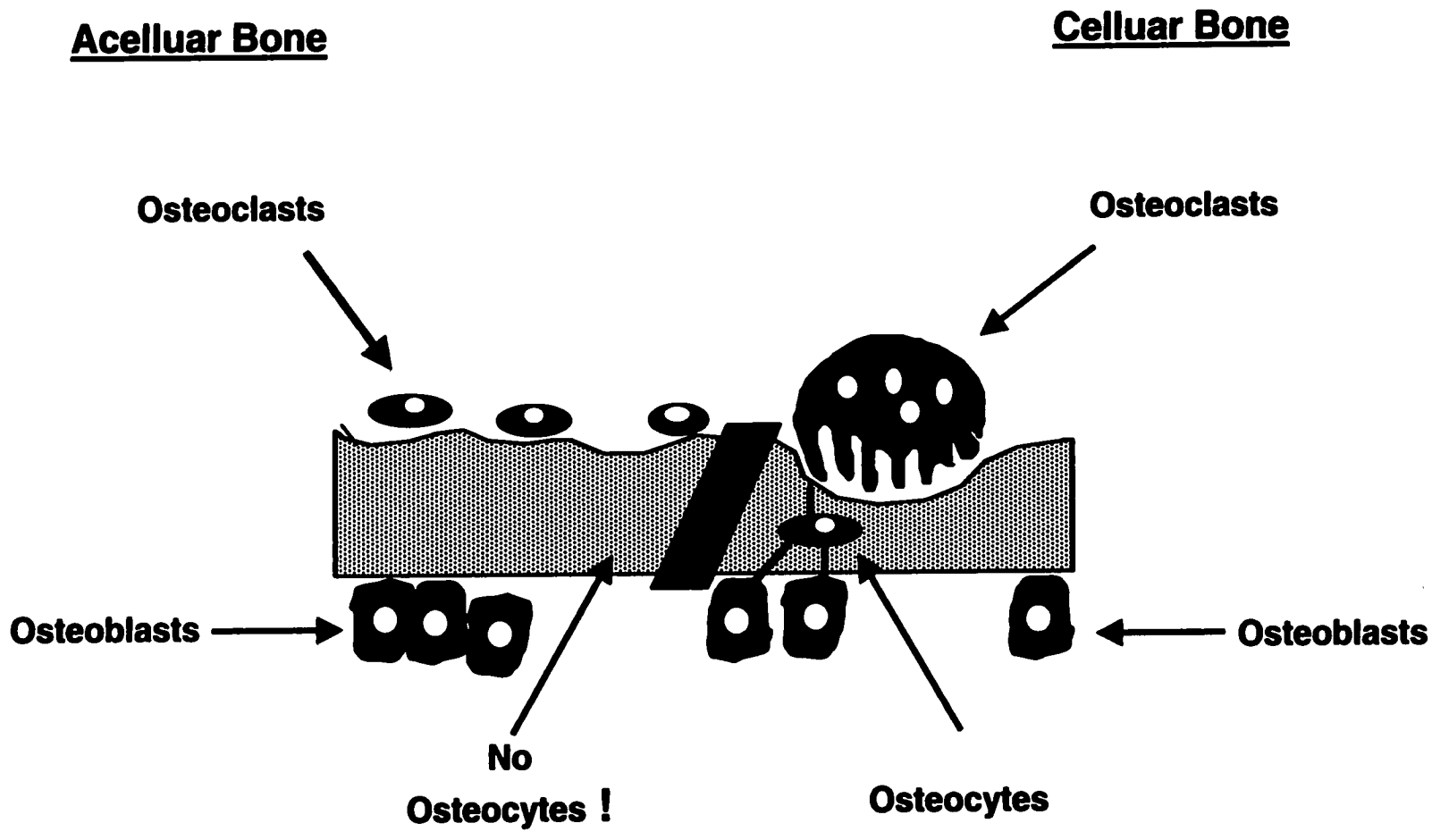

Fig. 1.1. Diagram of cellular system for vertebrate's bone (Figure presented by Witten, P.E.)

found to be resistant to tartrate in terrestrial vertebrates (Lindunger et al., 1990) as well as in fish (Persson et al., 1995; Witten and Villwock, 1997) and hence called tartrate-resistant acid phosphatase (TRAP).

In contrast, teleost bone is different histologically from mammalian bone. Two different types, cellular and acellular bones, are recognized in fish (Moss, 1961a, 1961b and 1963). With some exceptions, cellular bone is found only in the lower orders of Clupeiformes and Scopeliformes (Fleming, 1967) where it is similar to young mammalian bones in terms of cell counts and grades (Moss, 1965). Bones of higher-order teleosts are acellular and these acellular type of bone can be distinguished from cellular bone by lack of osteocytes (Starck, 1979; Ekanayake and Hall, 1987). The presence of osteoclasts in teleosts with acellular bone has been questioned because these fish lack parathyroid hormone (PTH; Taylor, 1985), a leading factor in the activation of osteoclasts and bone resorption in mammals (Vaes, 1988; Dickson, 1993). Only a few years also teleost acellular bone was considered to be metabolically inactive and it was thought that an endoskeleton of acellular bone may not be involved in Ca metabolism (Simmons, 1971; Urasa et al., 1984; Taylor, 1985). In contrast, a great deal of effort has been focused during the last two decades on understanding bone resorption and the remodelling of acellular bone. In acellular bone, the osteoblasts secrete matrix towards the bone which has been already deposited, allowing the cells to accumulate on the bone surface (Ekanayake and Hall, 1988). Acellular-boned teleosts are capable of bone resorption during Ca deficiency (Weiss and Watabe, 1979; Glowacki et al., 1986; Takagi and Kaneko, 1995) as well as under normal conditions (Sire et al., 1990; Sire and Huysseune, 1993; Hughes et al., 1994; Witten, 1997). The number of nuclei in osteoclasts shows considerable diversity in different animal and fish species. Several investigations have indicated that the number and type of nuclei are dictated by the demands of specific bone metabolic functions (Weiss and Watabe, 1979; Glowacki et al., 1986; Sire and Huysseune, 1993; Witten and Villwock, 1997). Most of the osteoclasts in cellular bones appear to be multinucleated and acellular bones have mono nucleated osteoclasts. However, both mono and multinucleated osteoclasts were recently demonstrated in cellular-boned zebra fish (Witten et al., 2001) and multinucleated

osteoclasts were found in acellular cichlid, *Hemichromis bimaculatus* (Sire et al., 1990). The function of mononucleated osteoclasts in acellular fish bone was a question. Studies in acellular bone of tilapia (*Oreochromis niloticus*) have indicated that the presence or absence of tartrate resistant acid phosphatase (TRAP) and related activity is tissue specific (Witten and Villwock, 1997). Weiss and Watabe (1979) reported that mononucleated osteoclasts in acellular bone (*Tilapia macrocephala*) are localized close to the osseous zone and they function seems to be demineralization. Histological variation has made it difficult to understand the reason of bone resorption in teleost acellular bone. A brief summary of the nature of bone resorption in various species is presented in Table 1.2. A review of present evidence in teleosts concludes that in fresh water systems mono and multinucleated osteoclasts may be involved in bone resorption in cellular and acellular bone for (a) growth and (b) mineral homeostasis. Similar to freshwater fish, bone deformities and demineralization is common in case of marine teleosts. Addition of Ca to both the freshwater and the diet restored the changes in bone cellular system induced by the calcium deficiency (Takagi and Yamada, 1993). Therefore, in marine fish where Ca and many other minerals are available to their surrounding water, participation of bone cells in mineral metabolism lead to enough doubt and may not be similar to the freshwater system. Moreover, involvement of mononucleated osteoclast's participation in mineral deficiency in the marine water environment is completely unknown. In addition temperature might be another factor for the functioning of osteoclasts in bone growth. Present knowledge is limited to warm water-cultured tilapia and we do not have any information on acellular marine cold-water species so far.

1.4 Role of phosphorus in bone metabolism

1.4.1. Effects on bone metabolism of terrestrial vertebrates

Phosphorus plays a vital role in bone metabolism (Day and McCollum, 1939; Freeman and McLean, 1941; Bloom and Flinchum, 1960; Copp and Suiker, 1962; Raisz, 1970). Raisz (1970) reported a relationship between matrix formation and the level of P in the diet. Phosphorus deficiencies in terrestrial animals (Freeman and McLean, 1941) and humans (Bloom and Flinchum, 1960) impair mineralization of skeletal tissues,

Table 1.2. Nature of bone resorption reported in various cellular and acellular-bone teleosts.

Species	Tissue	Reason for resorption	References
[4]Golden shiner (c) F *Notemigonus crysoleucas*	Scale	[e]To meet Ca and P deficiency after removal of scale	Weiss and Watabe, 1978
[3]Gold fish (c) F *Cararrius auratus*	Scale Vertebrae, Jaw and otolith	[e]Ca -deficiency No significant resorption	Mugiya and Watabe, 1977
[1,2]Gold fish (c) F *Carassius auratus*	Vertebrae	[e]Osteoclalstic resorption	Weiss and Watabe, 1979
[1,2] Salmonid (c) F *Salmo fario*	Dentary	[n]Multinucleated osteoclastic resorption for development	Sire et al., 1990
[1,2] Rainbow trout F *Oncorhynchus mykiss*	Opercula containing minerals	[e]Multinucleated osteclasts	Takagi and kaneko, 1995
[2]Carp (c) F *Cyprinu carpio*	Dentary vertebrae, neural arch	[n]Multinucleated osteclasts Flate mononucleated cells at endosteal , inside bone excavation and enchondral bone formation site	Witten et al., 2000
[1,2] Zebra fish (c) F *(Danio rerio)*	Lower jaw	[n]Mono and multinucleated osteoclasts for growth and remodelling	Witten et al., 2001
[4] Sunfish(ac) F *Lepomis macrochirus*	Scale	[e]To meet Ca and P deficiency after removal of scale	Weiss and Watabe, 1977
[1,2] Tilapia (ac) F *Tilapia macrocephala*	Vertebrae	[e]Mononucleated osteoclalstic resorption during calcium deficiency	Weiss and Watabe, 1979
[1,2] Cichlid (ac) F *Astatotilapia burtoni*	Tooth	[n]Mononucleated osteoclastic resorption of meackels cartilage and perichondral region for bone development	Sire et al., 1990
[1] Kelp bass (ac) M *Fundulus heteroclitus*	Scale, vertebrae, jaw and otolith	[e]Mononucleated osteoclastic resorption at low saline condition	Glowacki et al., 1986
[1,2]Substrate brooding cichlid (ac)F *Hemichromis bimaculatus*	Scale	[n]Multinucleated osteoclastic resorption for bone development	Sire et al., 1990
[1,2] Tilapia (ac)F *Oreochromis niloticus*	Pharyngeal bone	[e]Decreased in mononucleated osteoclastic bone resorption	Takagi and Yamada, 1991
[2] Tilapia (ac)F *Oreochromis niloticus*	Pharyngeal bone	[e]Increased mononucleated osteoclastic resorption for growth	Takagi et al., 1992
[2]Tilapia (ac)F *Oreochromis niloticus*	Pharyngeal bone	[e]Activate osteoclastic bone resorption at ca deficient water	Takagi and Yamada, 1992 and 1993
[2] Tilapia (ac)F *Oreochromis niloticus*	Neural arches, Dentary, Opercula, Preopercular shaft, Scale	[n]Mononucleated osteoclastic resorption for bone growth	Witten and Villwock, 1997
[2] Tilapia (ac)F *Oreochromis niloticus*	Pharyngeal bone	[e]Decreased mononucleated osteoclastic resorption of starved fish to retard bone growth	Takagi, 2001

(c) = Cellular bone; (ac) = Acellular bone, F= Freshwater species; M= Marine water species
Method of studies: [1] Electron microscopy; [2] Light microscopy; [3] Radioactive Ca^{45}; [4] Bone mineral measurement.
Condition of studies: e=experimental condition; n = normal condition

causing rickets or osteomalacia. Rats fed a low-P diet showed enlargement of the medullary cavity (Day and McCollum, 1939), which is a sign of increased endosteal osteoclastic resorption. Raisz and Niemann (1969) reported that a reduction of the level of P on rats in the incubation medium was associated with increased bone resorption. P deficiency causes a reduction in medullary bone and increased amount of osteoid in hens (Riddell et al., 1969; Wilson and Duff, 1990). Studies conducted on different animals suggest that changes in the activity of alkaline phosphatase and osteocalcin may be related to metabolic disorders of bone (Duda et al., 1988; Ohta et al., 1996). Recent studies on rats show that the dietary P deficiency affects the serum osteocalcin concentration and alkaline phosphatase activity (Hoshino et al., 1998). These markers are, however, non-specific and of limited sensitivity in bone formation (Duda et al., 1988). Some results of a survey on histological defects due to dietary P deficiency are summarized in Table 1.3.

1.4.2. Effects on fish bone metabolism

In mineral metabolism, fish bone functions as a reservoir of calcium, phosphate, magnesium, sodium, potassium, iron, zinc, copper, and sulfate (reviewed by Lall, 2002). Any change in metabolism and bioavailability of minerals in the body affects bone metabolism. In intensive culture systems, bone lesions are a frequently observed symptom related to rapid growth, without any clear reasons identified (Rath et al., 2000). Skeletal deformities, mainly shortness of the vertebral column in farmed Atlantic salmon have been well documented in Norway (Kvellestad et al., 2000; Vågsholm and Djupvik, 1998). A significant production loss caused by vertebral deformities (locally called as screamer disease) induced by a P deficient diet of Atlantic salmon has been noticed last few years in Chilli (Roberts et al., 2001). Lordotic character in the vertebral column and spinal cord has been reported in farmed gilthead sea bream (*Sparus aurata* L.) in a number of European countries (Andrades et al., 1996). Skeletal abnormalities in the caudal complex of the farmed Japanese flounder (*Paralichthys olivaceus*) have been reported in Japan (Hosoya and kawamura, 1998). The author considered that the amount of Ca and severity of abnormalities are correlated. It seems that normal bone growth is sensitive to nutrient supply and tight regulation of bone formation processes (Meunier

Table 1.3. Histological defects of bone associated with dietary phosphorus deficiency

Species	Histological defects	References
Hen	Reduced medullary bone and increased osteoid	Riddell et al., 1969
Hen	Resorbed medullary bone	Wilson and Duff, 1991
Rat	Decreased bone density of the lumbar spine	Hoshino et al., 1998
	Increased deoxypyrinoline level in collagen	Hoshino et al., 1998
Rat	Increased osteoid	Baylink et al., 1971
	Increased bone resorption rate	Baylink et al., 1971
Rat	Enlargement of medullary cavity	Day and McCollom, 1939
	Increased endosteal osteoclastic resorption	
Sheep	Impaired bone growth	Breves et al., 1985

and François, 1992). In P utilization studies, bone was found to be very sensitive tissue for both fresh water (Ketola, 1975; Watanabe et al., 1980a; Ketola and Richmond, 1994; Rodehutscord, 1996; Åsgård and Shearer, 1997; Baeverfjord et al., 1998; Jahan et al., 2001) as well as marine species (Sakamoto and Yone, 1978; Dougall et al., 1996; Borlongan and Satoh, 2001). Deformed bone has been reported as a common sign of P deficiency in carp, rainbow trout, and recently, Atlantic salmon (Ogino and Takeda, 1978; Shearer and Hardy, 1987; Lall, 2001 personal communication). In addition, the level of vertebrae ash and changes in bone P and Ca content found in deformed fish fed P-deficient diets have also been reported (Table 1.4.). It is obvious that bone deformity is an expression of poor bone mineralization which is related to the deficiency of P.

Histological characterization of the signs of P deficiency in fish has not been undertaken, and most reports include the description of gross vertebrae deformities and quantitative measurement of body ash content. Moss (1965) reported that the maintenance of Ca and P homeostasis was independent of bone type and aquatic environment (marine and or freshwater). The views on the role of acellular bone in Ca homeostasis are still contradictory. This is probably due to the fact that marine fish have unlimited access to water for Ca uptake through gills. However, diet is the major source of P. In addition, the histological variability in bone of acellular-boned teleost and the understanding of the mechanism of mineral homeostasis remains a complex dilemma. Reduced bone ash is a common indication of P deficiency in both terrestrial vertebrates and fish. The vertebrae ash reflects the total minerals in bone. Since Ca and P maintain a constant ratio in bone, their proportional variation during P deficiency is very limited.

1.5 Vitamin and hormonal control of bone metabolism in fish

Studies on vitamin and hormonal regulation of bone metabolism have mainly focused on the control of bone Ca in terrestrial animals. To date, no specific work on hormonal control of P in fish bone metabolism has been reported. Bone Ca and P metabolism, as well as bone formation and resorption, are controlled by some common and synergistic mechanism. A brief review of the vitamins and hormones involved in Ca metabolism will provide an understanding of the vitamin and hormonal control of bone P metabolism.

Table 1.4. Decrease* (%) in vertebrae ash, Ca and P content of fish fed P-deficient diets

Species	Ash	Ca	P	References
Carp	44.8	38.7	44.2	Ogino et al., 1979
(*Cyprinus carpio*)	13.4	24.7	21.3	Ogino and Takeda, 1976
	58.8	47.8	42.9	Ogino and Takeda, 1978
Red Sea Bream	28.3	28.7	26.9	Sakamoto and Yone, 1978
(*Chrysophrys major*)				
Channel cat-fish	19.9	14.9	17.1	Wilson et al., 1982
(*Ictalurus punctatus*)				
Rainbow trout	26.7	28.5	29.6	Shearer and Hardy, 1987
(*Salmo gairdneri*)				
Atlantic Salmon	36.0	2.2	3.3	Vielma and Lall, 1998a
(*Salmo salar*)				
	35.0	40.0	65.0	[a]Baeverfjord et al, 1998
	28.0	49.37	50.24	[b]Baeverfjord et al, 1998
Chum salmon	60.6	66.4	57.7	Watanabe et al., 1980
(*Oncorhynchus keta*)				
Sunshine bass				
(*Morone chrysops*)	not measured	13.48	8.0	Brown et al., 1993
Tilapia	11.23	11.03	11.17	Robinson et al., 1987
(*Oreochromis aureus*)				

*Values are calculated in relation to fish fed control diet and expressed as:

$$\text{Decrease in ash/Ca/P (\%)} = \frac{\text{Dry ash/Ca/P of control (\%)} - \text{Dry ash/Ca/P of deficient (\%)}}{\text{Dry ash/Ca/P of control (\%)}} \times 100$$

[a]Whole body ash reduction in fresh water
[b]Whole body ash reduction in sea water

1.5.1. Effects of vitamins on fish bone metabolism

In terrestrial mammals, vitamin D_3 (1,25-Dihydroxy Vitamin D) has the greatest effects on intestinal Ca and phosphate absorption, and it is considered that it may also have direct effects on bone and other tissue (Li et al., 1998). There is sufficient evidence to show that vitamin D_3 itself lacks biological activity and teleosts are able to convert vitamin D_3 and 25-$(OH)_2D_3$ to polar metabolites, including 1,25-$(OH)_2D_3$ (Hayes et al., 1986; Takeuchi et al., 1991). In *vivo* administration of 1,25-$(OH)_2D_3$ resulted in increased serum phosphate concentration in the European eel, *Anguilla anguilla* (MacIntyre et al., 1976), and in intestinal Ca absorption in the American eel *Anguilla rostrata* (Fenwick et al., 1984). In tilapia, the two vitamin D metabolites 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ seem to exert antagonistic effects on acellular bone tissue (Wendelaar-Bonga et al., 1983). Injection of 1,25-$(OH)_2D_3$ caused demineralization of the bones, in accordance with increased bone growth. Marine teleosts have large hepatic stores of vitamin D_3 (Urist, 1976; Takeuchi et al., 1984; Rao and Raghuramula, 1995). Atlantic salmon, Atlantic halibut and some tissues of Atlantic cod such as liver, kidney, gills, spleen and intestine all produce 25-$(OH)D_3$, 24,25-$(OH)D_3$, 1,25-$(OH)_2D_3$ as well as 25,26-$(OH)_2D_3$ (Graff et al., 1999). Sundell et al. (1992 and 1993) have identified 1,25-$(OH)_2D_3$ receptors in Ca-regulatory tissues such as the gills and intestine in Atlantic cod and observed increased Ca absorption after 1,25-(OH)2D3 administration in *vivo*. Larsson et al. (1995) suggested a hypocalcaemic role for 24,25-$(OH)_2$ *in vitro* in Atlantic cod. In contrast, Advani et al. (1998) have reported that 1,25-$(OH)_2D_3$ has no role in P metabolism in freshwater rohu (*Labeo rohita*). Graff et al. (2002) very recently reported that dietary 1,25-$(OH)_2D_3$ had no effect on bone formation of Atlantic salmon (*Salmo*) fry.

Research on the effect of vitamin K on bone development in fish is very limited. Recently, it has been reported that vitamin K plays an important role in bone tissue, in addition to its requirement for function in the process of blood clotting (Ferland, 1998). Due to phylloquinone deficiency vertebral abnormalities including fusion and irregularity of vertebral raw arrangement were reported in mummichong fish, *Fundulus heteroclitus* (Udagawa, 2001). The function of vitamin K in both systems is to carboxylate protein-bound glutamate (Glu) residues to gamma-carboxyglutamate (Gla). Osteocalcin is a

common non-collagenous Gla protein in the extracellular bone matrix, produced by osteoblasts. This small protein differs strongly from the Gla coagulation factors and is found in most animals. The function of this protein is to participate in the binding of the Ca in hydroxyapatite to the bone matrix, and serum concentrations of the protein reflect the osteoblast activity. Little is known on the efficacy of under-carboxylated osteocalcin and whether the bone mineralization depends on the vitamin K status.

According to recent findings in fish, vitamin A is considered as a key factor in bone development through regulation of cell differentiation and proliferation. Vitamin A is considered a potent skeletal toxin in rats (Brinkley and Krueger, 2000) and toxic levels of vitamin A in fish feed have caused bone deformities in fish (Primbs et al., 1971; Takeuchi et al., 1998) including vertebrae changes in Atlantic salmon (Øronsrud et al., 2001). Overdose of vitamin A caused abnormal chondrogenesis and osteogenesis in caudal hypural plates in Japanese flounder (Matsusato, 1986; Dedi et al., 1995, 1997; Hosoya and Kawamura, 1998). It was reported that larval flounder do not have the ability to convert β-carotene to vitamin A (Takeuchi et al., 1995). Biological studies in several species have demonstrated that an imbalance of vitamin A results in several types of organ malformations (Maden, 1994; Canlon, 1995; Zile, 1998, 2001). Effects of vitamin C deficiency in channel catfish diet were found to be impaired collagen formation (Wilson and Poe, 1973) and deformed veretebrae in rainbow trout, *Oncorhynchus mykiss* (Frischkencht et al., 1994). Sugiura et al., (2002) have shown reduction of phosphate in the diet increases the water solubility of the vitamin C.

1.5.2. Effects of hormones on fish bone metabolism

Parathyroid hormone (PTH) is a major Ca-regulating hormone in mammals. PTH stimulates bone resorption. High concentration of PTH produces acute inhibition of collagen synthesis, whereas prolonged intermittent administration of this hormone increases bone formation (Dempster et al., 1993). Plasma PTH increases with age, and this may produce an increase in bone turnover and loss of bone mass. However, teleost fish lack the ability to produce PTH's because they lack parathyroid glands. In contrast, parathyroid hormone-related proteins were detected in skin, kidney and skeletal muscle in cartilaginous and bony fish (Trivett et al., 1999). Other hormones may instead be

important in regulating skeletal growth of fish. Growth hormone can stimulate bone formation and resorption in terrestrial mammals (Rosen and Donahue, 1998) and rainbow trout (Takagi et al., 1992). Glucocorticoids may be necessary for bone-cell differentiation during development, but their greatest effect found so far has been the inhibition of bone formation (Advani et al., 1997) in teleost. Indirect effects of glucocorticoids on Ca absorption and sex hormone production may, however, increase bone resorption. Thyroid hormones stimulate bone resorption and formation and are critical for maintenance of normal bone remodeling (Kawaguchi et al., 1994). In the mouse, estrogen maintains normal bone turnover and its deficiency leads to an increase in the bone remodelling (Pacific, 1998). Under deficiency conditions in the mouse, bone resorption exceeds formation, resulting in a significant decrease in bone mass.

Hormones involved in phosphate regulation in fish include stanniocalcin and prolactin. Stanniocalcin inhibits the import of Ca from seawater via the gill (Wagner et al., 1997), stimulates phosphate reabsorption by the kidney in flounder, *Platichthys flesus (*Lu et al., 1994), and controls hyperphosphatemia in mudskipper, *Periophthalmodon schlosseri* (Fenwick and Lam, 1988). Calcitonin secreted from the ultramobranchial glands regulates bone mineralization in fish (Copp and Kline, 1989) but its effect on plasma Ca levels is not very clear (Bern and Madsen, 1992). A recent report on the effects of calcitonin on pharyngeal bone of gold fish, *Carassius auratus,* indicated that calcitonin is involved in the suppression of osteoclasts and enhancement of osteoblasts activity (Shinozaki and Mugiya, 2002). Calcium regulation in fish has been dominated by the hypercalcemic action of prolactin, a pituitary hormone, as well as by the hypocalcemic action of hypocalcin, a hormone produced by the corpuscle of stannius (Clement, 1992).

Skeletal deformities and poor bone mineralization are a reoccurring problem due to P deficiency that affects the growth and development as well as the market value of fish. The clinical data and the biochemical basis for the pathogenesis of bone malformation during the prevalence of this condition in both the freshwater and seawater phase of fish culture are not clearly defined. Several nutritional, physiological, environmental and genetic factors have been linked to this problem including P and/or micronutrient (vitamins A, C and D) deficiencies, water quality, infections, stress, systemic diseases,

pollutants, mechanical lesions and high temperature during the egg incubation. Although significant research efforts have been directed to minimize the incidence of skeletal deformities by changes in the feed formulations to prevent specific nutritional deficiencies and by providing better husbandry conditions, skeletal deformities caused by P deficiency have not been histologically characterized. One of the major bottlenecks is the lack of information on the role of P in fish bone metabolism especially, bone formation, resorption and mineralization during the various degrees of the deficiency period for bone development. Application of biochemical, radiography and histomorphometric methods in diagnosis of the effects of various levels of dietary P will provide sufficient evidence to characterize and establish the specific changes associated with P deficiency. Therefore, the present study focuses on P nutrition and characterization of its involvement in bone metabolism with the following objectives.

1.6 Objectives

This thesis covers the broad subject area of phosphorus nutrition in haddock. Several experiments were designed to gain new knowledge in the areas of P distribution in skeletal tissues and body, quantitative dietary requirements of this mineral, deficiency signs and the effects of excess P on bone mineralization of haddock. The specific objectives of this project were:

1. To determine the differences in mineral composition of vertebrae and muscle in wild and cultured haddock.
2. To determine the quantitative phosphorus requirement of juvenile haddock.
3. To measure the fecal and urinary phosphorus excretion in haddock.
4. To characterize phosphorus deficiency signs through biological, biochemical, histomorphometric and enzyme histochemical methods.
5. To determine the differences in bone mineralization in vertebrae, opercula, pre-opercula and ventral pharyngeal tissue induced by either deficiency or excess amounts of dietary phosphorus.

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CHAPTER 2

Mineral composition of muscle and vertebrae in wild and cultured haddock (*Melanogrammus aeglefinus* L.)

2.1 Abstract

A study was conducted to determine the macro and trace mineral composition of wild and cultured haddock to obtain preliminary information on the nutritional status of juvenile. Juvenile wild haddock (*Melanogrammus aeglefinus* L.) were collected near the south east coast of Halifax and cultured fish were obtained from a hatchery production stock from the NRC Aquaculture Research Station, Sandy Cove, Nova Scotia. Vertebrae and muscle of eight fish from each of the two groups were analyzed by inductively coupled plasma source mass spectrometry (ICP-Semi Trace –Scan – Metals in solid) to determine the concentration of major minerals of nutritional significance. The total ash and plasma P levels of these fish were also measured. There were no significant ($P>0.05$) differences in the vertebrae ash content between wild and cultured haddock. The average vertebrae ash concentration (56.5 ± 1.24 %) of haddock was higher than values reported for salmonids, however, plasma phosphate levels in haddock were relatively low (0.39 $mmol^{-1}$). The vertebrae P content of cultured haddock was lower than of wild fish whereas muscle P content was relatively high in cultured fish. Muscle iron content of cultured haddock was significantly ($P<0.05$) higher than of wild fish. The concentrations of Mg, K, Cu and Fe in vertebrae of haddock were significantly higher in wild fish than in cultured haddock, whereas Mn and Cr levels were low in cultured fish. These differences in trace element composition of wild and cultured haddock may have been due to the differences in mineral composition of diet and seawater intake and their metabolism. The significance of these findings in the formulation of mineral requirements, particularly P is discussed.

2.2 Introduction

All living organisms contain most naturally occurring elements and these are required for their normal life process. Of the 90 naturally occurring elements, 29 are known to be essential for normal growth and development. The higher components of

living matter constitute protein, lipid and carbohydrate and contain carbon, hydrogen, nitrogen and oxygen. The remaining elements are broadly classified into two groups namely macro and trace elements. The macro-elements, which include the structural elements calcium (Ca), phosphorus (P), potassium (K), manganese (Mn), sodium (Na), chloride (Cl) and sulfur (S), occur in fish body at concentrations ranging from 0.1 to 2 % of fish weight and their dietary requirement is generally higher than 100 mg/Kg dry diet. Trace elements occur in the body in low concentrations (milligrams or micrograms per kilogram). Fifteen trace elements (arsenic, chromium, copper, cobalt, cadmium, fluorine, iodine, iron, manganese, molybdenum, nickel, selenium, silicon, vanadium and zinc) are found in terrestrial vertebrates. Most of these elements have been detected in fish tissues, however, the requirements of only few of these elements has been discussed. In nature, marine fish absorb and retain most of the elements from water and food organisms. Generally, the concentration of minerals is not sufficient in either freshwater and seawater for normal growth and development and therefore, mineral supplementation in captivity is essential. Fish deposit minerals mostly in skeletal tissue, muscle and different organs (Lall, 2002). The major portions of minerals in the fish body are distributed in bone, especially vertebrae and scales (Roche and Bullinger, 1939).

Although mineral composition data of common foods for human consumption including fish are available, limited information exists on trace element composition of farmed fish and fishery products, particularly, whole body mineral distribution. An extensive report on the elemental composition of marine organisms was published by Vinogradov (1953). Studies conducted on the mineral composition of fish and some sea foods from 1930 to the mid 1960's were comprehensively reviewed by Causeret (1962). The concentration of minerals in fish is influenced by a number of factors such as seasonal changes, environmental factors (water chemistry, salinity, temperature and contaminations) and biological differences (species, age, sex and sexual maturity), and the composition of food consumed. The complete inorganic composition of cultured rainbow trout varies with fish size, stage of life cycle, and reproductive status (Shearer, 1984). Wide variations were observed in macro and trace element content of the same species. A sub optimal mineral supply in fish diets may not produce clinical signs of

mineral deficiencies but it may lead to biochemical changes and inadequate storage of minerals during short-term study (Hamilton, 1981).

Information on the mineral requirements of most marine fish is lacking. Whole body mineral composition, e.g. for magnesium, has been used as an indicator of mineral status in salmonids (Shearer, 1989). In order to obtain preliminary information regarding the mineral composition of haddock, particularly muscle and whole body, both wild and cultured haddock were analyzed. Another objective was to study whether natural and commercial diets have a significant effect on macro and trace element composition of these fish. The results were examined to determine differences between published values on salmonids and haddock, particularly P concentration, Ca to P ratio, and trace element concentration in relation to these two macro-elements.

2.3 Materials and methods

2.3.1 Location of wild and cultured haddock collection

Cultured haddock were reared at the NRC Aquaculture Research Station (Sandy Cove, NS, Canada) and eight fish (average weight 123.4 ± 1.1 g) were used in this study. The fish had been held in circular fiberglass tanks at a flow rate of 4 L^{-min} in a flow through system to ensure a renewal rate of once every hour. The water level in each tank was maintained at 250 L (holding capacity: 350 L). The rearing water was filtered and UV-treated with a temperature of 9 ± 2 °C and salinity of 32 ‰. Fish were held on a 12 h dark: 12 h light photoperiod with the light intensity at the water surface between 40 and 60 lux., and hand-fed a commercial diet. Eight wild juvenile haddock (average weight, 635.1 ± 1.3 g) were captured by a commercial fisherman using hand lines near the south east Atlantic coast of Halifax, Nova Scotia, Canada in December 2000. During the collection period, water temperature and salinity were 7 °C and 35 ‰, respectively. It is important to clarify the cause of enormous size variation between wild and cultured haddock in this investigation. As we first investigated the cultured haddock we set our aim to collect same size haddock from wild source. However, capture of a same size haddock from wild source was impossible.

2.3.2 Sampling and tissue collection

Blood was collected from the caudal vein of each fish and placed into a heparinized tube and centrifuged at 3000 × g. Plasma was collected and stored at – 20 °C until analysis. All fish were sacrificed with an overdose of tricaine methanesulphonate, rinsed in de-ionized water and individually dissected to remove vertebrae and muscle for bone ash and mineral concentration determinations

2.3.3 Analytical methods

Vertebrae were cleaned with de-ionized water. All samples were extracted twice with 5-ml chloroform-methanol (1:1, v/v) to remove lipids and then oven-dried at 110 °C for 24 h. Bone ash was determined by incineration in a muffle furnace at 550 °C for 18 h; cooled and digested according to the AOAC method (1995). Mineral concentrations of vertebrae and muscle samples were determined by inductively coupled plasma source mass spectrometry (ICP Semi-Trace Scan – Metals in solids) at the Norwest Labs of the Canadian Association for Environmental Analytical Laboratories (CAEAL). The ash was dissolved in a mixture of equal parts of concentrated HCl and HNO_3 and appropriately diluted with deionized water so that elemental concentrations remained within the analytical capabilities of the measuring instrument. All concentrations are reported as micrograms per gram wet weight. Plasma protein was removed by extraction with 10 % trichloroacetic acid before phosphate analysis and P was analyzed by a spectrophotometric method using molybdovanadate reagent (Taussky and Shorr, 1953).

2.3.4 Statistical analysis

Mineral concentrations of vertebrae and muscle were subjected to two sample t-tests using the Prism, version 3.0 software (Graphpad, San diego, CA). Cultured and wild haddock were considered as two treatments. Individual minerals, for example, P in vertebrae, were treated as separate variables. Phosphorus content in vertebrae of wild and cultured haddock was compared. Similarly, P content in muscle of wild and cultured haddock was compared. Confidence level for the confidence interval of the mean was 0.95

and the differences at with Bonferroni adjustment P<0.0036 was considered significant. Graphs are presented as mean and standard errors of the Least Square Means.

2.4 Results

The vertebrae ash content of wild and cultured haddock was 56.5 % and 54.8 %, respectively. Plasma P concentrations of cultured (0.39 $mmol^{-1}$) and wild (0.43 $mmol^{-1}$) haddock were not significantly different. Generally, mineral concentrations in vertebrae were higher than in muscle. However, Fe, K and S concentrations were found to be significantly (P<0.05) higher in muscle than in vertebrae. The mineral concentration in muscle and vertebrae are presented in figures 2.1. Major elements at the milligram per gram level were Ca, Fe, Mg, P, K, Na and S. The amount of Ca in vertebrae of wild and cultured haddock remained the same, although muscle Ca content of wild haddock was significantly higher than that of cultured haddock. In contrast, vertebra P of wild haddock was significantly higher than of wild haddock whereas muscle P in cultured haddock showed significantly higher levels than that in wild haddock. The amount of Ca in vertebrae was twice as much as that in vertebrae P. However, in muscle the amount of Ca and P did not show any relationship. Muscle Na varied between 6.3 to 4.3 mg/g in wild and cultured haddock, although no significant variation was observed in vertebra Na. Similarly, muscle K and Fe varied from 15.5 to 21.3 mg/g and 15.6 to 25.1 mg/g, respectively between wild and cultured haddock. Minor elements at the microgram per gram level were Al, Cr, Cu, Mn and Zn. The amount of Al in cultured haddock is significantly (P<0.05) higher (21.2 μg/g) and in muscle lower (5.4 μg/g) than wild haddock. The amount of Cr concentration in vertebrae lower amount (2.9 μg/g), although Cu concentration in vertebrae and muscle was higher (5.5 μg/g and 3.8 μg/g, respectively). The Zn concentrations in vertebrae and muscle of wild and cultured haddock remained similar, Mn concentration in cultured vertebrae was lower (50.9 μg/g) than wild (56.2 μg/g). Trace elements detected at a level less than in a microgram per gram were Co and Mo. The Mo concentration in vertebrae of cultured haddock was

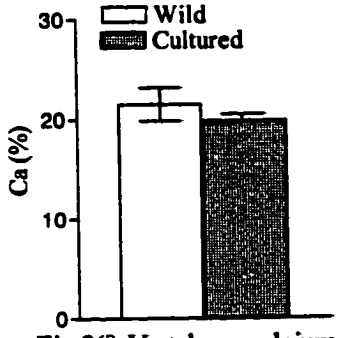

Fig 2(i).Vertebrae calcium of wild and cultured haddock are not significantly different (P>0.05) . Two tailed P= 0.38; t=0.92 with 14 df.

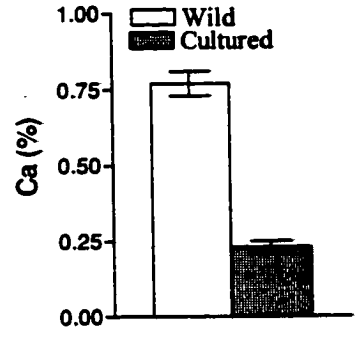

Fig 2(ii). Muscle calcium of wild and cultured haddock are significantly different (P<0.05). Two tailed P >0.0001; t=12.07 with 14 df.

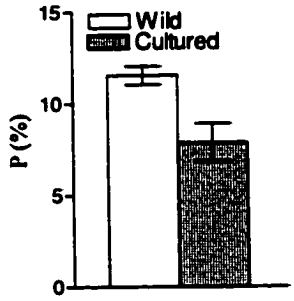

Fig 2(iii).Vertebrae phosphorus of wild and cultured haddock are significantly different. Two tailed P=0.007; t=3.14 with 14 df.

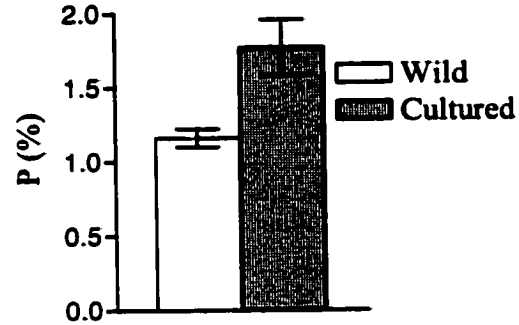

Fig 2(iv). Muscle phosphorus of wild and cultured haddock are significantly different (P>0.05). Two tailed P= 0.009; t=3.06 with 14 df.

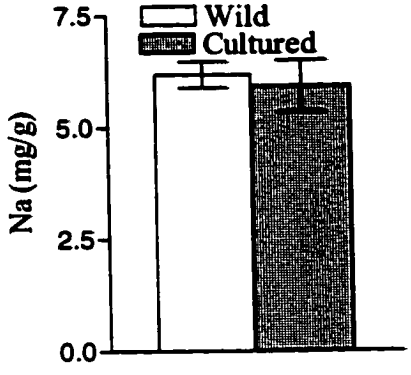

Fig 2(v).Vertebrae sodium of wild and cultured haddock are not significantly different (P>0.05). Two tailed P=0.7; t=0.4 with 14 df.

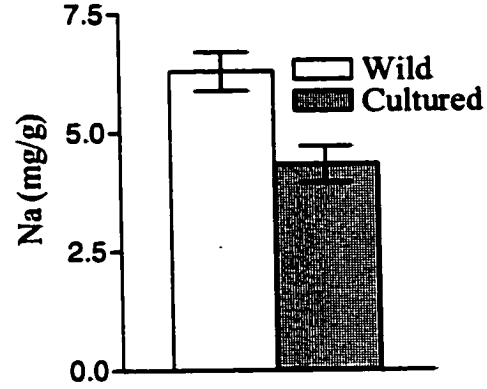

Fig 2(vi). Muscle sodium of wild and cultured haddock are not significantly different (P>0.05). Two tailed P= 0.004; t=3.51 with 14 df.

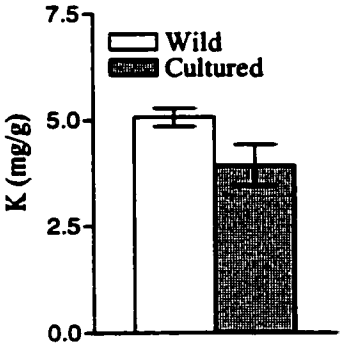

Fig 2(vii).Vertebrae potassium of wild and cultured haddock (P>0.05) are significantly different. Two tailed P=0.051; t=2.14 with 14 df.

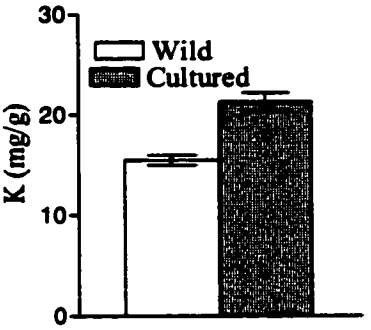

Fig 2(viii). Muscle potassium of wild and cultured haddock are significantly different (P>0.05). Two tailed P>0.0001; t=5.71 with 14 df.

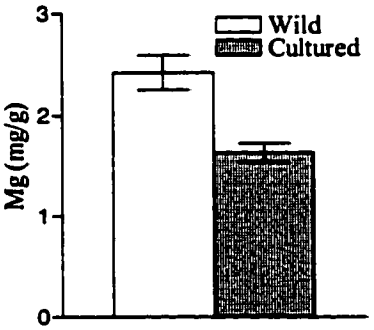

Fig 2(ix).Vertebrae megnesium of wild and cultured haddock are significantly different (P>0.05) . Two tailed P= 0.001; t= 4.11 with 14 df.

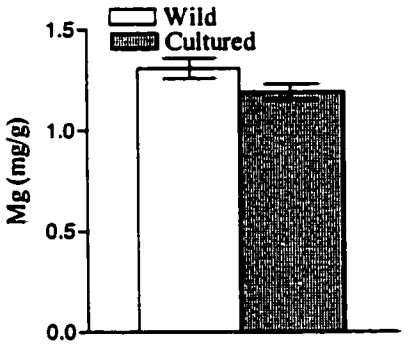

Fig 2(x). Muscle magnesium of wild and cultured haddock are not significantly different (P>0.05). Two tailed P=0.08; t=1.87 with 14 df.

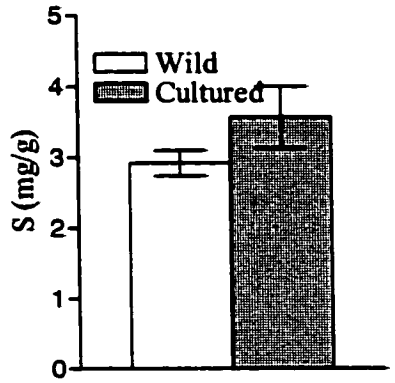

Fig 2(xi).Vertebrae sulfur of wild and cultured haddock are not significantly different. Two tailed P=0.2; t=1.37 with 14 df.

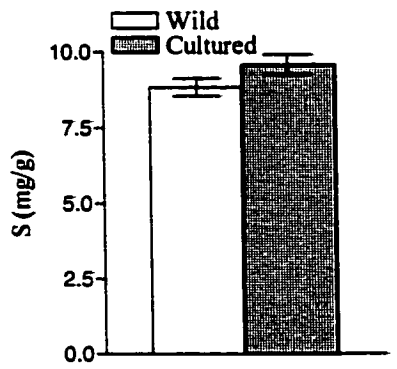

Fig 2(xii). Muscle sulfur of wild and cultured haddock are not significantly different (P>0.05). Two tailed P=0.11; t=1.71 with 14 df.

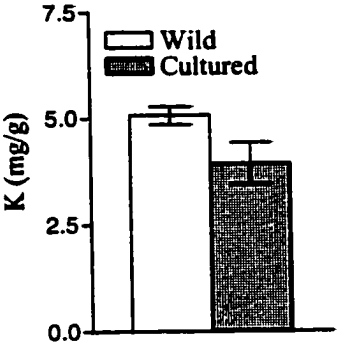

Fig 2(vii). Vertebrae potassium of wild and cultured haddock (P>0.05) are significantly different. Two tailed P=0.051; t=2.14 with 14 df.

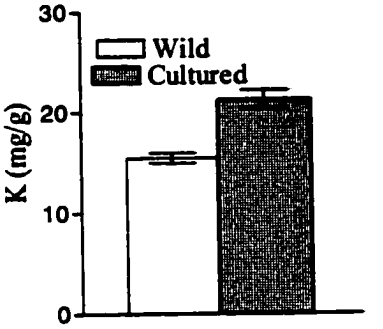

Fig 2(viii). Muscle potassium of wild and cultured haddock are significantly different (P>0.05). Two tailed P>0.0001; t=5.71 with 14 df.

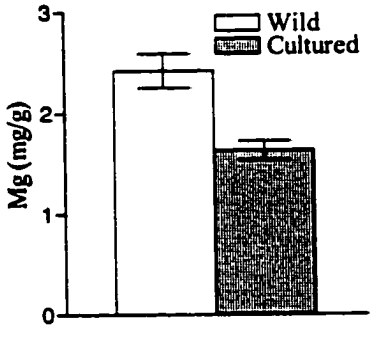

Fig 2(ix). Vertebrae megnesium of wild and cultured haddock are significantly different (P>0.05). Two tailed P= 0.001; t= 4.11 with 14 df.

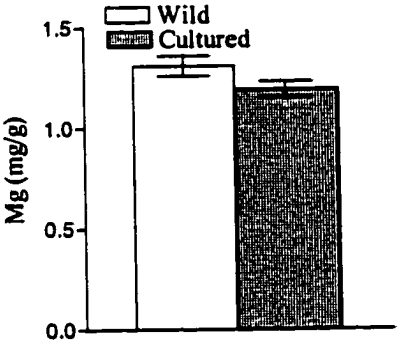

Fig 2(x). Muscle magnesium of wild and cultured haddock are not significantly different (P>0.05). Two tailed P=0.08; t=1.87 with 14 df.

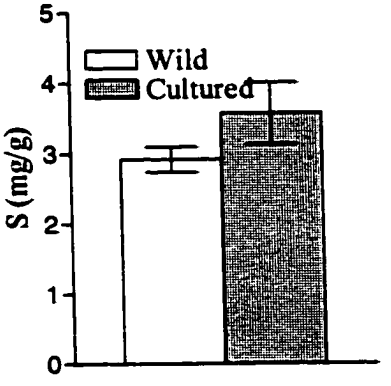

Fig 2(xi). Vertebrae sulfur of wild and cultured haddock are not significantly different. Two tailed P=0.2; t=1.37 with 14 df.

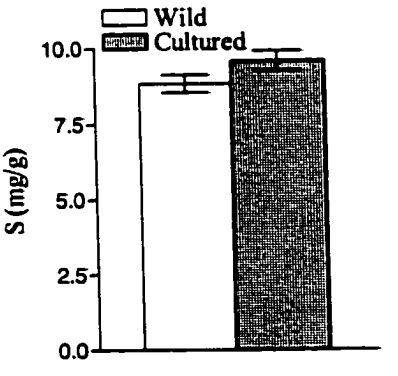

Fig 2(xii). Muscle sulfur of wild and cultured haddock are not significantly different (P>0.05). Two tailed P=0.11; t=1.71 with 14 df.

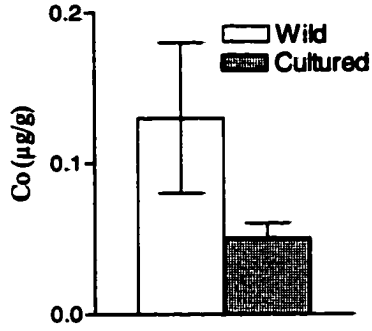

Fig 2(xix). Vertebrae cobalt of wild and cultured haddock are significantly different (P<0.05) . Two tailed P= 1.39; t=1.57 with 14 df.

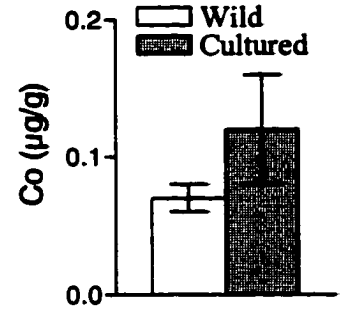

Fig 2(xx). Muscle cobalt of wild and cultured haddock are not significantly different (P>0.05). Two tailed P = 0.25; t=1.21 with 14 df.

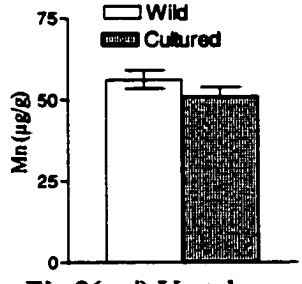

Fig 2(xxi).Vertebrae manganese of wild and cultured haddock are not significantly different (P>0.05). Two tailed P = 0.22; t=1.3 with 14 df.

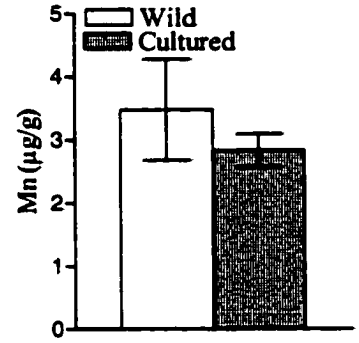

Fig 2(xxii). Muscle manganese of wild and cultured haddock are not significantly =different (P>0.05). Two tailed P=0.46; t=0.76 with 14 df.

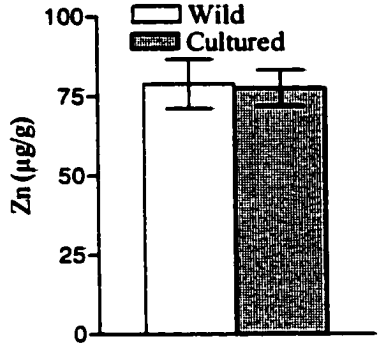

Fig 2(xxiii).Vertebrae zinc of wild and cultured haddock are not significantly different. Two tailed P=0.9; t=0.13 with 14 df.

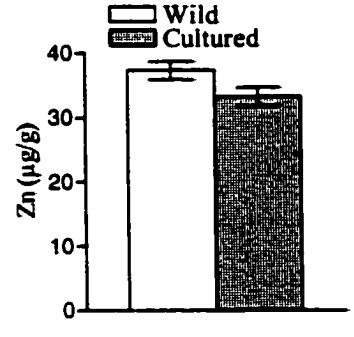

Fig 2(xxiv). Muscle zinc of wild and cultured haddock are not significantly different (P>0.05). Two tailed P=0.06; t=2.06 with 14 df.

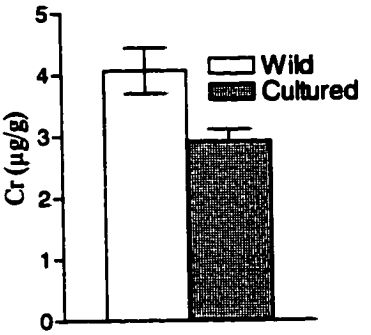

Fig 2(xxv).Vertebrae cromium of wild and cultured haddock are significantly different (P<0.05) . Two tailed P= 0.015; t=2.77 with 14 df.

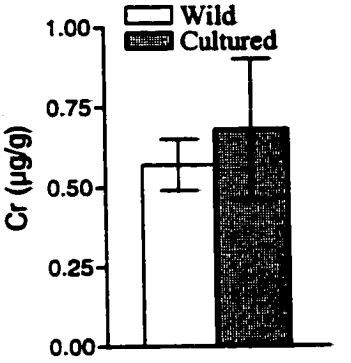

Fig 2(xxvi). Muscle cromium of wild and cultured haddock are not significantly different (P>0.05). Two tailed P = 0.65; t=0.47 with 14 df.

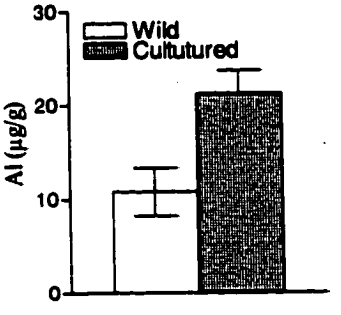

Fig 2(xxvii).Vertebrae aluminium of wild and cultured haddock showed significantly different (P<0.05) . Two tailed P= 0.0114; t=2.91 with 14 df.

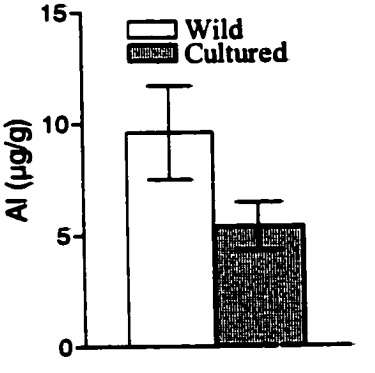

Fig 2(xxiii). Muscle aluminium of wild and cultured haddock are not significantly different (P>0.05). Two tailed P= 0.01; t=1.78 with 14 df.

significantly higher (2.3 μg/g) than in wild (0.7 μg/g). A few trace elements, for example, Se, As, I, B, Cd, Li, Ni, Sn were detected but the range of their variation among replicates samples was very high.

2.5 Discussion

The concentration of minerals in the body of fish depends on the food source, environment, species, stage of development, and physiological status of the animal. Most organisms accumulate and retain minerals from the environment; however, their incorporation is highly selective (Lall, 2002). Vertebrae ash content is widely used as an indicator of the mineral nutrition status of fish. Although no significant differences were observed in vertebrae ash content between wild and cultured fish, the ash content was approximately 10 % higher than values reported for salmonids. In a study designed to measure the optimum bone mineralization in Atlantic salmon (Vielma and Lall, 1998), the maximum vertebrae ash content was 46.2 %. Vertebrate ash content in rainbow trout was 50 % (Ketola and Richmond, 1994), in milkfish 38 % (Borlongan, and Satoh, 2001), in carp 50.1 % (Ogino et al., 1979) and in tilapia 32.8 % (Robinson et al., 1987). It appears that haddock deposit much higher concentrations of minerals, particularly Ca and P, than salmonids. Some of these differences could be associated with higher bone mass in the body as well as the differences in bone metabolism of fish. The ratio of Ca to P in haddock bone was 2:1. The ratio of these minerals in bones of common carp, pike, tilapia, sea bream, trout, and salmon ranges from 1.5 to 2.1 (Van Oosten, 1957; Ogino and Takeda, 1978; Ogino et al., 1979; Watanabe et al., 1980). The ratio of Ca to P in the whole body of various fishes ranges from 0.7 to 1.6 (Arai et al., 1975; Ogino and Takeda, 1976; Lovel and Li, 1978; Watanabe et al., 1980).

The vertebrae P level in haddock ranged from 5.3 to 8.7 %. These values were close to the P concentration measured in vertebrae of other fish species. Vertebrae ash content constitutes a major portion of the total body ash content and the P in the whole body of fish ranges from 0.4 to 0.5 % fresh weight. The estimated whole body P of haddock was 0.9 % which is higher than other fish species. Plasma phosphate levels of haddock were approximately six times lower than values reported for Atlantic salmon. Although haddock plasma P values would be considered a sign of deficiency in rainbow

trout (Satoh et al., 2002), haddock used in this study were maintained on normal diets and no gross deficiency signs were apparent. The low plasma P values in haddock were probably due to the differences in P homeostasis between the two species and the excretion of this element by haddock (see chapter 3). Comparing with salmon, a significantly higher muscle P content in both wild and cultured haddock was probably due to higher amounts of phospholipids in their muscle than in salmonids (Nanton et al., 2001).

Although the distribution of certain essential elements in various marine organisms has been reported (Vinogradov, 1953), information on complete elemental composition of vertebrae and other tissues of marine teleosts is limited. The mineral composition of the edible part of fish and shellfishes was recently reported (Lall, 1995), however, the concentration of trace elements showed a wide range of values. Other studies also show variations in tissue mineral composition of fish reared on commercial feeds (Eastblier, 1970; Galindo et al., 1986; Stoeppler and Brandt, 1979; Ganglitz et al., 1984). The mineral composition of rainbow trout varies with the stage of development particularly at sexual maturity (Shearer, 1984) and the concentration of Ca, Cu, Fe, K, Mg, Mn, Na, P, Sr and Zn were higher in juvenile fish than in adults. In the current study, although there was a difference in the size of wild and cultured haddock, none of the fish sampled were sexually mature.

The differences observed in macro, trace and ultra trace element composition of wild and cultured fish were probably due to the differences in distribution and absorption of these minerals from diet and water. Smith (1930) showed that marine fish swallow considerable quantities of seawater, which contains high concentrations of minerals such as calcium, sodium, chloride, and magnesium but very little phosphorus. The amount of P present in the diet of farmed fish is higher than in wild fish (Lall, 1991). The concentration of dietary Ca and P level also affect the bioavailiability of trace elements particularly Fe, Mn, Zn and Cu (Lall, 2002). It is possible that the differences observed in vertebrae and muscle trace element content of wild and cultured fish was due to mineral interactions and the differences in absorption and metabolism of various minerals in their diets. A wide range of potential mineral- mineral and mineral vitamin interactions has been reported in fish and terrestrial vertebrates (Hilton, 1989).

The data presented here clearly indicate that formulation of test diets for P requirement and metabolism studie requires special attention to dietary calcium and P supplementation. The high amount of total ash, Ca and P in vertebrae suggests a higher requirement for these elements for haddock than salmonids. Drinking seawater contributes significantly to satisfying the Ca requirement of most marine fish, however, there is a need to provide an optimum level of dietary P. Since the information regarding the metabolism of P, particularly absorption, excretion and retention, in haddock is limited, it is reasonable to suggest that P requirements of haddock may not be lower than salmonids. The test diets for P requirement studies should contain at least 0.6 % P and the trace element levels established for salmonids (NRC, 1993).

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CHAPTER 3

Dietary phosphorus requirement of juvenile haddock (*Melanogrammus aeglefinus* L.)

3.1 Abstract

A study was conducted to determine the quantitative requirement, excretion, availability and deficiency signs of phosphorus (P) in haddock. Triplicate groups of haddock (4.2 ± 0.01 g) were fed diets containing 0.42, 0.62, 0.82, 1.02 and 1.22 % P and 19 MJ digestible energy (DE) per g of diet to satiation for 12 weeks. The basal diet, containing 0.42 % P (0.08 g available P per MJ DE), was supplemented with graded levels of calcium phosphate, $Ca(H_2PO_4)_2$. H_2O, to formulate the five experimental diets. The fish were reared in seawater (salinity, 32 ppt) at a temperature of 12 ± 2 °C. The growth, feed conversion ratio, vertebrae and opercula ash and urinary P excretion were positively correlated with dietary P levels. Vertebrae ash increased from 44.5 to 56.6 % and operculum ash from 31.4 to 48.2 % of fat free dry matter with an increase in dietary P content. Phosphorus requirement was estimated by using a quadratic equation for vertebrae ash. The data suggest that a diet of 0.96 % total P, combined with 0.72 % or 0.34 g available P per MJDE, is required for haddock fingerlings. Serum and urinary phosphate excretion increased with increasing dietary P levels and ranged from 0.42 - 1.45 and 0.1 – 7.9 $mmol^{-1}$, respectively. The digestibility of organic P was 43.2 %, however, inorganic P was highly digestible (99 %). Phosphorus deficiency signs were characterized by poor growth, loss of appetite, poor bone mineralization, deformed vertebrae and an increase in body lipid content.

1. Introduction

Aquaculture of gadoid fishes including haddock (*Melanogrammus aeglefinus* L.) and cod (*Gadus morhua*) is expanding in Atlantic Canada and Europe. A paucity of information exists on their quantitative nutrient requirements, particularly the efficiency of mineral utilization from feed and seawater. Phosphorus (P) is an essential nutrient for growth, (Baverfjord et al., 1998) skeletal development (Åsgård and Shearer, 1997) and reproduction of fish (Hardy et al., 1985). Like in terrestrial vertebrates, a major

constituent of the structural components of skeletal tissues, P is located in every cell of the body (Lovell, 1978). The amount of P in feeds must be carefully balanced to prevent deficiency signs as well as to minimize the urinary and fecal excretions to reduce P discharge in natural waters (Lall, 1991; Wiesmann et al., 1988; Bureau and Choo, 1999). Feed is the main source of P for fish. Fish and other aquatic invertebrates have the ability to absorb P from water; however, the concentration of this element is low in both freshwater (Boyd, 1971) and seawater (NRC, 1993). Limited information is available on the metabolism, excretion and utilization of dietary P in fish (Lall, 1991).

Phosphorus deficiency signs in several fish species include poor growth (Andrews et al., 1973), reduced feed efficiency (Rodehutscord, 1996) and poor bone mineralization (Robinson et al., 1987). Carp fed a low P diet show an increase in the activity of certain gluconeogenic enzymes in their liver (Onishi et al., 1981), an increase in carcass fat with a decrease in carcass water content (Takeuchi and Nakazoe, 1981), reduced blood phosphate levels and a deformed head (Ogino and Takeda, 1978). A low P intake by red sea bream causes curved, enlarged vertebrae, increased serum alkaline phosphatase activity, higher lipid deposition in muscle, liver, and vertebrae, and a reduction in liver glycogen (Sakamoto and Yone, 1980 and 1981). A significant reduction in operculum and scale P concentration occurs in salmon and trout fed low P diets (Vielma and Lall, 1998). When the feed ingredients and/ or P supplements do not supply sufficiently available P to meet the metabolic need for bone mineralization of rapidly growing post-smolt Atlantic salmon, fish gradually develop soft bones and skeletal deformities (Baeverfjord et al., 1998). Although fish may not show obvious growth reduction, bone mineralization is significantly reduced.

Dietary P requirements ranging from 0.5 to 0.8 % have been reported for rainbow trout (Ogino and Takeda, 1978), Atlantic salmon (Ketola, 1975; Lall and Bishop, 1977; Åsgård and Shearer. 1997; Vielma and Lall, 1998), chum salmon (Watanabe *et al.*, 1980), carp (Ogino and Takeda, 1976) and red sea bream (Sakamoto and Yone, 1978). The P requirement of catfish and Japanese eel is approximately 0.45 % and 0.3 %, respectively. The P requirement of Atlantic salmon is not significantly influenced when reared in either freshwater (Ketola, 1975; Åsgård and Shearer, 1997) or seawater (Lall and Bishop, 1977). The following studies were designed to determine the dietary P

requirement, to measure P digestibility of experimental diets and urinary P excretions, and to characterize P deficiency signs in juvenile haddock.

3.3 Materials and methods

3.3.1 Experimental design, fish rearing system and feeding

Haddock fingerlings with an initial average weight of 4.3 ± 0.01 g, were hatched and reared at the National Research Council's Aquaculture Research Station, Sandy Cove, Nova Scotia. Seven hundred and fifty haddock were randomly distributed in fifteen circular fiberglass tanks (350 L holding capacity) located at this facility. Filtered and UV-treated water (salinity, 32‰) was supplied to each tank at a flow rate of 4 L^{-min} in a flow through system with the water renewal rate of once every hour. Fish were gradually acclimated over 3 weeks to experimental conditions and fed a low P basal diet containing no supplemental P. During the first four days of the experimental period, a commercial diet was gradually replaced with the low P basal diet. Fish were held on a 12 h dark:12 h light photoperiod with the light intensity at the water surface between 40 and 60 lux. Dissolved oxygen levels and water temperature were monitored each day and averaged 10 mg/L and 12 ± 2 °C, respectively. Fish were hand-fed one of five experimental diets to apparent satiation three times daily on weekdays and twice daily on weekends for 12 weeks.

3.3.2 Diet preparation

A basal diet was formulated using practical ingredients (Table 3.1), which contained 4.2 mg P/g diet. Phosphorus content in the feed ingredients is presented in Table 3.2. Five experimental diets were prepared by supplementing the basal diet with four levels of monocalcium phosphate {$Ca(H_2PO_4)_2.H_2O$ (Anachemic Science, Montreal, QC)} to reach the final concentrations of 4.2, 6.2, 8.2, 10.2 and 12.2 mg P/g diet. Calcium phosphate replaced cellulose to achieve the desired level of P in these five isonitrogenous and isocaloric experimental diets. Vitamin and mineral premixes were prepared using a twin-shell blender (Paterson-Kelly, East Stroudsburg, PA, USA), prior to adding them to the main ingredient mixture. All dry ingredients except the choline chloride and fish oil, were

Table 3.1. Composition of the basal diet

Ingredient	Amount (%)
Casein, vitamin free[1]	21
Wheat gluten meal[2]	10
Fish muscle[3]	9.5
Corn gluten meal[4]	8
Gelatin[1]	3
CPSP[5]	4.5
Krill meal	3
Corn starch, pre-gelatinized[7]	8
Corn starch	8
Cellulose[1]	9.7
Mineral premix[8]	1
Vitamin premix[9]	1
Choline chloride	0.3
Herring oil[10]	13
Chemical analysis (%DM)	
Protein	47.9
Lipid	14.5
Fibre	1.3
Ash	15.8
Nitrogen-free-extract[11]	36.3
Calcium	0.27
Phosphorus	0.42
Digestible energy (MJ/Kg)	19.0

[1]US Biochemical Corp. Cleveland, OH, USA.
[2]ADM Agri-industries Ltd., Montreal, QC, Canada.
[3]Composed of 83.7% protein and 0.8% lipid. Prepared in the lab. from bone and skin less cod fillet collected from local market.
[4]Corey Feed Mills Ltd., Fredericton, NB, Canada.
[5]Concentrated protein soluble de poisson (soluble fish protein concentrate) hydrolyzed fish meal (Sopropêche, France).
[6]Specialty Marine Product. West Vancouver, BC, Canada.
[7]National Starch and Chemical Company, Bridgewater, NJ, USA.
[8]minerals added to supply the following (per kg diet): manganous sulfate ($MnSO_4.H_2O$, 32.5% Mn), 40 mg; ferrous sulfate ($FeSO_4.H_2O.7H_20$, 20.1% Fe), 50 mg; copper sulphate ($CuSO_4.7H_2O$, 25.4% Cu), 10 mg; zinc sulfate ($ZnSO_4.7H_2O$, 22.7% Zn), 75 mg; cobalt chloride ($CoCl_2.6H_2O$, 24.8% Co), 5 mg; sodium selenite (Na_2SeO_3, 45.7% Se), 1 mg; sodium fluoride (NaF, 45.3% F), 4 mg.
[9]vitamin added to supply the following (per kg diet): Vitamin A (retinol acetate), 8,000 IU; vitamin D_3 (cholecalciferol), 4,500 IU; vitamin E (all-race-α-tocopheryl acetate), 300 IU; Vitamin K_3 (menadione sodium bisulfite), 40 mg; vitamin B_1 (thiamine HCl), 50 mg; vitamin B_2 (riboflavin), 70 mg; *d*- calcium pantothenate, 200 mg; biotin, 1.5 mg; folic acid, 20 mg; vitamin B12, 0.15 mg; Niacin, 300 mg; pyridoxine

HCl, 20 mg; vitamin C (ascorbic acid, stay C), 300 mg; inositol, 400 mg; butylated hydroxytoluene (BTH) 15 mg; butylated hydroxyanisole (BHA), 15 mg.
[10]stabilized with 0.06% ethoxyquin; Comeau Seafood Ltd, Saulnierville, NS, Canada.
[11]Nitrogen-free-extract = 100 – (protein + lipid + fibre + ash).

Table 3.2. Analysed phosphorus content in the dietary ingredients of the basal diet

Ingredients	Ash (%)	Phosphorus in ash (%)	Phosphorus in ingredient (%)
Casein	0.7 ± 0.01	23.4 ± 2.3	0.17 ± 0.02
Blood meal	6.9 ± 0.6	5.41 ± 0.4	0.37 ± 0.01
Krill meal	13.0 ±1.2	13.1 ± 0.5	1.7 ± 0.02
Sapropache	4.9 ± 0.4	15.0 ± 0.3	0.74 ± 0.04
Wheat gluten meal	1.1 ± 0.03	29.8 ± 1.2	0.33 ± 0.01
Corn gluten meal	1.8 ± 0.09	27.7 ± 0.9	0.49 ± 0.03
Gelatin	0.04 ± 0.0	10.1 ± 0.6	0.004 ± 0.00
Fish muscle	7.8 ± 0.8	15.7 ± 0.7	1.24 ± 0.03

Calculation were performed on the dry matter basis. The values are mean ± SE of four replicate samples.

Table 3.3. Calcium phosphate supplementation, and analyzed calcium and phosphorus contents of experimental diets (dry matter basis).

% P in diet	0.42	0.62	0.82	1.02	1.22
$Ca(H_2PO_4)_2.H_2O$, mg/g	0	8.2	16.3	24.4	32.5
Ca, mg/g	2.7	4	5.3	6.6	7.9
P, mg/g	4.2	6.2	8.2	10.2	12.2
	Diet #1	diet #2	diet #3	diet #4	diet #5

weighed and mixed in a Hobart mixer (model H600T; Hobart, Troy, Ohio, USA). Choline chloride was dissolved in water and mixed before the fish oil was added to the main mixture. Diets were steam pelleted, freeze-dried and stored at –18 °C until use. A portion of each of the five experimental diets was removed for gross energy determination and chemical analyses including protein, lipid, ash and P content (Table 3.1).

3.3.3 Biological sampling and tissue collection

At the beginning of the feeding trial, 20 fish were sacrificed with an overdose of tricaine methanesulphonate, rinsed in de-ionized water and freeze-dried. Of the 20 fish, ten were individually dissected to collect vertebrae for bone ash and mineral concentration estimation. The remaining 10 fish were finely ground for proximate analysis. At the beginning of the experiment and at every 3-week interval during the trial, fish were counted and bulk-weighed after a 24 h fast. At the end of 12 weeks, urine, serum, vertebrae and opercula were collected individually from 10 fish per dietary group for the determination of serum and urine phosphate, ash and mineral concentration. Urine was collected by internal catheterization using a spot sampling technique (Wood and Patrick, 1994) with some modifications (chapter II). Blood was collected from the caudal vein into a heparinized tube, centrifuged at 3000 × g and plasma was removed and stored at – 20 °C until analysis. Another 10 fish were collected and killed in a similar manner for body composition analyses and various biological measurements. After measuring the body weight and length, the liver was removed and weighed. Liver and carcass were pooled in four groups from each dietary treatment and immediately transferred to a freezer (–20 °C) until analysis. Haddock carcasses were later freeze-dried, ground and subjected to proximate analysis.

3.3.4 Gross examination of bone for deficiency symptoms

For the morphological examination of bone, five fish from each experimental group were anesthetized and rinsed in water. Fish were partially cooked in a microwave oven for 30 seconds to remove the flesh and soft tissues from the vertebral axis. Bones were air-dried and the gross differences in the shape of vertebrae and opercula of fish from each of the five experimental groups were determined.

3.3.5 Analytical methods

Vertebrae and opercula were washed with de-ionized water to remove any remaining muscle tissue. In order to determine the bone ash content on a fat free basis, bones were first extracted with 5-ml chloroform and methanol (1:1, v/v), air dried at room temperature in a fume hood, oven-dried at 110 °C for 24 h and incinerated in a muffle furnace at 550 °C for 18 h. Chemical composition of the experimental diets and lyophilized fish carcasses was determined using the following procedures: dry matter by drying in an oven at 110 °C (AOAC, 1995); crude protein (N x 6.25) according to Dumas method (Ebling, 1968) using an FP-228 nitrogen determinator (Leco, St Joseph, MI, USA); lipid by the Bligh and Dyer (1959) method modified by Iverson et al., (2001) and ; gross energy measured in an adiabatic bomb calorimeter (Model 1261, Parr Instruments, Moline, IL, USA); crude ash by incineration in a muffle furnace at 550 °C for 18 h (AOAC, 1995). Mineral concentration of vertebrae ash samples were determined by inductively coupled plasma source mass spectrometer (ICP Semi-Trace Scan–Metals in solids) at the Norwest Laboratory of Canadian Association for Environmental Analytical Laboratories (CAEAL,Vancouver, British Columbia). Chromium content of experimental diets and feces was analyzed according to Arthur (1970). Plasma protein was removed by extraction with 10 % trichloroacetic acid before P analysis. Ash samples from feed, carcass and bone were acid-digested according to the AOAC method (1995) to determine the P concentration. Phosphorus in all samples including plasma was analyzed by a spectrophotometric method using molybdovanadate reagent (Taussky and Shorr, 1953).

3.3. 6 Phosphorus digestibility measurement

Phosphorus digestibility of two experimental diets, one containing no P supplement (diet 1) and a diet containing 0.82 % P (diet 3) was determined to estimate the digestibility of organic and inorganic P. Because limited numbers of fish were available, the above two experimental diets were selected for the estimation of P digestibility. A new group of larger haddock was used in this study to collect sufficient amounts of feces required for various chemical analyses. One hundred and fifty juvenile haddock (initial weight, 60 ± 0.05 g) were randomly distributed in two circular rearing

tanks (75/tank) and adapted to the low P diet for a week. Chromic oxide (III) (Cr_2O_3) was added (0.5 % of diet) to the experimental diets as an inert digestion marker and these diets were fed for 3 weeks prior to fecal collection. Feces were stripped from all fish by applying gentle pressure in the anal area according to the procedure of Austreng (1978). After a 6 day interval, three samples were collected from each tank. Fecal samples were quickly frozen, lyophilized and stored at –20 °C until analysis. The percent P digestibility coefficient was calculated according to the formula described by NRC (1993). The following formula was used to calculate the digestibility of inorganic P in diet 3 by taking into the account the P supplied by the feed ingredients in diet 1 and the digestibility of this diet :

$$D = \frac{Q \times DD^1 - Q^1 \times D^1}{I} \times 100$$

Where,

Q = Total amount P/g diet

DD^1 = Amount of digestible P /g total P

Q^1 = Amount of P in diet 1/g diet

D^1 = Amount of digestible P in diet1 /g total P

I = Amount of inorganic P in diet 3 /g diet

D = Digestible inorganic P in diet 3/g P in diet

3.3.7 Statistical analysis and phosphorus requirement estimation

Vertebrae and opercula ash data were subjected to linear and four different types of non-linear regression models. For non-linear models quadratic, cubic, broken line regressions and piecewise equations were used. Dietary P requirement of haddock was estimated by using a non-linear quadratic regression model of vertebrae ash content of fish sampled after 12 weeks of feeding various experimental diets, against dietary P concentration. The quadratic equation used in this model was as follows: $Y = a + bx + cx^2$

where: Y= measured ash content

a= intercept,

b= co-efficient of the linear terms

c= co-efficient of the quadratic terms

x = dietary P content

Other biological and chemical data were subjected to analysis of variance (ANOVA). Five levels of P were considered as treatments. Individual parameters for example, ash content of vertebrae were treated as separate variables. Confidence level for the confidence interval of the mean was 0.95 and the differences with Banferrini adjustment at $P<0.01$ were considered significant. Statistical analyses of all data were performed using SYSTAT (version 10 for windows, SYSTAT Inc. Evanston, IL).

3.4 Results

3.4.1 Diets, Growth, feed utilization, hepatosomatic index and gross deficiency signs

Experimental diets were formulated to supply a low amount of P from the feed ingredients, and the analysis of diet showed slightly higher amounts than intended (Table 3.3). Krill meal was incorporated in the experimental diets to maintain palatability and optimum feed intake, however, the P content of this product was higher than of other feed ingredients (Table 3.2). Freeze dried, de-boned cod muscle also contained a higher amount of P probably due to a high amount of phospholipid found in the lipid fraction of gadoid muscle. The effects of dietary P levels on growth, feed conversion ratio and hepatosomatic index are presented in Table 3.4. The overall specific growth rate of haddock on all experimental diets was relatively high and these diets were well utilized by haddock. At the end of the 9-week experimental period, fish fed 0.42 % P showed the lowest weight gain (294.4 %) and the highest weight gain (443.3 %) was observed in haddock fed the diet containing 1.02 % P. The specific growth rate (SGR) of fish showed a significant increase with the increase in dietary P concentration from 0.42 to 0.82 % and decreased when the P content of the diet was 1.22 %. There was no significant difference in SGR or feed conversion ratio (FCR) of fish fed diets containing either 0.82 % or 1.02 % P. Fish fed either low (0.42 and 0.62 %) or high (1.22 %) P diets showed significantly higher FCR than the other two diets. During the 12 weeks, the average hepatosomatic index (HSI) of haddock increased from 5.65 to 10.6 %. The lowest HSI (9.1 %) was found in the group fed 1.22 % P and the fish given 0.62 % P showed the

Table 3.4. Growth[1], feed conversion[1], length[1], hepatomatic index[2] and proximate body composition[2] of haddock fingerlings fed five different concentration of phosphorus in the basal diet.

	% Phosphorus in diets					
	0.42	0.62	0.82	1.02	1.22	± SE
Performance of diet						
Initial weight (g)	4.3[a]	4.3[a]	4.3[a]	4.3[a]	4.3[a]	0.01
Final weight (g)	17.0[d]	19.9[c]	22.9[a]	23.2[a]	21.4[b]	0.43
Weight gain (%)	294.4	367.4	438	443.3	401.4	
SGR[3] (%)	2.2[d]	2.5[c]	2.7[a]	2.7[a]	2.6[b]	0.03
Final fish length (cm)	12.1[b]	13.2[a]	13.6[a]	14.0[a]	13.1[a]	0.81
Hepatosomatic index (HSI %)	10.8[b]	12.6[a]	10.5[b]	9.8[b]	9.1[c]	0.34
FCR[4] (as fresh basis)	0.77[a]	0.77[a]	0.68[b]	0.67[b]	0.74[a]	0.02
FCR (as dry basis)	2.8	2.8	2.5	1.8	2.7	
Proximate body composition (% dry matter basis) of the whole bodies						
% P in diets	Initial	0.42	0.62	0.82	1.02	1.22	± SE
Moisture	78.1	77.1[c]	79.1[a]	77.6[bc]	76.4[d]	72.8[e]	0.2
Lipid	4.9	6.3[b]	5.4[d]	5.3[d]	5.7[c]	7.1[a]	0.1
Protein	13.9	12.9[d]	12.5[d]	13.1[c]	13.5[b]	14.9[a]	0.1
Ash	2.6	1.8[c]	2.0[c]	2.4[b]	2.9[a]	2.8[a]	0.1

Values in the same row containing different letter superscripts were significantly different ($P<0.05$).
[1]Growth, feed conversion and length data are presented after 9 week feeding period.
[2]Hepatomatic index and proximate body composition data are presented after 12 weeks feeding period. HIS = 100 X wet liver wt./ body wt.; HSI of initial fish was 5.65
[3]Specific growth rate = 100 X {In (Final wt.) – In(initial wt.)}/ Duration (days).
[4]FCR is the feed conversion ratio and measured as, FCR = dry feed intake / wet weight gain.
The data is analyzed by one-way analysis of variance (ANOVA) and pair wise comparisons were performed with Beferroni adjustment at the $P<0.01$ level to determine significant differences.

highest HSI (12.6%). Other levels of dietary P did not show a consistent response on HSI. No mortality occurred during the 12-week experimental period.

Signs of P deficiency were observed in haddock fed a diet at a level of 0.42 % P. Growth was relatively low and feed intake nearly ceased after the 6-weeks of the commencement of this study and the fish showed the signs of lethargy. Gross examination of fish showed curvature of vertebrae, particularly near the caudal region. Bone deformities were apparent in the spine and the lower part of the vertebrae when flesh was removed from the bone.

3.4.2 Body composition

The average whole body lipid content of haddock increased significantly from 4.9 to 7.1 % during the 12-week experimental period (Table 3.4). However, the moisture content showed a decrease with the increase in body lipid content during this period in fish fed the various experimental diets except for the group fed 0.62 % P diet. Body moisture content of haddock fingerlings fed 1.02 % P in diet ($P<0.05$) decreased significantly from the initial value of 78.1 to 72.8 %. A significantly higher amount of lipid was also observed in fish fed low (0.42 %) and high (1.22 %) P diets. The effect of other dietary P levels on the body lipid or moisture content did not show a consistent trend. Body lipid content ranged between 5.3-5.7 % in haddock fed diets containing 0.62 – 1.02 % P. Body protein content ranged from 12.9 –14.9 % in fish fed various experimental diets and fish fed 1.22 % P showed the highest protein content. A significantly lower amount of body ash was found in fish fed a low P diet, however, the ash content gradually increased with the increase in dietary P concentration to 1.02 % P. There was no further increase in the ash content by increasing the dietary P concentration to 1.22 %.

3.4.3 Phosphorus digestibility

Phosphorus digestibility of the two experimental diets containing 0.42 and 0.82 % P were 43.2 ± 0.86 and 70.3 ± 1.23 %, respectively. Based on these values, P digestibility of the remaining diets was calculated. The estimates of P digestibility for the remaining diets are summarized in Fig 3.1. The feed ingredients present in the basal diet (diet 1)

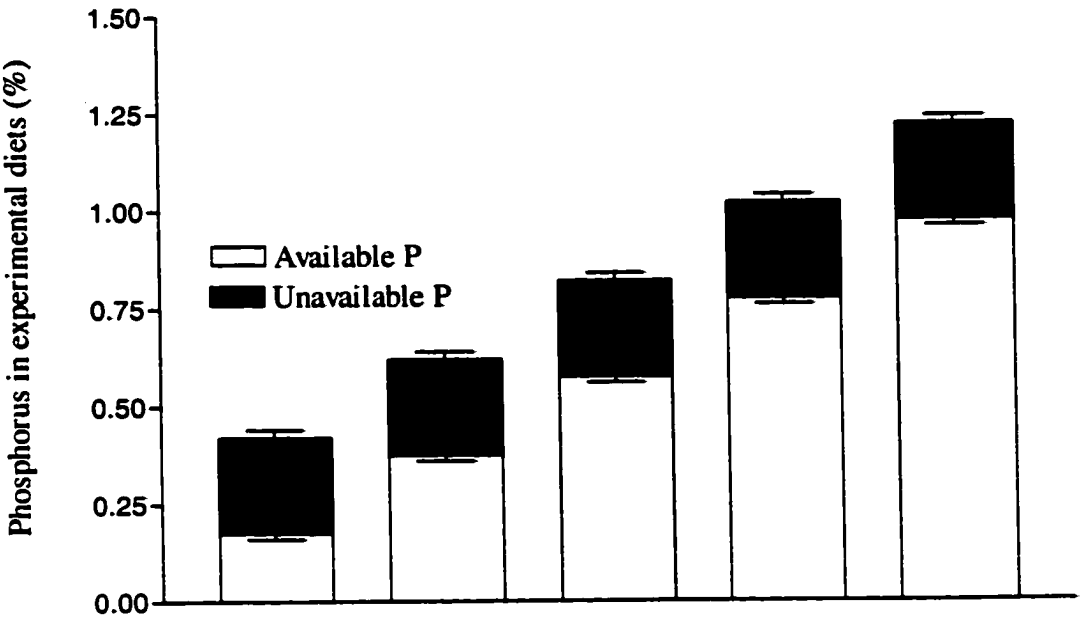

Fig. 1.1. Available and unavailable phosphorus in five experimental diets for haddock fingerlings.

supplied 0.42 % P mainly in the form of organic P, which was poorly digested (43.2 %) by haddock compared to the inorganic P supplement (calcium phosphate 99 % added to diet 3).

3.4.4 Vertebrae and opercula ash

A low amount of dietary P caused a significant ($P<0.05$) reduction in vertebrae and opercula ash content of haddock compared to other diets (Table 3.5). An increase in the dietary P concentration from 0.42 to 1.02 % resulted in a linear increase in vertebrae and opercula ash content. Further increase in dietary P content (1.22 % P) caused a significant reduction in vertebrae ash but this response was not significant in opercula ash content. The correlation co-efficient between vertebrae and opercula ash was 0.89. Vertebrae and opercula ash content data were subjected to non-linear regression analysis to determine optimum P requirement for haddock fingerlings. Five different models were tested. The mean corrected R^2 values for vertebrae ash were 0.858, 0.000, 0.489, 0.871 and 0.890 for piecewise linear, four parameter sigmoidal, simple linear, quadratic and cubic relation equation, respectively. Similarly, for opercula ash these were 0.694, 0.000, 0.694, 0.918 and 0.926 for piecewise linear, four parameter sigmoidal, simple linear, quadratic and cubic relation equation, respectively. Based on the measured R^2 we chose the quadratic model ($Y = a + bx + cx^2$; where: Y = measured ash content; a= intercept; b= co-efficient of the linear terms; c = co-efficient of the quadratic terms; x = dietary P content) with the best fit in both cases since it has a comparable R^2 to that of the cubic model, but provided a simpler description of the data. The quadratic analysis for vertebrae ash indicated that the dietary P requirement of haddock fingerling is 0.96 % total P of diet and 0.72 % available P. However, the dietary requirement based on opercula ash content was estimated as 1.03 % total dietary P or 0.79 % available P. In terms of per unit energy of diet, the haddock P requirement based on vertebrae ash content was 0.34 g available P/ MJ digestible energy.

3.4.5 Serum and urine phosphate concentration

Serum phosphate level significantly ($P<0.05$) increased from 0.4 to 1.5 $mmol^{-1}$ as the P content of the experimental diets increased from 0.42 to 1.02 %. However, a further

Table 3.5. Vertebrae and opercula ash (% of dry matter basis); serum phosphate concentration and urinary phosphate excretion of haddock fingerling fed diets containing five different levels of phosphorus for 12 weeks.

	% Phosphorus in diets					
	0.42	0.62	0.82	1.02	1.22	±SE
Vertebrae ash (%)	44.5^{e}	50.6^{d}	54.3^{b}	56.6^{a}	52.2^{c}	0.47
Opercula ash (%)	31.4^{e}	42.3^{d}	45.9^{bc}	48.2^{a}	47.2^{ab}	0.56
Serum PO_4^{-3} (m.mole/L)	0.4^{d}	0.8^{c}	1.2^{b}	1.5^{a}	1.5^{a}	0.03
Urinary PO_4^{-3} (m.mole/L)	0.1^{d}	0.3^{d}	2.5^{c}	4.3^{b}	7.9^{a}	0.20

Values in the same row containing different letter superscripts were significantly different with Bonferroni adjustment at the ($P<0.0125$).

Table 3.6. Mineral concentration (in dry matter) in vertebrae[1] of haddock fingerlings fed various concentration of dietary phosphorus for 12 weeks.

	% Phosphorus in diets						
	Initial	0.42	0.62	0.82	1.02	1.22	±SE
Ca (%)	14.35	15.3^{c}	16.2^{b}	16.3^{b}	17.6^{a}	17.8^{a}	0.13
Cr (μg/g)	2.8	3.7^{a}	3.4^{a}	2.6^{b}	2.8^{b}	3.1^{a}	0.04
Co (μg/g)	0.21	0.32^{a}	0.32^{a}	0.11^{c}	0.25^{b}	0.24^{b}	0.01
Cu (μg/g)	1.47	3.7^{a}	1.5^{b}	0.9^{c}	1.1^{b}	1.7^{b}	0.21
Fe (mg/g)	0.18	0.32^{a}	0.29^{a}	0.13^{c}	0.14^{c}	0.21^{b}	0.02
Mg (mg/g)	18.42	22.9^{a}	18.4^{b}	15.4^{c}	20.2^{a}	21.3^{a}	1.2
Mn (mg/g)	1.12	1.71^{a}	1.61^{a}	1.51^{a}	0.97^{b}	1.03^{b}	0.06
P (%)	5.53	6.82^{b}	7.51^{b}	8.03^{a}	8.71^{a}	9.33^{a}	0.51
K (mg/g)	0.82	1.72^{a}	1.69^{a}	1.08^{b}	1.68^{a}	1.90^{a}	0.14
Na (mg/g)	0.59	1.93^{a}	1.92^{a}	1.31^{b}	1.74^{a}	1.73^{a}	0.11
S (mg/g)	1.84	3.90^{a}	3.29^{a}	3.03^{a}	2.63^{b}	3.11^{a}	0.32
Zn (mg/g)	0.15	0.22^{a}	0.18^{b}	0.17^{c}	0.09^{d}	0.08^{d}	0.03

Values in the same row containing different letter superscripts were significantly different ($P<0.004$).
[1]The vertebrae of three fish were pooled together. The values are means ± SEM of three pooled samples of nine fish

increase in dietary P content did not affect the serum phosphate level. Urine phosphate data showed a linear (R^2=0.99, P<0.001) increase with the increase in dietary P intake. Phosphate level in urine ranged from 0.1 to 7.9 $mmol^{-1}$ in fish fed diets containing 0.42 and 1.22 % P, respectively.

3.4.6 Mineral concentration

Dietary P levels significantly affected the vertebrae mineral concentrations of haddock (Table 3.6). Calcium and P content of vertebrae increased significantly from 6.82 to 9.33 % and 15.3 to 17.8 %, respectively, as the P level of the diet increased from 0.42 to 1.22 %. Although the vertebrae ash, calcium and P content increased with dietary P supplementation, the calcium to P ratio in vertebrae remained close to 2:1 in fish sampled from all the experimental diets. Magnesium content of vertebrae showed a significant decrease when P content of the experimental diet increased from 0.42 to 0.82 %. However, no such decrease was observed in fish fed higher levels of dietary P. Vertebrae sodium, potassium and sulfur concentration were not significantly influenced by the increase in dietary P content. Zinc content of vertebrae decreased significantly with the increase in P content of the experimental diets. Iron content also decreased significantly with the increase in P content of the experimental diets up to 1.02% P. Copper content decreased in fish fed diet measure from 0.82% P. There was no clear effect of dietary P levels on vertebrae chromium concentration.

3.5 Discussion

The experimental basal diet promoted relatively good growth and the gross signs of P deficiency appeared within 12 weeks of the feeding study. A wide variety of P basal diets have been used for P requirement studies to supply low levels of this element from plant and animal protein supplements such as soybean meal (Ketola, 1975), casein-gelatin (Lall and Bishop, 1977), fibrin (Lovell, 1978), egg albumin (Watanabe et al., 1980), fish muscle (Brown et al., 1993) or a mixture of wheat gluten and free amino acids (Rodehutscord, 1996). A combination of several protein supplements was selected on the basis of a previous study conducted in our laboratory on Atlantic salmon (Vielma and Lall, 1998) to ensure optimum food consumption. After six weeks of normal feed intake,

haddock started to show a decrease in feed utilization due to dietary P deficiency. Phosphorus supplementation of the basal diet with a highly digestible mono-calcium phosphate in diets significantly improved growth and feed efficiency of haddock fingerlings. Lower growth and poor feed utilization due to dietary P deficiency have also been observed in rainbow trout (Ketola and Richmond, 1994) sunshine bass (Brown et al., 1993) channel catfish (Wilson et al., 1982) common carp (Ogino and Takeda, 1978) and red sea bream (Sakamoto and Yone, 1978). However, no significant changes in growth of sea bass (Chaimongkol and Boonyaratpalin, 2001) and Atlantic salmon (Vilema and Lall, 1998) fed low P diets have been observed. The requirement of essential elements affects feed efficiency (Shearer, 1995). Haddock fed diets containing 0.42 and 0.62 % P showed poor feed conversion. A significant improvement was observed when dietary P concentration was 0.82 and 1.02 % and the further increase causes a rise in FCR. Studies conducted on terrestrial vertebrates also show that growth and feed efficiency were not always affected by dietary P deficiency (Nelson and Walker, 1964; Ketaren et al., 1993). Several factors including age, stage of development, diet composition, duration of experiment, health and rearing condition may affect growth and feed utilization. Generally, young animals are more sensitive to nutrient deficiency than those at a later stage of development because during the rapid growth period dietary energy is utilized more efficiently. Redlip mullet fry (initial weight, 3.8 g) showed a clearer growth response to dietary P supplementation than large fish (initial weight, 26.5 g) under the same experimental conditions (Elzibdeh et al., 1995). Small fish were also more sensitive to P deficiency.

Dietary P levels had a significant effect on haddock bone ash content including vertebrae and opercula. Bone ash content is considered to be the most sensitive criterium for P utilization in terrestrial vertebrates (Nelson and Walker, 1964; Ketaren et al., 1993; Ravindran et al., 1995), and freshwater (Ketola, 1975; Watanabe et al., 1980; Ketola and Richmond, 1994; Rodehutscord 1996; Åsgård and Shearer 1997; Baeverfjord et al., 1998; Jahan et al., 2001) and marine (Sakamoto and Yone, 1978; Dougall et al., 1996; Borlongan and Satoh, 2001) fish. A close correlation was observed between vertebrae and opercula ash content, which indicates that vertebrae and opercula ash values could be

used to determine the efficiency of dietary P utilization and to estimate the P requirement of haddock.

On the basis of vertebrae and opercula ash content, the estimated dietary P requirement of haddock was 0.96% of the diet or 0.72% of digestible P or 0.34mg digestible P/MJ digestible energy kg^{-1}. Little variation was found between the requirement estimates based on vertebrae and opercula ash. The results were 91% similar and the best fit of data was with the quadratic equation. The estimated P requirement value was higher than reported values (0.5 to 0.8 % of diet) for rainbow trout, Atlantic salmon, chum salmon, carp and red sea bream (reviewed by Lall, 2002), but it was relatively close to the P requirement of tilapia (0.9 %) determined by Watanabe et al. (1980). Interestingly, both tilapia and haddock have acellular bone, whereas salmonids and cyprinids have cellular bone. Some differences in the estimated P requirement of various fish species may be due to the following factors: 1) species differences and variation in intestinal P absorption rate (Riche and Brown, 1999; Avila et al., 2000), 2) differences in the bioavailability of various inorganic and organic P sources (Satoh et al., 2002; Lall and Vielma, 2001), 3) fish size, condition factor and the stage of development (Shearer, 1984; El-Zibdeh et al, 1995; Rønsholdt, 1995), 4) dietary energy content and feed efficiency (Shearer, 1995), and 5) differences in experimental design (Shearer, 2000). Åsgård and Shearer (1997) reported that juvenile Atlantic salmon (1-5 g) required 0.8-0.9 % available P per kg diet when feed efficiency was 1.45, a value much higher than in other reports (Lall and Bishop, 1977; Ketola and Richmond, 1994; Vielma and Lall, 1998). The minimum dietary P requirement for rainbow trout has also ranged from 0.37 % (Rodehutscord and Pfeffer, 1995) to 0.8 % (Ogino and Takeda, 1978). The results of an extensive study on P requirement and metabolism in Atlantic salmon by Vielma and Lall (1998) indicated that available P per MJ of digestible energy content of the diet gave a better estimate of the P requirement of fish. When the quantitative P requirements were reported as available P per MJ available energy, these values were relatively close for two salmonid fish species; 0.25 for rainbow trout (Rodehutscord, 1996) and 0.28 for Atlantic salmon (Vilema and Lall, 1998).

Although plasma phosphate levels showed the same response as vertebrae ash content of fish fed different levels of dietary P, the concentration of plasma or serum phosphate is known to be influenced by several dietary and physiological factors (Cross et al., 1990). It represents P metabolized from various tissues as well as P absorbed from dietary sources. Rodehutscord (1996) observed a wide range in reported plasma phosphate concentration due to the different range of time lapsed after feeding when blood samples were collected. Our initial work was conducted to establish a reasonable time period for blood sample collection after feeding haddock and produced consistent results for this study on plasma phosphate measurements. The level of plasma phosphate significantly ($P<0.05$) increased from 0.4 mmol/L to 1.5 mmol/L as the P content of the experimental diets increased from 0.42 to 1.02 %; however, a further increase in dietary P content did not affect the serum phosphate level. The increase in plasma phosphate concentration also caused an increase in urinary P excretion. Urine phosphate data showed a linear ($R^2=0.99$, $P<0.001$) increase with the increase in dietary P intake. Unlike plasma phosphate concentration, urine phosphate level continued to increase with the dietary P concentration above 1.02 % of the diet and it did not reach a plateau with a high P diet (1.22 %). Additional research is needed on P homeostasis before recommendations could be made regarding the use of plasma phosphate as an indicator of P utilization or requirement.

Gross signs of P deficiency in haddock fed low P diets included poor growth, a compact body due to curvature of vertebrae, and sluggish movement. X-ray also confirmed bone deformities. Similar signs of P deficiency including bone deformities have been also reported in salmon (Watanabe et al., 1980), carp and rainbow trout (Ogino and Takeda, 1976; Ogino and Takeda, 1978 and Ogino et al., 1979) fed low P diets. However, the severity of P deficiency may vary with the duration of the feeding study, dietary factors (concentration , bioavailability and nutrient interactions etc.) species differences and mineral content of water, water temperature and rearing system (e.g. flow through or re-circulation). Redlip mullet (El-Zibdeh et al., 1995), milk fish (Borlongan and Satoh, 2001), carp (Ogino et al., 1979) and chum salmon (Watanabe et al., 1980) show deficiency signs when P levels are slightly below their requirement level;

however, rainbow trout (Rodehutscord, 1996), American cichlid (Chavez-Sanchez et al., 2000) and Gilthead sea bream (Pimentel -Ridrigves and Oliva-Tales, 2001) exhibited deficiency signs when the P level in the test diet was 20-30 % below their dietary requirement. In the present study, deficiency appeared within a short period because the P deficient diet contained approximately 50 % of the requirement.

Vertebrae and opercula ash levels were a good indicator of P deficiency and bone mineralization; however, the magnitude of decrease in the opercula ash was more pronounced. The opercula ash decreased from 48.2 to 31.4 % within the 12 week feeding study. Bone deformities did not appear in fish fed the diet containing 0.62 % P when vertebrae ash level dropped from 56.6 to 50.6 % and opercula ash decreased by 42.3 %. The duration of the experiment was probably not sufficiently long to deplete tissue P concentration, in order to produce P deficiency signs. Plasma and urine phosphate concentrations were significantly lower in fish fed a low P diet, indicating that effective biochemical mechanisms conserve body P when P intake is marginal to meet the metabolic needs of fish.

Fish growth, vertebrae ash and zinc content decreased significantly when P content of the diet exceeded the requirement of this element. Sakamoto and Yone (1978) observed a decrease in ash and plasma P content of red sea bream when dietary P level was 1.36 %. Formulation of high P experimental diets required additional amounts of monocalcium phosphate in the diet, which also increased the Ca content of the diet. Marine fish have an unlimited Ca supply from their environment and the excess amount of this element from dietary supplements or absorbed by drinking seawater may have caused physiological stress and affected the Ca metabolism in haddock. Marine fish regulate extracellular Ca by hypocalcaemia mechanisms. Stanniocalcin is considered the main hypocalcemic hormone in fish. It decreases the intestinal Ca uptake in Atlantic cod (Sundell et al., 1992) and reduces the Ca uptake through blocking of Ca channels in gills (Janz, 2000). The circulating level of stanniocalcin was reported to be three-fold higher in seawater than in freshwater adapted Atlantic salmon (Mayer-Gostan et al., 1992). A significant increase in urinary P excretion was observed in fish fed an excess amount of

P, the excretion of Ca was not measured. However, excess P in the diet had no significant effect on body Ca content. Additional studies are needed to explain the physiological mechanisms involved in absorption, excretion and metabolism of Ca and P in marine fish, including the hormones involved in these processes.

Calcium is also considered an important element for P utilization since Ca-binding protein is a carrier for both Ca and P from the intestine (Brody, 1994). Most fish maintain a constant ratio of Ca:P in bone as well as serum (NRC, 1993). An imbalance caused by changes in the level of either of these elements also affects the bioavailability of other minerals in terrestrial vertebrates (Bowen, 1966) and fish (Shearer et al., 1994). Excess P supplementation in rainbow trout and Atlantic salmon diets reduced the zinc and manganese utilization (Hardy and Shearer, 1985; Satoh et al., 1996; Vielma and Lall 1998) and caused cataracts in rainbow trout due to zinc deficiency (Ketola, 1979). A high P diet may chelate zinc and other trace elements and reduce their absorption and metabolism in fish (Lall, 2002). Zinc is an important trace mineral for the activation of metalloenzymes and is involved in the development of cartilage and bone in terrestrial vertebrates (Baker and Ammerman,1995) and it reduces bone mineralization in fish (Satoh et al., 1996).

A significant increase in calcium and P content of vertebrae with the increase in dietary P level was observed; however, their ratio and amount was not significantly affected when expressed as percentage of total minerals present in vertebrae ash. This indicates that bone matrix was either demineralized or under-mineralized. Bone matrix is an organic constituent and mainly composed of collagen, where inorganic minerals are deposited during the mineralization process as hydroxylated polymers of calcium phosphate $\{Ca_{10}(PO4)_6(OH)_2\}$ (Simkiss and Wilbur, 1989; Clark, 1955). Therefore, the estimation of mineral concentration as a percent of bone ash does not provide the true composition of a bone. A positive correlation between bone P and dietary P was found in rainbow trout (Ogini & Takeda, 1978), red sea bream (Sakamoto & Yone, 1978), chum salmon (Watanabe et al., 1980) sunshine bass (Brown et al., 1993), mirror carp (Schafer et al., 1995), Atlantic salmon (Baeverfjord et al., 1998), and American cichlids (Chavez-

sanchez et al., 2000). Further, a positive correlation between bone ash and dietary P level has been also reported in salmonids (Ketola, 1975; Åsgård and Shearer, 1998). A decrease in concentration of magnesium, zinc, iron and other cations in vertebrae with an increase in dietary P content may be caused by competitive inhibition of these cations during intestinal absorption. In fish, information on the effect of dietary P on the bioavailability of other elements is limited. High dietary P also decreased magnesium and zinc levels in common carp vertebrae (Satoh, et al. 1987a, 1987b) and Atlantic salmon (Vielma and Lall, 1998), and reduced body zinc content in rainbow trout (Hardy and Shearer, 1985).

An increase in the dietary P level caused a decrease in the whole body lipid and moisture content but increased the protein content. A similar relationship between dietary P and lipid was reported in common carp (Takeuchi and Nakazoe, 1980; Murakami, 1970) and red sea bream (Sakamoto and Yone, 1978). These authors speculated that inhibition of β-oxidation of fatty acids might have caused higher accumulation of body lipid. It is possible that uptake of extra-mitochondrial fatty acids into mitochondria involves an enzyme ATP-driven esterification of the free fatty acid with extra-mitochondrial CoA to yield fatty acyl-CoA. This step utilizes two high-energy bonds of ATP to activate one molecule of fatty acid. Lack of or insufficient inorganic phosphate may have inhibited this process, resulting in a lower utilization of lipid as an energy source. Higher HSI and lower protein in the fish fed a low P diet may explain the fact that haddock utilized protein for energy purposes as an alternative to lipid (lipid sparing effect). However, the role of dietary P in lipid utilization by haddock or any other marine fish has not been investigated.

Only 0.18 % P was available for haddock utilization in the basal diet and 0.24 % P was not utilized in every diet. Therefore, depending on growth data it appeared that haddock fingerlings might not show deficiency signs unless the level of P in the diet dropped below $0.82 - 0.24 = 0.58$ %. The highest growth and feed efficiency of haddock fingerlings indicated that a minimum of 0.58 % bioavailable P was required for optimum growth of haddock fingerlings.

Approximately 85 % of total dietary P in the low P basal diet was supplied by casein and corn gluten meal and 15% from fish meal. Phosphorus digestibility of this basal diet was 43.4 %, indicating that organic P present in feed ingredients was poorly utilized by haddock. A similar digestibility value has also been reported for rainbow trout fed a basal diet based on soybean meal and fish meal (Riche and Brown, 1999). Corn gluten meal contains phytic acid (1.37 %) and approximately 9.6 % P is available to rainbow trout (Satoh et al., 2002). Apparent P digestibility of casein in a basal diet used for rainbow trout was 87.1 % (Sugiura et al., 1999). Based on these values obtained for rainbow trout, it is estimated that in the basal diet used for haddock, 36 % digestible P was supplied by casein and the remaining 7.2% by fish meal. The high P digestibility value (99 %) of monocalcium phosphate obtained in this study is in agreement with the values reported for rainbow trout (Ogino and Takeda, 1978), common carp, (Ogino et al., 1979) channel catfish (Lovell, 1977) and Atlantic salmon (Lall, 1991).

It is obvious from these results that P is essential for growth, efficient feed utilization and bone mineralization of haddock. Excess P not only causes excessive excretion of this element but it has a negative effect on bone mineralization. Phosphorus requirements of haddock (0.96 % of diet or 0.72 % digestible P or 0.34 g P/MJ digestible energy kg^{-1}) were higher than in other fish species. Although gross deficiency signs have been characterized, there is a need for histological characterization of bone deformities associated with dietary P deficiency in haddock. Additional studies on biochemical mechanisms, particularly the role of hormones in Ca and P metabolism, may provide some clues to minimize P excretion in natural waters from aquaculture operations and also improve the retention of this element.

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CHAPTER 4

A cannulation technique for urine collection in haddock (*Melanogrammus aeglefinus* L.)

4.1 Abstract

Urine collection from fish is an integral part of metabolic studies designed to measure the excretion of various biochemical compounds. The present study was conducted to develop a cannulation technique for urine collection from haddock. Due to the anatomical differences between salmon and haddock, the procedure developed for salmonids could not be adopted for haddock. Juvenile haddock of three different size groups (123 ± 4.25, 324 ± 4.23 and 543 ± 5.32 g) were cannulated. The haddock urinary system consisted of a dorsally elongated single long canal originating from the dorsal kidney. The urinary canal originated with very little dilate organ and terminated directly into the uropore behind the anus without any pronounced urinary bladder. Just before the termination it had a U turn. The catheter was inserted in the direction of the tail to follow the U-shaped pocket while gently rotating from side to side until the catheter passed into the sphincter muscle. After insertion of the cannula into the urinary duct, the external segment of tubing was connected to a needle of a 100 μL Beckman measuring syringe. This technique was applied to twenty hatchery-produced haddock (346.23 ± 4.56 g). The reproducibility of urinary system and urine collection data indicates that the method is suitable for urine collection from haddock. Problems with other techniques for cannulation in haddock are also summarized

4.2 Introduction

The collection of urine from fish is important in studies designed to estimate the concentration of excretory products that originate from absorption of nutrients and other compounds from food or through metabolic transformation of nutrient reserves in the body. The use of cannulae to sample urine and blood from salmonids has been widely practiced (Iwama and Ishimatsu, 1994; Vielma and Lall, 1998). Proper timing for urine collection, along with proper handling of fish and obtaining the quantity of urine required

to allow for detection of compounds in urine without any contamination are all important factors that affect the results of urine studies. The cannulation for urine collection in fish is relatively complex because of the wide anatomical differences in the urinary system between marine and freshwater species. Therefore, the correct diameter of the catheter is essential. The anatomical differences in fish kidneys may also reflect the nature of their habitat (Hentschel et al., 2000) and differences in osmoregulation of various ions (Hentschel and Egler, 1989). A successful cannulation depends upon a through knowledge of the anatomy of the excretory system. Also the dose of the anesthetic must be within tolerance limits so that the fish has minimum stress on renal function. The timing of the urine collection to obtain a sufficient volume of urine is also important.

In studies designed to investigate P nutrition of haddock, several attempts to collect urine from haddock using the spot sampling technique developed for rainbow trout by Wood and Patrick (1994) were not successful. Haddock urinary bladder is not distinct well defined like salmonid fishes. Therefore, haddock have a limited capacity to store urine in the bladder The divided chamber method devised by Smith (1929) was not useful for cannulation because of the confinement stress and difficulty of measuring certain physiological parameters e.g. urine pH, urine flow and P composition. The anatomical differences compared to salmonids in the urine excretory system require modification of the cannulation technique. The aim of the present study was to develop a cannulation technique for haddock urine collection. The anatomy of the haddock urinary system, technique for urine collection and reproducibility of results obtained by this method are described.

4.3 Materials and methods

4.3.1 Description of the urinary system of haddock

Juvenile haddock of three different sizes (123 ± 4.25, 324 ± 4.23 and 543 ± 5.32 g; n=3) were used for describing the anatomy of the renal excretory system. Fish were anesthetized with TMS (tricaine methanesulfonate) and transferred to a V-shaped wooden trough with a foam embedded surgical table such that the ventral side of the fish

faced upward. In order to assess the anatomy of the urinary system cotton-blue stain was injected into the urethra. Fishes were carefully dissected in the uropore to display the urethra through to the kidney. The passage of the stain through the organs, including the urinary bladder, urethra and kidney, helped to define the anatomy of the urinary excretion system. The location of the two kidneys is fairly different. It appeared that the collecting duct of the kidney proceeded through the dorsal side of the fish and confluent as long as behind the urinary opening and took an U-shaped turn which emptied into a separate expanded, distensible and contractile pocket, called urinary bladder.

4.3.2 Cannulation

Three different sizes of fish described above (n =6) were investigated for cannulation. Two hours after feeding, three fish of each size were individually anesthetized by clove oil in ethanol or with TMS. The anesthetized fish was transferred to a V-shaped wooden trough (foam embedded surgical table) such that the ventral side of the fish was facing upwards. Attempts were made to insert three different sizes of catheter PE-25, 50 and 60. The end of the catheter was heated prior to insertion to reduce the sharpness. The catheter was inserted in the direction of the tail to follow the U-shaped pocket while gently rotating from side to side until the catheter passed into the sphincter muscle. After insertion of the cannula into the urinary duct, the external segment of the tubing was connected to a needle of a 100 μL Beckman measuring syringe. The external segment of the tubing was anchored to the base of two anal fin rays near the anterior end of the anal fin edge. It was fixed with a loop of thread passed with a straight needle and then tied off with a square knot. A second such anchor was made near the posterior edge of the anal fin. Urine was collected by aspiration with the syringe assisted with gentle pressure extended to the abdomen. The volume of the urine was measured and it was stored at room temperature in a 1 ml vial until analysis.

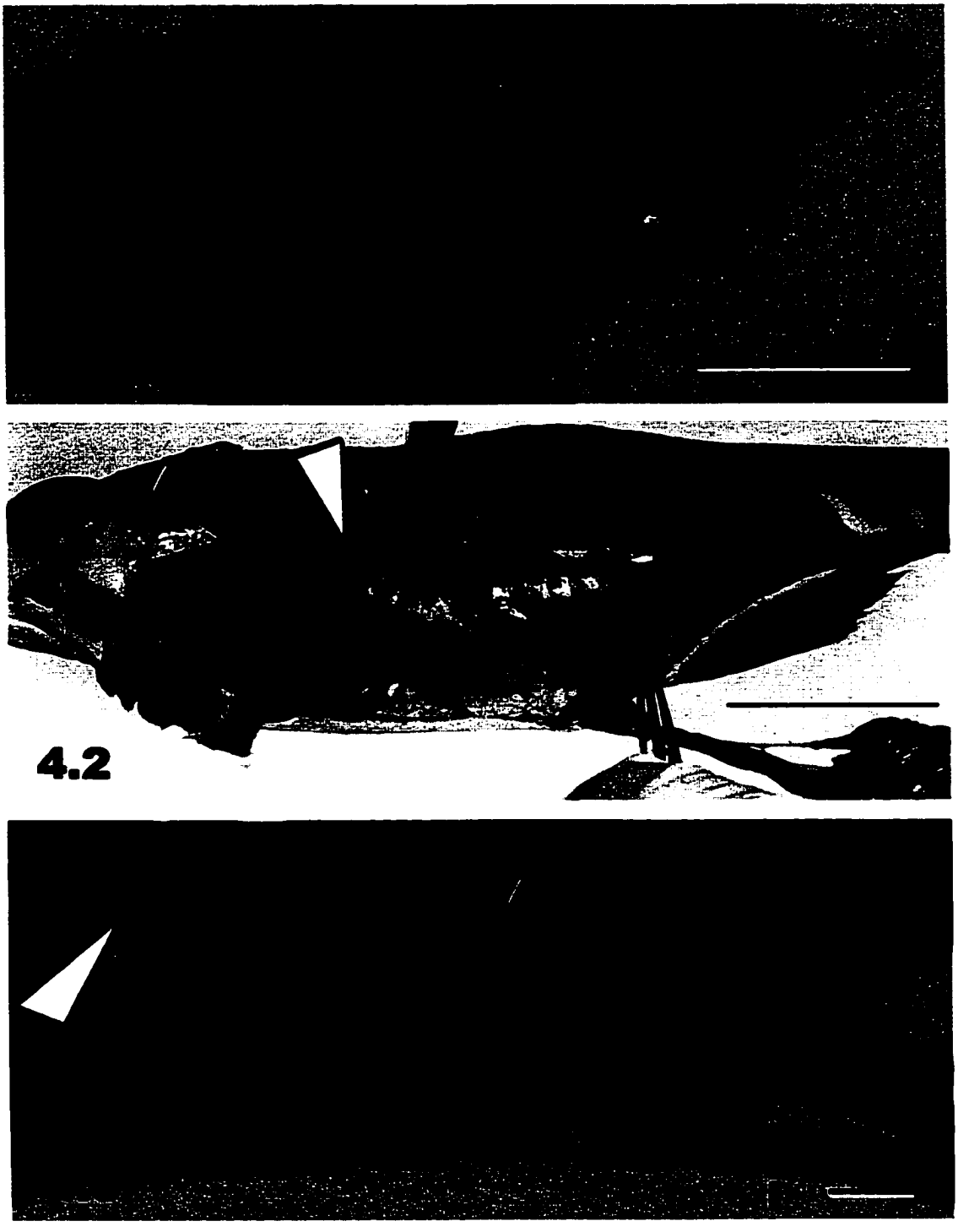
Similarly, twenty haddock (346.23 $\pm$ 4.56 g) were cannulated for urine collection. The urine volumes were measured. The phosphate concentrations of the collected urine were measured according to Taussky and Shorr (1953). The data were analyzed by one sample t-test using the Prism, version 3.0 (Graphpad software, San Diego, CA).

4.4 Results and Discussion

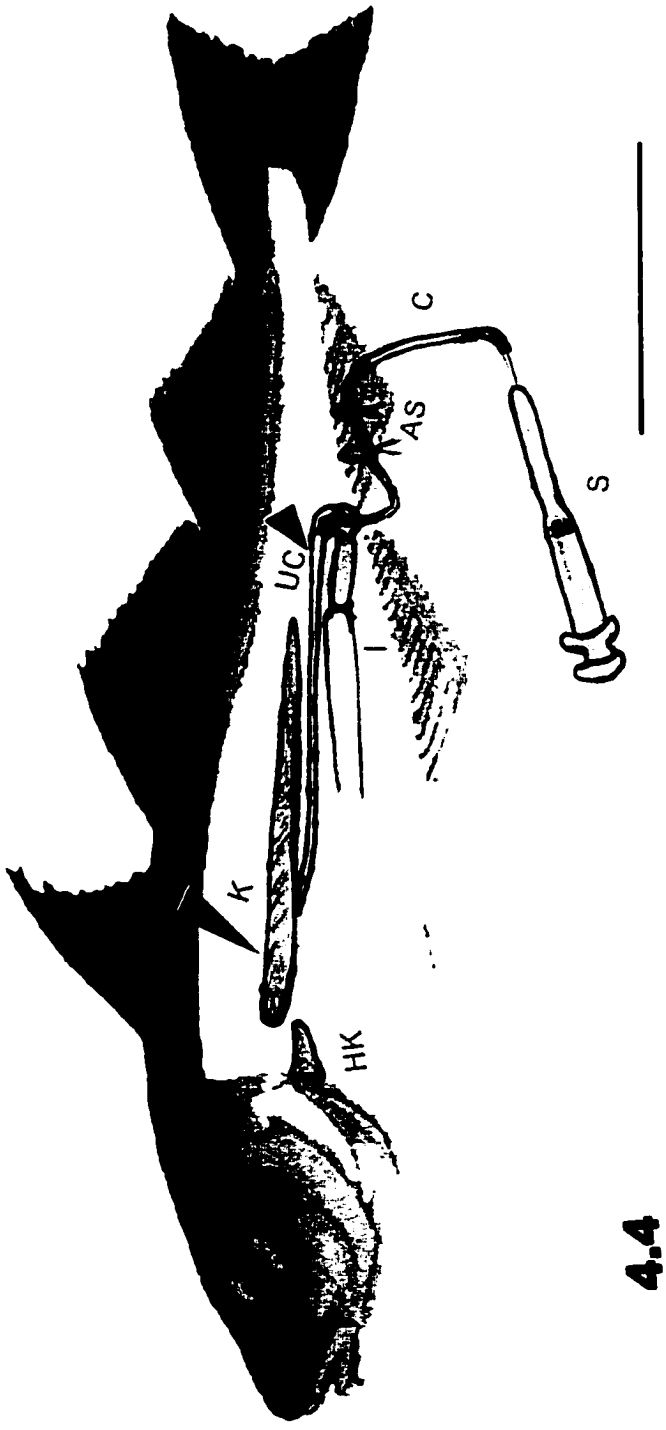
The anatomy of the urinary system visualized after the dissection is illustrated in Figures 4.1 to 4.3. A close examination of the urinary tract enabled the custom-fit of the catheter and avoided catheter insertion errors. The placement of the catheter during the cannulation is shown in Figure 4.4. The haddock urinary system consisted of a dorsal elongated single long narrow canal that originated from the kidney and slightly enlarged at the end terminating directly into the uropore behind the anus. The tract lacked any pronounced urinary bladder as seen with salmonid. Just before the termination it has a U turn. The quantity of urine and P concentration are presented in Figures 4.5 and 4.6, respectively. Mean urine volume and urine phosphate concentration were 34.85 ± 0.98 μL and 4.15 ± 0.28 $mmol^{-L}$, respectively. The data of urine quantity and urine phosphate concentration follow approximately Gaussian distribution and no data transformation was needed. The recurrence of anatomy and data suggested strong applicability of the method. The results were reproducible and consistent. The modified method effectively worked since the standard error of the data was not large.

Collection of urine from fish is difficult because of their continuous free-swimming behavior. Urine collection from fish has been practiced on both live and dead animals (Hentschel et al., 2000). Haddock are very sensitive to handling stress. The anchoring of a collecting bag recommended by many authors (Smith, 1929; Goldstein et al., 1964; Wood and Patrick, 1994) was not possible. Alternatively, a plugged cannula on the free swimming haddock with periodic draining of urine was also not applicable because the quantity of urine collected was very small. Also urine leaked into the water from the cannula and caused high methodological error. The physiological chamber method (Smith, 1929) was not suitable for haddock because the fish stopped feeding when placed in confinement and suffered from stress induced by the chamber. Moreover,

urine could be expected. The very narrow urinary canal in the haddock which terminated to the uropore which lacked a urinary bladder along with the complexity of the U shape before termination required modification of the spot sampling method for successful cannulation haddock. To some extent, the design of present method is similar to the naturally vented urine collection used for toadfish, *Opsanus beta* (Wood et al., 1995)



4.2



4.4

Fig. 1. Lateral view of a dissected male haddock (123 g) with internal features displayed: urinary system with other organs. (bar = 3.5 cm).

Fig. 2. Lateral view of a dissected haddock (324 g) urinary system, showing the location of kidney (white arrowhead), head kidney (black arrowhead), urinary canal and urinary opening. (bar = 3.5 cm).

Fig.3. Higher magnification of the site of the uropore where urinary bladder (black arrowhead) has terminated without any pronounced dilated bladder. White arrowhead indicating urinary canal (bar = 400 mm).

Fig. 4. Schematic drawing of cannulation technique for urine collection in haddock. weight = 543 g). (bar = 10 cm).

Abbreviations

AS	Anchoring system
C	Catheter
I	Intestine
HK	Head kidney
K	Kidney
S	Syringe
UB	Urinary bladder
UC	Urinary canal
UP	Uropore

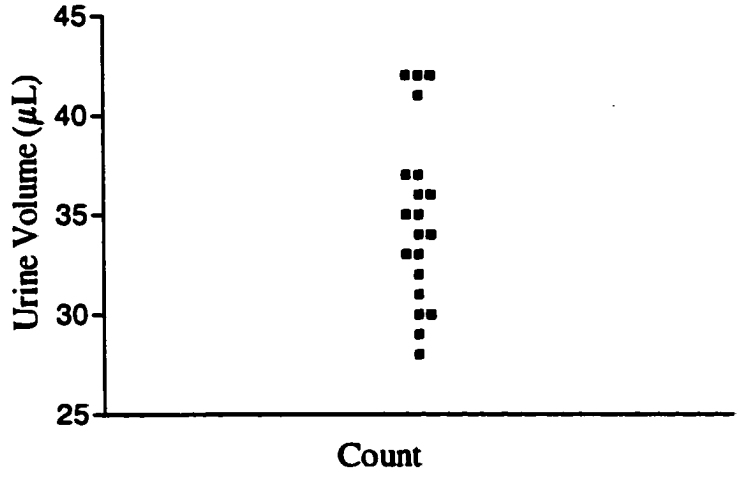


Fig.4.5. Urine quantity collected and its distribution. Normality test P>0.10. and passed with nonsignificant summary. One sample t = 0.154 with 19 df and 95% Cl of discripency -2.19 to 1.89.

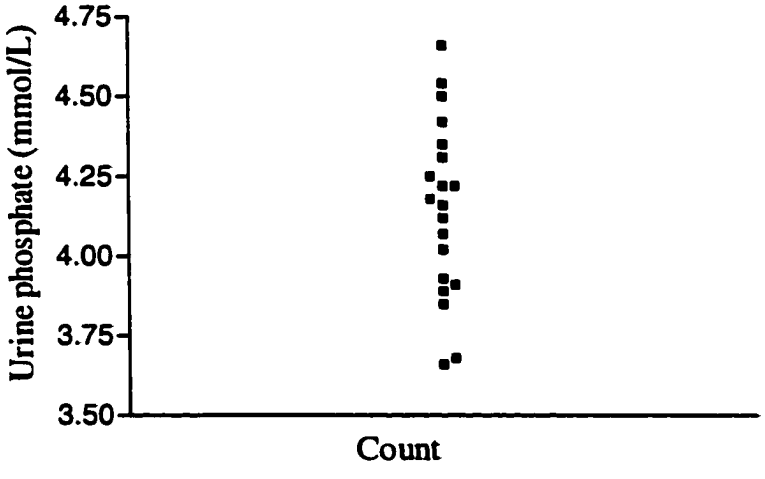


Fig.4.6. Urine phosphate and its distribution. Normality test P>0.10. and passed with nonsignificant summary. One sample t = 0.05 with 19 df and 95% Cl of discripency ± 0.13.

dilution and contamination was unavoidable which leads to error in the results. Urinary bladder cannulation techniques developed for most teleosts have been successful where the collecting duct from each side of the kidney empty into ureters. The urinary bladder cannulation of spot sampling technique developed by Wood and Patrick (1994) has been readily applied when the urinary bladder was more prominent and a sufficient quantity of because in both systems the catheter is heat smoothed and attached to a syringe. The present technique has overcome these problems and is suited for handling very small quantities of urine. The degree of reproducibility of the technique depends upon the desired tightness of the catheter in the uropore. Selection of correct size catheter was very important. Care must also be taken to remove air bubbles from the catheter as they will impede urine flow and are difficult to remove once the catheter is in place.

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CHAPTER 5

Urinary phosphorus excretion in haddock, (*Melanogrammus aeglefinus* L.) and Atlantic salmon (*Salmo salar*).

5.1 Abstract

Urinary and fecal phosphorus (P) excretions generate soluble waste in fish farms and their removal from farm effluents is a major concern. A study was conducted to compare the differences in urinary P excretion in haddock and Atlantic salmon. Twenty haddock and salmon were fed a commercial haddock feed for 3 weeks. Urine was collected 4 times a day (0h, 3h, 6h and 17h after feeding). The following parameters were measured: volume, phosphate concentration and pH of urine, and amount of food in the gut. The results were expressed on a per kg body weight (BW) basis. Haddock and Atlantic salmon excreted 83.0 ± 5.18 μL and 243.8 ± 10.51 μL^{-kg} body weight (BW) urine respectively. Urine phosphate concentration of haddock and salmon was 4.6 ± 0.08 and 1.0 ± 0.08 $mmol^{-L}$ and urine pH was 6.2 ± 0.25 and 7.5 ± 0.01 respectively. The maximum urine volume was observed after 3h post-feeding in haddock and 6 h in Atlantic salmon. The relationship between haddock urine collection time and the gut content data was best fitted by the quadratic equation: Haddock urine rate = 155.6 – 4.68 (gut content) + 0.049 (gut content)^2. In order to test the reproducibility of results, the experiment was repeated and similar results were obtained in each trial.

5.2 Introduction

Phosphorus is one of the six principal elements (carbon, nitrogen, hydrogen, oxygen and sulfur) essential for plant and animal life. Food is the main source of P because phosphate concentration is low in both freshwater and seawater (Lall, 1991). In recent years the pollution of water by fish farm and hatchery effluents, and the problems of P discharge are widely recognized. The form and the quantity of P excreted by fish have direct influence on their enrichment in the aquatic environment and the growth of algae. Generally, P is excreted in soluble and particulate forms: the soluble forms of P, consisting of organic P and PO_4^{3-}, affect the water quality directly, whereas the particulate

form settles to the bottom of the tank or accumulates in the sediment. Soluble P is readily available as a nutrient for plant growth.

Effective physiological mechanisms for P absorption, retention and mobilization exist in both freshwater and marine fish species. The amount of phosphate absorbed from the food is affected by the level of phosphate in the blood (Kudriavetz and Pora, 1958; Phillips et al., 1959; Phillips, 1962). The mechanisms of P absorption and transport in fish have not been well studied. In higher vertebrates, intestinal P absorption is dependent upon a sodium gradient caused by the active transport of sodium, and thus the transport of P from the intestinal lumen into the cells through the brush border membrane is secondary to that of sodium. A sodium-dependent absorption of inorganic PO_4^{3-} in carp intestine has been reported (Nakamura, 1985a,b). In terrestrial vertebrates (Cross et al., 1990) and fish (Vielma and Lall, 1998), P homeostasis is controlled through intestinal absorption, glomerular filtration and storage of this element in the bone. The absorbed P accumulates in soft tissues (heart, liver, kidney, muscle and blood, etc.) but the retention of P in the body, including skeletal tissues, is relatively low (Rodehutscord 1996). The main loss of phosphate from the body is in urine.

Balance studies conducted in fish show an indirect evidence of soluble phosphate loss, possibly via urine (Nakashima and Leggett 1980). An increase in renal excretion of phosphate after intra-venous administration of phosphate in Prussian carp (*Carassius auratus gibelio* Bloch) was demonstrated by Kaune and Hentschel (1987). Marine fish are capable of renal phosphate regulation (Hickman and Trump 1969). Quantification of urinary P excretion is important for the measurement of metabolic utilization of absorbed P (Coloso *et al.*, 2001). Although many studies have been carried out to evaluate P loading from salmonid farms in fresh and marine waters (Bergheim et al., 1991; Fivelstad et al. 1990; Forsberg, 1996), there are no data yet published for haddock.

Our previous study (Chapter 3) showed that availability of organic and inorganic P for haddock was 43.2 % and 99 %, respectively, when fed a basal diet. Moreover, urine P concentration was positively correlated with dietary P intake. In commercial feeds,

organic ingredients are the main source for P (Lall, 1991), which are unavailable to the fish (Beveridge, 1987). In order to ensure that the P requirement of fish is met without creating a deficiency sign, a high amount of P is maintained in commercial feeds (Lall, 1991; Tacon and de Silva, 1983). Moreover, the information on digestibility of P from common feed ingredients by most fish is limited as well as the urinary and fecal P excretion data. The aim of the present study was to determine the species differences in urine P concentration of haddock and Atlantic salmon fed the same commercial feed. At the preliminary stage of this study, an appropriate time to collect the maximum volume of urine was determined.

5.3 Materials and Methods

5.3.1 Experimental design, fish rearing system, feed and feeding

Juvenile haddock (312.2 ± 5.9 g) and Atlantic salmon (807.6 ± 8.3g), were reared at the National Research Council's Aquaculture Research Station, Sandy Cove, Nova Scotia. The experiment was conducted in 4500 L capacity circular fiberglass tanks supplied and maintained with a constant volume of 3000 L filtered seawater (salinity, 32 ‰) at a water temperature of 14 ± 2 ^{0}C. The flow rate was 6L^{-min} and seawater was continuously aerated. Forty haddock (*Melanogrammus aeglefinus*) were stocked in one tank and as 40 Atlantic salmon (*Salmo salar*) were stocked in the same size tank. Fish were gradually acclimated over 2 weeks to the experimental conditions and fed a low P basal diet without supplemental P. During the first four days of feeding trial the low P basal diet was gradually replaced by 25-100 % of the commercial diet produced by Zeigler Bros. Ltd. (Gardners, Pennsylvania, USA). This commercial diet was produced under the feed specification developed by the Institute for Marine Biosciences, National Research Council, Halifax. The photoperiod was controlled at 12-h light/dark. Fish were fed a commercial diet to apparent satiety three times daily on weekdays and twice daily on weekends for 3 weeks. The composition of the diet is presented in Table 1. The diet contained 1.4 % P and there was no inorganic P supplement in this diet.

Table 1. Composition of the Commercial Diet[a]

Ingredients	Amount (%)
Herring meal	37.2
Crab meal	15
Shrimp meal	10
Corn gluten meal	10
Wheat, ground	13.2
Binder (DAKA)	2
Choline chloride (50 %)	0.6
Mineral premix[1]	1.0
Vitamin premix[2]	1.3
Herring oil	13

[a] Produced by Zeigler Brothers, Inc., 2000. The diet contained (according to our analysis, dry matter basis per kg diet): 475.0 g protein, 156.0 g lipid, 158.0 g calculated carbohydrate, calculated fibre 25.0 g, 123.0 g ash, 14.0 g phosphorus, 18.2 MJ digestible energy.

[1]Mineral added to supply the following (per Kg diet): Manganous sulfate ($MnSO_4.H_2O$; 32.5 % Mn): 23.0 mg; Zinc sulfate ($ZnSO_4.7H_2O$; 22.7 % Zn) : 70.0 mg ; Copper sulfate ($CuSO_4.5H_2O$; 25.4 % Cu): 6.0 mg; Potassium iodide (KI; 76 % I): 2.0 mg; Carrier (wheat middlings or starch) to make 1 kg +. Total: 1000.0g.

[2]Vitamin added to supply the following (per Kg diet): Vitamin A (as acetate): 900 IU; Vitamin D_3: 400 IU; Vitamin E (dl-alpha tocopheryl acetate): 250 IU; Vitamin K_3 (menadione sodium bisulphite): 30 mg; Thiamin: 30 mg; Riboflavin: 40 mg; Pantothenic acid (as d-calcium salt): 12.0 g/ kg; Biotin: 0.1 g/ kg; Folic acid: 1.0 g/ kg; Vitamin B_{12}: 0.30 mg; Niacin: 150 mg; Pyridoxine: 40 mg; Ascorbic acid, phosphate 300 mg; Carrier (weat middlings or starch) to make 1 kg +. Total: 1000.0g.

5.3.2 Urine collection and gut content measurement

After 3 weeks of feeding the commercial diet, urine and the amount of food in the digestive tract (gut contents) were sampled for various measurements. Four samples were collected after 0, 3, 6 and 17 hours of feeding. Five haddock and five salmon were used at each sampling time. Before the sampling day fish were fed at 17:00h. On the first sampling day, urine and gut contents were collected at 9:00 AM. Fish were again fed the diets at 10:00 AM and subsequently sampled at 3, 6 and 17 hours of post-feeding. Five fish for each group of haddock and salmon were anesthetized with tricaine methanesulfonate (MS222) for urine collection and gut contents measurement. Haddock were cannulated by the method previously described (Chapter 4). Each fish was cannulated only one time under the approved guideline of Canadian Council on Animal Care, 1995 (Protocol I.D. # IMB2000.3). A polyethylene catheter (Clay Adams PE-50) was gently inserted into the urinary duct. The external segment of the tubing was connected to a 100 µl needle of a Beckman syringe and urine was aspirated by applying gentle pressure to the abdomen. Salmon were cannulated by the spot sampling technique of Wood and Patrick (1994) with some modifications. A polyethylene catheter (Clay Adams Pe-100) was inserted in the bladder and the urine was removed, by applying mild suction with a 2 ml syringe, and collected in pre-weighed glass vials. The weight of urine collected and pH of urine were determined prior to dilution with 1 ml deionized water for phosphate analysis. After urine collection the stomach of the fish was opened and food was removed. After the first sampling, the feeding trial was continued for another 3 weeks and the sampling process for urine and gut contents was repeated.

5.3.3 Analytical techniques

Phosphate of the diet was analyzed calorimetrically (Taussky and Shorr, 1953). Urine pH was measured using pH paper (indicator strips pH 5-10, colorpHast, Gibbstown, Germany). Crude protein (% N X 6.25) of the feed was determined by the Dumas method (Edling, 1968) using a Leco nitrogen determinator (model FP-228, Leco, St. Joseph MI). Lipid was extracted with chloroform and methanol (1:1) according to the method of Bligh and Dyer, (1959). Energy content of the diet was measured in an adiabatic bomb calorimeter.

5.3.4 Statistical analysis

Analytical data on urine volume, phosphate concentration and pH values, and gut content were analyzed by two way analysis of variance using the factors of post feed sampling time and species (Sokal and Rohlf, 1995), using the Prism, version 3.0 (Graphpad software, San diego, CA). Variance homogeneity was tested by Bartlett's test before ANOVA. Since heterogeneous variance was primarily due to outliers, data were not transformed. Comparisons of treatment means were carried out using Bonferroni's multiple comparison procedure. The individual effects of hours and species, and the dependence effects of sampling hours by species (treatment interactions) were considered for the measurement of the urine volume and P concentration. Confidence level for the confidence interval of the mean was 0.95 and differences at $P<0.0125$ was considered significant. Graphs are presented as mean and standard errors of the Least Square Means.

5.4 Results

After 3 weeks of feeding, haddock and salmon showed an 11.2 % ± 1.3 and 10.34 % ± 2.4 increase in their body weight, respectively, and the difference was not significant ($P>0.05$) between these two species fed the same feed. No mortality was observed during the experimental period. Atlantic salmon accepted the feed slowly during the acclamatisation period; however, they readily accepted the feed during the experimental period. The feed conversion ratio of haddock and salmon were 1.3 ± 0.2 and 1.4 ± 0.3 respectively and they were also not significantly ($P>0.05$) different.

At all collection times, the urine volume of Atlantic salmon was significantly higher ($P<0.05$) than of haddock. The highest quantity of urine was produced by haddock and salmon after 3 and 6 hours of the ingestion of a meal, respectively. The total volume of urine measured at these periods was 83.0 ± 5.18 μl^{-L} and 243.8 ± 10.51 μl^{-L}BW of haddock and salmon, respectively. The changes in the volume of urine produced at different time intervals are presented in Figure 5.1 and 5.2. The differences in urine volume between species, collection period as well as their interactions are statistically significant ($P<0.0001$).

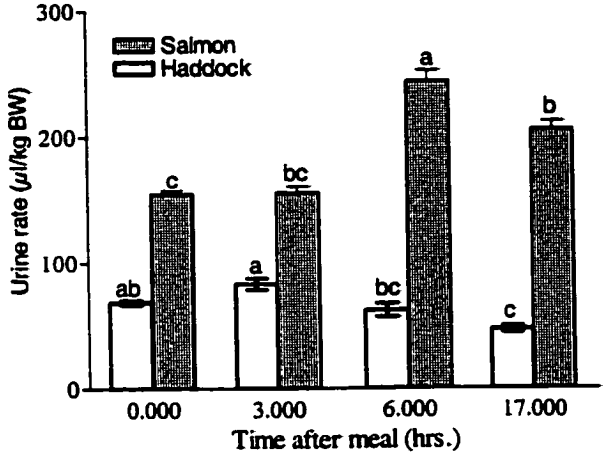

Fig 5.1. Pattern of urine excretion by haddock and Atlantic salmon. Species variation 81.69% (P<0.0001); meal variation 5.03% (P<0.0001); interaction 11.2% (P<0.0001). Bonferroni correction pooled measured of variability at 0:00 hrs. t = 12.22; at 3:00 hrs. t=10.34; 6:00 hrs. t= 25.72; at 17:00 hrs. t=22.52 with P<0.0001 in all cases.

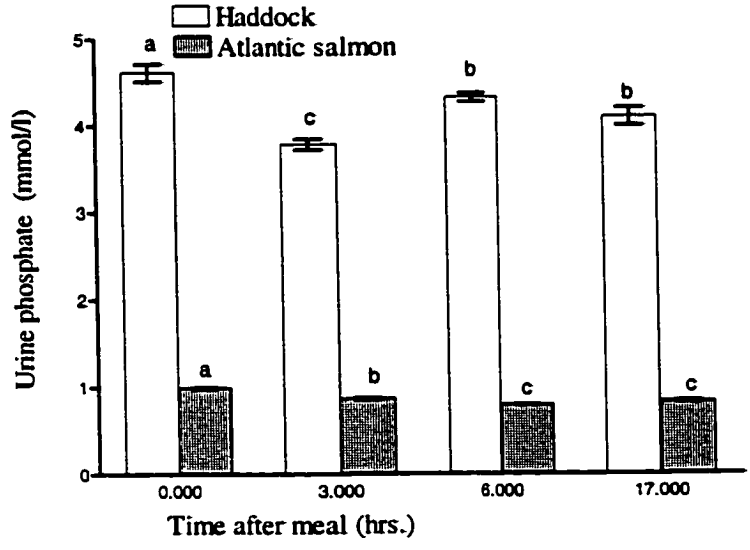

Fig.5.2. Changes in urine phosphate concentration between haddock and Atlantic salmon. Species variation 1.06% (P<0.0001); meal variation 97.81% (P<0.0001); interaction 0.65% (P<0.0001). Bonferroni correction pooled measured of variability at 0:00 hrs. t =43.61; at 3:00 hrs. t=35.16; 6:00 hrs. t= 42.48; at 17:00 hrs. t= 39.45 with P<0.0001 in all cases.

The urine P concentration of haddock was significantly higher ($P<0.05$) than the Atlantic salmon at all times when urine was sampled. The highest urine P was measured in haddock (4.6 ± 0.08 $mmol^{-L}$) and Atlantic salmon (1.0 ± 0.01 $mmol^{-L}$) prior to meal consumption and the P concentration was significantly higher in haddock than salmon urine. The urine P concentration during different time intervals showed a significant ($P<0.05$) decrease after 3 h feeding and then increased; however, it did not reach the initial level. There was a small change in the urine phosphate concentration of salmon, which showed a gradual decrease up to 6 hours of post-feeding and then reached a plateau. The urine pH (Figures 5.3) of Atlantic salmon (7.5 ± 0.22) was significantly higher than of haddock (6.2 ± 0.25) and there was no significant ($P>0.05$) change during the sampling period.

The gut food contents of haddock and Atlantic salmon showed significant changes ($P<0.05$) during different time intervals after the ingestion of the meal (Figure 5.4). The highest gut contents of both haddock and salmon was observed at 3 hours and 6 hours after the meal respectively and these were estimated as 76.2 ± 2.41 % and $71.2 \pm 2.41 \pm 2.41$ % for haddock and salmon respectively.

There was a close correlation between urine volume and gut contents of haddock. A polynomial second order-quadratic equation model was tested to fit both haddock and salmon data (Figures 5.5 and 5.6). The goodness of fit (R^2) in haddock was 0.99, however in salmon it was only 0.35. The standard deviation of the vertical distances of the points from the line in haddock was 2.32 whereas in Atlantic salmon it reached a high value of 60.1.

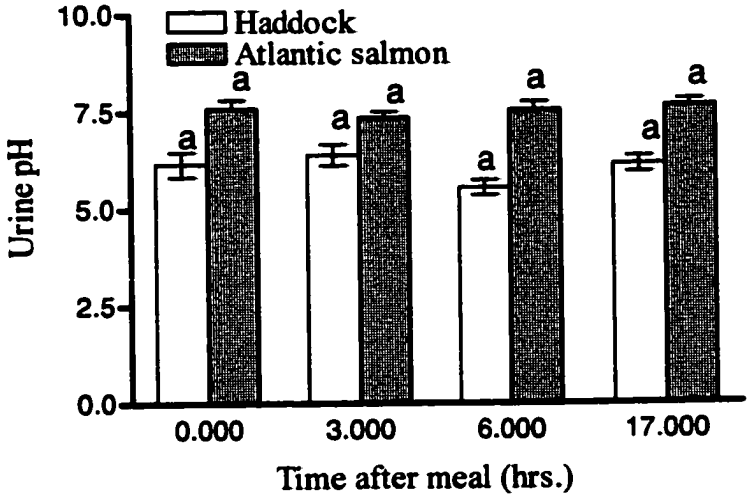

Fig. 5.3. Variation of urine pH between haddock and Atlantic salmon. Species variation 68.14% (P<0.0001); meal variation 2.58% (ns); interaction 4.25% (ns). Bonferroni correction pooled measured of variability at 0:00 hrs., t = 4.56 (P<0.001); at 3:00 hrs., t=3.04 (P<0.05); 6:00 hrs., t= 6.33 (P<0.001; at 17:00 hrs., t= 4.75 (P<0.001).

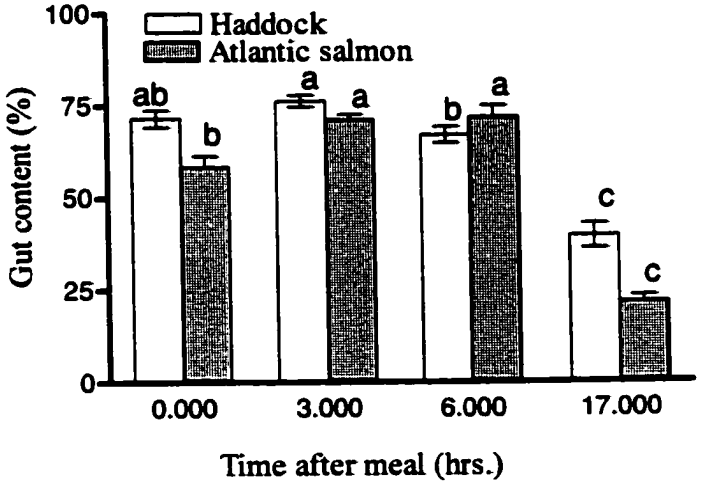

Fig. 5.4. Pattern of gut content after different period of meal. Species variation 4.42% (P<0.0001); meal variation 83.54% (P<0.0001); interaction 5.34% (P=0.0003). Bonferroni correction pooled measured of variability at 0:00 hrs., t = 3.86 (P<0.01); at 3:00 hrs., t=1.39 (ns); 6:00 hrs., t= 1.39 (ns); at 17:00 hrs. t= 5.25 with (P<0.001).

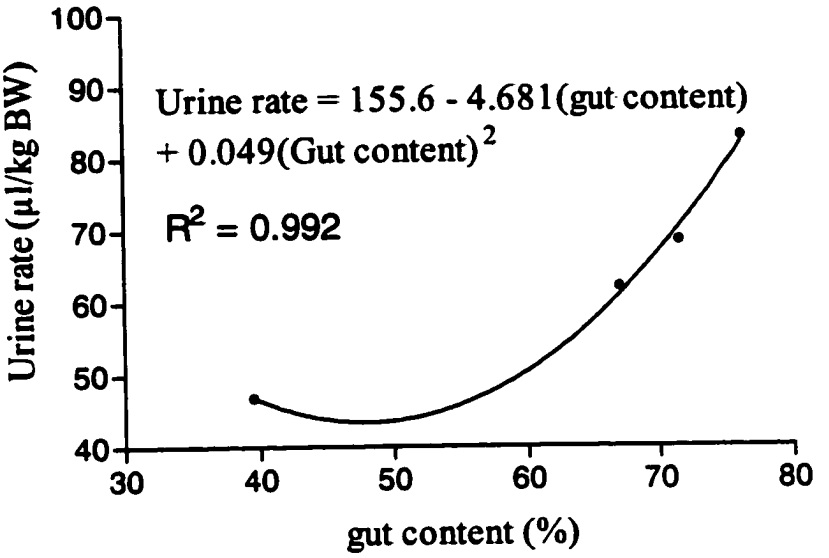

Fig. 5.5. Relation between gut content and urine excretion rate of Haddock. Values are means of 5 replicate fish. Urine rate is described by the function as Urine rate = 155.6 - 4.681(gut content) + 0.049(Gut content)^2; (R^2 = 0.992; Sy.x = 2.32).

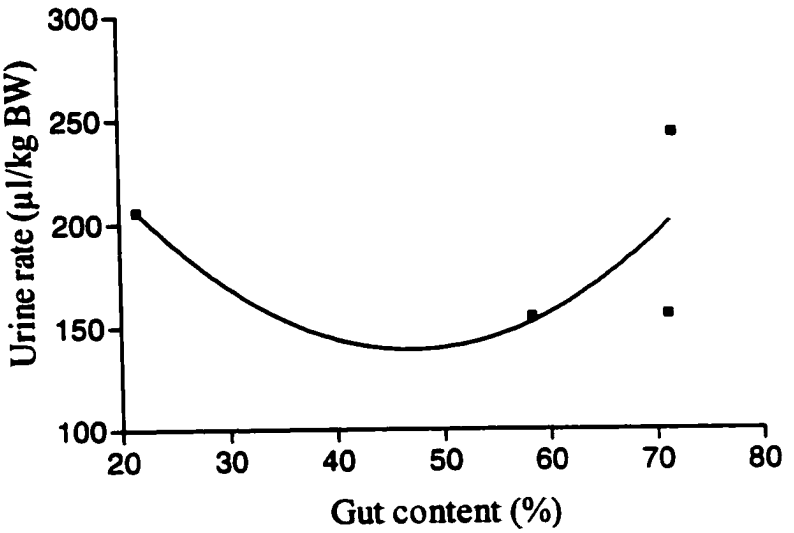

Fig. 5.6. Relation between gut content and urine excretion rate of Atlantic salmon. Values are means of 5 replicates fish. Since R^2 value is very law and none of non linear regression model including quadratic was fit the data unlike haddock. For R^2 = 0.35; Sy.x = 60.08).

5.5 Discussion

Atlantic salmon and haddock readily accepted the commercial diet, which allowed a relatively good estimate of P excretion. Body stores of nutrients have a profound influence on nutrient utilization (Baker, 1986), particularly during a short-term feeding study. A low P diet was used during the acclamation period to insure that a reasonable balance of P was reached prior to the urine collection.

The results clearly show that the volume of urine produced by salmon was higher than by haddock when both the species were reared in seawater environment. Moreover, urine volume of the same species showed different values during the 3, 6 and 17 hours of urine collection after the feeding. Some of the variations in urine volume over the different collection periods may have been due to the collection method used, as well as the anatomical differences in the urinary track of the two fish species (Marshall and Ferafflin, 1933). The quality of clinical results depends upon the physiological stress imposed on fish during the urine collection (e. g.) method of fish collection, dose of anesthesia and timing of urine collection and fish condition, etc. (Hunn and Willford, 1970; Formm, 1963; Holmes and Stainer 1966; McKim, 1966; Enomoto, 1967; Hunn, 1969). The cannulation technique developed as a part of our investigation (Chapter 4) was sensitive for collection of urine directly into the syringe. Therefore, the urine collection and measurement of volume in this experiment was not affected by the cannulation procedure, regardless of the small volume of urine obtained. The low urine excretion rate in haddock may not be associated with osmotic efflux across the gills since seawater contains very little P. Atlantic salmon are anadromous fish and their life cycle is spent in both fresh and seawater; however haddock are marine fish. The species differences may be associated with differences in osmoregulation and excretion of ions by these fish. The diurnal changes in urine excretion have been also observed in rainbow trout as due to the osmotic pressure effects from intestine to the urinary bladder after feeding (Shehadeh and Gordon, 1969). It is possible that similar mechanisms may have affected the urine volume in haddock and salmon.

Phosphorus excretion in urine depends upon kidney filtration rate, source of dietary P (Marshall and Ferafflin, 1933) and the metabolism of P in the body by various tissues (Lall, 2002). Fish require glomerular filtration (Marshall and Ferafflin, 1933) for inorganic P excretion through urine. The major proportion of P in feed ingredients in the commercial diet (Table 1) was in organic form and no inorganic feed supplement was added to this feed. Therefore, the inorganic P excretion by the kidney must be relatively low. Urine P concentration may be affected by dose of anesthesia used during urine collection (Hunn and Willford, 1970). Since we used the same dose-^{Kg} BW fish of tricaine methanesulfonate in both the species, the differences in urine P concentration were not due to the dose of anesthetic. Urine P excretion is determined by the glomerular filtration rate and reabsorption, which depends upon the plasma P level (Smith, 1939). The plasma P level is affected by the availability of dietary P (Lall, 1991, Ogino *et al.*, 1979; Lovell, 1978). The previous experiment (Chapter 3) showed that availability of P from organic feed sources for haddock was 43.2 %, which is lower than values obtained with rainbow trout (Rodehutscord, 1996). Moreover, the P requirement of haddock (Chapter 2) was higher than for Atlantic salmon (Vielma and Lall, 1998). Although we did not measure plasma P in this study, it is possible that higher plasma P level in haddock was associated with the differences in the utilization of P between the two species. Most of the P requirement and metabolism studied in salmonid fishes have been conducted in freshwater environment.

The high urine P concentration observed in haddock agrees with the value reported for phosphate in other marine fish (Smith 1939). High concentrations of other minerals such as magnesium and sulfate (Smith, 1930; Pitts, 1934), and sodium and chloride (Holmes, 1961; Formm 1963; Holmes and Stainer, 1966) have also been observed in marine fish. There is a consistent loss of water in haddock in hyperosmotic condition in seawater. They produce low volumes of urine and conserve absorbed salt for the maintenance of isosmotic body fluids, which may result in a low volume of urine. It is interesting to relate the urine rate and urine P of the two species. Although urine volume in salmon was double the amount in haddock, the concentration of P was nearly $1/4^{th}$ of the urinary P concentration of haddock. High amounts of P in haddock urine may be also

due to fluid re-absorption through the bladder. The differences observed in urine excretion rate and urine P between marine and salmonid fish species agree with a previous report (Howe and Gutknecht, 1978). The bladder of *Opsanus tau* reabsorbed 60 % of the urine excreted by the kidney.

The acidic urine of haddock observed in this study agrees with the values reported for dogfish (*Squalus acanthias*) by Smith (1939). Although no information is available on the effect of salinity on urine pH, the pH of intestinal fluid decreases with an increase in water salinity in rainbow trout (Shehadeh and Gordon, 1969). The pH of the urine is determined by the composition and concentration of the electrolytes (Na, K, Mg, Ca, SO_4, Cl etc.) and other unidentified anion and organic acids present in the bladder. A change in the anion and cation balance of urine may have affected the pH.

For estimation of total urine P excretion by fish under farm conditions, data on urine excretion rates, peak time of excretion and urine phosphate level in different fish species are important to develop nutritional strategies to manage the water quality and P discharged in natural waters. Although it was clear that urine excretion peaked in haddock and Atlantic salmon after 3 and 6 hours of meal ingestion, respectively, it was impossible to establish a specific statistical model to predict a relationship between them. Fitting quadratic equation $Y = a + bx + cx^2$ of urine rate and urine collection time the R^2 values of haddock and Atlantic salmon were 0.72 and 0.35, respectively. Nevertheless, the gut contents data indicated that the amount of food in the stomach was best suited with urine excretion rate. We have tested various nonlinear regression models available on Graphpad® and Systat® software. The quadratic equation model was best fitted for haddock urine rate and gut content since $R^2 = 0.99$. No published data were available to compare these results. Additional work is necessary to determine the peak period for P excretion as well as the chemical form of P present in urine to distinguish between metabolism of dietary and endogenous P excretion.

5.6 Conclusion

Phosphorus concentration of haddock urine was higher than in Atlantic salmon; however, urine volume of Atlantic salmon was higher than in haddock. The haddock urine excretion peak appears to be influenced by the gut food content rather than urine collection time. Species differences should be considered when estimating the urinary P soluble waste from commercial fish farms as well as experimental studies directed to measure P digestibility of potential feed ingredients.

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CHAPTER 6

Effects of dietary phosphorus in haddock (*Melanogrammus aeglefinus* L.) vertebrae: histology, histomorphometry and enzyme histochemistry.

6.1 Abstract

Skeletal tissue metabolism of fish and other vertebrates is directly affected by the dietary phosphorus (P) intake. A study was undertaken to examine the effect of dietary P levels on the bone metabolism of haddock (*Melanogrammus aeglefinus*), and to characterize bone deformities associated with P deficiency. Haddock fingerlings (average initial weight, 11.2 g) were fed basal diets containing low (0.42% diet), optimum (1.02 % diet) and excess (1.42 % diet) levels of P for 20 weeks. Vertebrae were subjected to histological, histomorphometry and enzyme histochemical examination. Tartrate resistant acid phosphatase (TRAP) staining employed for osteoclasts detection revealed that bone resorption occurs at the endosteal surfaces of the neural arches. Haddock juvenile diet deficient in P causes for the delay in bone mineralization. In contrast, excess amount of P in diet increased matrix deposition and an accelerated mineralization. Osteoclasts could be involved in homeostatic serum P balance in P deficient condition. Specific observations on the number of osteoblasts and osteoclasts, and relative amounts of mineralized bone and osteoid suggest that P deficiency in haddock bone initially increases bone resorption and subsequently decreases bone mineralization followed by a decrease in bone formation. Low bone minerals measured as reduced quantity of bone ash and deformed bone observed by direct examination and by X-radiograph may also be the result of a decrease in the mineralization of bone matrix. Other findings related to the requirement and excess intake of this element on bone metabolism of haddock are also summarized

6.2 Introduction

Phosphorus (P) is an essential mineral involved in energy metabolism and vertebrate bone development. The influence of dietary P intake on bone metabolism of teleosts (Urist et al.,1972; Lall, 1991, 2002) and skeletal deformities, resulting from P deficiency, have been reported in carp (Ogino et al., 1979), rainbow trout (Shearer and Hardy, 1987), and Atlantic salmon (Vielma and Lall, 1998). The amount of P in fish feeds must be carefully balanced to prevent deficiency signs as well as to

minimize the urinary and fecal excretions to reduce P discharge in natural waters. Excess amounts of P excretions from aquaculture operations contribute to increased P loads in the aquatic environment. Environmental regulatory agencies in several countries have set strict limits on the level of nutrients to be discharged in aquaculture effluents. Hence there is a need to reduce the dietary P level as well as to develop nutritional strategies to improve the bioavailability of this element (Lall, 1991; 2002).

Haddock and cod are emerging as potential new marine fish species for aquaculture in Europe and Canada. Recently, we found that the quantitative P requirement of haddock was 0.93 % of the diet (Roy and Lall, 2000), a requirement value significantly higher than in salmonids and other fish (NRC, 1993). Both hypophosphatemic and hyperphosphatemic haddock showed a decrease in total bone ash content. Growth and feed utilization were also reduced (Roy and Lall, 2000). To date, a histological characterization of signs of P deficiency in fish has not been undertaken; most reports provide only brief descriptions of gross vertebrae deformities and quantitative measurement of body ash content (Baeverfjord and Åsgård, 1998; Ogino and Takeda, 1976; Ogino et al., 1979; Shearer and Hardy, 1987). The present study was conducted to investigate the histological and histochemical changes in skeletal tissues of haddock fed low and excess amounts of dietary P. The main objective was to gain basic knowledge about the development of bone deformities in juvenile haddock under conditions of P deficiency and P surfeit. The results will be useful for further understanding the role of P in bone metabolism of fish.

6.3 Materials and method

6.3.1 Experimental design, fish rearing system and feeding

Three experimental diets were formulated to supply 4.2, 10.2 and 14.2 mg P/g diet and these levels were considered low, optimum and excess respectively based on our recent study (Roy and Lall, 2000). Calcium phosphate, $Ca(H_2PO_4).H_2O$ (Anachemia Science, Montreal, QC) was used as a P source in these diets. Cellulose replaced the calcium phosphate to adjust the total weight of feed ingredients in the two diets (4.2 and 10.2 mg P/g) containing lower amounts of P. The diets were steam pelleted, freeze-dried and stored at -18^0C until use.

The feeding trial was conducted in 350 liter capacity circular fiberglass tanks supplied with seawater (salinity, 32 ‰) at a water temperature of 12 ± 2 ^{0}C. Three hundred haddock fingerlings (initial weight 11.2 ± 0.06 g) were randomly distributed in six tanks (50/tank). Fish were gradually acclimated over 3 weeks to experimental conditions and fed a low P commercial diet containing no supplemental P. During the first four days of the experimental periods the commercial diet was gradually replaced by 25-100 % of experimental diets. The photoperiod was controlled at 12-h light/dark. Fish were fed the three experimental diets to apparent satiety three times daily on weekdays and twice daily on weekends for 20 weeks.

6.3.2 Sampling for serum and tissue phosphorus measurement

Plasma and vertebrae samples were collected at 8 and 20 weeks for P and ash determination. At each sampling period, 10 fish from each of the three dietary groups were used for vertebrae ash and serum P measurements. Blood was collected into heparinized tubes from the caudal vein after anaesthetizing the fish with tricaine methanesulfonate (TMS 222). The plasma was separated from the blood cells by centrifugation at 3000 × g for 10 min at 25 °C. Subsequently the plasma was stored at –40^{0}C until analysis. After blood collection, anesthetized fish were killed by a sharp blow to the head. The vertebrae column was removed for the determination of bone ash.

6.3.3 Tissue collection, fixation and histology

To diagnose the short and long term effects of dietary P on haddock bone, sampling followed the schedule described above. Four fish of each dietary level were killed with an overdose of TMS 222. The vertebrae were removed for histological and enzyme histochemical examination.

For Masson's trichrome and for tetrachrome staining, vertebrae were fixed for 24h with a solution containing 10 % para-formaldehyde and 50 mmol tris buffer in water, pH 7.2, rinsed overnight, decalcified in tris buffer (100 mmol) containing 10 % EDTA for 4 days, dehydrated in ethanol and embedded in paraffin. To examine osteoblasts, samples were serially sectioned (sagittal through the midline of the spine) at 10 µm thickness. Specimens were stained with Massons trichrome procedure (Weigerts iron haematoxylin for 10 minutes, 1 % Ponceau-acetic acid solution for 5

minutes, 1 % phosphomolybdic acid for 5 minutes, 1 % phosphomolybdic acid, light green for 1.5 min). To distinguish between mineralized bone and osteoid, sections were stained with tetrachrome staining (osteoid staining) as described by Ralis and Watkins (1992).

6.3.4 Enzyme histochemical procedures

Preparation of specimens, fixation, dehydration, infiltration with glycol methacrylate, and TRAP staining were carried out following the protocol of Witten and Villwock (1997). Fixation and embedding of the specimens were carried out at 4°C. All specimens were fixed for 1h with 10 % formaldehyde (methanol free) in 50 mmol Tris hydroxymethyl-aminomethane (Tris) buffer, pH 7.2. Samples were rinsed in tap water for 1 h and subsequently decalcified for 48 h in Tris buffer (100 mmol, pH 7.2) containing 10 % EDTA. After decalcification, specimens were dehydrated for 2.5 h in graded acetone solutions (30, 50, 70, 100 %). Vertebrae were first impregnated with the following glycol methacrylate monomer solution for 60 min: 80 ml (2-hydroxyethyl)-methacrylate + 200 ppm p-methoxyphenol + ethylene glycol monobuthyl ether 12 ml + benzoyl peroxide 270 mg. In the second step of impregnation, a monomer solution was used for 24 h. For embedding, a 2 % catalyst mixture (N,N-dimethylaniline 1 ml + poly-ethylenglycol-200 10 ml) was added to the monomer solution. Specimens were embedded in polyethylene jars with tight snap-on lids. The polymerization process continued for 24 hrs at 4°C and the samples were kept for another 24 h at room temperature to complete the process. The tissue blocks were stored at 4°C. Sections of 5µm thickness were floated on demineralized water (25 °C), mounted on uncoated slides, and dried at 25 °C.

Prior to the determination of TRAP activity, specimens were preincubated for 1 h at 20° C in 0.1 M acetate buffer (pH 5.5) containing 50 mM $^{-L}$ (+) di-sodium tartrate dihydrate. TRAP was detected with napthol AS-TR phosphate (N-AS-TR-P) as substrate, and hexazotized pararosaniline (PRS). The PRS was prepared by the following procedure: 1 g PRS (chloride) [C I. 42500] dissolved in a solution containing 20 ml de-ionised water and 5 ml 37 % HCl by heating at 70°C for 2 minutes, and stored in the dark at 4 °C. For hexazotiation, 2 ml 4 % $NaNO_2$ (0.58 M) was added to 1 ml of PRS-solution at 20 °C. The incubation medium was prepared by mixing the various solutions in the following order: 1 ml hexazotized PRS, 600 µl 2

% $MgCl_2$ solution, 2 ml enzyme substrate solution (2 mg N-AS-TR-P dissolved in 2 ml N,N –dimethylformamide), and 30 ml of 0.1 M acetate buffer (pH 5.5) containing 100 mM di-sodium tartrate dihydrate. Incubation lasted for 1 h at 20 °C. Subsequently, slides were rinsed in demineralized water. Specimens were counterstained with Mayer hematoxylin for 10 min, and mounted with DPX (Fluka, Buchs, Switzerland). For controls, slides were either heated at 90°C for 10 min prior to incubation or incubated without substrate.

6.3.5 X-radiographs

For the detection of morphological bone alterations resulting from P deficiency, five fish on each diet were X-rayed at the end of the experiment of 20 weeks duration. X-rays were taken using a portable "Mini X-ray HF80+" machine (Mini X-ray inc., Northbrock, USA) and "Kodak Industrex M Film Ready Pack II" (Kodak Industry, France) without a screen. The fishes were exposed for 2 seconds at 70 KV at 42 cm distance between the beam source and the film. Films were developed in Kodak chemicals, following the manufacturer protocol.

6.3.6 Gross examination of bones and histomorphometric measurements

For the morphological examination of vertebrae, five fish from each experimental group were anesthetized and rinsed in demineralized water. Fish were partially cooked in a microwave oven for 30 seconds to remove the flesh and soft tissues from the vertebral axis. The lipid from vertebrae was removed by a chloroform-methanol (1:1 v/v) extraction procedure (Vielma and Lall, 1998). Specimens were air-dried and photographed to demonstrate gross bone deformities. Percent bone in fish and bone density was calculated by the following formulas: Relative amount of bone (%) = total skeleton weight (g) / total fish weight (g) x 100.

6.3.7 Bone histomorphometry and image analysis

Osteoblasts: For osteoblasts, vertebrae samples were serially sectioned (sagittal section) starting from the midline of the vertebrae and sections were collected from the both side of the midline. Osteoblasts populations were counted on the surface of the vertebrae growing zone.

Osteoclasts: Preliminary investigations have shown that osteoclasts are concentrated at the neural arch. Therefore, in the whole subsequent slides we used for cross section. The neural arch of vertebrae samples were serially sectioned (cross section) starting from the midline. Osteoclasts, as the number of TRAP-positive cells, were counted along a minimum of 20000 μm bone surface following the criteria of Kaye (1984) for counting human osteoclasts, as applied to fish bone by Witten (1997).

The arrangement of osteoclasts was different from that of osteoblasts. Osteoclasts are located only along the endosteal surface and forming one lining layer in the neural arch, which allowed counting the cells per unit of length of the neural arch surface in only one direction, that is, by length of the neural arch. Osteoclasts counts were expressed per 1000μm. Conversely, the osteoblasts as well as their projenitors, from a population in a certain area on the bone growing plate. Therefore, the counting was done in two directions and osteoblasts counts were expressed per 100^2μm.

Osteoid blue colour is highly specific for osteoid layer and different from that of connective tissue and bone marrow elements. The PCI software of CIMAGING System (Model 1280, Compix Inc., Cranbberry, PA) was applied to measure osteoid on sections of tetrachrome stained vertebrae. First, the total area of bone of that vertebral body was captured and it's surface measured (giving the region of interest – ROI). Then, within the ROI the deep blue colour area of osteoid was captured and its area measured (giving the object area). The software-controlled measurement was based on the following equation: the object area is equivalent to the sum of the area measurement for each object. The area fraction of the osteoid was expressed according to the following formula:

Area fraction (%)= Object area/ Region of interest (ROI) X 100.

6.3.8 Statistical analysis

Homogeneity of variance was tested by Bartlett's test and the error residuals by Lillifers test. Data were analyzed by two way analysis of variance with three different levels of P and two different time periods (Sokal and Rohlf, 1995) using the Systat 10 (2000). Comparisons of treatment means were carried out using Bonferroni's multiple comparison procedure. The individual effects of dietary P

during each period, and the dependence effects of dietary P by period (interaction effects) were considered for the measurement of the gradual change of P effects on bone metabolism. Confidence level for the confidence interval of the mean was 0.95 and differences at $P<0.007$ was considered significant. Data are presented as mean and standard errors of the Least Square Means.

6.4 Results

6.4.1 Effects of dietary phosphorus on growth, vertebrae ash and serum phosphate level

The growth, vertebrae ash and serum phosphate levels were significantly ($P<0.05$) influenced by the increase in the dietary P concentration from 4.2 to 14.2 mg g^{-1} (Table 1). After 8 and 20 weeks, the growth of haddock fed low amounts of P was significantly lower than in fish fed diets containing 10.2 and 14.2 mg P g^{-1}. Fish fed low, optimum and high levels of P during the 20 week feeding experiment showed 361, 666 and 544 % increase in their body weight, respectively. However, the increase in body weight for the low P group was only 28 % during the 8-20 week period. Fish fed the high level of P gained significantly less weight than the fish fed the optimum amount of dietary P. The interaction between dietary P level and feeding period (P X 'Period') was significant ($P<0.05$) indicating a change in the magnitude of growth response in fish fed the three levels of dietary P during these two periods (0 - 8 and 8 - 20 weeks).

The vertebrae ash content of fish sampled before feeding the experimental diets was 52.7 ±1.6 %, as expressed on dry matter basis. A significant ($P<0.05$) decrease in the vertebrae ash content was observed in fish fed the low P diet for 8 weeks and it continued to decrease until the termination of the experiment at 20 weeks (Table 1). Fish fed the high P diet showed a similar trend, but the magnitude of decrease was lower than fish fed the P-deficient diet. There was a significant ($P<0.05$) increase in the vertebrae ash content of fish fed the diet containing 1.02 % P (Optimum P) and the ash content did not show a significant increase by continuing the experiment to 20 weeks. The correlation between vertebrae ash content of fish fed

Table 6.1. Growth, vertebrae ash, serum phosphate level, osteoblasts, osteoclasts and osteoid in fish fed three different dietary P for 8 and 20 weeks feeding period.

Period	8 weeks			20 weeks			±SE
P (% in diet)	0.42	1.02	1.42	0.42	1.02	1.42	
Initial weight (g)	11.2	11.2	11.2	11.2	11.2	11.2	0.06
Weight gain[1](g)	40.8^{e}	51.4^{cd}	50.9^{d}	52.2^{c}	86.7^{a}	72.9^{b}	0.36
Feed utilization[2] (as fresh basis)	0.95^{b}	0.75^{c}	0.82^{e}	1.23^{a}	0.76^{c}	0.93^{d}	0.02
Vertebrae ash[3] (%)	49.3^{d}	60.6^{a}	57.4^{b}	44.3^{e}	61.9^{a}	54.1^{c}	0.7
Serum Phosphate[4] (mmol^{-L})	0.6^{b}	1.4^{a}	1.6^{a}	0.4^{b}	1.5^{a}	1.7^{a}	0.16
Osteoblasts[5] (per 100^{2}µm)	8^{b}	9^{b}	8^{b}	3^{c}	8^{b}	14^{a}	1.13
Osteoclasts[6] (per 1000µm)	201^{b}	76^{c}	79^{c}	239^{a}	82^{c}	85^{c}	6.09
Osteoid[7](%)	42.8^{c}	42.8^{c}	21^{d}	73.3^{b}	39^{c}	8^{e}	2.81

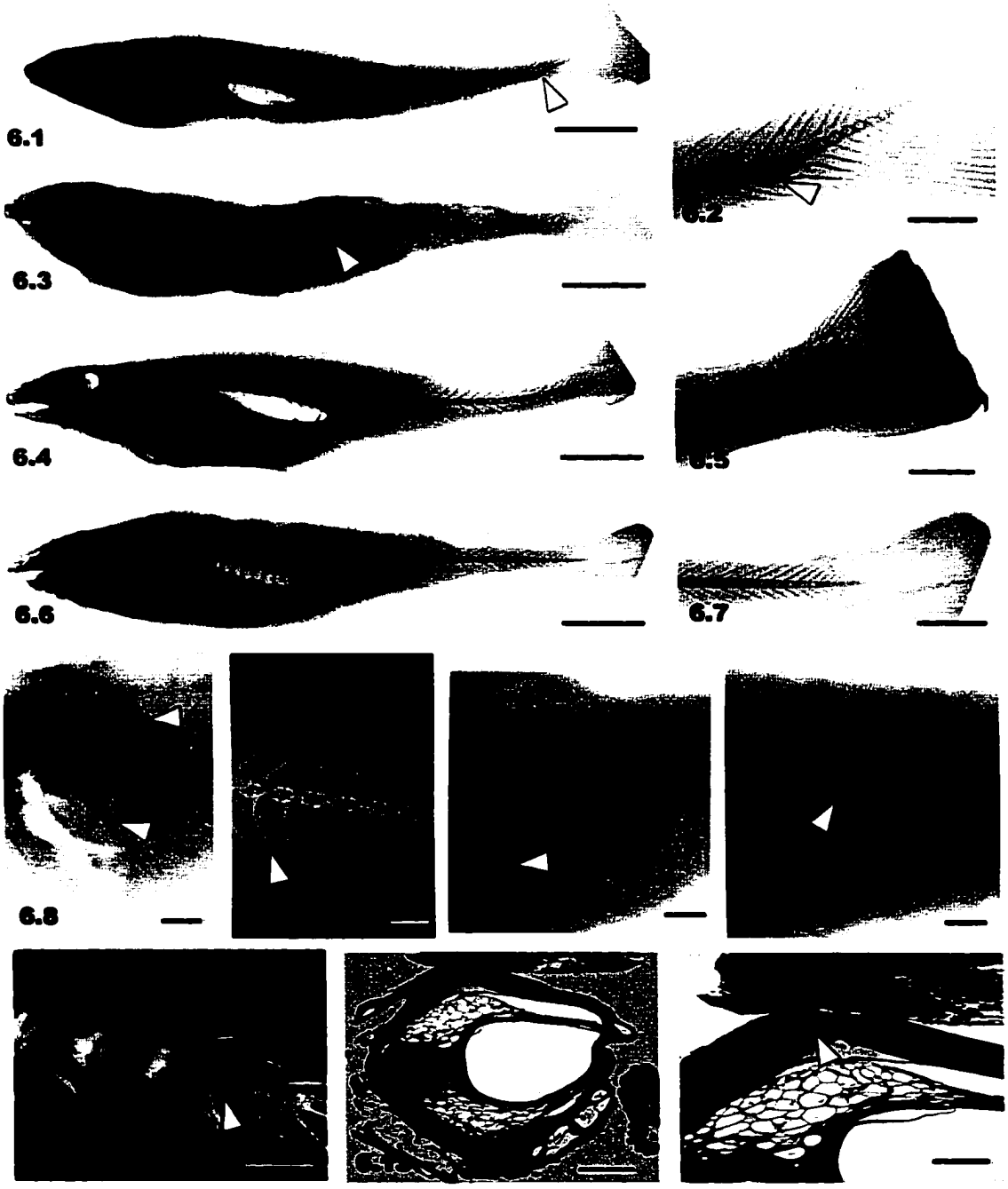
	Growth	Serum P	Ash	Osteoblasts	Osteoclasts	Osteoid
Phosphorus	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05
Period	P<0.05	NS	P<0.05	NS	P<0.05	P<0.05
Interaction (P X Period)	P<0.05	NS	P<0.05	P<0.05	P<0.05	P<0.05
R^{2}	0.98	0.78	0.9	0.73	0.98	0.95

[1]Mean wet weight gain of the 40 experimental fish.
[2] Feed utilization was measured as amount of feed intake per fresh fish weight gain.
[3]Mean vertebrae ash content of 10 experimental fish. This is measured on dry matter and fat free basis.
[4]Mean serum phosphate level of the same 10 fishes those were used for the measurement of vertebrae ash.
[5]Osteoblasts counts were made of 100^{2}µm X 3 (area) of vertebrae trabecula (n = 10). Samples were collected from the middle of the bone through serial section of paraffin blocks.
[6]Osteoclasts counts were made along a line 20000µm in length of neural arch (n=10). Samples were collected from the middle by serial section of plastic blocks.
[7] Osteoid were measured as percent of blue area of 'Alcian Blue stain' of total vertebrae (n = 10). Samples were collected from the middle by serial section of paraffin blocks. The similar letters in the same raws are not significantly different (P>.007).

diets containing the three levels of P were significant (R^2=0.98; P<0.05), including the interaction between P X 'Period". At 8 weeks the vertebrae ash of P deficient fish, fish fed the optimum P diet and fish fed excess P were 49.3 ± 2.4 %, 60.64 ± 3.0 %, 57.4 ±1.11 % respectively. After the 20 week feeding period, the vertebrae ash of the P deficient fish, fish fed optimum P diet and fish fed excess P, were 44.32 ± 1.4 %, 61.85 ± 2.98 % and 54.12 ± 1.61 % respectively. Similar to growth rates, the vertebrae ash content of P deficient fish was significantly lower (P<0.05) than in the fish fed optimum P and excess P diets. Also the vertebra ash of the fish fed the excess P diet was significantly (P<0.05) lower than the fish fed an optimum P diet. A pairwise comparison at P deficient fish suggested that the duration of experimental time appears to determine the quantity of vertebra ash. This indicates that the effects of P on vertebrae ash were not independent. Similarly, at 20 weeks the vertebrae ash content were significantly (P<0.05) lower in fish fed excess P diet than in the fish fed at 8 weeks. The differences among dietary P effects on serum phosphate level were significant (P<0.05). The serum phosphate levels in the experimental fishes increased with increasing dietary P level in both the 8 and 20 week study period. At 8 weeks of feeding, the serum phosphate level was 0.6 ± 0.1, 1.4 ± 0.2 and 1.6 ± 0.2 $mmol^{-L}$ respectively. At 20 weeks these were 0.4 ± 0.1, 1.5 ± 0.1 and 1.7 ± 0.2 $mmol^{-L}$. Although there was apparent variation in serum phosphate levels these was only significant for P deficient fish. Since the 'P' X 'Period' interactions were insignificant (P>0.05), it appears that the effects of dietary P on serum phosphate can be measured either at 8 or 20 weeks.

6.4.2 Gross examination and x-ray diagnosis

Dietary P affected the morphology of the experimental haddock skeleton. Radiographs indicated scoliotic changes and the impairment of the vertebrae bodies in P deficient fish. Figures 6.1 to 6.12 show the effect of dietary P levels on fish skeletons after 20 weeks of feeding the three experimental diets. The relative amount of bone in fish fed low, optimum and high amounts of dietary P was 35.8, 40.6 and 39.3 %, respectively. Smaller inter- vertebra spaces were demonstrated in X-radiographs of P deficient fish and fish fed excess P diets. The vertebrae became compressed and lost their defined structure, and in some places the radiograph did not show whether the vertebrae were connected with each other. This may be due to the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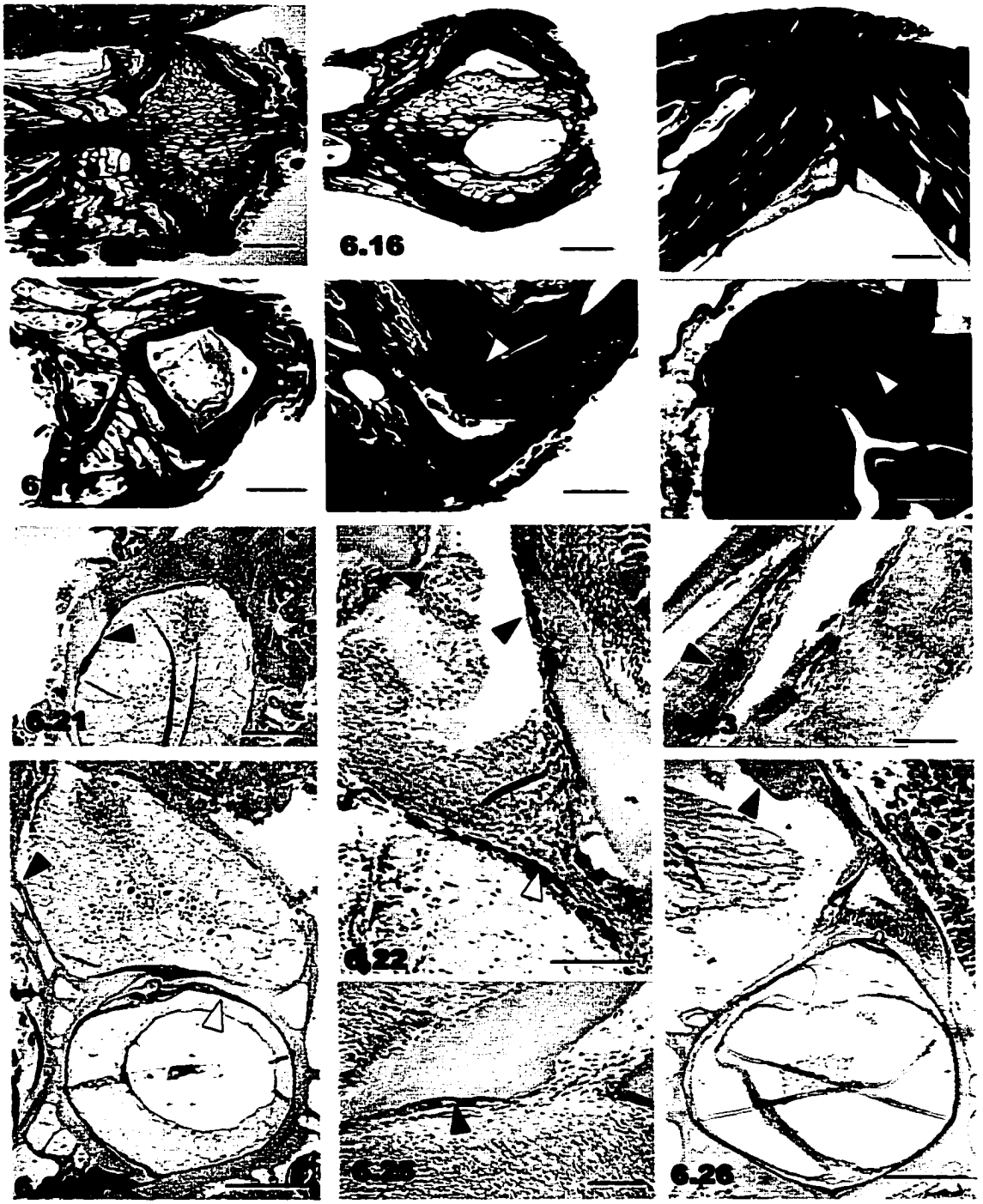
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Fig. 6.1. X-radiograph of a P deficient fish. Curvature of tail due to deformity in vertebrae (arrowhead) (bar = 1500 mm).

Fig. 6.2. Higher magnification of the tail of figure 1 (bar = 500 mm).

Fig. 6.3. X-radiograph of a P deficient fish. The vertebrae convexity in the trunk region as viewed from the side. This is appreciable lateral deviation for P deficient fish when comparing with the fish fed optimum P diet (bar = 1500 mm).

Fig. 6.4. X-radiograph of a fish fed optimum P diet. The vertebral column is normal (bar = 1500 mm).

Fig. 6.5. Higher magnification of the tail of figure 4. No deformities of vertebrae are visible in the tail region (bar = 500 mm).

Fig. 6.6. X-radiograph of a fish fed excess P diet (bar = 1500 mm). No abnormality is visible in bone growth.

Fig. 6.7. Higher magnification of the tail of figure 6 (bar = 500 mm). No curvature is visible.

Fig. 6.8. The vertebrae bodies from the 18^{th} (count starting from head) to the 23^{rd} vertebra of P deficient fish. The 19^{th} vertebra (black arrowhead) appeared cracked and is not clear visible. The neural and haemal spines (white arrowheads) become curled (bar = 200 mm).

Fig. 6.9. The vertebrae bodies from the 18^{th} (count starting from head) to the 23^{rd} vertebra of another P deficient fish. Both haemal (white arrowhead) and neural arch (black arrowhead) become curled (bar = 200 mm).

Fig. 6.10. The vertebrae bodies from 18^{th} (count starting from head) to 23^{rd} number of fish fed optimum P diet. The haemal (black arrowhead) and neural spines (white arrowhead) are normal (bar =200 mm).

Fig. 6.11. The vertebrae bodies from the 18^{th} (count starting from head) to the 23^{rd} verteba of fish fed excess P diet. The haemal and neural spines (arrowhead) are normal. The bone density between inter-vertebrae space (white arrowhead) appears to be comparatively low (bar = 200 mm).

Fig. 6.12. The deformation of vertebrae bodies at the tail region of a P deficient fish. The bone was collected and fat was removed by rinsing in chloroform and methanol. Showing diffused vertebrae (bar = 500 mm).

Fig. 6.13. The vertebra of P deficient fish. (Masson's trichrome method, X 4; bar = 1000 μm). Osteoblasts indicate is lower than in the fish fed optimum P diet.

Fig. 6.14. Higher magnification of a figure 13. The osteoblasts density are visible (white arrowhead) is lower when comparing to the fish fed optimum P diet (Masson's trichrome methodX25; bar = 250 μm.

Fig. 6.15. A vertebra body of a P deficient fish. The vertebra is underdeveloped, especially with the continuation of the chorda dorsalis, which represents an early developmental stage and hence bone appeared comparatively weak. (Masson's trichrome method, counterstaining with light green; X4; bar = 1000 μm).

Fig. 6.16. The vertebra body of a fish fed optimum P diet. (Masson's trichrome method; counterstaining with light green; X4; bar = 1000 μm). Osteoblast density is higher than in the P deficient fish.

Fig. 6.17. Higher magnification of the dorsal side of the bone growing area of figure 16. Osteoblasts (white arrowhead) well displayed along the bone growing area. (Masson's trichrome method, counterstaining with light green; X25; bar =250 μm).

Fig. 6.18. The vertebra body of a fish fed excess P diet. Highest osteoblast density in the bone growing area. (Masson's trichrome method, counterstaining with light green; X4; bar = 1000 μm.

Fig. 6.19. Higher magnification of the ventral bone growing area of figure 18. High-density osteoblast (white arrowhead). (Masson's trichrome method X25; bar = 250 μm).

Fig. 6.20. Osteoblasts of another vertebra of a fish fed excess P diet. Dense growing osteoblasts (white arrowhead) display in the bone growing area. (Masson's trichrome method X25; bar = 250 μm).

Fig. 6.21. Cross section reveals bone resorption at the neural arch of a P deficient fish. TRAP is located as red colour (black arrowhead). The cells are lining the endosteal area

(Demonstration of TRAP with PRS and N-AS-TR, counterstaining with haematoxylin, X 10; bar=500 μm).

Fig. 6.22. Huge bone resorption at the dorsal tip of the neural arch of a P deficient fish (black arrowheads). White arrowhead indicates pigment. The product of TRAP staining is highly concentrated in the neural arch (Demonstration of TRAP with PRS and N-AS-TR, counterstaining with haematoxylin, X 25; bar = 100μm).

Fig. 6.23. Bone resorption at the dorsal tip in the neural arch of a fish fed P deficient fish. The product of TRAP staining is highly concentrated in osteoclast. The osteoclasts number is high (Demonstration of TRAP with PRS and N-AS-TR, counterstaining with haematoxylin, X 40; bar = 100μm).

Fig. 6.24. Cross section of a vertebra body of a fish fed optimum P diet. The product of TRAP staining in osteoclast is very limited (black arrowhead). The osteoclasts number is low. (Demonstration of TRAP with PRS and N-AS-TR, counterstaining with haematoxylin, X 4; bar = 1000μm).

Fig. 6.25. Bone resorption in the dorsal tip of the neural arch of a fish fed optimum P diet. Showing limited osteoclast's resorption. (black arrowhead). (Demonstration of TRAP with PRS and N-AS-TR, counterstaining with haematoxylin, X 10; bar = 500μm).

Fig. 6.26.Cross section of a vertebra body of a fish fed excess P diet. The product of TRAP staining in osteoclasts is very limited (black arrowhead). The osteoclast number is low (Demonstration of TRAP with PRS and N-AS-TR, counterstaining with haematoxylin, X 10; bar = 1000μm.

Fig. 6.27. Bone resorption at the very end of the dorsal tip of the neural arch (black arrowhead) of a fish fed excess P diet. Demonstration of TRAP with PRS and N-AS-TR, counterstaining with haematoxylin, X 40; bar=100μm).

Fig. 6.28. A vertebra body of a P deficient fish. Deep colour osteoid (non mineralized bone matrix) in the vertebra centrum. Wall of the osteoid is thick (between black and white arrowhead). Highly active osteoblasts (top white arrowhead) did not become osteocytes after forming the new bone matrix (Osteoid) (Modified tetrachrome method, X 25; bar= 25μm).

Fig. 6.29. The dorsal side of the bone growing area of a vertebra of P deficient fish. The osteoid (white arrowhead). (Modified tetrachrome method, X 25; bar= 250μm).

Fig. 6.30. A thick layer (between arrowheads) of osteoid at the lateral side of the vertebra of a P deficient fish. Modified tetrachrome method, X 100; bar= 25μm).

Fig. 6.31. Bone growing area of vertebra in fish fed optimum P diet. The newly formed osteoid at the zone beneath the osteoclasts (arrowheads) become mineralized bone. Modified tetrachrome method, X 25; bar= 250μm).

Fig. 6.32. A thick layer (arrowheads) of new bone of a fish fed optimum P diet. The newly formed osteoid is mineralized. Modified tetrachrome method, X 100; bar= 25μm).

Fig. 6.33. Bone growing area of a fish fed an excess P diet. No osteoid at the zone of bone formation, beneath the osteoblasts (arrowheads). Bone is highly mineralized. Modified tetrachrome method, X 25; bar= 250μm).

Fig. 6.34. A thick layer (between arrowheads) of new bone of a fish fed an excess P diet. Similar to the old bone the newly formed osteoid has been highly mineralized (Modified tetrachrome method, X100; bar = 25).

irregularity of the vertebral column. In contrast to fish fed the optimum P diet, the neural arches and haemal arches of P deficient fish appeared curled and shorter.

6.4.3 Effects of dietary P on bone formation and resorption

Osteoblasts were detected on paraffin sections (sagittal) stained with Masson's trichrome based on the pale greenish colour of the cell cytoplasm and the location of the cells in the growing area of the vertebrae bodies. Histomorphometric data are presented in Table 1. The number of osteoblasts at the growing zone of vertebrae bodies did not show any significant changes ($P>0.05$) at 8 weeks, however after 20 weeks the number of osteoblasts significantly increased ($P<0.05$; $R^2 = 0.73$) with increasing dietary P concentration. The highest number ($14/100^2\mu m \pm 4$) of osteoblasts was found in fish fed excess P for 20 weeks whereas the lowest number ($3/100^2\mu m \pm 1$) was observed in the P deficient group. The number of osteoblasts in fish fed optimum levels of P were $9/100^2\mu m \pm 1$ and $8/100^2\mu m \pm 2$ at 8 weeks and 20 weeks, respectively. The effect of dietary P was not dependent on the duration of the period because 'P X Period' interaction was significant ($P<0.05$) and only the effect of 'Period" was not significant ($P>0.05$). Pairwise comparison over the period suggests that the number of osteoblasts was significantly ($P<0.05$) decreased in P deficient fish but increased significantly ($P<0.05$) in fish fed excess amount of dietary P. Figures 6.13- 6.20 show the osteoblasts in the bone growing area of fish fed diets containing the three levels of P.

Since osteoclasts could not be identified on the basis of standard histological procedures, the osteoclast marker TRAP was used to identify these cells. The TRAP positive cells were detected in the endosteal surface of the neural arches. The statistical analyses of the data indicates that dietary P had a significant ($P<0.05$; $R^2 = 0.98$) influence on the number of osteoclasts in the neural arch. The osteoclast numbers significantly ($P<0.05$) increased in P deficient fish, after 8 ($201/1000\mu m \pm 23$ in length of neural arch) and after 20 weeks ($239/1000\mu m \pm 18$). No significant variation of the osteoclasts at 8 and 20 weeks of feeding were detected in fish fed an optimum P diet versus fish fed an excess P diet. The effect of dietary P was not independent of period because the P X 'Period' interaction on the number of osteoclasts was significant ($P<0.05$). Pairwise comparison over time suggests that the number of osteoclasts significantly ($P<0.05$) increased in P deficient fish. This

indicates that the amount of bone resorption is connected to dietary P over time. Figures 6.21 to 6.27 show osteoclasts at the neural arches of P deficient fish, fish the fed optimum P diet and fish fed the excess P diet after 20 weeks of feeding.

6.4.4 Effect of dietary phosphorus on bone mineralization

Tetrachrome staining of paraffin sections indicates osteoid by its dark blue colour and normal mineralized bone by its red colour. The amount of osteoid within vertebra measured is presented in Table 1. The data indicate that dietary P significantly ($P<0.05$; $R^2=0.98$) influenced bone mineralization. At 8 weeks, the amount of osteoid in P deficient fish (42.8 ± 5.2 %) was the same as in fish fed the optimum P diet (42.8 ± 4.1 %). At 20 weeks, the highest quantity of vertebrae osteoid was found in P deficient fish (73.3 ± 12 %) and the lowest amount of osteoid was found in haddock fed the excess P diet (8 ± 4.3 %). The percentage of osteoid decreased in vertebrae of haddock fed excess P for 8 (21 ± 3 %) and 20 weeks (8 ± 3.7 %). The effect of dietary P was not independent of period because the 'P X Period' interaction on the amount of osteoid was significant ($P<0.05$). Pairwise comparison of dietary P over time suggests that osteoid was significantly ($P<0.05$) increased in P deficient fish and decreased in fish fed the excess P diet. This indicates that bone mineralization may be controlled by dietary P intake over this feeding period. The vertebrae osteoid of fish fed the P-deficient diet, optimum P diet and excess P diet are presented in figures 6.28 to 6.34.

6.5 Discussion

Bone formation by osteoblasts, mineralization of the bone matrix and bone remodelling, involving bone resorption by osteoclasts, are considered the three most important steps for bone development and growth in fish (Huysseune, 2000; Witten et al., 2000). The radiological, biochemical and histological findings of this study clearly indicate that dietary P influences all these three processes in haddock: bone formation, mineralization, and resorption. The major characteristics of the pathogenesis of P deficiency, in this advanced marine teleost species with acellular bone, are summarized in Table 2.

Table 2. Signs of P deficiency and effects of excess dietary P in haddock, (*Melanogrammus aeglefinus* L.) detected by biochemical, histological and X-ray analysis

P in diet (%)	P-deficient fish (0.42 % of diet)		Excess-P fish (1.42 % of diet)	
Period (Week)	8	20	8	20
Osteoblasts	no change	decreased	no change	increased
Osteoclasts	increased	increased	no change	no change
Osteoid	no change	increased	decreased	decreased
Serum phosphate	decreased	decreased	increased	increased
Urine phosphate[a]	decreased	not conducted	increased	not conducted
Gross deformities	no change	present	no change	absent
X-radiograph	not conducted	deformed bone	not conducted	no change

[a] Roy and Lall, 2000

6.5.1 Phosphorus in vertebrae development and deformities

A reduced amount of total vertebrae ash was measured in fish fed low and excess amounts of dietary P and these observations are consistent with our earlier study (Roy and Lall, 2000). Although reduced vertebrae ash was found in both hypophosphatemic and hyperphosphatemic conditions, the vertebrae deformities were recorded only in hypophosphatemic fish. Similar morphological changes of bone as a result of P deficiency have been previously reported in carp head (Ogino and Takeda, 1978), in vertebrae of Atlantic salmon (Baeverfjord et al., 1998) and striped bass (Dougall et al., 1996). A skeletal abnormality in the caudal complex of the farmed Japanese flounder (*Paralichthys olivaceus*) has also been reported (Hosoya and Kawamura, 1998). The author considered that the amount of Ca^{++} and the severity of the abnormalities were correlated. It seems that normal bone growth is sensitive to nutrient supply and tight regulation of bone formation processes (Meunier and François, 1992). In P utilization studies, bone was found to be a very sensitive tissue for both fresh water (Ketola, 1975; Watanabe et al., 1980; Ketola and Richmond, 1994; Rodehutscord 1996; Åsgård and Shearer, 1997; Jahan et al., 2001) as well as marine species (Sakamoto and Yone, 1978; Borlongan and Satoh, 2001). Our recent finding (Roy and Lall 2002) indicated that P is the second major mineral in haddock bone. From the present findings of P deficient fish, the impairment of vertebrae detected by gross examination as well as x-radiography indicates that the signs of bone deformities could be the result of failure in maintaining isometric relation of fish growth. The subject of vertebral fractures in fish has been reviewed by Bengtsson (1975) and it appears that demineralization of the skeleton is the most likely cause of bone deformities. Nutritional deficiencies are known to induce spinal deformities in fish (Wilson and Poe, 1973; Kloppel and Post, 1975; Lim & Lovell, 1978, Walton et. al., 1984; Akiyama et al., 1986; Roberts & Bullock, 1989). Unlike salmonids, the life cycle of haddock, a gadoid, is restricted to the marine environment where Ca^{++} is readily absorbed by fish. Phosphorus is a limiting nutrient in sea water for fish (Lall, 2002) and an adequate amount of this element is necessary to prevent P deficiency in haddock. Present findings indicate that 0.42 % of dietary P is insufficient to prevent the spinal deformities in haddock. Deformities occur overt physical signs of P deficiency including poor growth, curvature vertebrae, sluggish movement, was

observed only in haddock fed P deficient diet. Signs of P deficiency can vary with the severity of deficiency.

6.5.2 Relation of dietary phosphorus with bone formation

The number of osteoblasts increased with the increase in dietary P concentration from 4.2 to 1.42 mg P g^{-1} in fish fed these experimental diets for 20 weeks; however this increase was not apparent at 8 weeks. Although the effects of dietary P on bone development of salmonid fish have been previously reported (Vielma and Lall, 1998a and b), the relationship between dietary P and bone forming cells in teleosts (both seawater and freshwater) and different types of bone (cellular, acellular) is not known. However, a decrease in osteoblasts in starved tilapia has been reported by Takagi (2001). Conversely, an increased area of bone formation in the ventral pharyngeal bone has been reported in the Nile tilapia (*Oreochromis niloticus*) fed P deficient diets for 20 days (Takagi and Yamada, 1991). Thus, the pattern of vertebral bone formation in haddock contrasts with the ventral pharyngeal bone of tilapia. Haddock is a marine species while tilapia are cultured in freshwater, although both have a similar type of acellular bone. It is possible that the concentration of other micronutrients in the experimental diets, water chemistry, and mineral absorption rate through the intestine might be different because of the osmotic variation between fresh and marine water. Moreover, the osteoblasts measured in the case of tilapia as a covering the surface perimeter do not always reflect the correct number of either osteoblasts. Also the experimental period required to deplete a nutrient may be important. Most of the animals can tolerate nutrient deficiency up to a certain period through conservation of the nutrient in specific tissues and cells to prevent total depletion (Jobling, 1994). The results clearly showed that the effects of dietary P on the number of osteoblasts also varied over time.

An administration of PTH (Parathyroid hormone) has been reported to increase bone formation in terrestrial vertebrates (Dempster et al., 1993). However, teleosts lack the ability to produce PTH's because they lack parathyroid gland. In contrast, parathyroid hormone-related protein was detected in skin, kidney and skeletal muscle in cartilaginous and bony fish (Trivett et al., 1999). Moreover, growth hormone has also been reported to stimulate bone formation and resorption in terrestrial mammals (Rosen and Donahue, 1998) and rainbow trout (Takagi et al.,

1992). Glucocorticoids might be required for bone-cell differentiation during development, but their greatest effect is the inhibition of bone formation (Advani et al., 1997) in teleosts. In tilapia, injection of $1,25(OH)_2D_3$ caused demineralization of the acellular bones, in accordance with increased bone growth (Wendelaar-Bonga et al., 1983). Marine teleosts have large hepatic stores of vitamin D_3 (Urist, 1976). Atlantic cod produce $25(OH)_2D_3$, $24,25(OH)D_3$, $1,25(OH)_2D_3$ as well as $25,26(OH)_2D_3$ (Graff et al., 1999) and $1,25(OH)_2D_3$ receptors have been identified in Ca^{++}-regulatory tissues to increase Ca^{++} absorption after $1,25(OH)_2D_3$ administration in *vivo* (Sundell et al., 1992, 93). Larsson et al. (1995) suggested a hypocalcaemic role for $24,25(OH)_2$ in vitro in Atlantic cod. Haddock is very similar to cod. Presumably, the increase in the osteoblasts in haddock vertebrae is mainly due to an increase of vitamin D_3 and its metabolites during hyperphosphatemic condition.

6.5.3 Phosphorus deficiency and osteoclastic resorption

Enzyme histochemical studies showed that the number of osteoclasts increased in fish fed P deficient diet after 8 and 20 weeks. It is well known that osteoclastic bone resorption results in matrix degradation as well as release of bone minerals (Persson et. al., 1998; Takagi and Yamada, 1991; Mugiya and Watabe, 1977; Moss, 1965). TRAP positive osteoclasts occur in cellular and acellular teleosts bone during bone growth and development (Persson et al., 1995 and 1999; Witten, 1997; Witten and Villowck, 1997; Witten et al., 1999, 2000 and 2001). Acellular-boned teleosts are capable of bone resorption during Ca^{++} deficiency in a freshwater (Weiss and Watabe, 1979; Takagi and Kaneko, 1995) and marine environment (Glowacki et al., 1986) as well as under normal conditions (Sire et al., 1990; Sire and Huysseune, 1993; Hughes et al., 1994; Witten, 1997). Investigations previously indicated that the number of nuclei were dictated by the demands of specific bone metabolic functions (Weiss and Watabe, 1979; Glowacki et al., 1986; Sire and Huysseune, 1993; Witten and Villwock, 1997). The information of relation between numbers of osteoclasts and levels of P in diet is very limited. A decrease in osteoclastic resorption has been reported in Nile tilapia fed P deficient diets for only 20 days (Takagi and Yamada, 1991), which is not in agreement with our findings. Conversely, increased bone resorptive cell surface in Ca^{++} deficient (Takagi and Yamada, 1992, 1993) and starved tilapia (Takagi, 2001) have been reported. Moreover, haddock is a marine fish, which

is different from Nile tilapia for their environment. Although Takagi and Yamada (1991) measured the bone eroded area, they did not measure the number of osteoclasts. The surface of eroded bone area is not necessarily connected to the number of TRAP positive cells.

6.5.4 Bone mineralization

Effects of P deficiency on osteoid were not apparent at the 8th week of the feeding trial. However, the excess amount of dietary P caused a decrease in osteoid in vertebrae at 8 and 20 weeks. At 20 weeks, the amount of osteoid increased in fish fed a P deficient diet but decreased in fish fed a high P diet. A similar increase in osteoid has been also reported in P deficient Nile tilapia (Takagi and Yamada, 1991). The amount of newly formed osteoid is the result of a balance between the rate of matrix deposition and the rate of bone mineralization (Malluche and Faugere, 1986). The increased amount of osteoid after 20 weeks of feeding a P deficient diet together with the decrease in the amount of osteoblasts is rather indicative of a delay in mineralization. In contrast, the decreased amount of osteoid in the excessive P diet, together with the increase of osteoblasts in rather suggestive of increased matrix deposition and an accelerated mineralization. Moreover, statistical analyses of the osteoid data suggest that the effect of dietary P on osteoid is dependent on the duration of the feeding period. Bone mineralization might not be hindered in fish fed for 8 weeks. Indeed, excess osteoclastic resorption could maintain the homeostatic balance of serum P because total bone ash was declined and osteoclasts increased after 8 weeks of feeding. The amount of osteoid in the fish fed the excess P in these diet was significantly lower than in fish fed optimum P and deficient P for the same period. Minimum osteoid, highly mineralized bone, high serum phosphate and excess urinary phosphate excretion in fish fed the excess P diet raised the question of whether excess P in these fish may be initially deposited in the bone during the mineralization process and later demineralized and excreted in urine. Bone mineral accretions, demineralization and excretion of Ca^{++} and P through urine have been also reported previously in salmon (Wendelaar Bonga and Pang, 1991; Janz, 2000). Demineralization could be caused by the imbalance of minerals and the affinity between minerals to chelate. Mineral interaction and their effects on body mineral composition are well documented (Vielma and Lall, 1998a; Ãsgärd and Shearer,

1997). Yet several studies (Vielma and Lall, 1998b; Flick et. al., 1995; Bern and Madsen, 1992) suggest that the utilization of dietary P is not influenced by dietary Ca^{++}. Environmental factors as well as several vitamins and hormones may control both P and Ca^{++} utilization in fish are not clear particularly during the mineral imbalance.

6.5.5 Hyperphosphatemic response

A high amount of dietary P caused a significantly lower vertebra ash content of haddock. Although the ash content was decreased in hyperphosphatemic fish as well as under hypophosphatemic conditions, there were some distinct differences in bone histology, serum phosphate level and urinary P excretion. Hyperphosphatemic haddock showed an increase in the number of osteoblasts, decreased amount of osteoid and an increase of serum phosphate content and a higher amount of urinary P excretion. In hypophosphatemic fish, serum P level did not reach the saturation level; P excretion was reduced and fish maintained a high amount of osteoid in their bone. The serum phosphate level and urinary P excretion data of fish fed an excess amount of P clearly show that P absorption was not affected and serum P was derived either through absorption or bone demineralization. Higher concentration of urinary phosphate in hyperphosphatemic fish suggests that fish efficiently absorbed P by their digestive tract and finally was excreted in the urine.

A high amount of dietary P and Ca^{++} affects the bioavailability of certain minerals, e.g., manganese, zinc and copper in both fresh and marine water fish (Lall, 2002) and other terrestrial vertebrates (McDowell, 1992). Growth and bone ash data indicated that the high level of dietary P significantly reduced the growth and total vertebrae ash content. It is possible that the high P diet reduced the bioavailability of trace elements, which are also involved in the bone mineralization process. Based on these results as well as on the results of our previous study (Roy and Lall, 2000), it is apparent that negative mineral interactions caused low vertebrae ash content. Like terrestrial vertebrates, fish maintain a constant ratio of Ca: P in bone as well as serum and it varies 2: 1 to 1.5: 1 regardless of the changes in total ash content (NRC, 1993). Mineral interactions are complex (Bowen, 1966) and poorly defined in fish. Changes in the dietary mineral concentration affects the bioavailability of several minerals in terrestrial animals (Bowen, 1966) as well as in Atlantic salmon (Shearer et al., 1994).

Although marine fish have unlimited access to Ca^{++} from their aquatic environment, they regulate extra cellular Ca^{++} by hypocalcaemia mechanisms. Stanniocalcin is considered as a main hypocalcemic hormone in fish, reducing the Ca^{++} uptake through blocking Ca^{++} channels in the gills (Janz, 2000). The circulating level of stanniocalcin was three-fold higher in seawater than in freshwater adapted Atlantic salmon (Mayer-Gostan et al., 1992). Hormones as well as control mechanisms involved in excretion and reabsorption of P through kidney of marine fish remain to be investigated. Attempts should be made to avoid excess levels of P in marine fish diets.

6.6 Conclusion

The acellular bone of the haddock, a marine gadoid, is sensitive to the changes in dietary P content. The number of osteoblasts and osteoclasts, and relative amounts of mineralized bone and osteoid in the vertebrae suggest that P deficiency in haddock bone initially increases bone resorption and subsequently decreases the bone mineralization followed by a decrease in bone formation. Low bone minerals measured as reduced quantity of bone ash and the deformed bone observed by direct examination and by X-radiograph may also be the result of a decrease in the mineralization of bone matrix. Bone deformities, increased the amount of osteoid and caused a significant decrease of bone mineral co-existence during P deficiency, indicating that haddock vertebrae demineralization is likely to be a halastatic process. The cause of the very localized occurrence of osteoclasts at the endosteal surface of the haddock neural arches and the increased osteoclastic resorption in P deficient fish requires further clarification. The decreased number of osteoblasts after prolonged P deficiency suggests that bone formation might be inhibited because of P deficiency. Bone minerals are released into the plasma to compensate for the low P level during the deficiency and when P intake remained low for an extended period, the P in bone may have continued to deplete, resulting in the cessation of matrix formation under the influence of certain hormones. An excess P level may have contributed to enhance the bone mineralization process. The need to further examine the role of certain micronutrients (e.g. vitamins K and D) that are closely linked to P metabolism as well as hormones that control P homeostasis is imminent. Such research will allow development of nutritional strategies to improve P utilization and to get more insights

into the mechanisms involved in bone metabolism of haddock during low and excess P intake.

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CHAPTER 7

Effects of dietary phosphorus in haddock (*Melanogrammus aeglefinus* L.) bone: Variation of mineralization between different bone.

7.1 Abstract

Recent studies conducted on dietary phosphorus (P) requirements and histological examination of skeletal tissues showed that P is an essential mineral for bone formation and mineralization in juvenile haddock (*Melanogrammus aeglefinus*). This study investigated the changes in ash, Ca^{++} (calcium) and P content of vertebrae, opercula, pre-opercula and ventral pharyngeal bone in haddock fingerlings (average initial weight, 11.2g) fed diets containing deficient (4.2mg/g), optimum (10.2 mg/g diet) and excess (14.2 mg/g diet) levels of P for 20 weeks.

Haddock fed a low P diet caused a significantly ($P<0.05$) low amount of ash content in all bone tissues. Conversely, a diet containing high amounts of P caused a significantly ($P<0.05$) low amount of ash in vertebrae only. Ash content in opercula, preopercula and ventral pharyngeal bone were remaining the same as of haddock fed an optimum P in their diet. Mineral analysis data indicated that the amount of Ca^{++} and P content of opercula, pre-opercula and ventral pharyngeal bone increased with increasing dietary P. On the contrary, the amount of Ca^{++} and P content in vertebrae remaining the same as of haddock fed an optimum P in their diet. The results suggest that low P affects in all bone tissues equally, although excessive P has negative effect only in vertebrae. Based on the present finding it could be concluded that P metabolism of different skeletal tissues of haddock may have different regulatory mechanisms.

7.2 Introduction

Bone is a highly organized hard tissue of vertebrates characterized by a bone matrix, composed mainly of collagen and minerals. Inorganic minerals are hydroxylated polymers of Calcium phosphate, $Ca_{10}(PO_4)_6(OH)_2$ (Simkiss and Wilbur, 1989; Clark, 1955); the collagen is mainly of type I. In addition, trace amounts of other organic

compounds such as GAG, proteoglycans, phospholipids and other organic compounds are present and are considered as basic substances (Fourman and Royer, 1968). Bone requires continuous remodelling for growth and metabolism. Any change in metabolism and bioavailability of minerals in the body affects bone cellular metabolism. The processes of bone formation, mineralization and remodelling are controlled by growth hormones, physiological conditions and bone mineral metabolism (Huysseune, 2000).

Dietary Ca^{++}, P and other trace elements have essential roles in growth, development and maintenance of healthy bones in fish (Lall, 2002). Marine fish depend much more on dietary P supply than Ca^{++} because fish readily absorb Ca^{++} by drinking seawater. Bone deformities develop when insufficient amounts of Ca^{++} and P are present either in diet or water. In farmed fish, deformed bones retard normal growth rate and render fishes more susceptible to stress and disease, causing considerable economic loss. Phosphorus deficiency has been produced experimentally in several fresh water and marine fish species, where they exhibit poor growth, reduced feed utilization, and poor bone mineralization (Sakamoto and Yone, 1973; Ogino and Takeda, 1976; Onishi et al., 1981; Takeuchi and Nakazoe, 1981). A significant reduction in operculum and scale P concentration occurs in salmon and trout fed low P diets (Vielma and Lall, 1998a). Phosphorus deficient carp show an increase in liver gluconeogenic enzyme activity, accumulation of body fat and deformed heads. Reports in the literature concerning the impact of the water Ca^{++} content on the fish bone metabolism are controversial. Although Ca^{++} is abundant in seawater, dietary supplementation is necessary for red sea bream (Sakamoto and Yone, 1973). Calcium deficiency has not been observed in carp and catfish held in fresh water (Ogino and Takeda, 1976; Andrews et al., 1973) or in Atlantic salmon grown in seawater (Lall and Bishop, 1977). Although a low concentration of Ca^{++} was required in the diet of red sea bream (Sakamoto and Yone, 1973), eel (Arai et al., 1975) and tilapia (Robinson et al, 1984) for optimum growth, skeletal deformity or any other signs of gross deficiency were not detected.

Phosphorus deficiency affects total mineral (ash) content of vertebrae and opercula bones and an excessive amount of dietary P reduces the ash content only of vertebrae (Roy and Lall, 2000). Haddock bone is acellular type. Moreover, histomorphometric data showed that TRAP positive osteoclasts increased in P deficient

juvenile haddock. Therefore, the present study was conducted to investigate differences in the amount of minerals distribution between vertebrate, opercula, preopercula and ventral pharyngeal

7.3 Materials and methods

7.3.1 Experimental design, fish rearing system and feeding

Three experimental diets were formulated to supply 4.2, 10.2 and 14.2 mg P/g diet and these levels were considered low, optimum and excess P levels, respectively, based on our recent study (Roy and Lall, 2000). Calcium phosphate, $Ca(H_2PO_4).H_2O$ (Anachemia Science, Montreal, QC), was used as a P source in these diets. Cellulose replaced the calcium phosphate to adjust the total weight of feed ingredients in the two diets (10.2 and 14.2 mg P/g) containing a lower amount of P. The diets were steam pelleted, air-dried and stored at –18°C until use.

The feeding trial was conducted in 350 liter capacity circular fiberglass tanks supplied with seawater (salinity, 32 ppt) at a water temperature of 12 ± 2°C. Three hundred haddock fingerlings (initial weight, 11.2 ± 0.06 g) were randomly distributed in six tanks (50/tank). Fish were gradually acclimated over 3 weeks to experimental conditions and fed a low P basal diet containing no supplemental P. During the first four days of the experimental period the commercial diet was gradually replaced by 25-100 % of the low P basal diet. The photoperiod was controlled at 12-h light/dark. Fish were fed one of three experimental diets to apparent satiety three times daily on weekdays and twice daily on weekends for 20 weeks.

7.3.2 Sampling and tissue selection for minerals

Tissue samples were collected at 8 and 20 weeks. At each sampling period, 10 fish from each of the three dietary groups were used for ash, Ca^{++} and P estimation of vertebrae, opercula, preopercular and ventral pharyngeal bones. For histology, three replicate fish of each P level diet were taken at each sampling period. Fishes were anesthetized with tricaine methanesulfonate.

7.3.3 Analytical technique for mineral analysis

Vertebrae, opercula, preopercular and ventral pharyngeal bones were collected and cleaned with deionized water. Samples were extracted twice with 5-ml chloroform-methanol (1:1, v/v) to remove lipids and then oven-dried at 105°C for 24 h before ashing. Bones were ashed at 550°C for 18 h cooled and digested according to the method of AOAC (1995). Phosphorus was analyzed colorimetrically (Taussky and Shorr, 1953) and Ca was analyzed by AAS (Perkin-Elmer 5000).

7.3.4 Statistical analysis

Homogeneity of variance was tested by Bartlett's test and the error residuals by Lillifers test. Data were analyzed by two way analysis of variance with three different levels of P and two different periods (Sokal and Rohlf, 1995) using the Systat 10 (2000) version. Comparisons of treatment means were carried out using Bonferroni's multiple comparison procedure. The individual effects of dietary P and period, and the dependence effects of dietary P by period (interaction effects) were considered for the measurement of the gradual change of P effects on bone composition. Confidence level for the confidence interval of the mean was 0.95 and differences with Bonferroni adjustment at $P<0.125$ were considered significant. Data are presented as mean and standard errors of the Least Square Means.

7.4 Results

The effects of dietary P on bone minerals (P, Ca^{++} and ash) are presented in Table 7.1. Phosphorus concentration significantly ($P<0.05$) decreased in vertebrae of fish fed the P deficient diet and no significant differences were found between P content in vertebrae of fish fed the optimum P diet or the excess P diet. However, P concentration of opercula, preopercula and ventral pharyngeal bones increased with increasing P levels in the diets. P concentration in the preopercula of fish fed the excess P diet for 20 weeks was significantly higher ($P<0.05$) than for the same diet and same tissue for 8 weeks.

Table 7. 1. Variation of P, Ca^{++} and ash (in dry matter) in haddock bones fed three different dietary P for 8 and 20 weeks feeding period.

Period	8 weeks			20 weeks			
P in diet (%)	0.42	1.02	1.42	0.42	1.02	1.42	±SE
Phosphorus (%)							
Vertebrae	6.3^{b}	8.9^{a}	9.4^{a}	5.8^{b}	9.2^{a}	9.2^{a}	0.23
Opercula	5.5^{c}	6.2^{b}	7.0^{a}	5.2^{c}	6.4^{b}	7.3^{a}	0.12
Preopercula	5.7^{d}	6.4^{c}	6.9^{b}	5.8^{d}	6.4^{c}	7.4^{a}	0.14
Ventral pharyngeal bone	4.9^{c}	5.8^{b}	7.4^{a}	4.9^{c}	5.5^{b}	7.6^{a}	0.11
Calcium (%)							
Vertebrae	15.5^{b}	17.8^{a}	17.5^{a}	13.3^{b}	17.8^{a}	18.5^{a}	1.02
Opercula	11.8^{c}	13.7^{a}	15.3^{a}	11.5^{c}	13.8^{a}	15.7^{a}	0.63
Preopercula	12.5^{c}	13.3^{b}	14.7^{a}	11.8^{c}	12.9^{ab}	15.4^{a}	0.32
Ventral pharyngeal bone	10.2^{e}	10.5^{d}	15.1^{b}	9.9^{e}	11.2^{c}	16.2^{a}	0.21
Ash (%)							
Vertebrae	49.3^{d}	60.6^{a}	57.4^{b}	44.3^{e}	61.9^{a}	54.1^{c}	0.7
Opercula	38.8^{b}	49.7^{a}	47.9^{a}	36.7^{b}	48.4^{a}	48.4^{a}	0.92
Preopercula	31.8^{b}	42.8^{a}	44.0^{a}	27.8^{c}	43.7^{a}	44.5^{a}	0.87
Ventral pharyngeal	32.8^{b}	40.8^{a}	41.4^{a}	29.2^{c}	40.2^{a}	43.7^{a}	1.02

The similar letters in the same raw are not significantly different. The observed significant levels were adjusted with Bonferroni ($P>0.0125$).

Calcium concentration was significantly ($P<0.05$) lower in vertebrae and opercula of fish fed the P deficient diet. However, variations in Ca^{++} content between vertebrae of fish fed the optimum P diet and those fed the excess P diet were not significantly different. ($P>0.05$). Calcium concentration of preopercula and ventral pharyngeal bones increased with increasing P levels in the diets.

Different levels of P in the diets affected the vertebrae ash content. In comparison to the vertebral ash of fish fed the optimum P diet, the vertebral ash in fish fed the P deficient and an excessive P diets were lower. Ash content was significantly ($P<0.05$) lower in opercula, preopercula and ventral pharyngeal bones of fish fed the P deficient diet. The ash contents of fish fed the optimum P diet and the excessive P diet were not significantly different ($P>0.05$).

7.5 Discussion

Total bone ash has been considered the most sensitive indicator of bone mineralization in terrestrial vertebrates (Nelson and Waker, 1964; Ketaren et al., 1993; Ravindran et al., 1995; McDowell, 1992) as well as in fish (Lall, 1991; Vielma and Lall, 1998a; Äsgård and Shearer, 1997; Lall, 2002). Recently, we found that dietary P affects vertebrae ash content of haddock (Roy and Lall, 2000). The present work has examined the changes in bone mineralization in vertebrae, opercula, preopercula and ventral pharyngeal bones with changing amounts of P in the diet. Similar to our previous work, this study shows that dietary P significantly affected bone mineralization. However, the effect in vertebrae, opercula, preopercula and ventral pharyngeal bones was not the same. Although deficient P caused decreased bone ash in all bone tissues, excess P had no detrimental effect on bone mineralization in opercula, preopercula and ventral pharyngeal bones. Thus, it appears that the vertebra is very sensitive to dietary P and is the site causing an imbalance in mineral content of bone due to the excess P, since excess P decreased vertebrae ash. It is worthwhile to mention here that excess P in vertebrae causes decreased ash without affecting Ca^{++} and P levels. This indicates that the cause of decreased bone ash may be the result of a decrease in other minerals in bone (Zn, K, Na Mg etc.) without affecting Ca^{++} and P. Further, a decrease in Mg concentration

(Hardwick et al., 1991) inhibits Zn with an increasing dietary P content in fish (Vielma and Lall, 1998b; Hardy and Shearer, 1985; Porn-Ngam et al., 1993, Satoh et al., 1992 and 1993; Shearer et al., 1992). The effect of dietary P on other minerals is also supported by the present finding in preopercula and ventral pharyngeal bones, where excess P caused increased Ca^{++} and P without changing the quantity of total ash. Quantitative variations of minerals due to different P levels have also been reported in Atlantic salmon, *Salmo salar* (Vielma and Lall, 1998b), carp, *Cyprinus carpio*, (Ogino et al., 1979), red sea bream, *Chrysophrys major* (Ogino and Tekeda, 1978), and rainbow trout, *Salmo gairdneri* (Shearer and Hardy, 1987), but variation of the tissue response to the same dietary P level is not known. Because the endocrine hormones control the Ca^{++} and P simultaneously (Wagner et al., 1997; Lu et al., 1994; Fenwick and Lam, 1988), both have a common feature for their metabolism. It is important to point out the differences in the level of Ca^{++} in the different bone tissues. The present findings have shown that the effect of P on Ca^{++} concentration within vertebrae and opercula were similar. On the contrary, the effect of P on Ca^{++} concentrations in preopercula and ventral pharyngeal bones is also similar, but different from that in vertebrae and opercula. In vertebrae and opercula, the effect of excess P did not change the Ca^{++} level, whereas Ca^{++} concentration increased in preopercula and ventral pharyngeal bone. Moreover, Ca^{++} concentration in preopercula and ventral pharyngeal bones of fish fed excess P increased with increased duration of feeding period. Variation of tissue Ca^{++} content between vertebrae and scales is reported by Vielma and Lall (1998b) in Atlantic salmon reared in fresh water with different dietary Ca^{++}, although dietary Ca^{++} had no significant influence on P utilization. Marine fish have unlimited access to Ca^{++}, which is transported across the intestinal epithelium (Flick et al., 1995). Haddock, a marine gadoid, readily absorbs Ca^{++} from seawater. The cause of the variation in Ca^{++} content of different bone tissues could be partly due to the variation in the utilization capacity of this element, which may be related to the efficiency of mineral mobilization and distribution of these elements in different parts of the body.

7.6 Conclusion

The present investigation on the effect of dietary P on vertebrae, opercula, preopercula and ventral pharyngeal bones has clearly shown that excess dietary P affects the ash content only in vertebrae, though deficient P affects all bone tissues equally. It could be concluded that biochemical mechanisms involved in P metabolism of different skeletal tissues of haddock may have different regulatory mechanisms and the examination of a single tissue may not be sufficient to make meaningful conclusions regarding the P metabolism of this species.

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CHAPTER 8

General discussion, conclusion and future research direction

8.1 General discussion

In phosphorus (P) nutrition of fish, the major challenge to a nutritionist is to establish the optimum dietary requirement of this mineral for normal growth, reproduction and health, and to promote efficient feed utilization and bone mineralization while minimizing the P output from aquaculture operations to natural waters. Although significant progress has been made in the past decade in determining the P requirements of salmonids and several freshwater fish species, limited information exists on P requirement and metabolism in marine fish as well as the efficiency of P utilization from various feed sources and seawater (Lall, 2002). The nutritional strategies to minimize P excretion from marine fish and the estimates of P load generated by fish farms are scarce. The amount of P in haddock diet must be carefully balanced to prevent deficiency signs (e.g. skeletal deformities) as well as to minimize urinary and fecal P excretions. To date published literature on P nutrition of most fish does not provide adequate information on sensitive biochemical methods to determine the nutritional status of P in most fish or the proper histological or histochemical technique to characterize early signs of P deficiency, particularly under farm conditions. In recent years several cases of bone abnormalities in farmed Atlantic salmon and other fish have been reported in several countries particularly in Europe and South America (Chile) and they may be closely linked to P deficiency (Lall, 2001). Bone deformities may be also linked to other nutrients and environmental factors (Chapter 1). Obviously, there is a need to develop sensitive techniques to detect signs of P deficiency or excess in skeletal tissues as well as to distinguish the deficiency of P from that caused by other nutrients e.g. vitamin K or vitamin A. The present studies were designed to gain new knowledge on P nutrition of haddock, a marine fish species which has a good potential for aquaculture in Atlantic Canada. In addition to establishing the quantitative P requirement of haddock, biochemical and histological techniques were used to study the role of dietary P in fish bone metabolism, especially bone formation, resorption and mineralization at early stages of growth and development in juveniles. We have also identified the most sensitive skeletal tissue to dietary P deficiency and excess in

haddock by biological, biochemical, histomorphometry, enzyme histochemical and radiography methods. Moreover, estimates of fecal and urinary P excretion in fish fed experimental and commercial diets were also obtained. These findings are discussed in more detail in the following paragraphs.

A relatively high amount of vertebrae ash and low serum phosphate concentration observed in haddock compared to Atlantic salmon during the preliminary investigation (Chapter 2) indicated that P utilization by these two species might be different. A study designed to measure the quantitative P requirement of haddock confirmed this view (Chapter 3). Growth, feed efficiency, body composition and bone mineralization of haddock were significantly affected by the amount of P in the diet (percent amount of P in the diet were 0.42, 0.62, 0.82, 1.02 and 1.22 %).

A correlation was observed between vertebrae and opercula ash content, which indicates that vertebrae and opercula ash values could be used to estimate the quantitative need of this element as well as to measure the efficiency of dietary P utilization by haddock. On the basis of vertebrae and opercula ash content, the estimated dietary P requirement of haddock was 0.96 % of the diet or 0.72 % digestible P or 0.38 g digestible P/MJ digestible energy kg^{-1}. Vertebrae ash content has been also used to measure the P utilization or dietary requirement of terrestrial vertebrates (Nelson and Walker, 1964; Ketaren et al., 1993; Ravindran et al., 1995), and freshwater (Ketola, 1975; Watanabe et al., 1980; Ketola and Richmond, 1994; Rodehutscord 1996; Åsgård and Shearer, 1997; Baeverfjord et al., 1998; Jahan et al., 2001) and marine (Sakamoto and Yone, 1978; Dougall et al., 1996; Borlongan and Satoh, 2001) fish.

The current estimated P requirement values are higher than reported values (0.5 to 0.8 % of diet) for rainbow trout, Atlantic salmon, chum salmon, carp and red sea bream (reviewed by Lall, 2002), but it is relatively similar to those of tilapia (0.9 %) determined by Watanabe et al. (1980). Both tilapia and haddock have acellular bone, whereas salmonids have cellular bone. It cannot be stated that fish that have acellular bone require high levels of P because the P requirement of gilthead sea bream is 0.75 % (Pimentel-Rodrigues and Oliva-Teles, 2001). Åsgård and Shearer (1997) reported that juvenile

Atlantic salmon required 0.8-0.9 % available phosphorus per kg diet when feed efficiency was 1.45, a value much higher than in other reports (Lall and Bishop, 1977; Ketola and Richmond, 1994; Vielma and Lall, 1998). The minimum dietary phosphorus requirement for rainbow trout ranges from 0.37 % (Rodehutscord and Pfeffer, 1995) to 0.8 % (Ogino and Takeda, 1978). The differences in the phosphorus requirement between various fish species or among the same species may be due to the following factors: 1) species differences and variation in intestinal P absorption rate (Riche and Brown, 1999; Avila et al., 2000), 2) differences in the bioavailability of various inorganic and organic P sources (Satoh et al., 2002;Lall and Vielma, 2001), 3) fish size, condition factor and the stage of development (Shearer, 1984; El-Zibdeh et al, 1995; Rønsholdt, 1995), 4) dietary energy content and feed efficiency (Shearer, 1995), and 5) differences in experimental design (Shearer, 2000). There is a need to standardize the methodology and experimental design to measure the quantitative mineral requirement of fish, as was recently emphasized by Schwartz (1995). The studies conducted on P requirements in this thesis were carefully designed and will serves a useful values in feed formulation and diet development for haddock.

Signs of phosphorus deficiency were characterized in haddock fed a diet at 0.42 % phosphorus level (Chapter 3, 6, and 7). The growth rate was relatively low and feed intake nearly ceased after the 6-weeks of the commencement of the study and the fish became lethargic. Gross examination of fish showed curvature of vertebrae particularly near the caudal region. Bone deformities were apparent in spine of the vertebrae. Similar signs of P deficiency including bone deformities have been also reported in salmon (Watanabe et al., 1980), carp and rainbow trout (Ogino and Takeda, 1976; Ogino and Takeda, 1978 and Ogino et al., 1979) fed low phosphorus diets. The gross appearance of P deficiency is influenced by several factors including the duration of feeding a deficient diet, dietary mineral interactions and absorption, species differences and mineral content of water, water temperature and rearing system etc. Redlip mullet (Elzibdeh et al., 1995), milk fish (Borlongan and Satoh, 2001), carp (Ogino et al., 1979) and chum salmon (Watanabe et al., 1980) show deficiency signs when phosphorus levels are slightly below their requirement level, however, rainbow trout (Rodehutscord, 1996), American cichlid

(Chavez-Shnchez et al., 2000) and Gilthead sea bream (Pimentel -Ridrigves and Oliva-Tales, 2001) exhibited deficiency signs when phosphorus levels in the test diet were 20-30 % below their dietary requirement. In the present study, deficiency appeared within a short period because the P deficient diet contained only about 50 % of the requirement.

Chemical analysis of mineral content in vertebrae, opercula, pre-opercula and ventral pharyngeal bone (Chapter 3 and 7) showed that ash was significantly decreased when fish were fed a P-deficient (0.42 % P) diet. This effect was pronounced because vertebrae ash decreased from 56.6 to 44.5 %, opercula ash dropped from 48.2 to 31.4 %, pre-opercula ash dropped from 42.8 to 31.8 % and ventral pharyngeal bone ash dropped from 40.8 to 32.8 %. Bone deformities did not appear in fish fed the diet containing 0.62 % P when vertebrae ash level decreased from 56.6 to 50.6 %. Therefore, the present work has clearly established that bone deformities and low growth depend upon the severity of deficiency. Haddock bone can tolerate until certain limit of mineral content dropped noticeably. Plasma and urine phosphate concentration were significantly lower in fish fed a low P diet indicating an effective biochemical mechanism to conserve body P when P intake is marginal to meet the metabolic needs of fish.

The bone deformities were also apparent by X-radiography (Chapter 6). Morphological changes of bone have been previously reported in carp head (Ogino and Takeda, 1978), in vertebrae of Atlantic salmon (Baeverfjord et al., 1998) and striped bass (Dougall et al., 1996). It seems that bone formation and development is sensitive to nutrient supply and controlled by other physiological factors e.g. hormones (Meunier and François, 1992). In the present study, the impairment of vertebrae detected by gross examination, as well as x-radiography, suggests that the signs of bone deformities could be also the result of failure to maintain a balance between skeletal and overall somatic growth. Phosphorus is a limiting nutrient in seawater for fish (Lall, 2002) and an adequate amount of this element is necessary to prevent P deficiency in haddock. It is clear that 0.42 % dietary P was insufficient to prevent the spinal deformities in haddock. Overt physical signs of P deficiency, including poor growth, curved vertebrae, and sluggish movement were observed only in haddock fed a P deficient diet.

An extensive histomorphometrical and enzyme histochemical study (Chapter 6) on serial sections through vertebrae have indicated that dietary P influences haddock bone formation, mineralization, and resorption. The number of osteoblasts decreases with a decrease in dietary P concentration in fish fed for 20 weeks, however this change was not apparent after the 8 week period. For the first time, a relationship between dietary P and bone formation in a marine fish has been demonstrated. Information on the relation of dietary P with osteoblasts is very rare. Decreased osteoblast numbers in starved tilapia were reported by Takagi (2001). An increased area of bone formation in ventral pharyngeal bone of the Nile tilapia (*Oreochromis niloticus*) fed a P deficient diet has been also observed (Takagi and Yamada, 1991). However, a limited effort was directed to fully characterize the histological changes. Although, deficiency signs of their studies were not severe enough to detect all the changes.

Several dietary factors as well as hormones influence bone mineralization process. Intravenous administration of $1,25(OH)_2D_3$ in tilapia caused demineralization of the acellular bones, in accordance with increased bone growth (Wendelaar-Bonga et al., 1983). Marine teleosts have large hepatic stores of vitamin D_3 (Urist, 1976). Atlantic cod produce $25(OH)_2D_3$, $24,25(OH)D_3$, $1,25(OH)_2D_3$ as well as $25,26(OH)_2D_3$ (Graff et al., 1999). $1,25(OH)_2D_3$ receptors in Ca-regulatory tissues are influenced by *in vivo* administration of $1,25(OH)_2D_3$ where there was an increase in calcium absorption (Sundell et al., 1992 and 1993). Larsson et al. (1995) suggested a hypocalcaemic role for $24,25(OH)_2D_3$ in Atlantic cod. Haddock is very similar to cod and it is possible that the increase in the osteoblasts in haddock vertebrae was mainly due to increase in vitamin D_3 and its metabolites during hyperphosphatemic condition. Additional studies are needed to define the role of vitamin D and its metabolites in P and Ca metabolism of haddock.

Enzyme histochemical studies employed to detect the effect of dietary P and osteoclasts have indicated that the number of osteoclasts increased in P deficient fish after 8 and 20 weeks. Acellular-boned teleosts are capable of bone resorption during Ca deficiency in freshwater (Weiss and Watabe, 1979; Takagi and Kaneko, 1995) and marine environments (Glowacki et al., 1986) as well as under normal conditions (Sire et al., 1990; Sire and Huysseune, 1993; Hughes et al., 1994; Witten, 1997). Most of the

experimental studies have speculated that mineral bone loss due to Ca deficiency might be performed by bone resorption. However, the relationship between numbers of osteoclasts and dietary P levels was never investigated. Yet, a decrease in osteoclastic resorption was observed in P deficient Nile tilapia (Takagi and Yamada, 1991), which does not agree with our findings. An increase in bone resorptive cell surface was observed in Ca deficient (Takagi and Yamada, 1992, 1993) and starved tilapia (Takagi, 2001). TRAP positive osteoclasts were detected in the neural arch but not in the opercula, preopercular and ventral pharyngeal bone of the experimental fish in haddock. This indicated that osteoclastic resorption might have restricted to the endosteal surface of the neural arches of haddock. The limited distribution of osteoclastic resorption in haddock indicates that cells cannot contribute to the overall body mineral metabolism. However, the present findings for the first time have established histologically that the number of osteoclasts reflects P deficient signs. This information and histomorphometric technique can be used in the nutrition laboratory as well as in aquaculture farms to diagnose early signs of P deficiency in fish.

At 20 weeks, the amount of osteoid increased in fish given P deficient diet although these differences were not pronounced at the 8th week of the feeding period. This suggests that the effect of dietary P on osteoid is dependent on the duration of the feeding period. Bone mineralization might not be hindered in fish fed for 8 weeks and excess osteoclastic resorption could maintain the homeostatic balance of serum P because total bone ash declined and osteoclasts increased after 8 weeks of feeding. A similar increase in osteoid surface has been also reported in P deficient Nile tilapia (Takagi and Yamada, 1991). The amount of newly formed osteoid is considered to be an indicator of bone mineralization (Malluche and Faugere, 1986). The increased amount of osteoids show poor bone mineralization whereas decreased amounts of osteoid may cause be result of higher bone mineralization (Parfitt et. al., 1987). This demonstrates the applicability of osteoid measurements in severely P deficient farmed fish or when experimental fish have been maintained on P deficient diet for an extended period of time.

The severity of P deficiency may have direct influence on the magnitude of decreased growth and feed utilization in fish (Lall, 1991; Shearer, 1995). Although growth and feed efficiency of haddock fed diets containing 0.42 and 0.62 % was reduced, bone deformities were observed only in fish fed low P diet. Like terrestrial vertebrates the effect of dietary P on fish growth of various species was not consistent. Several factors such as differences in fish size, diet composition, duration of study, fish health and culture condition may have affected the growth performance. A relationship between deficient P diets and low growth was found in redlip mullet (Elzibdeh et al., 1995), although there was no significant effect from P deficiency in sea bass (Dougall et al., 1996) and also in a study on Atlantic salmon (Vielma and Lall, 1998).

Fish growth, vertebrae ash and zinc content decreased significantly when P content of the diet exceeded the requirement of this element (Chapter 3, 6 and 7). However, high amount P had no negative effect on the ash content of the opercula, pre-opercula and ventral pharyngeal bones. Sakamoto and Yone (1978) observed a decrease in vertebrae ash and plasma P content of red sea bream when dietary P level was 1.36 %. It is important to point out the differences of the level of Ca in the different bone tissues. The present results show that the effect of P on Ca content of vertebrae and opercula were similar. On the contrary, the effect of P on Ca concentration in preopercula and ventral pharyngeal bones was also similar but this was not the case in the vertebrae and opercula. In the vertebrae and opercula, the effect of excess P did not change the Ca level, whereas Ca concentration increased in preopercula and ventral pharyngeal bones. Moreover, Ca concentration in the preopercula and ventral pharyngeal bones of fish fed excess P increased with increased duration of the feeding period. Haddock is a marine gadoid and has an unlimited supply of Ca from seawater. The differences in Ca distribution in skeletal tissues may be due to the differences in utilization of this mineral. It may be affected by the mobilization and distribution of minerals in different parts of the body. Formulation of high P experimental diets required additional amount of monocalcium phosphate in the diet, which also increased the Ca content of the diet. Excess amount of Ca from dietary supplements or Ca absorbed by drinking seawater may have caused physiological stress and affected the Ca metabolism in the haddock.

Most fish maintain a constant ratio of Ca: P in bone as well as serum (NRC, 1993). An imbalance caused by changes in the level of either of these elements also affects the bioavailability of other minerals in terrestrial vertebrates (Bowen, 1966) and fish (Shearer et al., 1994). Excess P supplementation in the haddock diet may chelate zinc and other trace elements in haddock bone. Similar chelating effects of high P in rainbow trout and Atlantic salmon causing zinc deficiency have been observed (Hardy and Shearer, 1985; Satoh et al., 1996; Vielma et al., 1998a and Ketola, 1979). Therefore, it is well understood that P metabolism in *Melanogrammus aeglefinus* in different skeletal elements is regulated differently whereby only investigating one tissue is not always sufficient to make precise conclusions about species.

Histomorphometry and enzyme histochemical studies (Chapter 6) have indicated that a high amount of P in haddock diet increased bone forming cells when fish where fed for 20 weeks. However, this change was not apparent at 8 weeks period. Conversely, the number of osteoclasts remained the same at both the 8^{th} and 20^{th} week of feeding an excess P diet. The area of osteoid decreased with an increase of the feeding period in haddock when fed high amount of dietary P. It is clear that bone formation was not affected by the high amount of P in the diet and that the bone was highly mineralized. The decrease in vertebrae ash content may be caused by mineral imbalance in the diet as pointed out before in this section.

Phosphorus fecal excretion data of fish fed two experimental diets (Chapter 3) indicated that P digestibility of haddock fingerling was low from feed ingredients high in calcium phosphate. A similar value (43.4 %) of P digestibility was obtained in same size (68g) rainbow trout fed combination of plants protein (soybean meal) and fish meal (Riche and Brown, 1999). High P digestibility of monocalcium phosphate has agreed with the digestibility measured in rainbow trout (Ogino et al., 1979), common carp, (Ogino et al., 1979) and channel cat fish (Lovell et al., 1977), and Atlantic salmon (Lall, 1991). Thus, it clear that P digestibility is very poor from feed ingredient although, it is high in inorganic sources. The P digestibility data will be useful in formulation of

haddock experimental diets for both laboratory research and commercial aquaculture diets.

Urinary P concentration increased linearly (Chapter 3) with increasing dietary P levels. Feeding of a commercial diet to haddock and salmon (Chapter 5) indicated that amount of urine, urine phosphate concentration and pH of haddock urine was significantly different from salmon. The volume of urine excreted in salmon was almost double than that of haddock, whereas the urinary P concentration was almost half the amount found in haddock. It appears that re-absorption of P and other fluids was much more efficient in haddock. Similar differences in the volume of urine and urinary P concentrations have been observed in marine and salmonid fish (Howe and Gutknecht, 1978). The bladder of *Opsanus tar* reabsorbed 60 % of the urine excreted by the kidney. The present study demonstrated that haddock like other marine fish excrete a significantly higher amount of P in the urine. Also the urinary P concentration is much higher in this species as compared to Atlantic salmon maintained in seawater or other freshwater fish species.

In this thesis, new knowledge has been acquired on the requirement, digestibility and excretion of P in haddock as well as the role of this mineral in bone metabolism. Results clearly show that dietary P deficiency or excessive amount has detrimental effects on fish health. The skeletal tissue metabolism of haddock like other vertebrates is directly affected by the dietary phosphorus (P) intake. Phosphorus deficiency affects various physiological processes probably at the cellular level, which in turn influence growth, feed utilization, bone development and skeletal tissue mineralization. The severity of P deficiency depends on the duration of feeding a P deficient diet and on the dietary P concentration. The number of osteoblasts and osteoclasts, and relative amounts of mineralization and osteoid suggest that P deficiency in haddock bone initially increases bone resorption and subsequently decreases the bone mineralization followed by a decrease in bone formation. Low bone minerals measured as reduced quantity of bone ash and deformed bone observed by direct examination and by X-radiograph may also be the result of a decrease in the mineralization of bone matrix. Phosphorus metabolism may

differ in various skeletal tissues and the vertebrae ash was a sensitive indicator of the P nutrition status in haddock. Little is known about the mechanism of P in causing decreased vertebrae ash in haddock. It appears that several physiological and dietary factors e.g. hormones and vitamin D can influence and control the P metabolism in fish. These findings will be useful in characterizing P deficiency and toxicity as well as to improve the health of farmed haddock.

8.2 Conclusion

1. Phosphorus is an essential nutrient for growth, efficient feed utilization and bone development as well as to maintain normal physiological processes. The estimated quantitative dietary phosphorus requirement of haddock is 0.96 % P or 0.34 g digestible P per MJ of diet.
2. The vertebrae ash content is the most sensitive indicator of phosphorus nutritional status in haddock. Excess dietary P reduces the vertebrae ash content, however, P deficiency reduces the vertebrae, opercula, preopercula and ventral pharyngeal bone ash content equally.
3. The P digestibility of organic constituent presents in the test diet was 43.2 %. The P digestibility of mono-calcium phosphate used as P supplement in experimental diets was 99 %.
4. Urine in haddock is acidic and the concentration of phosphate within the urine for is much higher haddock than for Atlantic salmon.
5. Haddock bone is of the acellular type.
6. Osteoclasts detected by tartrate resistant acid phosphatase (TRAP) at the endosteal surfaces of the neural arches could be involved in P homeostasis balance.
7. Osteoclasts were mononucleate and smooth with ruffled border. They were distributed singly or aggregated in-groups or bands.
8. Dietary phosphorus intake had significant influence on bone metabolism of haddock including, bone formation, mineralization, and resorption.
9. Gross, clinical and histopathological signs of P deficiency in haddock includes:
    - Poor growth and deformed bone
    - Low serum phosphate and low bone ash content
    - Initially bone resorption followed by decrease in bone mineralization and bone formation
10. Excess phosphorus not only causes higher P excretion but it also reduces the total mineral content of vertebrae.

8.3 Future Research Direction

The present research was focused on P requirement and metabolism in haddock and an investigation on other nutrients closely linked to P metabolism was not possible. Several physiological factors and micronutrients are involved in bone mineralization of terrestrial vertebrates. In particular the role of two fat soluble vitamins, D and K in P metabolism in aquatic animals is not clear. In mammals, the vitamin D metabolite, $1,25(OH)_2D_3$ is the most potent factor in the intestinal uptake of Ca, reabsorption by the kidney and bone remodelling. In addition to the findings of similar hypercalcemic effects in fish injected with these metabolites, the exact role of vitamin D metabolites in bone formation and resorption in teleosts also should be clarified. The change in blood circulatory vitamin D_3 metabolites in rapidly growing fish, examination of relative importance on intercellular P and Ca regulation and interaction of stanniocalcin with vitamin D_3 will clarify the role of Vitamin D_3 in fish bone metabolism.

Little is also known about the efficacy of under-carboxylated osteocalcin, and whether bone mineralization depends on the vitamin K status. In humans sub-clinical vitamin K deficiency is manifested by under-gamma-carboxylation (ucOC) protein osteocalcin of the bone matrix (Brinkley et al., 2000). Addition of vitamin K decreases ucOC, although there is no effect either on N-teleopeptide of type I collagen or bone-specific alkaline phosphatase, markers of osteoclast activity. Does vitamin K deficiency reduce the Ca-binding properties in the bone tissue and do the present nutritional regimes and metabolism prevent the supply of vitamin K to the target cells? Matrix osteocalcin needs to be carboxylated by vitamin K to function properly in bone mineralization. Biochemical techniques to determine the volume and composition of the matrix components collagen, osteocalcin and the degree of osteocalcin carboxylation; and the status of vitamin K in liver, lipoproteins and target tissue will provide the essentiality of the role of vitamin K in bone development and mineralization. Future research in these areas will provide better understanding of the role of these key nutrients in bone and P metabolism of fish.

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