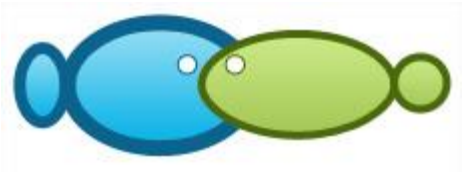

Vitamin D3 effect on the micro-crystal phase and atomic mineral content of bone: Incidence of spinal malformation in *Epinephelus fuscoguttatus* X *Epinephelus polyphekadion* hybrid grouper juveniles

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Abstract. The issue of bone anomalies in marine-cultured fish, particularly the hybrid grouper *Epinephelus fuscoguttatus* X *Epinephelus polyphekadion* (EFEP), has not been yet resolved. This study aims to determine the microcrystal structure-forming phase of bone and the atomic mineral content in normal and malformed bones treated with vitamin D3 (VD3). VD3 has a role in maintaining bone normocalcemia homeostasis, so that the formation of the extracellular matrix, the primary component of bone, may occur normally. This study focuses on the larvae of EFEP fed a VD3-enriched diet. VD3 enrichment is achieved through bioencapsulation of rotifers and spray-coating of formulated feed. The control treatment was without the addition of VD3. The crystal structure of normal bones differs from that of deformed bones, according to an X-ray diffraction (XRD) investigation. Merrillite predominates in the crystal structure of the EFEP bone, as it does in normal bone. Normal bones and deformed bones have very different atomic minerals. Mineral atoms such as Br, Ca, K, and S can distinguish between a normal and a fractured bone. VD3 therapy produces positive outcomes by preserving the majority phase of normal bone components and Ca content under ideal settings.

Key Words: bone malformation, bone micro-crystal phase, bone mineral atom, hybrid grouper.

Introduction. Intensively farmed marine fish often show morphological defects due to improper bone development (bone deformities). Bone deformities occur not only in captive-bred, but also in fish living in their natural environment (Sambraus et al 2014; Fjelldal et al 2021). Bone deformities in groupers are diverse and can be categorized according to head and body position. Jaw deformities and an underdeveloped, hollow-looking operculum are the most common anomalies of the head. On the body, spinal anomalies (lordosis, kyphosis, scoliosis, saddle-like syndrome) predominate, and the caudal and dorsal fins are underdeveloped. The effects of skeletal anomalies lead to economic losses as the morphological architecture of the fish does not meet sales criteria. Due to the disrupted swimming movements, the growth rate of fish with bone anomalies is slow (Başaran et al 2009), resulting in operational losses (Noble et al 2012). However, the problems of bone malformation, its etiology, and how to treat it are current important issues.

Bone is hard and rigid, and has a structure that enables it to fulfil its role as body building scaffolding. This includes supporting and protecting soft tissues/organs and acting as levers for muscle work (movement), supporting blood formation and storing calcium, phosphate, and the spinal cord (Harada & Rodan 2003; Datta et al 2008). Four cell types make up bone cells: osteoblasts, bone lining cells (lining cells), osteocytes, and osteoclasts (Buckwalter et al 1996; Downey & Siegel 2006). Bone is a highly active organ, with constant resorption by osteoclasts and remodeling by osteoblasts (Clarke 2008). Osteocyte cells serve as sensors that provide feedback. Bone contains many bone-lining cells,

although these cells are not yet well understood. They most likely take part in healthy bone remodeling, respond to osteoanabolic osteoporosis therapies, and play significant roles in skeletal healing following injury (Wein 2017). Recent studies utilizing lineage tracing techniques have revealed that bone lining cells in a quiescent state have the ability to undergo direct conversion into osteoblasts that actively form the bone matrix. This phenomenon has been observed in response to treatments with parathyroid hormone and anti-sclerostin antibody (Wein 2017).

Bone cells are not the primary component in bone formation. The extracellular bone matrix (ECM) of bones is a biomaterial composed of 60% inorganic minerals, 30% organic minerals, and 10% water (Feng 2009). The inorganic component is identified as carbonate-hydroxy-apatite, because it is a nanocrystalline solid with an apatite structure and a chemical element with a carbonated calcium phosphate composition. The remainder of the bone mass is composed of biological matter and water (Vallet-Regi & González-Calbet 2004). The inorganic components of the combination give it rigidity, strength, and stress resistance. An organic matrix comprising collagen fibers, glycoproteins, and mucopolysaccharides provides elasticity and resistance to stress, bending, and fracture (Alford et al 2015). The symbiotic relationship between these two substances gives the bone a unique quality that allows it to support loads, resist with significant strength, and bend without breaking within predetermined bounds (Vallet-Regi & Navarrete 2016). Bone preserves mineral homeostasis and provides mechanical support for propulsion and organ protection (Feng 2009). In mineral homeostasis regulation, bone is a buffer for cations and anions (Vallet-Regi & Navarrete 2016).

The inorganic matrix of the ECM is primarily composed of calcium-phosphate in the form of apatite compounds. In general, the chemical formula for apatite compounds is $[M(1)]_2[M(2)]_3(TO_4)_3X$. M(1) and M(2) are frequently divalent cations (e.g., Ca^{2+}), T is a pentavalent cation (e.g., P^{5+}), and X is a monovalent anion (e.g., OH^-). Bones have a hexagonal crystal structure composed of hydroxyapatite molecules with the chemical formula $[Ca_3(PO_4)_2]Ca(OH)_2$ (Datta et al 2008). According to the periodic system chart, many elements can react with one another to produce apatite compounds. The presence of particular mineral atoms in the bone induces substitution interactions with other mineral atoms, resulting in alterations in bonds and organic structures that comprise bone microstructure (Noor et al 2013). The crystallization by particle attachment (CPA) theory is used in the deposition of inorganic minerals (bone mineralization) to generate bone matrix. The particles in question are ions, dimers, oligomers, complex and ionic clusters, liquid phases and amorphous precursors, nanoparticles with poor crystallinity, and nanocrystals (Wallace et al 2013; De Yoreo et al 2015). The numerous complex ions (phases) involved in the bone mineralization process can affect the crystal architecture of the bone, resulting in crystal defects.

Vitamin D3 (VD3) is essential for regulating calcium and phosphate balance and preserving bone integrity. The binding of this hormone to the vitamin D receptor (VDR) stimulates the production of calcium-binding and transport proteins in the gut, hence sustaining normocalcemia and, indirectly, bone mineralization. In addition, VD3 acts directly on osteoblasts to stimulate bone cell synthesis in the skeletal system by suppressing proliferation, regulating differentiation, and regulating extracellular matrix mineralization (Sutton et al 2005). As with other hormones, VD3 is activated not only by binding to the VDR, but also through second messenger pathways (adenylate cyclase/cAMP/PKA, PLC/DAG + IP3/PKC, intracellular Ca2+ and MAPK cascades) (Boland 2011). In this study, we evaluated the effect of VD3 therapy on the bone micro-crystal phase and atomic mineral composition of malformed and normal bones of *Epinephelus fuscoguttatus* X *Epinephelus polyphekadion* (EFEP) larvae. Due to the small size of the larvae, only the spinal vertebrae was investigated in this study. This is the first attempt to determine what causes bone deformities in young EFEP hybrid groupers and how to treat them.

Material and Method

Treatment and research subject. The research occurred at the Institute of Mariculture Research and Fisheries Extension (IMRAFE) in Gondol, Buleleng, Bali, Indonesia. This study

examined EFEP hybrid grouper larvae treated with VD3. The therapy consisted in the addition of VD3 (VD3-500 *Rovimix DSM) at a dosage of 19.2 IU g^{-1} feed (Darias et al 2010) in combination with an IMRAFE fortification. The control treatment consisted of IMRAFE fortification without VD3. Technical vitamin C, *Selco (commercial fortification), and taurine were included in IMRAFE fortification (Melianawati & Astuti 2012). Bioencapsulation was used to apply VD3 between the ages of 3-25 days of the larvae, and pellet coating was used between the ages of 25-35 days of the larvae. Bioencapsulation of rotifers and coatings on artificial feed were employed in enrichment applications. Bioencapsulation enrichment was accomplished by blending the materials until an emulsion formed, after which the mixture was placed in a rotifer incubation container for six hours (Sagala et al 2010). The coating was accomplished by combining vitamin D with Selco to make an emulsion, which was sprayed onto the artificial feed. The vitamin D content of rotifers and artificial feed treated with VD3 and control samples was determined using HPLC (Table 1). Initially, the rotifer samples and formulated feed were freeze-dried.

Table 1

Vitamin D content in rotifers and feed

Treatment	*Feed*	*Vitamin D content (µg 100 g^{-1})*
Control	Rotifer	73.5
	Artificial feed	7.7
Vitamin D3	Rotifer	331
	Artificial feed	20.3

Rearing was carried out until 35 days of age (juvenile). Management of larval rearing followed the IMRAFE Standard Operating Procedure (Sugama et al 2012). Every morning, larvae were offered green water with *Nannochloropsis*, which influenced their visual foraging. Rotifers were administered twice a day (morning and evening), whereas artificial feeding occurred between 4 and 8 times a day, depending on the age of the larvae (Table 2). Meanwhile, *Artemia* was administered once a day before the administrations of artificial feed. On the eighth day of upkeep, water-change management commenced (Table 2).

Table 2

Rearing hybrid grouper larvae management

Age of larvae (days)	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	…	*20*	*21*	…	*25*	…	*30*	…	*35*
Feeding management																				
Nannochloropsis																				
Rotifer			5 - 6 ind mL^{-1}								10 - 15 ind mL^{-1}									
Pellet																				
Brine shrimp																				
Water exchange management																				
Fish oil																				
Water exchange									10%				20%			50%			100%	
Syphoning																				

Research sample preparation. The target of the investigation was the spinal vertebrae of 35-day-old hybrid grouper juveniles with 21.52±3 mm total length rate and 25.21±6.59 mg weight, which were classified based on normal performance and bone abnormalities. Before collecting the bones, every individual was euthanized with an anaesthetic (MS-22). The bone samples were freeze-dried and ground into powder. The minimum amount of bone powder sample was 1 g. The bone powder was calcined at 900°C for five hours (Mutmainnah et al 2017).

Bone micro-crystal structure phase and bone mineral content. The formation of bone microstructure is contingent upon the function of mineral atoms. Mineral atoms can substitute or be incorporated into the crystal structure of hydroxyapatite (HA) in the bone matrix. This allows them to serve as complementary components to the positions of calcium and phosphorus, ultimately leading to the replacement of these elements. X-ray diffraction (XRD) was utilized to observe the crystallization profile of hydroxyapatite (HA) in the bone. The experiment was conducted at an angle of 2θ ranging from 20O to 90O, using a Cu-Kα radiation source with a wavelength of 1.54056 Å, and a current and voltage of 40 mA and 40 kV, respectively. The data obtained from XRD were subjected to analysis using Profex software version 4.3.6. The X-Ray Fluorescence (XRF) technique was employed to determine the atomic composition of bone minerals. The State University of Malang conducted the XRD and XRF analyses.

Results. The X-Ray Diffraction (XRD) examination confirms the hypothesis that the phases of the microcrystalline matrix of normal and abnormal bone are distinct. The peak crystallization pattern differed between the structures of deformed and normal bone matrix structures (Figure 1). The crystalline phase that formed the backbone of the EFEP hybrid grouper with normal control architecture consisted of numerous types, including merrillite, $Ca_9CrP_7O_{28}$ (TCP-Cr0.29), and hydroxyapatite, $Sr_{0.25}Ca_{2.75}P_2O_8$, corresponding to the peak crystallization pattern (Table 3). Merrillite, TCP-Cr0.29, Cu-hydroxyapatite, and oxyapatite phases were found in normal bone-VD3. The primary crystalline mineral phases making up normal bone are merrillite and TCP-Cr0.29. TCP-Cr0.29, Cu-hydroxyapatite, $Ca_{10}NaP_7O_{28}$ (TCP-Na0.29), and K_3PO_4 were the phases in the malformation-control bone. Merrillite, Cu-hydroxyapatite, and $Sr_{0.25}Ca_{2.75}P_2O_8$ were found in the VD3 malformed bones. TCP-Na0.29 and merrillite dominated the mineral phase composition of the control bone and malformation VD3 bone, respectively.

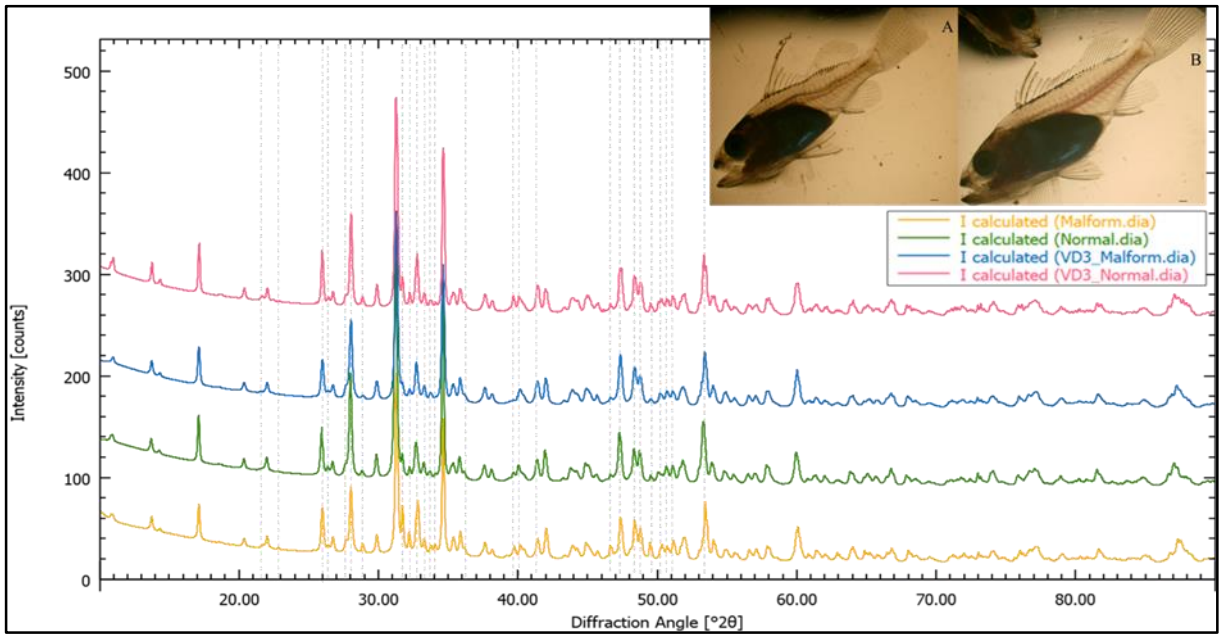

Figure 1. Normal and deformed bone XRD; grey dash lines show differences in bone crystallization; A - malformed bone malformation; B - normal bone.

From the point of view of mineral constituents in bone (XRF), the mineral composition of normal bones was nearly identical in both the control and VD3 groups. Bromine (Br) mineral atoms were discovered in the distorted bones of both treatment groups. Control therapy revealed mineral atoms of magnesium (Mg) in both normal and deformed bones (Figure 2). Based on the total composition of the mineral atoms in the bone, calcium (Ca), potassium (K), and sulfur (S) can distinguish between normal and deformed bones. Normal bones had a 2.31% greater average Ca concentration than control-malformed bone.

Meanwhile, VD3-malformed bones contain the same amount of calcium as normal bones. Normal bones contain less K (14.97%) and S (26.15%) mineral atoms than malformed bones (Figure 2).

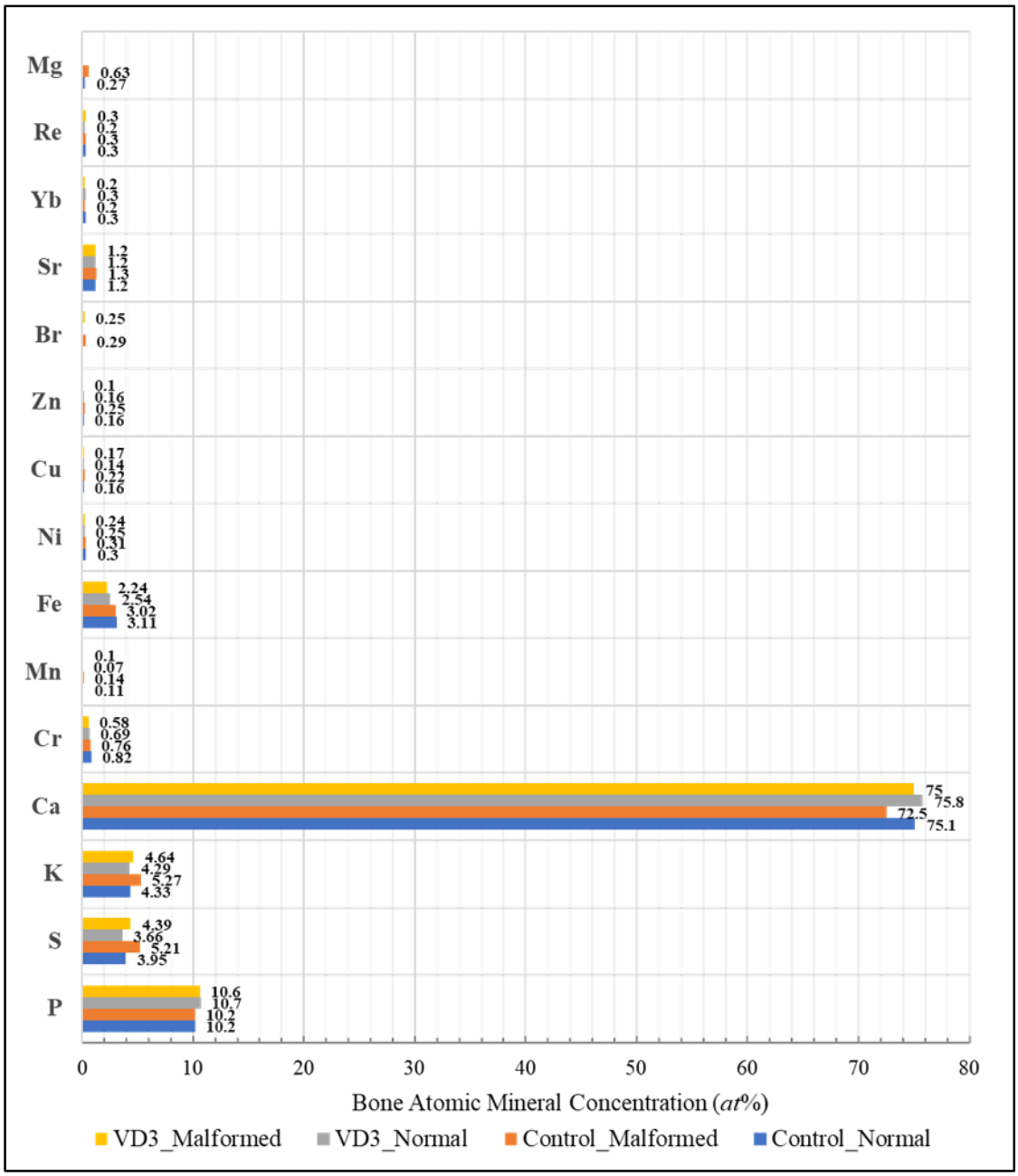

Figure 2. Bone mineral atomic composition in *Epinephelus fuscoguttatus* X *Epinephelus polyphekadion* hybrid grouper juveniles.

Table 3

X-ray Diffraction (XRD) crystal structure of normal and deformed bone mineral constituents

Treatment	*Architecture*	*Phase*	*Formula*	*Mineral*	*Quantity*		*Crystallinity*		*GoF**	*X^2***
					%	*Std*	*Diameter (nm)*	*Std*		
Control	Normal	Merrillite	$Ca_{18.88}(Mg_{1.87}Fe_{0.13})(PO_4)_{13.77}(PO_3(OH))_{0.23}$	Merrillite	75.9	1.8	86.6	3.9	1.11	1.23
		$Ca_9CrP_7O_{28}$	$Ca_9Cr(PO_4)_7$	TCP-Cr0.29	10.9	1.1	211	36		
		Hydroxyapatite	$Ca_5(PO_4)_3(OH)$	Hydroxyapatite	7.5	0.5	65.8	8.6		
		$Sr_{0.25}Ca_{2.75}P_2O_8$	$Sr_{0.25}Ca_{2.75}(PO_4)_2$	TCP-Sr0.25	5.6	1	191	54		
	Malformed	$Ca_9CrP_7O_{28}$	$Ca_9Cr(PO_4)_7$	TCP-Cr0.29	17	2	163	23	1.1	1.2
		CuHydroxyapatite	$Ca_5Cu_{0.27}(PO_4)_3O_{0.54}(OH)_{0.32}$	Hydroxylapatite	15.9	0.6	68.4	5.9		
		$Ca_{10}NaP_7O_{28}$	$Ca_{10}Na(PO_4)_7$	TCP-Na0.29	64	2	76.7	3		
		K_3PO_4	K_3PO_4	K3PO4	2.5	0.5	348	216		
VD3	Normal	Merrillite	$Ca_{18.88}(Mg_{1.87}Fe_{0.13})(PO_4)_{13.77}(PO_3(OH))_{0.23}$	Merrillite	79.7	2	76.9	2.6	1.11	1.23
		$Ca_9CrP_7O_{28}$	$Ca_9Cr(PO_4)_7$	TCP-Cr0.29	10.5	1.9	174	25		
		CuHydroxyapatite	$Ca_5Cu_{0.27}(PO_4)_3O_{0.54}(OH)_{0.32}$	Hydroxylapatite	1.62	0.64	148	55		
		Oxyapatite	$Ca_{10}(PO_4)_6O$	Oxyapatite	8.2	0.71	196	53		
	Malformed	Merrillite	$Ca_{18.88}(Mg_{1.87}Fe_{0.13})(PO_4)_{13.77}(PO_3(OH))_{0.23}$	Merrillite	86.5	1.7	62.3	2.1	1.09	1.18
		CuHydroxyapatite	$Ca_5Cu_{0.27}(PO_4)_3O_{0.54}(OH)_{0.32}$	Hydroxylapatite	5.92	0.54	51.2	9.7		
		$Sr_{0.25}Ca_{2.75}P_2O_8$	$Sr_{0.25}Ca_{2.75}(PO_4)_2$	TCP-Sr0.25	7.6	1.6	177	49		

Note: * - ideal category if GoF (goodness of fit index) =1; ** - acceptable if $X^2<1.5$.

Discussion. The bone mineral is structurally related to hydroxyapatite (HA), a naturally occurring geological mineral, but differs in crystal size, purity and the type of impurities present. According to the crystallization structure, the main phase composition of the normal bone microstructure ranges from 74.9 and 79.7% merrillite and 10.5 to 10.9% TCP-Cr0.29 (Table 3). In contrast, VD3-affected bone has only one dominant phase, merrillite, which is 7.63-12.25% higher than in normal bone. However, the crystal size of the merrillite phase in deformed VD3 bone was between 23.43 and 39% smaller than that of normal bone. Unlike other bones, the malformation control bone had a distinct dominant phase composition, containing a TCP-Cr0.29 phase, which was the second dominant phase in the normal bone, with a composition 35.88 to 38.24% larger than that of the normal bone and a crystal size 6.75 to 29.45% smaller than that of normal bone. Several conclusions can be drawn from the results of these observations.

Firstly, the predominant phase of normal bone formation in the EFEP hybrid grouper fry was merrillite. The other phase is an impurity phase of bone formation. This impurity phase is related to substituted minerals in the bone. The bone crystal matrix is formed by crystallization by CPA. The particles in question are ions, dimers, and oligomers, complex and ionic clusters, liquid-phase and amorphous precursors nanoparticles with poor crystallinity, and nanocrystals (Wallace et al 2013; De Yoreo et al 2015). Those particles will go through a process of polymerization, reorganization, phase transformation, and (oriented) aggregation. That regulation itself is controlled by numerous parameters, including energy kinetics, phase stability, and the presence of additives such as gels, polymers, and small molecules. Contrary to what was previously thought, the crystalline phase of the bone is not simply composed of hydroxyapatite [$(Ca)_{10}(PO_4)_6(OH)_2$] (Scheele 1931; Eliaz & Metoki 2017). Referring to the CPA theory, it provides opportunities for other phases to participate in bone formation. Octacalcium phosphate (OCP) and tetracalcium phosphate (TCP) phases were discovered to play a role in the formation of human bone and tooth structure (Brown 1966). According to Pasteris et al (2008), bone is composed of mineral components, organic fractions, and water molecules. On this basis, the hydroxyapatite crystal structure is capable of substituting with other minerals and varies in composition, which influences the micro-crystal architecture of the bone (Noor et al 2012). During the mineralization process, the bone tissue undergoes maturation, transforming the initial phase of bone constituents into a crystalline structure (Aufort et al 2019). The manifestation of the substitution of these minerals affects the mineral phase of bone production, causing it to become an impurity in the primary phase of bone formation.

Secondly, the deformed bone crystals of the hybrid grouper are much smaller than those of normal bone. This indicates that the deformed bone matrix has a lower hardness than the normal bone. Together with increased crystallinity, stiffness and bone density also improve (Yerramshetty & Akkus 2008). On this basis, crystallinity size can be used to determine normal bone minerality characteristics and deformities in hybrid EFEP groupers. The crystallinity index is also used as a variable to determine a new angle for the assessment of bone mineral characteristics in osteoporotic patients (Farlay et al 2010).

Thirdly, the contribution of VD3 to bone mineralization was evident in both VD3-malformed and malformed-control bones. In VD3-malformed bones, VD3 maintained the primary phase of normal bone development. In this respect, it is clear that mineral imbalances can lead to skeletal abnormalities. A study found that mineral imbalances can interfere with metabolism and lead to osteodystrophy, bone abnormalities. This is one of the reasons why mammoths went extinct (Leshchinskiy 2017).

In addition to the crystal phase, bone mineral content can be used to differentiate between normal and deformed bones. Ca and P levels in VD3-treated EFEP hybrid grouper were greater than in control fish. This suggests that adding VD3 can increase the Ca and P mineral content of bones, which play a role in mineralization. The control-defective bone had less calcium than the control-normal and VD3-normal fish. Ca ion absorption into the bones of control-malformed fish appears to be more inhibited than P ion absorption. VD3 may enhance intracellular Ca levels, since it facilitates Ca absorption from the environment via both the second messenger (Boland 2011) and the VDR pathways (Sutton et al 2005). Even though the Ca and P concentrations of VD3-deficient juveniles were equal to those of normal juveniles in both treatments, bone abnormalities were discovered. In comparison

to VD3-normal, the Ca/P ratio is also insignificant. This implies that factors other than Ca mineral deficiency cause bone deformities.

It was discovered that EFEP hybrid grouper treated with VD3 did not contain the mineral Mg. This is possible because vitamin D is activated by the absorbed magnesium (Uwitonze & Razzaque 2018). Magnesium is a cofactor in renal enzymatic processes. Because vitamin D metabolism requires enzymatic actions that need Mg, Mg's role is restricted to that of a cofactor. Mg is essential for bone biomineralization to maintain bone density and prevent brittleness (Kim et al 2014). In the control treatment, Mg is transformed into a mineral deposited into the bone matrix. Thus, additional research must be conducted to determine the impact of VD3 on bone matrix synthesis. Consideration must be given to the dose and duration of VD3 administration on bone formation.

Based on the mineral composition of the bones, the mineral Br can be utilized to distinguish between normal and deformed bones. Br can only be found in malformed bones. Not all minerals that are deposited in the bone have a positive influence on bone regulation. In general, apatite has a hexagonal structure with the chemical formula $[M(1)]_2[M(2)]_3(TO_4)_3X$, which allows for the interaction of various monovalent X anions. Br mineral atoms are classified as reactive halogen elements. In one study, apatite mimetites were synthesized with Br-, which replaced the OH- anion and reduced the c/a ratio. This will reduce the interatomic distance and the T-O distance (T = pentavalent cation) when the variable turning angle of the meta-prism shrinks (stenosis) (Sordyl et al 2020). Topologically, this causes a narrower bone structure. Various chemical components can be substituted for anion X, causing the c/a ratio to change. The c/a ratio can be used to identify structural changes in the bone matrix. The normal hydroxyapatite c/a ratio range is $(2\sqrt{2})/(\sqrt{3})$; thus, if the c/a ratio range is close to the normal value, the hydroxyapatite structure has no significant effect on the bone matrix structure.

According to this research, K and S are minerals that distinguish between normal and malformed bones. K is required for suppressing osteoclastogenesis, collagen degradation, and the expression of RANKL-mediated osteoclast differentiation genes (Granchi et al 2017). K minerals play a function in maintaining the pH equilibrium in bones during bone regulation by suppressing Ca excretion (Lambert et al 2015; Dawson-Hughes et al 2015). Under low pH conditions, it will induce bone resorption activity (osteoclastogenesis), particularly in mineral substances and non-collagenous protein (NCP) (Granchi et al 2017). A low pH will influence the solubility of chemicals, causing the minerals that comprise the bone matrix to degrade and the bones to lose their rigidity and become pliable. This is because collagen protein cannot be destroyed under acidic circumstances, but only through an enzymatic reaction (Mort et al 2016). Bone cells will not experience Ca deficit due to the suppression of urinary Ca excretion, and bone matrix development will proceed normally. Mineral K can also stimulate osteoblast proliferation and alkaline phosphatase expression.

S also plays an important role in the development of bone tissue. Methionine and cysteine are formed when sulfur binds to amino acids (Total Sulfur Amino Acids, or TSAA). Methionine is more critical than cysteine because only methionine can satisfy methionine demands, although cysteine can be replaced by methionine, which can be permanently transformed into cysteine (Stipanuk 2004). Adding TSAA to the diet of livestock can improve bone mineral density (BMD) of trabecular and cortical bone and bone mineral content (Castro et al 2019; Castro et al 2020). TSAA concentrations are correlated with NCP. NCP regulates bone remodeling and mineralization during skeletal development (Morgan et al 2015). 2% of the total NCP of the total bone protein is osteonectin (Clarke 2008), which is rich in cysteine and plays a role in calcium binding (Heinegrd & Oldberg 1989). This demonstrates that mineral S is essential for the formation of bone architecture. Although the presence of K and S minerals is essential for bone management, the activity of osteoblasts and osteoclasts must also be balanced. Therefore, adequate levels of K and S minerals must also be present in the bones. This was also supported by Dawson-Hughes et al (2015), who found that the need for potassium in low amounts has a beneficial effect compared to high doses. This study demonstrated that minimal levels of the minerals K and S are required for normal bone development.

It was recognized that malformed bones had a low calcium content, an excess of K and S minerals, and a deposition of Br. Ca, K, and S minerals play a significant role in the control of bone. Ca is the primary mineral in the formation of the apatite matrix. Hence, its presence is essential (Chang et al 2000). Therefore, a Ca deficit will have a significant impact on the structure of bone hydroxyapatite. Ca, in the form of amorphous calcium phosphate (ACP) rather than ionic bonding from solution, functions as a precursor to the production of apatite (Roohani et al 2021). Solution and renucleation processes transform the amorphous phase into crystal apatite. Nucleation is the process of formatting crystals from supersaturated clusters of dissolved complex molecules. This supersaturation condition is significantly affected by temperature, which impacts solvent evaporation and reaction or diffusion rates (Prayuga 2022). This indicates that water temperature will greatly affect the bone mineralization process. Considering that saltwater contains numerous types of minerals, the synthesis of multiple minerals to form complex compounds is highly possible. Therefore, the bone matrix may have many crystalline phases. This is further confirmed by the results of XRD analysis, which revealed that the crystal matrix of normal and deformed bones is composed of distinct phases. During the treatment process applying VD3 in the larval to juvenile stage, it was shown that the bone crystal matrix forming phase could be maintained. This is connected to the function of VD3 in maintaining bone normocalcemia (Sutton et al 2005), as evidenced by the identical Ca mineral concentration in deformed and normal bones. This also shows that not all malformed bones are induced by a Ca deficiency but by other variables, such as the deposition of Br minerals and an excess of K and S minerals.

Conclusions. The micro-crystal phase of normal and deformed bones is different. Merrillite dominates the microcrystalline component of normal bone and malformations treated with VD3, whereas TCP-Na0.29 dominates deformed bone without treatment. The addition of VD3 to the diet of grouper larvae EFEP improved the microcrystal structure-forming phase in VD3-malformed bone. In addition to their distinct structures, deformed bones have a different mineral composition than normal bones. A deficiency of Ca minerals and an excess of K and S minerals in the bones of EFEP hybrid groupers resulted in skeletal deformities. In addition, the deposit of Br minerals in the bones of EFEP hybrid groupers causes bone abnormalities. The addition of VD3 can raise the calcium content in the bone, as demonstrated by the VD3-malformation treatment. According to the findings of this study, VD3 plays a crucial role in bone mineralization, as VD3 can maintain the microcrystalline phase, type, and mineral content of bones under optimal conditions. Bone architecture is affected by the microcrystalline phase and the mineral concentration and types deposited in the bone.

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Conflict of Interest. The authors declare that there is no conflict of interest.

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