Myocardial calcification in captive grouper  
*Epinephelus aeneus*

**Calcificazione del miocardio in cernia**  
*Epinephelus aeneus d’allevamento*

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SUMMARY - A case of myocardial calcification is described in the Mediterranean white grouper, *Epinephelus aeneus*, cultured as broodstock in Eilat (Israel, Red Sea). Large calcified nodules and scar areas were observed in the ventricular tissues. The chemical composition of the nodules, characterized by Fourier Transform Infrared (FTIR) microspectroscopy in reflectance mode in a position resolved fashion and powder X-ray diffraction, was consistent with carbonate apatite, the inorganic constituent of bone in higher vertebrates. The nodules, however, were virtually protein-free, and of a higher crystallinity than in bone and dentin and lower than in enamel. X-ray microcomputed tomography revealed the calcified nodules as three-dimensional roundish structures. Absence of collagen in the nodules, however, seems to rule out a cell-regulated, osteogenic calcification. As no calcification was detected in any other organ, it is hypothesized that an unidentified lesion occurred in the grouper’s heart acting as a predisposing factor and that the calcification was a flawed attempt of compensatory repair. In it, calcium and phosphate ions precipitating from the bloodstream, possibly as the result of deficiency in physiological calcification inhibitors, gradually impregnated the heart tissue. With the crystalline nodules embedded in the ventricular tissues growing in size and number, the fish presumably suffered from a prolonged cardiac insufficiency, culminating in heart rupture and consequent lethal hemorrhage.

RIASSUNTO – È stato osservato un caso di calcificazione del miocardio in una cernia bianca mediterranea, *Epinephelus aeneus*, allevata come riproduttrice a Eilat (Israele, Mar Rosso). Sono stati evidenziati numerosi noduli calcificati e aree di cicatriceizzazione nei tessuti ventricolari. La composizione chimica dei noduli, caratterizzata specialmente mediante microspettroscopia all’infrarosso in riflessione (utilizzando la trasformata di Fourier) e diffrazione X, è risultata compatibile con l’apatite carbonata, il costituente inorganico delle ossa e dei vertebrati superiori, ma pressoché priva di materiale proteico e con una cristallinità più elevata rispetto a quella ossea e della dentina, ma meno elevata di quella dello smalto. La tomografia microcomputerizzata ha rivelato la struttura tridimensionale rotodeggiante dei noduli. L’assenza di collagene nei noduli sembra poter escludere una calcificazione di tipo osteogenico-cellulare. Dal momento che la calcificazione non è stata rilevata in altri organi del pesce, si avanza l’ipotesi che una presunta lesione al cuore della cernia abbia agito da fattore predisponente e che la calcificazione sia sopravvenuta a seguito di un fallimento tentativo di riparazione compensatoria. Ioni calcio e fosfato provenienti dal circolo sanguigno sarebbero precipitati nella sede della lesione principale impregnando gradatamente i tessuti cardiaci, forse a causa di una deficienza di inibitori fisiologici di calcificazione. Col tempo, i noduli formatisi nei tessuti ventricolari sarebbero cresciuti di numero e dimensioni. La cernia avrebbe così sofferto di una prolungata insufficienza cardiaca, culminata in una emorragia letale.

**Key words:** Myocardial calcification; Grouper; *Epinephelus aeneus*; Carbonate apatite; FTIR microspectroscopy; X-ray diffraction; X-ray microcomputed tomography.

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through 50 m³ tank. Seawater salinity in this system is fairly constant at 40 ppt ± 0.5 ppt, and sea surface water temperature at the time was 24°C ± 0.5°C. The grouper was fed mainly squid, and occasionally fresh or frozen fish and pelleted feed. Heart, liver, spleen, kidney, eye and swim bladder tissues were fixed in buffered neutral formalin (BNF). The heart was cut into two halves. One half was processed for histological examination and, due to its extensive calcification, was treated in formic acid (Sheehan & Harpach, 1980) by an exceptionally long demineralization procedure. The second half was analyzed with FTIR-RM, XRD and μCT. It was imaged first, then lyophilized, and finally imaged again. After lyophilization, the white nodules became brittle and chalky, and a small amount of particles was collected for powder XRD. The specimen was then embedded in acrylic embedding medium (Ultra-Mount™, Buehler, Lake Bluff, IL, USA) with its wide cross section parallel to the flat bottom of the mold. After curing at room temperature for 24 hours, both sides of the specimen were subjected to dry polishing with abrasive papers sequentially to 4000 grit.

**Histology**

Fixed heart, kidney and liver tissues were cut to 5 μm sections (Leica RM2245 microtome, Wetzlar, Germany), stained with hematoxylin and eosin (H&E) and viewed under a Nikon light microscope (Labophot-SA, Japan).

**FTIR Microspectroscopy**

Fourier Transform Infrared (FTIR) microspectroscopy was used to map the composition and the two dimensional (2D) spatial distribution of the calcified nodules in the entire cross section of the heart. The analyses were performed using a Nicolet Magna-IR 550 FTIR spectrophotometer interfaced with a Nic-Plan IR microscope operated in reflectance mode (FTIR-RM). The microscope is equipped with a video camera, a liquid nitrogen-cooled mercury cadmium telluride (MCT) detector (Nicolet Instrumentations Inc. Madison, WI, USA) and a computer-controlled translation stage (Spectra-Tech, Inc., Shelton, CT, USA) programmable in the x and y directions. The spectra were collected from the surface of the polished acrylic embedded native heart in the 650 cm⁻¹ to 4000 cm⁻¹ region with 8 cm⁻¹ spectral resolution; 32 scans and beam spot size of 200 μm x 200 μm of the full cross section (Figures 2f and 2g) and with 120 μm x 120 μm (Figures 2h and 2i) when zoomed on the more calcified region. The spectral point-by-point mapping of the surface of the cross section of the heart was performed in a grid pattern with the use of the computer-controlled microscope stage and Atlas mapping software. The reflectance spectra were proportioned against the background of an Al mirror and transformed to absorbance-like spectra using the Kramers-Kronig transform algorithm (Chalmers et al., 1996) for dispersion correction. The FTIR-RM maps were processed as mineral maps (area under the v₁PO₄ peaks at 750 cm⁻¹ to 1150 cm⁻¹ spectral region) and 1495 cm⁻¹ to 1700 cm⁻¹ amide I and II peaks, and displayed as color contour maps. Individual spectra extracted from the maps were compared to bovine bone and human dentin and enamel spectra obtained from the surfaces of the respective specimens, and to spectra obtained from pressed pellets of synthetic apatite (prepared under physiological conditions: 37°C, pH 7.4, 5% CO₂) and from highly crystallized hydroxyapatite that was prepared at 900°C in a water vapor atmosphere (Fowler, 1974), all obtained in reflectance mode and corrected in the same way as those of the fish heart.

**X-ray Diffraction**

X-ray Diffraction (XRD) was utilized to characterize the chemical structure of the mineral. The powdered particles that were scraped from the white nodules in the lyophilized fish heart were packed in an Al holder with suitable cavity depth and width and were subjected to XRD analysis. The XRD pattern was recorded in the 4° to 60° 2θ range in intervals of 0.010°.
Figure 1 - Histology images (H&E) of various tissues of the grouper: (a) Myocardium ventricle: scar tissue (arrowed), which would be typical of myocardial infarction in higher vertebrates, is evident. (b) Renal tissue: severe interstitial nephritis, lymphocytic infiltration (arrows) and sporadic ceroid deposits (arrowheads) are seen, but no signs of nephrocalcinosi. (c) Liver tissue: area of fatty degeneration shows severe vacuolation (circled) and cosinophilic inclusion bodies (arrows).

Figura 1 - Sezioni istologiche (EE) di vari tessuti di cernia: (a) Venticolo miocardico: è evidente il tessuto cicatrizzato (frecce) che, nei vertebrati più evoluti sarebbe tipico di infarto del miocardio. (b) Reni: sono visibili una grave nefrite interstiziale con infiltrazione di linfociti (frecce) e sporadic depositi ceroidi (punte di freccia), ma nessun segno di nefrocalcinosi. (c): Fegato: Area di degenerazione lipidica con severa vacuolizzazione (nel cerchio) e corpi inclusi cosinofili (frecce).
Figure 2 - Images and FTIR-RM maps of the cross section of the halved grouper's heart: (a) Fixed - native. (b) After freeze drying. (c) After embedding in acrylic and polishing. (d) Visual map of the whole specimen (obtained with the FTIR microscope). (e) Visual map of the main calcified region (dotted box in d). (f) and (g) FTIR-RM mineral (PO₄) and proteins (amides) maps of the whole cross section (200 µm x 200 µm spatial resolution). (h) and (i) FTIR-RM mineral (PO₄) and proteins (amides) maps of the main calcified region (dotted...
Figure 3

(a) Schematic representation of the experimental setup.

(b) Photograph of the sample under study.

(c) Raman spectra showing the relative absorbance against wavenumbers (cm⁻¹).

(d) X-ray diffraction patterns of various materials.
DISCUSSION AND CONCLUSION

Despite the different ecological environments, the pathways leading to the pathology in our grouper may have been similar to that observed by Prior et al. (1968) and Evans (1974) in trout, i.e. starting with a cardiac injury. In humans, hydroxyapatite is found in cardiovascular calcification often at sites of myocardial infarction (Towler et al., 1998; Tintut et al., 2000; Ribeiro et al., 2006). Since the abundant scar tissue in the fish heart (Figure 1a) suggests that the replacement fibrosis had started at least several weeks before death, if comparable to human disease, the damage produced by a predisposing lesion may have triggered the precipitation of calcium and phosphate ions from the blood. Gradually increasing in size and number, the hard crystals of carbonate apatite embedded in the cardiac tissue presumably compromised cardiac functions. The peritoneal edema, in which gelified serous exudate filled the abdominal cavity, may have been the indirect consequence of congestive heart failure, with a domino effect on the branchial and renal circulation. The grouper's interstitial nephritis and copious lymphocytic infiltration (Figure 1b) were an indication of a possible latent infection. Although renal failure is known to predispose to calcification of tissues, if this were the case, metastatic calcification of other organs, including nephrocalcinosis or urolithiasis, should have been expected. The heart rupture and extensive hemorrhage observed in the pericardial cavity seems to be the final outcome of the physiological impairment of the calcified heart.

The smaller, more resolved nodules seen in the cross section of the heart after embedding and polishing (Figure 2c) might have been either the result of the embedding material penetrating through the porous tissue surrounding the nodules, or due to their different distribution and sizes in the polished surface, which is slightly below the plane of the unprocessed surface of the heart after freeze-drying (Figure 2b). Comparison between the dark orange spots (lowest IR intensity, as the background) in the protein map (Figure 2g) and the blue spots (highest intensity) in the mineral map (Figure 2f) reveals that the protein map is almost a negative image of the mineral map. This indicates that the granules contained primarily carbonate apatite without proteins and are thus very different from bone and dentin (see Figure 3c). The relatively larger blue areas seen in the nodules in the mineral map that was acquired with higher spatial resolution (Figure 2h) compared to those in the map with lower spatial resolution (Figure 2f) indicate that the higher spatial resolution captured even more calcified regions and that many of the “individual” calcified spots were larger than 200 μm x 200 μm (had they been any smaller, after mapping with higher spatial resolution, the blue spots would have appeared smaller, indicating that the individual nodules would have been below the spatial resolution). The optical images obtained from large blue spots in the FTIR-RM mineral (PO₄) map (Figure 3a) revealed that these calcified nodules are even larger than 940 μm, which is the largest individual image that can be obtained by the lowest lens (x10) of the FTIR microscope. These continuous 2D calcified regions were found to be continuous 3D nodules inside the heart by μCT (Figure 4a to 4l). The absence of collagen (amides) peaks from the FTIR-RM spectra of the whitish nodules while that of bone contained large amide peaks suggests a different formation mechanism.

The XRD results corroborated and complemented the FTIR-RM findings. It confirmed that the only inorganic compound present in the calcified heart is apatite and added important information about its crystallinity. Comparison of the main reflections of apatite at the 25° to 37° range (Figure 3d) indicates that the phase collected from the calcified heart was more crystallized than bone, dentin and physiological synthetic apatite (narrower and more resolved peaks) but less crystallized than enamel and highly crystallized hydroxyapatite. This was most clearly seen at the 34° reflection and the split in the 32° region. Sharper and more resolved peaks represent higher crystallinity (Klug & Alexander, 1974). The 1/FWHM
Watabe, 1979; Zylberberg & Nicolas, 1982). Calcium can also be reabsorbed, as in starving or pre-spawning salmon, during which time depletion from scales occurs (Bruno & Poppe, 1996). Calcium homeostasis is maintained in teleostean fish by the hypocalcemic factor stanniocalcin (STC), a glycoprotein hormone produced from the corpuscles of Stannius (Hazon & Balment, 1998), a structure embedded in the kidney tissue (Wagner, 1994). Although the STC-producing corpuscles of Stannius are fish-specific organs, in mammalian intestines and kidneys the hormone is known to regulate calcium and phosphate absorption (Wagner, 1994).

The absence of proteins and any other organic components in the calcified nodules in the grouper’s heart clearly indicates that the calcification mechanism in the presented case was different from the cell-regulated, osteogenic scale and bone formation which involves collagen. The mechanism of formation of apatite in human cardiovascular system because of local deficiency of natural calcium phosphate inhibitors (e.g., pyrophosphate, Mg) in certain organs has been proposed by several investigators (Eidelman et al., 1987; Ketteler et al., 2003; Speer & Giachelli, 2004). On this ground, bisphosphonates, derivatives of pyrophosphate known as calcification inhibitors, were attached to artificial heart valves (Connolly et al., 2005). This mechanism was listed in a review by Giachelli (2004) as the first out of four mechanisms that in humans may arise from several different, non-mutually exclusive pathways (Speer & Giachelli, 2004), each ending in vascular calcification:

1) Spontaneous vascular calcification as a result of loss of mineralization inhibitors, such as pyrophosphate and matrix GLA protein that are expressed normally in blood vessels in human was hypothesized by Rutsch et al. (2003). Spontaneous calcification of arteries and cartilage occurred in mice that lacked the matrix GLA protein (Luo et al., 1997).

2) Osteogenic mechanisms through induction of bone formation by bone morphogenetic proteins expressed in human atherosclerotic lesions as osteopontin (Giachelli et al., 1993), osteocalcin (Levy et al., 1983), and BMP2 (Bostrom et al., 1993). In vitro studies showed that cells derived from vascular media underwent bone-like phenotypic change and calcification (Tintut et al., 1998; 2000; Jono et al., 1998; 2000; Parhami et al., 2002).

3) Circulating nucleational complexes: link between vascular calcification caused by circulating nucleational complexes released in bone turnover and osteoporosis in postmenopausal women (Price et al., 2002).

4) Cell death: phospholipid-rich membranous debris and apoptotic bodies can nucleate apatite, especially in atherosclerosis (Tanimura et al., 1983; Schoen et al., 1986; Proudfoot et al., 2000).

Elevated Ca, P, and Ca × P thermodynamically promote apatite nucleation and crystal growth and can enhance vascular calcification initiated by any of the four mechanisms (Giachelli, 2004). A recent in vitro study (Olsson et al., 2007) showed that nanocrystals of carbonate apatite with composition and morphology analogue to atherosclerotic plaque formed in vivo, precipitated from human serum-like solutions that did not contained inhibitors, supporting the mechanism of spontaneous precipitation of carbonate apatite in the absence of physiological inhibitors. The relatively high levels of ionized Ca and inorganic P in fish blood, and the absence of proteins and any other organic components in the calcified nodules in the grouper’s heart in this study, support the hypothesis that the reported calcification might have followed the first mechanism.

Protein-free carbonate apatite was not reported to have been found in cardiovascular deposits retrieved from human patients most probably because, to the best of our knowledge, no position resolved characterizations of cardiovascular deposits were ever made. We mapped the intact native deposits with high spatial resolution whereas in human
study shows how FTIR-RM, XRD and X-ray μCT used on the same specimen can help fully characterize the chemical composition, crystal structure, morphology, shape and three-dimensional distributions of calcified deposits in any mineralized tissues specimens.

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Disclaimer

Certain commercial materials and equipment are identified in this work for adequate definition of the experimental procedures. In no instance does such identification imply recommendation or endorsement by the National Institute of Standards and Technology or the American Dental Association Foundation or that the material and the equipment identified is necessarily the best available for the purpose.

REFERENCES


cardiovascular studies, the specimens were reportedly crushed or homogenized or powdered without charting the position of the nodules in the heart. The mineral deposits that were isolated from atherosclerotic plaque of human calcified aorta and crushed before the chemical analyses contained 71% calcium apatite, 9% carbonate and 15% proteins (Schmid et al., 1980). However, the proteins might have originated from the surrounding tissues. A small amount of calcified material that looked as single-phase was removed from an excised aortic calcified valve, ground finely, subjected to XRD, and found to be Ca deficient hydroxyapatite (Ortlepp et al., 2004). The excised calcified valves were homogenized, dissolved in sulfuric acid and the calcium content was determined by atomic absorption. Similar methods were used in a study that found a correlation between lower serum calcium levels and higher calcium hydroxyapatite deposition in native aortic valves of male patients with severe calcific aortic stenosis (Ortlepp et al., 2006). In these two last studies, the mineral that was deposited in the valves was not separated from possible attached soft tissues. Local calcification in various regions of the calcified valves (position resolved) was not carried out either. Since no FTIR analysis was done, presence or absence of proteins in these deposits could not be established. Had we used the same procedure, then the tissue surrounding the heart (blue and deep yellow in the amides map, figure 2g) would have been included in the homogenate and we would have seen amides in the FTIR-RM spectra as well. This artifact was avoided in our study by keeping the integrity of the heart specimen and the nodules.

This grouper had been held captive at the NCM facility for six years, and was presumably at least 3 years old upon capture off the Mediterranean coast of Israel. Like other living organisms, fish do not elude degenerative processes that naturally occur with aging. Groupers in particular, due to their relatively large size, social behavior and, as yet, only partially understood nutritional requirements, show poor adaptation in captivity, so that the unnatural conditions of confinement and the cumulative effects (whether by deficiency or excess of some factor) of an unchanging or rarely modified diet would probably exacerbate any health problem. It is noteworthy that a second large (7.5 kg) E. aeneus individual from the same tank died three months later with pericarditis, myocarditis and infiltration of connective (scar) tissue into the pericardium. The calcification case presently reported may have developed from a similar cardiac lesion. In fish, the spongiotic ventricular myocardium is a common site for lesions of various nature (Ferguson, 1989). Granulomatous lesions by Mycobacterium marinum in sea bass Dicentrarchus labrax (Colorni, 1992) and lymphocystis cells in red drum Sciaenops ocellatus and sea bream Sparus aurata (Colorni & Diamant, 1995) - but no sign of calcification - have been observed in the fish heart ventricle, during epizootics at the IOLR-NCM facilities. Sanguinicoldis too have occasionally been observed in the gills of our captive E. aeneus in Eilat. The adults of these flukes are present in the fish heart and major blood vessels, and release embryonated eggs into the blood stream, which lodge in and clog narrow capillary networks. The necrotic areas in the grouper liver (Figure 1c) were possibly due to incipient enzymatic autolysis as the fish had been dead for some hours before the post-mortem analysis was performed.

In conclusion, it is hypothesized that an unidentified lesion occurred in the grouper's heart acting as a predisposing factor, triggering calcification in a flawed attempt of compensatory repair. In it, calcium and phosphate ions precipitating from the bloodstream, possibly as the result of a deficiency in the natural calcification inhibitors, gradually impregnated the heart tissue. With the crystalline nodules of carbonate apatite embedded in the ventricular tissues growing in size and number, the fish presumably suffered from a prolonged and progressively more severe cardiac insufficiency, culminating in heart rupture and consequent lethal hemorrhage. The study of cardiovascular diseases in fish is still in its infancy. Increasing awareness of these pathologies may soon reveal a higher incidence. The present
values of the 002 (25.8°) peaks (Table 1) obtained from the XRD patterns presented in figure 3d, and their values relative to those of bone and the highly crystallized hydroxyapatite reveal that the crystallinity of the apatite present in the calcified nodules is 1.4 times higher than that of bone and dentin and corroborate quantitatively the above observations that the crystallinity of the apatite in the nodules was higher than that of the physiological apatite, but lower than enamel and about half of the highly crystallized solid-state thermally prepared hydroxyapatite.

<table>
<thead>
<tr>
<th>Sample</th>
<th>1/FWHM</th>
<th>Relative to bone</th>
<th>Relative to OHAp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physiological apatite</td>
<td>2.50</td>
<td>1.30</td>
<td>0.42</td>
</tr>
<tr>
<td>Dentin</td>
<td>1.99</td>
<td>1.03</td>
<td>0.33</td>
</tr>
<tr>
<td>Bone</td>
<td>1.93</td>
<td>-----</td>
<td>0.32</td>
</tr>
<tr>
<td>Calcified nodules</td>
<td>2.70</td>
<td>1.40</td>
<td>0.45</td>
</tr>
<tr>
<td>Enamel</td>
<td>3.55</td>
<td>1.83</td>
<td>0.59</td>
</tr>
<tr>
<td>OHAp</td>
<td>5.95</td>
<td>3.09</td>
<td>-----</td>
</tr>
</tbody>
</table>

Table 1 - Crystallinity (1/FWHM) values of the calcified nodules, various calcified tissues and apatite powders (obtained from their XRD patterns), and their relative values to those of bone and the highly crystallized hydroxyapatite (OHAp).

Tabella 1 - Valori di cristallinità (1/FWHM) dei noduli calcificati, di vari tessuti calcificati e di polveri di apatite (ottenuti in base ai loro profili XRD), in relazione a quelli di osso e di idrossiapatite (OHAp) ad alto grado di cristallizzazione.

In summary, in this study we used three complementary techniques: the first, FTIR-RM, revealed the presence of a phosphate band similar to that of apatite, of carbonate and only negligible traces of amides. The second technique, XRD, confirmed that the inorganic phase in the nodules is indeed apatite but with significantly higher crystallinity than in bone. The third, μCT, showed that the calcified nodules, seen as 2D structures in the FTIR-RM and visual maps, are continues in all dimensions. Together, the three techniques supplied complementary information that allowed us to characterize the calcified inclusions as being three-dimensional roundish nodules composed of protein free highly crystallized carbonate apatite.

In fish, calcium and phosphate are present in circulating blood and fluids in free ionic form and as structural part of bone and scales. The concentration of inorganic phosphorous in the blood of juvenile Malabar grouper (Epinephelus malabaricus) (Cheng et al., 2005), for example, is 48.5 mg/L (1.6 mmol), and the ionized calcium concentration in the blood of sea bream (Sparus aurata) is 1.5 mmol (Guerreiro et al., 2002). These values are higher than the average concentrations in human serum, which are about 1.2 mmol for both inorganic P and ionized calcium (Eidelman et al., 1987; Kratz & Lewandrowski, 1998). No data are available for the calcium concentration in blood of groupers. Mineralization of elasmoid scales of teleost fish occurs continuously throughout the fish life. Needle-like or flaky crystals of calcium-deficient hydroxyapatite containing small amounts of sodium and magnesium ions and carbonate anions that were incorporated into the hydroxyapatite crystal lattice in the phosphate ions sites (Ikoma et al., 2003) permeate a matrix of collagen fibers (Onozato &
The top and bottom of the nodules near the polished surfaces of the specimen are cut (arrows in image 4d for example). This image, as well as that shown in Figure 4l, was obtained from the plane perpendicular to the wide cross section that was mapped with FTIR-RM. The projected views illustrate different planes of the nodules as the specimen was rotated from 0° to 270°. Those images show that the calcified nodules, seen as cross sections in the FTIR-RM and visual maps, are continues in all dimensions, as seen especially in certain projected images (Figure 4: a to c and f to j).

**Figure 4**

![Figure 4](image-url)

Figure 4 - Projection of μCT images rotated around the X axis parallel to the plane of the FTIR-RM map. The images are arranged in a “chain order” or “alternate” left to right and right to left directions.

Figura 4 - Proiezione di immagini μCT ruotate attorno all’asse X parallelo al piano della mappa FTIR-RM. Le immagini sono disposte in un ordine “a catena” o in direzione “alternata” da sinistra a destra e viceversa.
box in d, f and g) acquired with 120 µm x 120 µm spatial resolution (about 3 times than in d, f, and g). Blue in the FTIR-RM maps represents the highest intensity of the related functional group and pink represents the lowest. The blue-green nodules in the mineral maps (f and h) represent carbonate apatite spectra. They are correlated with the light spots in the corresponding visual maps (d and e). The blue-green-yellow spots in the proteins maps (g and i) represent proteins-tissue spectra. Size scale bars are indicated in the images and maps.

Figura 2 - Immagini e mappe FTIR-RM della sezione trasversale di una metà del cuore della cernia: (a) Fissato - non processato. (b) Dopo lisoilizzazione. (c) Dopo inclusione in resina acrilica e lucidatura. (d) Visione d'insieme del campione (ottenuta al microscopio FTIR). (e) Mappa della regione principale calcificata (riquadro punteggiato in d). (f) e (g) mappe FTIR-RM di minerale (PO₄) e di proteine (amidi) dell'intera sezione trasversale (risoluzione spaziale 200 µm x 200 µm). (h) e (i) mappe FTIR-RM della regione principale calcificata (riquadro punteggiato in d, f e g di minerale (PO₄) e di proteine (amidi) acquisite con risoluzione spaziale 120 µm x 120 µm (3 volte circa quella di d, f e g). Il colore blu nelle mappe FTIR-RM rappresenta l'intensità più alta del relativo gruppo funzionale, mentre il rosa rappresenta quella più bassa. I noduli verde-blu nelle mappe minerali (f e h) rappresentano spettro di apatite carbonata e sono correlati con i punti chiari nelle corrispondenti mappe visuali (d ed e). Le macchie giallo-verdi nelle mappe delle proteine (g ed i) rappresentano spettro di tessuti proteici. I rapporti in scala sono indicati dalle barre nelle immagini e nelle mappe.

X-ray Microcomputed Tomography
A 2D µCT image obtained from ~180 µm below the top surface of the specimen that was mapped first with FTIR-RM, is shown in Figure 3b. The orientation of this image was adjusted to best match the FTIR-RM map (Figure 3a).

The small disparity in the shapes and areas of the calcified nodules between the FTIR-RM map and the µCT image, might be attributed to the fact that the µCT image was obtained at a slightly lower location in the specimen, relative to that of the FTIR-RM, and to the higher resolution of the µCT. Selected 3D µCT projection images of the calcified heart specimen are shown in Figure 4. These images are projected views of the specimen rotated around the X axis which is parallel to the long side (x-axis) of the FTIR-RM map (Figure 3a).

Figure 3 - (a) Images (obtained by the FTIR microscope) of specific calcified regions (1, 2 and 3) in the FTIR-RM mineral (PO₄) map (200 µm x 200 µm spatial resolution) of the whole cross section. Blue represents the highest intensity of the mineral (PO₄) and pink represents the lowest. (b) 2D µCT image obtained at approximately 180 µm below the plane of the FTIR-RM map. (c) FTIR-RM spectra that were obtained from the calcified nodules (average of five spectra) and the surrounding tissue of the fish heart in comparison to those obtained from bovine bone and human dentin and enamel specimens, and from pressed pellets of physiological synthetic apatite and highly crystallized hydroxyapatite. The functional groups seen in the spectra of the calcified nodules are PO₄ and CO₃. (d) Pattern of particles from the nodules in the calcified heart in comparison to those of bovine bone, human dentin and enamel, physiological synthetic apatite and highly crystallized hydroxyapatite.

Figura 3 - (a) Immagini ottenute al microscopio FTIR delle specifiche regioni calcificate (1, 2 e 3) nella mappa minerale (PO₄) FTIR-RM (risoluzione spaziale 200 µm x 200 µm) dell'intera sezione trasversale. Il colore blu rappresenta la più alta intensità del minerale (PO₄), mentre il rosa quella più bassa. (b) Immagine bidimensionale µCT ottenuta a circa 180 µm al di sotto del piano della mappa FTIR-RM. (c) Spektri FTIR-RM ottenuti dai noduli calcificati (media di cinque spettri) e del tessuto circostante al cuore del pesce confrontati con quelli ottenuti da campioni di osso bovino, dentina e smalto umani, e da granuli pressati di apatite fisiologica sintetica e di idroxiapatite ad alto grado di cristallizzazione. I gruppi funzionali evidenti negli spettri dei noduli calcificati sono PO₄ e CO₃. (d) Profilo di particelle ottenute dai noduli del cuore calcificato confrontato con quelli di osso bovino, dentina e smalto umani, apatite fisiologica sintetica e idroxiapatite ad alto grado di cristallizzazione.
FTIR Microspectroscopy

Images of the cross section of the grouper's ventricle are presented in Figure 2. The “open” ventricle preserved in BNF is shown in Figure 2a. The whitish granules were irregularly distributed in the ventricular muscle but appeared to be more densely concentrated at its core. These granules became more evident after freeze drying (Figure 2b). The tissue that surrounded them (arrows) shrunk and the drying process caused them to bulge slightly. Subsequent embedding and polishing (Figure 2c) revealed more resolved and yet smaller granules. Visual and FTIR-RM maps (200 μm x 200 μm spatial resolution) of the whole embedded and polished cross section are shown in Figures 2d, 2f and 2g. The FTIR-RM mineral map (Figure 2f) was obtained from the area under the PO₄ peaks. The blue-green nodules correspond to spectra that contained IR absorptions originating from PO₄ whose shape is typical of apatite (see spectra in Figure 3c) and carbonate that was incorporated into the apatite lattice. These spectra represent carbonate apatite. The blue-green nodules correlated with the light spots in the visual map (Figure 2d). The FTIR-RM protein map (Figure 2g) was obtained from the area under the amide peaks. The blue-green-yellow spots represent protein-tissue spectra. The tissue that surrounded the heart can be seen (arrows) in blue and deep yellow colors. There is no correlation between the blue-green nodules in the mineral map and the blue-green-yellow spots in the protein map. Furthermore, wherever blue-green colors that represent the highest intensity (area under the PO₄ peaks) appear in the mineral map, an orange area that represents the lowest intensity (area under the amide peaks) appears in the protein map. Dark orange spots (corresponding to the lowest IR intensity, as the background) correlate with the blue spots (corresponding to the highest intensity) of the mineral map.

Visual and FTIR-RM maps with approximately 3 times higher spatial resolution (120 μm x 120 μm) of the heavier calcified region of the cross section are shown in Figures 2e, 2h and 2i. As in Figures 2f and 2g, the blue-green nodules that represent carbonate apatite spectra are correlated with the light spots in the visual map (Figure 2e) and the blue-green-yellow spots in the amide map represent protein-tissue spectra. Comparison between the FTIR-RM mineral (PO₄) maps of the whole and the partial cross section of the calcified heart (acquired with higher spatial resolution, dotted box in Figure 2f) reveals that the blue areas in the respective blue-green nodules in Figure 2h are relatively larger. Images of specific calcified regions in the FTIR mineral (PO₄) map (200 μm x 200 μm spatial resolution) of the whole cross section are shown in Figure 3a. FTIR-RM spectra obtained from the calcified nodules and the surrounding tissue of the fish heart, and those obtained from bone, dentin and enamel specimens, and from pressed pellets of physiological synthetic apatite and from highly crystallized hydroxyapatite are presented in Figure 3c. The functional groups that are seen in the spectra of the calcified nodules are those of PO₄ (typical of apatite) and CO₃ (type B replacement of phosphate by carbonate in the apatite lattice). There are negligible traces of amides (the main functional groups of proteins) in the slight elevation at the 1500 cm⁻¹ to 1700 cm⁻¹ region. The FTIR-RM results indicate that the calcified nodules are composed of carbonate apatite containing negligible traces of proteins.

X-ray Diffraction

The XRD pattern of particles that were retrieved from the whitish granules in the calcified heart is shown in Figure 3d along with XRD patterns of bone, dentin, physiological synthetic apatite and highly crystallized hydroxyapatite at the 9° to 37° 20 range. All the reflections of the highly crystallized hydroxyapatite were present in the pattern of the calcified nodules, but they were not as resolved and not as sharp. There were no additional peaks to those of hydroxyapatite in the whole measured range, indicating that the only inorganic compound that was present in the calcified nodules was apatite.
20 with CuK visibly radiation (λ = 0.154 nm) using a Rigaku 2200 D-Max X-ray diffractometer (Rigaku/USA Inc., Danvers, MA, USA) operating at 40 kV and 40 mA. The powders of bone and dentin, those of the physiological apatite and those of the highly crystallized hydroxyapatite that were used earlier for the FTIR-RM measurements were scanned under similar conditions, for comparison. The estimated standard uncertainty in 20 measurements was 0.01°. The full width at one half the maximum height (FWHM) above background values of the well resolved 002 diffraction peak (25.8°) of the mineral that was retrieved from the white nodules in the calcified heart, as well as those of bone, dentin, enamel, physiological and highly crystalline apatite powders were determined with the Jade 6.1 software (Materials Data, Inc., Livermore, CA, USA) using the Pseudo Voigt function to model the peak shape. Since FWHM correlates inversely with crystal size and lattice perfection (Klug & Alexander, 1974), the reciprocal of the FWHM values (1/FWHM) for the 002 peak and their values relative to those obtained from bone and OHAp patterns, were used as a comparative quantitative measures of the crystallinity of the mineral present in the calcified heart.

**X-ray Microcomputed Tomography**

X-ray microcomputed tomography (μCT) was applied to the calcified heart to obtain the 3D internal distribution of the nodules. The embedded and polished heart segment (5 mm thick) - whose wide cross section was mapped with FTIR-RM - was imaged with an X-ray microcomputed tomography scanner (Scanco Medical μCT 40, Sweden) to acquire the 3D structures of the calcified nodules. The micro-focus X-ray source was set at 75 kV and 114 μA and the specimen was scanned at 18 μm line resolution with an integration time of 300 s. The specimen was placed horizontally with the larger cross section upright and fixed at marked positions in the μCT sample holder. The surface of the larger cross section was mapped earlier with FTIR-RM. A series of 2D images were collected and reconstructed into 3D images using the manufacturer’s complete imaging and evaluation software and ImageJ image analysis software (version 1.39), downloaded from the website of the National Institute of Health (NIH) website.

**RESULTS**

**Gross pathology and histology**

Gross pathology revealed a large clot of coagulated blood in the pericardial cavity. A gelified colorless exudate filled the abdominal cavity. The fish presented extensive calcification of the myocardium, most severe in the spongy tissue of the ventricle, with calcified whitish granules, often coalesced, of various size embedded in it. Scar tissue (Figure 1a), which would be typical of myocardial infarction in higher vertebrates, was evident. Severe interstitial nephritis with lymphocyte infiltration and ceroidosis were observed in the kidney (Figure 1b). The liver presented areas of fatty degeneration (Figure 1c). Spleen and eye tissues did not present any particular pathology, but the swim bladder had greatly and abnormally thickened walls. No signs of spongiosis in the retina, typical of viral encephalitis and retinopathy (VER) infections which previous stocks of groupers had suffered, were evident.
INTRODUCTION

Cardiomyopathies in fish have been poorly studied and most of the available information is on salmonid species (Robertson et al., 1961; Ferguson et al., 1986; Amin & Poppe, 1989; Ferguson et al., 1990), where particular attention was devoted to coronary lesions and arteriosclerosis (Van Citters & Watson, 1968; Maneche et al., 1972; Müller, 1983; Farrell et al., 1990; Saunders et al., 1992). Nutritional arteriosclerosis and cardiomyopathy have been diagnosed in a variety of fish, especially under conditions of intensive culture (Farrell et al., 1986; Ferguson, 1989). Johansen & Poppe (2002) reported pericarditis and myocarditis in farmed halibut (Hippoglossus hippoglossus). Very few cases of cardiac calcification have been reported in fish, and all of them from salmonids in freshwater. Such a disorder affecting the bulbus arteriosus of brown trout (Salmo trutta morpha fario) and rainbow trout (Oncorhynchus mykiss) caught in various lakes, rivers and streams of New Zealand was described by Prior et al. (1968). The cause of those lesions, however, was not identified. Extensive calcification and necrosis of the heart was reported by Evans (1974) in cutthroat trout, Salmo clarki, experimentally infected seven months before with the digenean blood fluke Sanguinicol a klamathensis. In neither case was the chemical nature of the mineral determined. The present study reports a case of myocardial calcification in a Mediterranean grouper, Epinephelus aeneus, cultured in Eilat (Israel, Red Sea). To the best of our knowledge, ours is the first report of such a disorder occurring in a marine fish, and the first time that Fourier Transform Infrared Microspectroscopy in Reflectance Mode (FTIR-RM), X-ray Diffraction (XRD) and X-ray microcomputed tomography (μCT) were applied in combination to determine the composition, chemical structure and internal distribution of cardiovascular calcified inclusions.

FTIR-RM, a non-contact, non-destructive mapping method for characterizing surface chemical composition both qualitatively and quantitatively (Chalmers et al., 1996; Eidelman et al., 2004) has been used previously to determine the distribution of different chemical components in human gallstones (Wentrup-Berne et al., 1995), the mineral and collagen in dentin (Tesch et al., 2001), the mineral and resins in dental composites (Skrtic et al., 2004), to map combinatorial curing gradients of epoxy films (Eidelman et al., 2004) and combinatorial composition library of biodegradable polyanhydride copolymers (Vogel et al., 2005). It was applied for the first time in the present study to map cardiovascular calcification. The advantage and uniqueness of the FTIR-RM mapping are that both the inorganic component (phosphate) and the proteins (amide groups) can be determined at the same time in the same spots from the native biological specimen with high spatial resolution without the need for either decalcifying the specimens to determine the protein contents or deproteinize them to determine the inorganic phase.

μCT that obtains the three dimensional (3D) internal structure of small objects with high spatial resolution, has been used to determine the mineral distribution and content of bones (Rueggsegger et al., 1996), teeth (Stock et al., 2002; Plotino et al., 2006) and juvenile dermatomyositis deposits (Stock et al., 2004), but was not used prior to our study to examine cardiovascular calcified deposits.

MATERIALS AND METHODS

Necropsy was conducted on an 8 kg white grouper Epinephelus aeneus Geoffrey Saint-Hilaire, 1817 (Serranidae, Epinephelinae) cultured as broodstock at the National Center for Mariculture (NCM), Israel Oceanographic and Limnological Research Institute (OOLR) in Eilat (Israel, Red Sea). The fish, a female, died in July 2005 while kept in a seawater flow-