Mechanical Behavior of Bioceramic Materials: Case Study of The Skeleton of The Staghorn Coral, Acropora cervicornis and The Tibia of The Laboratory Mouse, Mus Musculus

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MECHANICAL BEHAVIOR OF BIOCERAMIC MATERIALS: CASE STUDY OF THE SKELETON OF THE STAGHORN CORAL, ACROPORA CERVICORNIS AND THE TIBIA OF THE LABORATORY MOUSE, MUS MUSCULUS

by

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ABSTRACT

I studied two types of bioceramics: (A) the aragonite CaCO$_3$ skeleton of the staghorn coral, *Acropora cervicornis* and (B) the hydroxyapatite Ca$_{10}$(PO$_4$)$_6$(OH)$_2$ bones of the laboratory mouse, *Mus musculus*. The research aimed to analyze coral deformation behavior and study dietary and estrogen depletions effects on bone strength. I compared the mechanical and structural properties of staghorn coral skeletons cleaned by chemical bleaching and biological processes. Optical microscopy and computed tomography (CT) revealed a sophisticated microstructure of non-homogenously distributed pores. Staghorn coral skeletons showed ‘’gracious’’ fracture, where the unique pore arrangements resisted catastrophic crack growth and prevented instantaneous failure. Elastic moduli measured by uniaxial compression was 0.19 ± 0.16 GPa or 7.34 GPa, if displacement was measured by a crosshead on the universal testing machine or a clip-on extensometer, respectively. These values were substantially lower than Young’s modulus measured by nanoindentation as 86 ± 7.26 GPa, which was explained by the effect of porosity during uniaxial loading. Computed tomography scans revealed porosity of skeleton branches decreased from tip (67 ± 5 %) to base (31 ± 5 %), which indicated that coral skeletons calcify as they age. Furthermore, the deformation of skeleton increased during cyclic loading when temperature of the environment increased. Four different diets (control, high saturated fat [HSF], high fat diet [HFD] and high polyunsaturated fat [HPUF] affect the structure and strength of mice tibia. Mice raised on a HSF diet had greater tibial diameter, area and moment of inertia but lower tibial strength compared to the other groups. Moreover, the HFD was associated with enhancing the tibial strength as a result of increasing the adipose marrow tissue. However, such HFD intake
did not prevent bone loss in the osteoporotic model. Both bone density and strength of OVX+oil group were significantly lower compared to OVX+E2 and sham groups.
This dissertation is dedicated to my brother, Dr. Mazin Omer, for his endless encouragement, support and dedicated partnership for success in my life.
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CHAPTER 1  INTRODUCTION

1.1 Overview of bioceramics

Bioceramics are biocompatible ceramics materials which are very important and have been employed in the medical and dental applications [1, 2]. Bioceramics can be categorized into man-made or nature made. Man-made bioceramics include polycrystalline aluminum oxide $\text{Al}_2\text{O}_3$, zirconium oxide $\text{ZrO}_2$ and hydroxyapatite $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ composites. Such man-made bioceramics can be produced in different forms (e.g., powders, coating and bulk) and have been extensively studied for their application in repairing, reconstruction or replacement of damaged parts of the human body [3, 4, 5]. Nature made bioceramics materials includes coral skeletons and bones. Coral skeletons, which consist of aragonite $\text{CaCO}_3$ ceramic, are the inorganic part of the coral reefs [6], while the inorganic component of bone consists of the hydroxyapatite $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ ceramics [5]. Coral reefs are very important and provides many benefits ranging from protecting the coastlines from the hurricane damage and tropical storms to supporting the commercial and recreational fisheries, boating, diving and other recreational activities [7, 8, 9]. In addition, although the chemical and phase compositions of the coral skeletons and bones are very different, however, the attempts were made to convert the $\text{CaCO}_3$ aragonite structure of the coral skeleton into hydroxyapatite $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ [10, 11, 12], in order to use their complex porous structure in the medical field as bone graft substitute. Bones play an important role and provide many functions to the human body, besides providing the shape of the body, bones also protect the internal organs, provide the movement by anchoring the muscles, and store the essential minerals for the human body such as calcium and phosphorus throughout the body [13]. Nutrition intakes
play an important factor on bone health, where different dietary intake such as high saturated fat and low saturated fat influences the bone strength and overall health. Furthermore, other factors such as deficiency of estrogen hormone in the females affects the bone health and metabolism as it results in the bone loss, thus contributing to osteoporosis diseases. Over the recent decades, a lot of biomedical research has been done on the mechanical properties of animal models such as mice models due to their easy handling, small size and availability of their genetic resources. Therefore, the study of the structure and mechanical behavior of these nature made ceramics materials are of high importance as it allows to understand the structural integrity of coral skeletons, and to better predict skeleton response to changing environmental conditions. In addition, understanding the effect of diet and estrogen depletion on the mice bone strength is also of importance to maintain healthy bones and prevent bone resorption. The mechanical and structural behavior of coral skeletons and murine bone is the subject of this research. The literature overview on the properties of those nature made materials: coral skeletons and mouse bone are included in the section below.

1.2 Overview of coral reefs: corals and coral skeletons

Coral reefs are very diverse and are considered as the largest biological structure on Earth [14]. Coral reefs provide coastlines for more than 100 countries worldwide [14] and almost more than one third of the world’s marine fish species use coral reefs for their habitat. Therefore, by supporting more than 35% of the fish caught worldwide [15], coral reefs are considered as a huge source of protein intake for humans [16]. Corals consists of both inorganic and organic components. The inorganic component of the coral is their skeleton, which is built of aragonite
CaCO₃. Such a skeleton is formed and inhabited by the organic component ‘polyps’ and play an important role as it bears repeated cyclic loads applied on corals by the ocean and waves. Unfortunately, in the last decades many environmental factors bring the corals population to decline. Sedimentation is one of the important factors, which photosynthetically and physically impacts the growth of coral reefs [17]. Sedimentation photosynthetically reduces the quality/quantity of the sunlight that is required for coral nutrition which will impact their reproduction [18]. Moreover, sedimentation can also result in physical SiO₂ sand coating of the feeding polyps’ surfaces, thus, preventing the sun-light to provide solar energy to the corals [17]. Another factor that contributes to the decline of coral reefs is the presence of toxic materials in sewage and sunscreen products. Such toxic materials affect the coral reefs as they reduce the access of the sun light to the corals’ polyps, thus, impacting their growth and reproduction [19]. It was also discovered that the toxic substances in sewages result in anthropogenic water eutrophication, thus, causing bacterial infections and other diseases which results in the coral degradation [20]. Temperature rising, water quality, destructive fishing practices are other factors that affect the reproduction and growth of the corals [17]. At least 75% of the world’s coral reefs are endangered [21] and coral species that once were widespread and abundant now are listed as threatened or endangered on the International Union for Conservation of Nature’s (IUCN) Red List of Threatened Species.

1.2.1 Different coral reef species

Depending on different biomineralization and growth processes of coral skeletons in different coral reef species, they can be classified as porous perforate or dense imperforate corals [22, 23].
Perforate corals grow relatively quickly and have porous skeletons with polyps connected by extensive, deeply penetrating canal systems lined with living tissue. Imperforate corals grow more slowly and have dense skeletons without subsurface connections among polyps. The difference in biomineralization and growth rate between different coral species is determined by light, depth, and exposure to hydrodynamic stress, sedimentation, and other factors which corals experience in their natural habitat [24]. Examples of the perforate (porous) corals are *Acropora cervicornis*, *Acropora millepora*, *Acropora palmata*, *Siderastrea radians* and *Montastrea annularis*, and their descriptions are provided in the sections below.

### 1.2.1.1 *Acropora cervicornis* coral

The staghorn coral *Acropora cervicornis* is a reef-building coral, common in the shallow waters (up to 30 m) throughout the Caribbean Sea [25, 26], as well as in other locations such as south east Asia (Figure 1) [27]. *Acropora cervicornis* coral tends to branch trichotomously and has a rapid growth rate of 12 cm/year [25, 28]. In addition, *Acropora cervicornis* corals reproduce asexually, where branch fragments are able to form new colonies. Unfortunately, *Acropora cervicornis*, like all other coral species, face an uncertain future and having experienced a significant decrease in their population due to the anthropogenic forces ranging from global climate to widespread disease [29, 30, 31]. One of the most common diseases that has been affecting the acroporid corals is the white band disease (WBD) [32] which is caused by bacterial infections resulting in the removal of the tissue from the skeletons and formation of white skeletons within the colony [33]. The white band disease affects the colony in its base and spread towards the tip at an approximate rate of 0.3 mm/day causing an excess damage to the entire colony. Many factors affect the growth of these
bacteria in the corals which includes: the elevated water temperatures, dissolved organic carbon and the exposure to the different chemical products [32]. An example of a colony affected by white band disease bacterial infection is shown in Figure 2. The decline in *A. cervicornis* coral reef population has been affecting the reef community as it increased the microalgae abundance and decreased the coral reef accretion [34, 35]. Therefore, *A. cervicornis* coral has been listed as a threatened species under the USA endangered Species Act. Recently, coral nurseries in Florida, Puerto Rico, the Dominican Republic, Jamaica and Honduras are raising colonies to transplant them back onto reefs by coral gardening process [36]. During the process, laboratory raised coral fragments are planted on reefs after removing the tissue from the dead skeletons, it was observed that the gardening process does not cause significant damage on the artificial colonies as they behave similarly to the wild colonies once they are planted [37].

Figure 1 *Acropora cervicornis* coral in its natural habitat [38]
1.2.1.2 *Acropora millepora* coral

*Acropora millepora* is a ‘‘weedy’’ coral that grow in a very shallow water with low waves exposure in the Red Sea, Indian Ocean and Western pacific (up to 12 m depth). They can be found in different colors which gives them their unique appearance. The homogenous shape of the colony enhances the feeding process for the corals as it reduces the velocity of the flowing water which creates small vortices that cause the particles to fall into the polyps [40].

The *Acropora millepora* population has significantly decreased during the past 30 years due to diseases, increasing water temperatures and human fishing and recreation activities, therefore, it was listed as near threatened on the IUCN red list of threatened species [41].
1.2.1.3 Acropora palmata coral

Acropora palmata corals are found in shallow water (up to 20 m depth) throughout the Caribbean Sea. Acropora palmata colonies can grow up to 4 m in diameter and their antler-like branches are considered very thick and strong [42]. The reproduction process of the corals is asexual, as the branches fragment readily and produce new colonies. Acropora plamata branches grow 2-4 inches per year, reaching its maximum length at 12 years old [43].

Acropora plamata provides several ecosystem services and habitat to many marine species. However, 90% reduction in their population was observed as a result of diseases, ocean acidification, hurricane damage and human recreation activities. Therefore, Acropora palmata is listed as threatened under the U.S. Endangered Species Act and critically endangered by IUCN red list of threatened species [42].

Figure 3 Acropora millepora coral in its natural habitat [41]
1.2.1.4 *Siderastrea radians* coral

*Siderastrea radians* is a common coral throughout the shallow water of the West Indian and the Caribbean Sea [45] with large colonies that can grow up to 10 m in diameter [46]. *Siderastrea radians* colonies are hemispherical in shape and most frequently seen connected to a hard substrate.

*Siderastrea radians* coral is continuously subjected to severe wave stresses which causes significant damage to the colonies. However, it was observed that *Siderastrea radians* coral has the ability to recover and grow new tissues in their corallites, which is used as an important surviving mechanics for the corals [47]. Different stages of tissue recovery in *Siderastrea radians* are shown in figure 6.

![Acropora palmata coral in its natural habitat](image)

**Figure 4 Acropora palmata coral in its natural habitat [44]**
Figure 5 *Siderastrea radians* coral in its natural habitat [48]

Figure 6 Recovery stages of the skeleton of *Siderastrea radians* coral. (A) after 2 weeks, (B) after 3 weeks and (C) after 6 weeks of losing the tissue [47]

1.2.1.5 *Montastrea annularis* coral

*Montastrea annularis* coral grows in shallow and deep water throughout the Caribbean Sea and Western Atlantic. It has the largest colony size among all other coral reef species. Depending on
the water depth, currents intensity and minerals concentration, *Montastrea annularis* colonies can be found in different morphological shapes such as columns, domes or flat structures [49].

In waters with high current intensity, colonies were found to form a flat shape structure while in shallow waters and deep waters they were found in columnar shape and dome shape respectively [50]. As a result of its large colony size, *Montastrea annularis* forms a barrier to protect the coastline from tropical storms and hurricanes, it also provides a habitat to a large number of fish and marine species. However, *Montastrea annularis* has been listed as endangered species in the IUCN red list of threatened species after its population decreased significantly due to diseases, sedimentation and hurricanes [51].

![Montastrea annularis coral in its natural habitat](image)

**Figure 7 Montastrea annularis** coral in its natural habitat [52]

In addition to the perforate (porous) coral reefs detailed in the section above, examples of the imperforate (dense) coral reefs include: *Montastrea cavernosa, Duncanopsammia axifuga, Oculina patagonica, Oculina varicose, Phyllangia mouchezii* and *Phyllangia americana* [53].
1.2.2 Structure of perforate coral skeletons

1.2.2.1 Microstructure

Coral skeletons structure is very complex [54, 55] and consists of aragonite crystals organized as the primary skeletal elements [56]. *A. cervicornis* skeletal morphology is considered radially symmetric with a consistent sequence of different types of skeletal elements and cavities in between them. Marfenin [57] has described the morphology of *Acropora muricata* (*A. muricata*) in details which has a similar microstructure to *Acropora cervicornis*. A cross-section of *A. muricata* branch is shown in Figure 8, it can be observed that the structure is highly porous with non-homogenously distributed cavities along the coral. The main skeletal elements of the cross-section as corresponding to the numbers in Figure 8 are:

1- The axial corallite 
2- 1\textsuperscript{st} order radial corallite 
3- 2\textsuperscript{nd} order radial corallite 
4- The axial canal 
5- Subsystem of circumaxial cavities 
6- Radial cavities 
7- Subsystem of interradial cavities 
8- Subsystem of surface cavities

The axial and radial corallites are skeleton cups formed and inhabited by an individual coral polyp, which are tiny animals that build up the coral reef. The polyps extract the calcium and carbonate ions from seawater to build the corallites in order to protect their body. There are several millions of tiny algae called “zooxanthellae” living inside the tissue of the polyps, which provide nutrition through photosynthesis and produce the pigment which gives the corals its unique color. Cross-section of a coral polyp is shown in figure 9.
As one can see from Figure 9, the corallite also consists of the septum and the theca. The septum is a skeletal plate which radiate from the corallite wall, and it was observed that the septum formation occurs in two different steps: the first step involves producing spines at the top edge of the septa as the initial framework, such spines are further embedded and covered by a fibers tissue in the second step of the septum formation as shown in figure 10 [59].
Another skeletal element of the corallite is the theca which represents the outer wall of the corallite. The development in corallite wall (theca) structure of the skeleton of Caryophyllia salinaria coral was studied by Storalrski [60], and it was concluded that the thecal formation process occurs in three different stages: initial stage, juvenile stage and adult stage. During the initial stage the primary elements of theca and protosepta are arranged vertically and consists of minitrabecular elements. The initial skeleton is then covered by thick layers of tissue, which is developed in the next stage, while during the juvenile stage, the diameter of the corallite increased and the formation of the central structure of the corallite (center of calcification) began as a result of fusion of lower septum elements. Later in the juvenile stage, layer of the fiberous tissue (tectura) consists of aragonite crystals bundles which forms the second step of the septa are deposited on the outer ring of the theca. Finally, during the adult stage the corallite has reached its maximum diameter, and the wall is developed by thickening the outer part of the septa (septotheca) [60]. The three stages of the corallite wall (theca) development are shown in Figures 11 and 12.
Figure 11 Microstructure of *Caryophyllia salinaria* corallite showing (A,B) adult stage of the corallite with partially destroyed septothecal wall. (C) Formation of the fibers tissue (tectura) in the outer ring of the theca during the juvenile stage. (D) transverse section of the corallite, (E,F) distal and lateral views of corallite in the late juvenile stage [60]

Figure 12 Model showing the variation in *Caryophyllia salinaria* corallite wall type during the different formation stages [60]

The corallite of *Acropora cervicornis* is built of a series of skeletal elements which forms in a cylindrical shape and subsequently connected to each other by bars as show in in Figure 13A [56], the skeleton extension process is by the growth of these cylindrical shape skeletal elements. The
wall of the axial corallite is covered by 3 layers: a layer of calicoblastic ectoderm (CEC), thin layer of mesoglea and a layer of flagellated endoderm (Figure 13B). The 3-dimensional gastrovascular canal system which is responsible for fluid recirculation inside the corals is highly divaricated and connects the radial and axial polyps together, the canal also extends throughout the pores of the skeleton [56].

![Figure 13](image)

Figure 13 (A) SEM image of ramified tissue system of the corallite of *Acropora cervicornis*. (B) Enlargement image of A [56].

There are two hypotheses describing how coral reefs build their skeletons. The first hypothesis claims that coral skeletons are built by a physiochemical process, where inorganic crystals precipitate from a supersaturated solution of calcium carbonate (CaCO₃) [54, 61]. The crystals growth in the supersaturated solution of CaCO₃ may be classified as syntaxial or nonsyntaxial growth. In case of syntaxial growth, the solution is not sufficiently saturated, thus, it will tend to lose molecules to the lattice of crystals in order to reach the saturation state. These crystals will further grow while maintaining their original orientation [54], while in the nonsyntaxial growth, the solution is sufficiently saturated, therefore, new crystals will nucleate and grow in random
orientations on the surface of the original crystals [54]. However, recent studies of coral reef biomineralization, hypothesized that the aragonite CaCO$_3$ crystal growth is a biologically driven process [62, 63, 64]. In a study by Von Euw et al. [62], the biological formation of coral skeleton was investigated by studying the corallite of *Stylyphora pistillata* coral using Scanning Helium Ion Microscopy (SHIM). SHIM revealed that amorphous CaCO$_3$ (ACC) nanoparticles form both in the columella and septum edge of the corallite (Figure 14), and they further grow and crystalize by forming large anisotropic aragonite crystals, almost fibers [62]. Such aragonite crystals are considered as the primary skeleton element of the coral reefs [65, 62].

Figure 14 SHIM micrographs obtained on the intact surface of a skeletal branch. The region in (A) is a single corallite, in which a growing columella and six growing septa are observable. (B1-B3) and (C1-C3) are successive magnifications that progressively reveal the morphology and size of the mineral particles at the top of the growing columella and on the edge of one of the growing septa, respectively [62].
The formation of needle-like aragonite crystals in the skeleton of *Acropora cervicornis* coral was studied using Scanning Electron Microscopy (SEM) in [56]. It was observed that the needle-like aragonite crystals are formed within the corallites and can be oriented either individually or in a random group in several directions (Figure 15) [56]. Typically, the needle-like crystals are in the range of 1.5-3 µm and 3-6 µm in length and diameter respectively [56]. Further growing and extension of the aragonite crystals form another skeletal element called “tufts” which grow in an equal rate and parallel to each other as shown in Figure 15 [66].

Figure 15 SEM image for the tip of *A.cervicornis* axial corallite. (A) reference view to show the position of (B-E). (B) Tip of the corallite showing various needle-like aragonite crystals oriented in different directions. (C) Older part of the corallite showing the “tufts”. (E) The fasciculi formation as a result of the combination of the “tufts” [56].
1.2.2.2 Crystal structure

The three different polymorphs of calcium carbonate (CaCO$_3$) are: calcite, aragonite and vaterite. While each polymorph has its own crystal structure, different techniques such as: Raman spectroscopy and X-Ray powder diffraction can be used to distinguish between them [67]. Corals build their skeleton of aragonite, considered as one of the most widespread bio-minerals and has a significant role in many biogenic composites [54]. The aragonite structure is pseudo-hexagonal with four molecules in each orthorhombic unit cell (space group $Pmcn$ according to JCPDS (PDF 00-005-0453)) as shown in figure 16. The crystal lattice parameters for the aragonite structure are $a = 4.960$ Å, $b = 7.964$ Å and $c = 5.738$ Å [68].

![Figure 16 The aragonite orthorhombic unit cell [69]](image)

The crystal structure of the three different CaCO$_3$ polymorphs was studied in Ni and Ratner [70] using X-ray Diffraction (XRD) techniques. The XRD patterns of the three different CaCO$_3$ polymorphs are shown in Figure 17. From figure 17, one can clearly observe that each of the polymorphs exhibited different diffraction pattern, therefore, the $2\theta$ peak position of the XRD pattern can be easily used to distinguish between aragonite, calcite and vaterite structures.
The CaCO$_3$ aragonite structure can be easily distinguished using Raman spectroscopy which can provide information about the vibrational frequencies of the atoms in the crystal [67]. It was observed that the vibrational response of the CO$_3^{2-}$ molecule in the crystalline lattice to the Raman spectrum can be divided into: internal (molecular) modes and external (lattice) modes [67]. The molecular modes consist of four different vibrations which occurs due to symmetric stretching ($\nu_1$), antisymmetric stretching ($\nu_3$), in-plane bending ($\nu_4$), out-of-plane bending ($\nu_2$) of the CO$_3^{2-}$ molecule in the lattice as shown in Figure 18.
Figure 18 Schematic representation of the four different vibrations for the internal modes [67].

While the molecular modes are the vibrations of the $\text{CO}_3^{2-}$ molecule in the crystalline lattice, the lattice modes incorporate the vibration of the entire crystalline lattice, which includes both the linear motion and the rotation of the $\text{CO}_3^{2-}$ molecule. Generally, the lattice modes can be detected at frequencies lower than the molecular modes. Transitional and rotary lattice modes of the aragonite crystalline are shown in figure 19 and 20 respectively.
Behrens [67] has studied the Raman spectrum of the aragonite crystalline both for molecular modes and lattice modes. It was observed that the strongest Raman peak corresponded to the symmetric stretching of the CO$_3^{2-}$ molecule ($\nu_1$) in the crystalline lattice at 1085 cm$^{-1}$. The in-plane bending vibration ($\nu_4$) was split into two bands at 701 and 705 cm$^{-1}$. Raman shift of out-of-plane bending ($\nu_2$) and antisymmetric stretching ($\nu_3$) vibrations were too weak to be detected by Raman spectra, however, $\nu_2$ vibration was expected to be at Raman shift of 870 cm$^{-1}$. Raman spectra of molecular vibrations in aragonite is shown in (figure 21).
Figure 21 Raman spectra of molecular vibration in calcite, aragonite and vaterite crystalline [67]

A Raman spectrum of the lattice modes is shown in (figure 22) and has peaks of 142 cm$^{-1}$, 152 cm$^{-1}$, 179 cm$^{-1}$, 189 cm$^{-1}$, 205 cm$^{-1}$, 213 cm$^{-1}$, 273 cm$^{-1}$ and 282 cm$^{-1}$, such peaks can be used to distinguish between the aragonite and calcite structure as there are only two peaks at the same Raman shift range for the calcite structure [67].
Figure 22 Raman spectra of lattice vibration in calcite, aragonite and vaterite crystalline [67]

The spectral vibrational response of the skeleton of *Acropora cervicornis* coral was studied in [6]. The Raman spectroscopy technique was used to verify the aragonite structure of the coral skeleton and to determine the response of the skeleton to the applied stresses. The Raman spectrum revealed the aragonite structure of the coral skeleton, as it had eleven active Raman bands of 142 cm\(^{-1}\), 154 cm\(^{-1}\), 183 cm\(^{-1}\), 195 cm\(^{-1}\), 206 cm\(^{-1}\), 215 cm\(^{-1}\), 273 cm\(^{-1}\), 278 cm\(^{-1}\), 701 cm\(^{-1}\), 705 cm\(^{-1}\) and 1083 cm\(^{-1}\).
cm$^{-1}$ peaks (Figure 23). Such Raman bands are considered characteristics of the aragonite structure [6] (Behrens et al. 1995; Kontoyannis and Vagenas 2000; Weiner et al. 2003).

Figure 23 Raman spectrum of the skeleton of a staghorn coral (Acropora cervicornis) showing (a) the signature peak characteristic of an aragonite structure. (b) optical micrograph and corresponding 2D maps of (c) 1083 cm$^{-1}$ Raman peak of peak intensity, (d) peak position, and (e) peak full width at half maximum. The dotted white line in (b) is the area of the impression and the black dashed line is the mapped area. [6]

To determine the coral skeleton response to the applied stress performed using Vickers hardness test, half of the Vickers impression was selected for 2D Raman maps of peak intensity, peak position and full width at half maximum (FWHM) (Figure 23). The v1 (1083 cm$^{-1}$) Raman active band with the strongest intensity was selected for collecting the 2D Raman maps. From figure 23,
one can clearly notice that peak intensity, peak width and FWHM parameters were affected by the presence of the stresses introduced during the indentation event. The intensity of the peak decreased substantially, from 11000 – 12000 arbitrary units (a. u.) outside of the impression to 5000 – 5500 a. u. in the center of the impression, where residual compressive stresses were highest. However, the position of the peak barely changed, from 1083.6 cm\(^{-1}\) outside the impression to 1083.9 cm\(^{-1}\) in the center. The peak broadened from 7 cm\(^{-1}\) of FWHM outside of the impression to 11 cm\(^{-1}\) in the center of the impression. Thus, the aragonite structure responded to the applied stress and especially to the concentrated load, which might occur when objects like marine debris impact the coral skeleton during storms or other events [6].

The spectral vibrational response of the skeletons of *Favia stelligera* and *Mussa angulosa* corals were studied in Cuif and Dauphin [72]. The Raman spectrum showed Raman active bands of 143 cm\(^{-1}\), 153.4 cm\(^{-1}\), 180.3 cm\(^{-1}\), 191.1 cm\(^{-1}\) and 205.8 cm\(^{-1}\) and 153.8 cm\(^{-1}\), 191.1 cm\(^{-1}\), 206.7 cm\(^{-1}\) and 273.3 cm\(^{-1}\) for *Favia stelligera* and *Mussa angulosa* corals, respectively (Figures 24 and 25). Such Raman bands revealed the aragonite structure of the skeleton of both *Favia stelligera* and *Mussa angulosa* corals, as they are all characteristics of aragonite structure [67, 73, 74] (Behrens et al. 1995; Kontoyannis and Vagenas 2000; Weiner et al. 2003).
Figure 24 Raman spectrum of the skeleton of *Favia stelligera* coral [72]

Figure 25 Raman spectrum of the skeleton of *Mussa angulosa* coral [72]
1.2.4 Mechanical properties

1.2.4.1 Hardness and Young’s modulus

The hardness (H) and Young’s moduli (E) of the skeletons of *Balanophyllia europaea* (*B.europaea*) and *Stylophora pistillata* (*S. pistillata*) corals were studied using nanoindentation techniques in [75]. As a reference of comparison, two aragonite-based materials of nacreous layer of the seashell *Atrina rigida* and geogenic single crystal geogenic aragonite were also examined. Nanoindentation tests were performed in different orientation sections within the samples, as the longitudinal and transverse sections of the coral skeletons, parallel and perpendicular sections to the nacreous layer, perpendicular sections to the 001 and 122 planes of the single crystal aragonite were tested. Nanoindentation load-depth plots, elastic modulus and hardness values obtained from the nanoindentation tests are shown in Figures 26-28 and Table 1, respectively.

Figure 26 Plots showing load-displacement nanoindentation measurements of (a) longitudinal, (b) transverse sections of the skeleton of *Balanophyllia europaea* coral (c) resulting average for the two sections combined [75]
Figure 27 Plots showing load-displacement nanoindentation measurements of (a) longitudinal, (b) transverse sections of the skeleton of *Stylophora pistillata* coral, (c) resulting average for two sections combined [75]

Figure 28 Plots showing load-displacement nanoindentation measurements of (a) nacreous layer of *A.rigida* seashell, (b) geogenic single crystal aragonite [75]
Table 1 Elastic modulus and hardness values obtained from nanoindentation tests for the skeletons of *B. europaea* and *S. pistillata* corals, *A. rigida* seashell and single crystal aragonite [75]

<table>
<thead>
<tr>
<th>Material</th>
<th>Section Orientation</th>
<th>E (GPa)</th>
<th>H (GPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skeleton of <em>B. europaea</em> coral</td>
<td>Longitudinal</td>
<td>77.0</td>
<td>4.87</td>
</tr>
<tr>
<td></td>
<td>Transverse</td>
<td>76.3</td>
<td>4.97</td>
</tr>
<tr>
<td>Skeleton of <em>S. pistillata</em> coral</td>
<td>Longitudinal</td>
<td>76.6</td>
<td>5.04</td>
</tr>
<tr>
<td></td>
<td>Transverse</td>
<td>76.0</td>
<td>5.10</td>
</tr>
<tr>
<td><em>A. rigida</em> seashell</td>
<td>Perpendicular</td>
<td>55.0</td>
<td>3.49</td>
</tr>
<tr>
<td></td>
<td>Parallel</td>
<td>67.4</td>
<td>3.25</td>
</tr>
<tr>
<td>Single crystal aragonite</td>
<td>{001} plane</td>
<td>100.8</td>
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<tr>
<td></td>
<td>{112} plane</td>
<td>82.7</td>
<td>4.15</td>
</tr>
</tbody>
</table>

From Table 1, one can notice that there is no significant difference in the elastic modulus and hardness values between the longitudinal and transverse sections of *Balanophyllia europaea* and *Stylophora pistillata* corals, which reflects the isotropic mechanical behavior of the coral skeletons, such behavior is attributed to homogenous arrangement of the aragonite crystals along the coral skeleton [75] as shown in figure 29.

Figure 29 Atomic force microscopy (AFM) images showing arrangement of aragonite crystals for the skeletons of *B. europaea* and *S. pistillata* corals in both longitudinal (ln) and transverse (tr) directions. Scale bar is 50 nm [75]
In the case of *A. rigida* seashell, a significant difference in elastic modulus and hardness values between the parallel and perpendicular sections to the nacreous layers, resulting from the anisotropic mechanical properties of the nacre were observed. Generally, elastic behavior of seashell strongly dependent on the thickness of the aragonite platelets [75], as *A. rigida* has a thin nacreous layer, young’s modulus and hardness values are significantly lower than the pure aragonite, while, for the species with thicker nacreous layers, similar values of aragonite’s elastic modulus and hardness were obtained [76].

The elastic and plastic mechanical behavior of the single crystal aragonite appeared to be anisotropic, as both young’s modulus and hardness values obtained from the tests are significantly different between the {001} and {122} planes. In addition, from Table 1, one can notice the similarity of elastic modulus and hardness values between the skeletons of *Balanophyllia europaea* and *Stylophora pistillata* corals and the geogenic single crystal aragonite. Such similarity is attributed to the presence of the very little porosity in the areas where the impressions were placed in the coral skeletons [75].

The hardness of the skeleton of *Acropora cervicornis* coral was studied in [6, 77] using Vickers hardness test. It was determined that the average Vickers hardness was measured to be equal to 3.56 ± 0.31 GPa. Most of the Vickers impression of the coral skeletons had almost no radial cracks originating from the corners of the impression (Figure 30a); However, lateral cracks were observed in many impressions, causing chipping and uplifting of the surfaces surrounding the impressions [6] (Figure 29b).
Figure 30 Optical micrographs of Vickers hardness impressions (50g load) in staghorn coral skeleton showing (a) Indent with no chipping or lateral cracks. (b) Indent with lateral cracks [6, 77]

1.2.4.2 Uniaxial stress-strain deformation behavior

Chamberlain [78] studied the compressive strengths of skeletons of Acropora palmata, Siderastrea radians and Montastrea annularis corals using the uniaxial compression tests. In Chamberlain [78], it was stated that the pattern of the skeleton fracture upon the uniaxial compression test depended on the polyp growth direction with respect to the axis of the skeleton axial canal. When the polyp grew parallel to the axial canal, the fracture developed in a direction parallel to the applied stress, while when the polyp grew inclined to the axial canal, the fracture plane was also inclined to the applied stress, while for some skeletons, when the polyps grew in different
directions within the sample, the fracture developed in a more complex pattern as shown in Figure 31.

![Figure 31 Images showing the fracture pattern with respect to the polyps growth direction in skeletons of (A,B) *Montastrea annularis*, (C) *Acropora palmata*, corals [78]](image)

The fracture structure of skeleton of *Acropora palmate* coral after the uniaxial compression test was observed in more detail using scanning electron microscopy [78], where the presence of aragonite crystals and different step-like depressions along the fracture surface was noticed as shown in Figure 32, such step-like depressions resulted from the fracture of the aragonite crystals on the fracture surface of the coral skeleton.
Figure 32 SEM images of a fracture surface of the skeleton of *Acropora palmata* coral showing (A) overview of the fracture surface, (B) magnification of the fracture surface, (C) step-like appearance of the aragonite crystals, (D) details of fracture showing relation between crystals, steps and pits. [78]

The compressive strength and elastic modulus of skeletons of *Acropora palmate*, *Siderastrea radians* and *Montastrea annularis* corals after the uniaxial compression test were measured to be equal to (47.7 ± 17.3 MPa, 21.5 ± 8.3 GPa), (32.0 ± 8.2 MPa, 15.1 ± 2.1 GPa) and (22.4 ± 12.9 MPa, 10.6 ± 7.0) GPa respectively. From the stress-strain deformation plot (Figure 33), Chamberlain [78] observed that the behavior of the skeletons under the compression load was mostly linearly elastic, however, several sharp pop-ins events were formed as a result of the cracks.
formation and propagation. Such pop-ins are explained by the ability of the skeleton’s structure to retard crack growth, however, after a few cracks developed and grew sufficiently long upon increased applied loading, the skeleton failed completely by reaching a critical stress.

Figure 33 Stress-strain deformation plot after uniaxial compression for the skeleton of *Montastrea annularis* coral [78]

In particular, since coral skeleton microstructure contains non-homogenous distributed porous, it was observed that small stresses were able to develop cracks in the high porous region of the skeletons, while less porous regions resisted the cracking until higher stresses obtained [78].

The compressive strength of the skeletons of *Porites compressa* and *Porites lobata* corals was studied in [79]. In order to investigate the effect of the wave exposure on the skeletal strength, the colonies of these two coral species were obtained from areas with different wave exposure. It was
found that the skeletons of *P. lobata* coral which were collected from high wave exposure areas obtained a higher compressive strength than *P. compressa* which was collected from areas with low wave activity, as the compressive strength values were $15.8 \pm 4.7$ MPa and $6.28 \pm 1.74$ MPa for skeletons of *P. lobata* and *P. compressa* corals, respectively. The study results confirm the fact that corals with higher skeletal strength are found in areas with higher wave exposure [79].

1.2.5 Thermal properties

The thermal properties of *Acropora*, *Goniopora* and *Porites* corals species was studied in [80] using Differential Scanning Calorimetry (DSC) techniques. Before starting the thermal experiments, the powder samples of the coral skeletons were examined using X-Ray powder diffractometer (XRD). The XRD pattern revealed that all three coral skeleton samples were composed of aragonite, as only XRD peaks corresponding to the aragonite structure were detected (Figure 34) [80]. Moreover, powder samples of the coral skeletons were then heated in order to determine their decomposition. The high-temperature thermal analysis revealed that the aragonite structure of the coral skeleton samples underwent a phase transition between 290°-325° C, such phase transition can be attributed to the transformation of the aragonite structure to calcite phase (Figure 35) [80].
Figure 34 X-Ray diffraction patterns of Acropora, Geniopora and Porties coral species [80]

Figure 35 Differential scanning calorimetry of Acropora, Goniopora and Porties coral species [80]

To the best of the author’s knowledge, the thermal behavior of coral skeletons has not been studied in many research settings, however, the thermal behavior of aragonite CaCO₃ ceramic derived from anadaragranosa seashell was studied in [81] using Thermogravimetric (TGA) and Differential
Thermal (DT) analyses. It was observed that the calcium carbonate CaCO$_3$ structure of the *anadaragranosa* seashell decomposed to calcium oxide CaO between 600 °C and 800 °C with weight loss of 42% at the end of the TGA experiment as shown in Figure 36.

![Figure 36 TGA and TDA curves of CaCO$_3$ structure of *anadaragranosa* seashell [81]](image)

In addition, the reduction in the DTA curve with the decomposition of CaCO$_3$ to CaO (Figure 36), resulted from the endothermic decomposition reaction process [81]. Moreover, XRD analysis was performed on the decomposed calcium oxide CaO both at 800 °C and 900 °C, which revealed the CaO diffraction peaks of 32.24°, 37.39°, 53.88°, 64.18°, 67.40°, 79.66° and 88.50° (Figure 37). From Figure 37, one can notice that in addition of the calcium oxide diffraction peaks, CaCO$_3$ peaks of 29.35° and 47.12° and calcium hydroxide Ca(OH)$_2$ peaks of 17.87°, 28.55°, 34.18°, 47.51° and 50.71° were also presented. The presence of CaCO$_3$ peaks reflects the fact that the decomposition process was not entirely completed at 800 °C. Such partial decomposition of the
CaCO$_3$ to CaO can be attributed to the thick shell layer of *anadara granosa* seashell, therefore, it is difficult to fully decompose to CaO at 800 °C. In addition, the presence Ca(OH)$_2$ peaks resulted from the reaction between CaO and the water vapor H$_2$O in the air (Figure 37) [81].

![Figure 37 XRD pattern of the decomposed CaO structure of *anadara granosa* seashell [81](image)](image)

1.2.6 Fluidic behavior

The fluidic behavior of the skeletons of branching coral *Acropora intermedia* was studied in [82] using Computational fluid dynamic (CFD). The CFD analysis permitted the estimation of velocity vectors and different forces associated with fluid movement around the skeleton, which supports a relatively thin “veneer” of living polyps [82]. Their models assumed water velocity in the range of 1 to 5 m/s, which is an approximation of the pure current velocity that corals experience in their natural habitat [82]. The drag forces were in the range of 0.8-12.2 kN and increased with increased
water velocity. The CFD stress analysis was performed on three different sections (A, B and C) of the branching coral *A. intermedia* as shown in Figure 38.

![Diagram of branching coral](image)

Figure 38 Geometry of the branching coral *A. intermedia* showing sections A, B and C which was used in the stress analysis (Ballock et al. 2014)

The CFD analysis revealed that the stress of the coral stem, represented by section A, exceeded the failure tensile stress of 10 MPa at water velocities above 1 m/s. In reality, the stem of the coral branch (section A) would have a larger cross-section and stiffness than sections B and C (Figure 5). Therefore, potential failure of the coral branch at multiple locations (sections B and C) is also expected at a water velocity lower than 1 m/s [82].

The flow-field around the skeleton of *Acropora cervicornis* coral was studied using Particle Image Velocimetry (PIV) analysis in [6, 77]. The coral skeleton was accelerated in a large aquarium (1 m long, with a cross section of 45 cm x 45 cm) to a speed of 0.1 m/s for 1 second, then towed to a constant speed of 2 seconds before stopping. The PIV measurements were carried out using a high-speed camera which moved simultaneously with the coral skeleton sample.
Figure 39 Plots showing normalized velocity ($\omega_c/U_\infty$) in the wake of staghorn coral skeleton. $c$ denotes the equivalent radius of the coral skeleton’s cross-section. a) after acceleration was competed, two vortices formed in the wake, b) smaller vortices started to form and two main vortices began disintegrating, which continued further as shown in c and d [6, 77]

The flow-field of the coral skeleton at different instants of motion during a fluidic flow experiment is presented in Figure 39. After 1 s from motion initiation, two counter-rotating vortices formed in the wake of the moving skeleton, still attached to it at that moment and which had not separated.
yet from the coral surface (Figure 39a). The shape of the coral skeleton - a circular cylinder with significant surface roughness - created several smaller-scale vortices that were dragged into the wake. Vortices formed earlier during the motion, then disintegrated, and small-scale vortices appeared in the wake (Figure 39b). This pattern continued at the later stages of motion (Fig. 39c and d), where smaller-scale vortices prevailed.

From Figure 39, it was observed that surface roughness of the coral skeleton likely promoted formation of small-scale instabilities in the wake [6, 77], which quickly ruptured the bigger, Kármán type vortices [83]. Such vortices around coral skeletons are vital for helping bring food to polyps and exchange respiratory gases, thereby allowing a coral colony to thrive [84].

1.3 Problem statement for staghorn coral skeletons

The skeleton of *Acropora cervicornis* corals bear the cyclic mechanical load constantly imposed on this branching coral by ocean waves, and thus, the mechanical behavior of coral skeletons is of a great importance for the understanding their structural integrity and predicting whether they will survive wave action, hurricanes, impacts from divers and marine debris, and other environmental factors. In addition, understanding the mechanical behavior of coral skeletons is vital to ensure successful coral reef restoration and delivery of key ecosystem services, including providing habitat and protecting coastlines and beachfront properties from waves and storm surge [6]
1.4 Conversion of CaCO₃ coral skeleton into hydroxyapatite for use as bone graft substitute

Over the recent decades, a lot of research has been done in order to convert the porous CaCO₃ coral skeleton structure into hydroxyapatite Ca₁₀(PO₄)₆(OH)₂ in order to use it in the biomedical applications as a bone graft substitute [10, 11, 12]. Coral skeletons have been shown to be a biocompatible material, which can resemble the bone structure because of the complex open porosity, and therefore, contributes to becoming a successful bone graft substitute [85]. Coralline hydroxyapatite (CHA), which is produced from converting the coral skeleton to hydroxyapatite, has been extensively studied as a bone graft substitute in different rodent animal models including mice and rats [86, 87], therefore, understanding the mechanical and structural properties of murine bone is also of importance to relate between coral skeletons and bones properties.

1.5 Diet and Its Importance for Human Health

Nutrients, such as fats, sugars, and proteins, play a primary function in bone metabolism where high fat or high sugar (fructose, glucose, or sucrose) diets have a significant impact on bone structural integrity [88]. Previous studies [89, 90] have reported that high saturated fat diet consumption is associated with a decrease in bone mineral density (BMD) and as bone strength diminishes, adverse microstructural changes occur in the cancellous bone compartment. In addition, with our increasing life expectancy, human consumption of high-fat food is expected to increase [91], therefore, diseases such as osteoporosis, muscle loss and obesity are predicted to significantly increase and become a major public health problem [92].
Over the recent decades, animal models such as murine models, have been extensively used in biomedical research to investigate the effect of diet on bone health [93, 94]. The benefits of using a murine model for research purposes is represented in their easy handling, small size and availability of their genetic resources. In addition, mice are considered an ideal animal due to the anatomical and physiological similarities between them and humans [93].

1.5.1 The effect of diet on the properties of bone

The intake of different dietary patterns has indeed a significant impact on bone composition, microarchitecture, strength and overall health. Adequate nutrition is important for maintaining healthy bone and in preventing bone resorption. The effect of diet on the structural, geometrical, spectral vibrational and mechanical properties of bone, are discussed in the sections below.

1.5.1.1 The effect of diet on bone structure and geometry

The effect of a fat diet on the structural properties of the murine cortical bone was studied in [91]. C57BL/6J mice were first assigned to receive either a high fat sucrose diet (HFS) or low fat, complex carbohydrate (LFCC) diet, which they remained on for 10 weeks. Tibiae were separated from the knee and ankle joints, and the disarticulated bones were then cleaned using phosphate buffer (PBS) solution in order to prepare them for the biomechanical and morphological testing. It was observed that at the end of the 10-week diet period, the overall body mass of the high-fat diet group was 40% higher than the body mass of the low-fat group. As the overall body mass affects bone size, bone mineral density and thus, overall bone strength, it was determined that the tibial
cortical thickness in the HFS group was 28% lower than the cortical thickness of the mice fed a LFCC diet. In addition, the HFS diet led to a 25% reduction in the tibial cross-sectional area as compared to the LFCC diet group. Furthermore, the 3-point-bending mechanical testing revealed that the fracture load was 23% lower in the mice fed HSF diet relative to the LFCC diet (Figure 40) [91].

![Bar graph showing the results of LFCC and HFS mice groups cortical thickness, mid-shaft cross-sectional area and maximal load adjusted for total body mass [91].](image)

The effect of the high fat diet (HFD) on the structural properties of femora of the mouse, *mus musculus* was studied in [95]. C57BL/6 male mice were fed either a high fat diet (60 kcal% fat) or a standard laboratory chow (control) diet (21.6 kcal% fat) for a duration of 19 weeks. The mice were then euthanized, and the femora were retrieved for further geometrical analysis. It was observed that all geometrical parameters of the mice fed a HFD were greater compared to the mice
fed a control diet (Figure 41). Results showed that the cortical thickness was increased by 10.5%, outer cortical radius by 6.1%, inner cortical radius by 4.8%, cortical cross-sectional area by 11.4% and moment of inertia by 26.7%. Overall, the femoral length was the same between HFD and control groups (Figure 41) [95].

Figure 41 femoral geometrical parameters of HFD and CHOW groups [95]

The findings in [95] demonstrate that the high fat diet induced obesity in mice, and as a consequence, a clear increase in the femoral structural properties of the HFD was observed in comparison with the chow (control) diet group. Such anabolic effect of the HFD on bone structure
is considered temporary as the bone response depends on kcal% fat content as well as the length of duration of the feed.

1.5.1.2 The effect of diet on the spectral vibrational response of bone

Raman spectroscopy has been extensively used to understand the chemical composition and analyze the mineral properties in bone [96, 97]. The spectral vibrational response of murine cortical bone was studied in [97] using Raman spectroscopy. The Raman spectrum showed 33 different Raman bands (Table 2) which corresponded to the mineral and matrix components of the cortical bone (Figure 42) [97]. The most characteristic mineral and matrix bands of cortical bone are the phosphate ($\nu_1$ PO$_4^{3-}$) at ~959 cm$^{-1}$ and amide I at ~1660 cm$^{-1}$ Raman shifts, respectively. These two Raman bands can be further used to determine the mineral to matrix ratio, and therefore, the bone mineral density. It was determined that the bone mineral density has a significant impact on the overall bone health. Research studies [98, 99] showed that as the bone mineral density decreases, the bone becomes brittle and more likely to fracture. The mineral to matrix ratio of osteoporotic bone was significantly lower compared to the mineral to matrix ratio of the healthy bone [98, 99].
Figure 42 Raman spectrum of murine cortical bone showing mineral and matrix Raman bands

Table 2 Mineral and matrix Raman bands of cortical mouse bone [97]

<table>
<thead>
<tr>
<th>Raman shift, cm⁻¹</th>
<th>Assignment</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>430</td>
<td>ν₃PO₄²⁻</td>
<td>Strong band</td>
</tr>
<tr>
<td>450</td>
<td>ν₅PO₄⁻</td>
<td>Shoulder on 430 cm⁻¹ band</td>
</tr>
<tr>
<td>584-590</td>
<td>ν₅PO₄⁻</td>
<td>Multiple partially resolved components</td>
</tr>
<tr>
<td>609</td>
<td>ν₅PO₄⁻</td>
<td>Shoulder on 590 cm⁻¹ band</td>
</tr>
<tr>
<td>668</td>
<td>ν(C–S)</td>
<td>Cysteine</td>
</tr>
<tr>
<td>756</td>
<td>ν₃CO₅⁻</td>
<td>B-type carbonate, very weak</td>
</tr>
<tr>
<td>853</td>
<td>ν(C–O)</td>
<td>Collagen proline, may include δ(C–C–H) contribution from tyrosine</td>
</tr>
<tr>
<td>872</td>
<td>ν(C–O)</td>
<td>Mostly collagen hydroxyproline</td>
</tr>
<tr>
<td>920</td>
<td>ν(C–O)</td>
<td>Shoulder, mostly collagen proline</td>
</tr>
<tr>
<td>937</td>
<td>ν(C–O)</td>
<td>Proline and protein backbone</td>
</tr>
<tr>
<td>955</td>
<td>ν₅PO₄⁻</td>
<td>Transient bone mineral (P–O) phase, usually seen in immature bone.</td>
</tr>
<tr>
<td>957</td>
<td>ν₅PO₄⁻</td>
<td>Bone mineral containing extensive HPO₄²⁻, usually immature</td>
</tr>
<tr>
<td>959-962</td>
<td>ν₅PO₄⁻</td>
<td>Bone mineral, mature</td>
</tr>
<tr>
<td>1003</td>
<td>ν(C–O)</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>1035</td>
<td>ν₅PO₄⁻</td>
<td>Overlaps with proline ν(C–C) component</td>
</tr>
<tr>
<td>1048</td>
<td>ν₅PO₄⁻</td>
<td>Overlaps with proline ν(C–O) component</td>
</tr>
<tr>
<td>1060</td>
<td>Proteoglycan</td>
<td>Overlaps with lipids, collagen and components of ν₅PO₄⁻</td>
</tr>
<tr>
<td>1070</td>
<td>ν₃CO₅⁻</td>
<td>Overlaps with component of ν₅PO₄⁻</td>
</tr>
<tr>
<td>1076</td>
<td>ν₃PO₄⁻</td>
<td>Overlaps with component of ν₃CO₅⁻</td>
</tr>
<tr>
<td>1176</td>
<td>ν(C–O–C)</td>
<td>Tyrosine, phenylalanine</td>
</tr>
<tr>
<td>1204</td>
<td>ε(CH₂)</td>
<td>Tyrosine, hydroxyproline</td>
</tr>
<tr>
<td>1242</td>
<td>Amide III</td>
<td>Protein β-sheet and random coils</td>
</tr>
<tr>
<td>1272</td>
<td>Amide III</td>
<td>Protein α-helix</td>
</tr>
<tr>
<td>1293-1305</td>
<td>δ(C=H)</td>
<td>Lipid band, sometimes seen in fresh untreated bone</td>
</tr>
<tr>
<td>1340</td>
<td>Amide III</td>
<td>Protein α-helix, sometimes called CH₃CH₂ wag</td>
</tr>
<tr>
<td>1365</td>
<td>Pentosidine</td>
<td>Overlap with lipid 1369 cm⁻¹ band</td>
</tr>
<tr>
<td>1375</td>
<td>Proteoglycan</td>
<td>Representative of glycosaminoglycans</td>
</tr>
<tr>
<td>1446</td>
<td>δ(CH₂)</td>
<td>Protein CH₂ deformation</td>
</tr>
<tr>
<td>1585</td>
<td>ν(C–C–H)</td>
<td>Weak band, aromatic ring</td>
</tr>
<tr>
<td>1609</td>
<td>δ(C≡C)</td>
<td>Phenylalanine, tyrosine</td>
</tr>
<tr>
<td>1640</td>
<td>ν(C≡C)</td>
<td>Shoulder to 1660 cm⁻¹ band</td>
</tr>
<tr>
<td>1660</td>
<td>Amide I</td>
<td>Strongest amide I ν(C=O) component, polarization sensitive</td>
</tr>
<tr>
<td>1690</td>
<td>Amide I</td>
<td>Shoulder, prominent with immature cross-links</td>
</tr>
</tbody>
</table>

*The band also relates to β-sheet or disordered secondary structure*
The effect of a high sugar diet on the mineral properties of the bone was studied using Raman spectroscopy in [100]. Rats were divided into two groups: Sprague-Dawley control rats (CD) and Zucker Diabetic Sprague-Dawley rats (ZDSD). The CD rats were fed a control diet, while the ZDSD rats were fed a high sugar diet, for a duration of 30 weeks. The rats were then euthanized, and the tibiae were retrieved for further spectral analysis. The Raman spectrum of a rat tibia along with the band area ratios used to determine the mineralization of the bone is shown in Figure 43.

It was determined that the presence of high sugar nutrients in the rats increased their bone mineral density, as the ($v_1$ PO$_4^{3-}$/ Amide I) and ($v_1$ PO$_4^{3-}$/ CH$_2$ deformation) mineral to matrix bands ratios for the ZDSD rats was significantly higher compared to the CD rats. The mineral to matrix ratio ($v_1$ PO$_4^{3-}$/ Amide III) was still higher in ZDSD rats, however, no significant difference was observed compared to the CD rats (Figure 44) [100]. In addition, it was observed that the presence of the advanced glycation end products (AGEs) in the high sugar ZDSD rats, reduces the collagen

Figure 43 Raman spectrum of rat’s tibia showing the band area ratios [100]
(matrix) band areas significantly, therefore, increasing the bone mineral density [100]. Thus, according to Hammond et al. [100], the high sugar diet consumption leads to increase in the bone mineral density and overall bone strength compared to the control diet.

![Figure 44](image)

Figure 44 Mineral to matrix ratio of ZDSD rats compared to CD rats, * indicated the significant difference between the two groups (p<0.05) [100]

1.5.1.3 Diet effect on the mechanical properties of bone

The effect of a fat diet on bone’s mechanical properties was studied using 3-point-bending techniques in [101]. 3-week-old and 15-week-old C57BL/6 mice were fed either a high fat diet (HFD) (60 kcal% fat) or low-fat diet (LFD) (10 kcal% fat) for a duration of 16 weeks. The mice were termed as young HFD (yHFD), adult HFD (aHFD), young LFD (yLFD) and adult LFD (aLFD) depending on their age at the end of the diet. The mice were then euthanized, and the femora and tibiae were retrieved for further mechanical, structural and histological analysis. The results of the 3-point-bending test on the femora showed a degradation of the mechanical
properties associated with the high fat diet compared to the low-fat-diet (Figure 45). From figure 45, one can observe that elastic modulus, yield stress, ultimate stress, and maximum load were all lower in both yHFD and aHFD compared to yLFD and aLFD. However, the fracture toughness of the yHFD, measured from the length of cracks originated from the corner of impression during indentation, was non-significantly higher compared to yLFD (Figure 45).

Figure 45 Comparison of the mechanical properties of the femora cortical bone between HFD and LFD groups. (A) Elastic modulus, (B) Maximum load, (C) Yield stress, (D) Maximum stress and (E) Fracture toughness [101]
To better understand the effect of a high fat diet on the bones’ mechanical properties, both histological and structural properties of the bone had to be further explored. The HFD induced obesity in mice, as both aHFD and yHFD groups were significantly heavier than aLFD and yLFD groups at the end of the diet duration. Furthermore, total fat body mass, insulin-like growth factor (IGF-I) and leptin hormone were all affected with the obesity, as they were significantly greater in the HFD groups compared to the LFD groups (Figure 46).

Figure 46 Plots showing the variation in (A) body weight, (B) Lean body mass, (C) fat body mass, (D) Serum Leptin and (E) growth factor (IGF-I) between HFD and LFD groups [101]
It is possible that reduction in mechanical properties of HFD was affected with the higher fat body mass and (IGF-I) levels in the bone as a result of obesity. It was observed that higher (IGF-I) level is associated with larger bone size – as shown in the structural analysis (Figure 47)- and therefore, more likely to fracture at the lower stress in comparison LFD group [101]. Furthermore, contrary to [100], which stated that the presence of the advanced glycation end products (AGEs) in the rats which were fed a high sugar diet increased the bone mineral density, and therefore, their bone strength, authors in [101] observed that the reduced mechanical properties in HFD group might be explained by the increase in (AGEs) products as a result of obesity induced with the HFD. Moreover, the structural results of the femora revealed that indeed the obesity in HFD groups affected the ability of the bone to increase in size with increase in mouse age, which can further affect their mechanical properties (Figure 47). Both femoral thickness and femoral diameter are lower in the aHFD compared to the yHFD. The findings in [101] revealed that both young and adults are at a high risk for bone fracture when obesity is present.
Figure 47 Structural analysis of cortical bone in femora showing the variation in (A) Femoral thickness, (B) Femoral diameter and (C) Femoral length between HFD and LFD groups [101]

1.5.2 Estrogen depletion and the effect on bone properties

Estrogen plays an important role in bone growth and has a significant impact on bone’s overall health and strength [102]. Previous studies [103, 104] showed that estrogen deficiency leads to the reduction in bone mineral density, and therefore, bone strength. The major consequence of the estrogen deficiency is causing an increase in the number and activity of the osteoclast thereby enhancing bone resorption. In addition, estrogen deficiency is responsible for a significant decrease of the Fas/Fasl protein in osteoblast, which are primarily responsible for bone formation [105]. Osteoporosis due to estrogen deficiency is a widespread disease and highly common within the population. According to National Health and Nutrition Examination Survey (NHANES), 13-18% of postmenopausal women in the United States suffer from osteoporosis disease [106].
Therefore, understanding the relationship between the estrogen level and bone health is vital to enhance bone strength and structural integrity. The effect of estrogen depletion on bone’s structural, spectral and mechanical properties is described in the sections below.

1.5.2.1 Estrogen depletion effect on bones’ structure and geometry

The effect of estrogen loss on the structural properties of murine tibiae was studied in [107]. 8-week-old C57BL/6 mice underwent either ovariectomy (OVX) or sham (SHAM) surgery, and the mice were euthanized at 2, 4 and 8-weeks post-surgery. The tibiae were then retrieved for further structural analysis. It was determined that murine bone maturity is reached at 16 weeks of age [108], thus, authors in [107] studied the influence of estrogen depletion not only on the tibiae of fully mature mice, but also, on the tibiae of mice still undergoing bone maturation.

The structural results revealed that the tibia of both OVX and SHAM groups was still developing until 12-weeks-age (i.e., 4 weeks post-surgery). In both surgical treatments, there was an increase in tibial length and cortical cross-sectional area until 12 weeks age, however, after 12 weeks age of, no significant difference in tibial length or cross-sectional area was observed (Table 3). In addition, the tibial length and cortical cross-sectional area did not differ significantly between OVX and SHAM groups, except for the cortical area at 10-weeks age, as the OVX group demonstrated significant lower cortical cross-sectional area compared to their age-matched SHAM group (Table 3).
Table 3 Tibial structural properties of OVX and SHAM mice groups [107]

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>Weeks post-surgery</th>
<th>Treatment</th>
<th>Tibial length (mm)</th>
<th>Cortical area (mm²)</th>
<th>Cavity area (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>2</td>
<td>SHAM</td>
<td>16.59 ± 0.11</td>
<td>0.47 ± 0.01</td>
<td>0.30 ± 0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OVX</td>
<td>16.52 ± 0.18</td>
<td>0.31 ± 0.06</td>
<td>0.31 ± 0.06</td>
</tr>
<tr>
<td>12</td>
<td>4</td>
<td>SHAM</td>
<td>16.98 ± 0.11</td>
<td>0.55 ± 0.02</td>
<td>0.29 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OVX</td>
<td>17.24 ± 0.12</td>
<td>0.59 ± 0.02</td>
<td>0.33 ± 0.02</td>
</tr>
<tr>
<td>16</td>
<td>8</td>
<td>SHAM</td>
<td>17.06 ± 0.13</td>
<td>0.54 ± 0.01</td>
<td>0.30 ± 0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OVX</td>
<td>17.23 ± 0.19</td>
<td>0.56 ± 0.01</td>
<td>0.32 ± 0.03</td>
</tr>
</tbody>
</table>

Overall, the findings in [107] demonstrated that the estrogen loss in the OVX mice didn’t have any effect on the bone structural properties, as the bone continuously grew as the mice aged. Such growth can be clearly explained by the increase of tibial length and cortical area (Table 3).

1.5.2.2 Estrogen depletion and the effect on the spectral vibrational response of bone

Estrogen deficiency has a significant impact on bone resorption and formation. The effect of estrogen loss on the spectral response of murine tibiae was studied in [109] using Raman spectroscopy. 12-weeks old mice underwent either ovariectomy (OVX) or sham (SHAM) surgery, mice were then euthanized, and tibiae were retrieved for further spectral analysis. Raman spectrum of murine tibiae of OVX and SHAM groups are shown in Figures 48 and 49, respectively.
Figure 48 Raman spectrum of the tibia of OVX mice [109]

Figure 49 Raman spectrum of the tibia of SHAM mice [109]
This study [109] quantified the bone mineral density (mineral to matrix ratio) and the lipid content for tibia of both OVX and SHAM groups. The mineral to matrix ratio and relative lipid content were calculated by dividing the intensities of ($\nu_2$ PO$_4^{3-}$ at $\sim$ 450 cm$^{-1}$ Raman shift) / (Amide III at $\sim$ 1272 cm$^{-1}$ Raman shift) and (lipid bands at $\sim$ 1293-1305 cm$^{-1}$ Raman shift) / (Amide III at $\sim$ 1272 cm$^{-1}$ Raman shift), respectively. It was determined that the bone mineral density was significantly higher in the SHAM group (1.07 $\pm$ 0.13) compared to the OVX group (0.37 $\pm$ 0.04) [109], which indicates that estrogen loss in the OVX group resulting from ovariectomy reduced mineral content in the bone by increasing the number of osteoclasts [110]. The relative lipid content was much higher in the OVX group (1.07 $\pm$ 0.23) compared to the SHAM group (0.26 $\pm$ 0.06). Such higher values of the lipid content in the OVX group were in a good correlation with their significantly lower mineral to matrix ratios. As a higher value of lipid content is responsible for increasing formation and accumulation of the advanced glycation end products (AGEs) in the bone, which results in reducing the bone mineral density, and therefore, diminishing the bone strength [110].

The authors in [111] studied the effect of ovariectomy on the bone mineral density of rats’ cortical bone using Raman spectroscopy. Forty-eight rats underwent either ovariectomy (OVX) or sham surgery, rats were then euthanized at 2-, 4-, and 8-months post-surgery, and mandible cortical bone were retrieved for further spectral analysis. The average difference in Raman spectrum of the cortical bone from SHAM and OVX groups at 2, 4 and 8 months after surgery are shown in Figure 50.
Figure 50 Average difference spectrum from rats’ mandibular cortical bone from SHAM and OVX groups. (A) 2 months, (B) 4 months and (C) 8 months, post-surgery [111]

From Figure 50, one can notice that the intensity of minerals (ν₁ PO₄³⁻ at ~ 959-960 cm⁻¹ Raman shift) in the cortical bone of the OVX group 2 months post-surgery, was higher compared to the minerals of the SHAM groups. However, the intensity of the minerals in the OVX groups eventually decreased and was smaller than that of the SHAM group 4 months post-surgery onward. Furthermore, the Raman spectrum revealed that the intensity of the organics (Amide III at 1250 cm⁻¹ Raman shift) in the OVX groups was continuously lower compared to the organics’ intensity in the SHAM group at each time period (Figure 49). The overall mineral to matrix ratio was higher in the OVX group compared to the SHAM group in the first 2 months, then continuously decreased and became lower from 4 months onward (Figure 51) [111]. The findings in [111] suggests that estrogen loss as a result of the ovariectomy doesn’t change the bone mineral density immediately, however, the degradation in bone minerals and organic content occurs gradually with time [111].
1.5.2.3 Estrogen depletion and the effect on the mechanical properties of bone

The estrogen loss effect on the mechanical properties of murine tibia was studied using 3-point-bending techniques in [107]. 8-week-old C57BL/6 mice underwent either ovariectomy (OVX) or sham surgery, the mice were euthanized at 2, 4 and 8-weeks post-surgery. The tibias were then retrieved for further mechanical analysis. The purpose of the study was to investigate whether the age and the surgical treatment influenced the mechanical properties of bone. It was determined that the Young’s modulus in the SHAM group was continuously higher compared to their age matched OVX animals at each time point (Figure 52a). From figure 52a, one can notice that the Young’s modulus of the SHAM group at 16-weeks of age was significantly higher than OVX group; otherwise, no significant difference in Young’s modulus between both groups was observed at the younger ages (Figure 52a). Moreover, results of the fracture stress revealed that the SHAM
group were able to withstand significantly higher stresses at each time point compared to their age matched OVX group (Figure 52b). Such higher stress values in the SHAM group were expected, as the estrogen depletion in the OVX group significantly decreased the bone mineral density, and therefore, their overall strength [110].

Figure 52 variation mechanical properties of the mice tibiae between OVX and SHAM groups. (a) Modulus of elasticity, (b) Fracture stress [107]

Furthermore, the findings in [107] demonstrated that as the mice age, a continuous decrease in the Young’s modulus of the OVX tibia was observed. In addition, the fracture stress of the OVX mice was only reduced in the tibiae of 10-weeks of age mice (Figure 52b). Similarly, it is clearly observed that age has an influence on the mechanical properties of the SHAM group, as both the Young’s modulus and fracture stress were significantly higher in the fully mature mice (8-weeks
post-surgery) (Figure 52). Overall, this study [107] revealed that the estrogen loss in the OVX mice indeed has a significant impact on reducing the mechanical strength of their tibiae, however, such impact on the mechanical strength aggravated once the tibiae reached full maturity at 16-weeks of age.

The effect of estrogen depletion on the mechanical properties of a rat femur was studied using 3-point-bending techniques in [112]. 12 weeks old female rats underwent either ovariectomy (OVX) or sham (SHAM) surgery, rats were euthanized at 0-, 2-, 4-, 8-, 16-, 28- and 40-weeks post-surgery, and the femurs were then retrieved for mechanical analysis. The 3-point bending results revealed that the fracture load of the SHAM group was significantly higher than the age-matched OVX group at each time point. However, although the fracture load of the SHAM group increased gradually at younger ages, it further decreased after 16-weeks post-surgery and became similar to the fracture load of the OVX group at 40 weeks post-surgery. In addition, the fracture stress of the OVX group was almost the same during the follow-up time of the mechanical test, as no significant difference in femoral fracture stress between different aged- rats was observed. In contrary to the study in [107], the findings in [112] demonstrated that age did not influence the mechanical strength of the OVX bone. The consistency of the femoral fracture stress of OVX animal at each time point might be explained by possible geometric changes in the cortical bone of the rats (e.g., increase in the moment of inertia) as their age increases [112].
1.6 Problem statement for diet and estrogen depletion and the effect on the structural and mechanical properties of bone

Dietary intake plays an important role in bone metabolism and indeed has a significant impact on bones strength and structural integrity. Adequate nutrients are very important to maintain healthy bones and reduce the fracture risk. In addition, estrogen deficiency has shown to be associated with decreasing the bone mineral density, thus, reducing overall bone strength. Therefore, understanding the relationship between obesity, osteoporosis and dietary intake is vital to enhance our scientific knowledge and facilitate the development of new treatment strategies that enhance bone and prevent bone resorption, when under these degenerative conditions.
CHAPTER 2  RESEARCH METHODOLOGY

2.1 Experimental set-up for testing of the staghorn coral skeletons

The experimental methodology for testing the skeletons of *Acropora cervicornis* corals to better understand their structural, mechanical and thermal properties are described in the sections below.

2.1.1 Samples preparation

The skeletons of *Acropora cervicornis* corals retrieved from Nova Southeastern University’s coral nursery off Broward County, FL, USA were studied. These colonies either failed or were knocked loose from the lines suspending them from the coral “tree” structure during storms, and their skeletons were collected for future use. The skeletons were not diseased and appeared normal to visual inspection. The algae and other materials were removed from the skeletons using the bleaching and the sanding processes. During the bleaching process, the skeletons were soaked overnight in a bleach solution. The skeleton’s pigmentation was removed during bleaching and sanding, resulting in discoloring of the structure from the normal yellow to the white color. In the sanding process, the skeletons were buried overnight underneath sand which was used as a filtration media for the proteins. After cleaning, a diamond blade cutting saw (Series 15LC blade, Buehler Ltd. Lake Bluff, Illinois, USA) was used to cut skeletons into cylinders with 1:2 to 3:1 height to diameter ratios (dimensions were approximate due to the irregular skeletal geometry). Top and bottom surface of the cylindrical samples were grounded and polished using P1000-P3000 SiC sandpaper to achieve a mirror surface suitable for the mechanical tests.
2.1.2 Structure

The structure of the staghorn coral skeleton was studied using several techniques such as optical microscopy, computed tomography (CT) scans, thermogravimetry (TG), powder x-ray diffraction (PXRD), surface area and pore size distribution by Brunauer-Emmett-Teller (BET) method, Raman spectroscopy. The description of the experimental setup or each technique is provided in the sections below.

2.1.2.1 Optical microscopy

Optical micrographs of the cross-sectional surfaces of skeleton samples cut from the original colonies were made using a Zeiss Axio Lab.A1 microscope (Carl Zeiss AG, Jena, Germany). The full cross-sectional surfaces of the coral skeleton samples were photographed using a Canon SX530 digital camera (Canon Inc., Tokyo, Japan).

2.1.2.2 Computed tomography (CT) scans

X-ray computed tomography GE Phoenix Nanotom-M™ (GE Sensing & Inspection Technologies GmbH, Hamburg, Germany) was used to produce images/animation of the coral skeleton microstructure and determine the porosity of the skeleton samples. The skeleton samples were imaged at different voxel sizes because differences in sample dimensions affected the field of view and subsequent achievable voxel size. The X-ray nanofocus tube used for emission had an acceleration voltage of 90 kV and beam current of 140 μA. 1,801 angular radiographs (projections) were collected at 0.33° angular intervals through a 360° rotation. For each 0.33° angular interval,
three 2D radiographs was collected and averaged as one positional 2D X-ray radiograph. The 3D volume was produced by reconstructing all averaged 2D radiographs using Phoenix Datos software (GE Sensing & Inspection Technologies GmbH, Hamburg, Germany) and rendered using VG Studio Max software (v 2.1; Volume Graphics GmbH, Heidelberg, Germany). The 2D images were characterized with greyscale histograms using the software’s Volume Analyzer tool in VG Studio Max software, and the material boundaries were further defined using the global threshold technique. The air surrounding the 3D rendered coral skeleton samples was removed by segmentation methods and two analyses were performed to estimate porosity. First, specific histogram regions that contained the lowest grayscale values were isolated (i.e., the most pores) and the pore volume percentage was computed. Then, histogram regions that contained the highest greyscale value (i.e., the most skeletal material) were selected and the pore volume percentage was determined by subtracting the material volume percentage from 100%. The porosity values from these two analyses were averaged to provide an overall measure of coral porosity. Isolation of specific regions (pores or coral skeleton material) depended on the resolution, proper segmentation and isolation of the coral, and its interior, because some pore and coral material voxels overlapped.

2.1.2.3 Thermogravimetry (TG) and Powder X-ray Diffraction (PXRD)

Thermogravimetric analysis (TGA) was used to characterize the thermal stability and to determine the temperature of phase transition from the aragonite CaCO$_3$ coral skeleton’s structure to calcite phase. Skeleton samples were broken into small pieces about 1-2 mm diameter and a micro thermogravimetric analyzer (Shimadzu TGA-50H, Kyoto, Japan) was used to determine the
thermal stability of the skeleton sample. Approximately 40 mg of broken skeleton sample was heated up to 1000°C with a ramp speed of 5°C/min.

PXRD was used to verify the skeletons’ CaCO$_3$ structure before and after decomposition. An X-ray diffractometer (Rigaku miniflex 600, Tokyo, Japan) was used to study the room-temperature crystal structure of broken skeleton samples, before and after heating in air up to 1000°C. The diffractometer had 2θ Bragg-Brenatno geometry, a 300 mm goniometer diameter and a 600 W X-Ray tube source using Ni-filtered CuK\(\alpha\) radiation.

2.1.2.4 Surface area and pore size distribution by B.E.T

A physisorption chemisorption analyzer (Quantachrome Autosorb-iQ, Micromeritics, Norcross, GA, USA) and the Brunauer-Emmett-Teller (BET) method [113] were used to analyze the surface area and pore size distribution of the coral skeleton samples. Before testing, samples were outgassed at 200 °C for 3 h to remove adsorbed molecules from the surface. The nitrogen adsorption–desorption isotherm was measured using pressure intervals of 0 < \(\frac{P}{P_0}\) < 1 with 20 adsorption and 20 desorption steps. The BET surface areas were calculated using adsorption points in the relative pressures between 0.05 and 0.3, and the non-local Density Functional Theory (DFT) method [114] was used to determine pore size distributions.

2.1.2.5 Raman spectroscopy

Micro-Raman Spectroscopy was used to evaluate the vibration of atoms within the CaCO$_3$ skeleton and the influence of applied stress. Raman spectra of the skeleton were collected using a
Renishaw® inVia Raman microscope system (Renishaw, Gloucestershire, UK), which consisted of a 532 nm Si laser with maximum power of 300 mW, and a spectrograph with holographic notch filter attached to a Leica microscope (Leica Microsystems, Wetzlar, Germany) with a motorized XYZ-stage. This system was used to 2D map the sample’s surface within an area of 588 μm x 433 μm.

Before data collection, the spectrometer was calibrated using a 520.3 cm$^{-1}$ Raman peak of Si. In total, we acquired 4624 points with a collection time of 10 s per point using an objective lens of 5x at a working distance of 14 mm. Autofocus was used for map collection because it provided better focused results during area mapping. To determine the spectral variation in structural properties in the coral skeleton, the v1 (1083 cm$^{-1}$) Raman active band with the strongest intensity was selected to collect 2D Raman maps. The maps were produced using Renishaw WiRE software with mixed Gaussian and Lorentzian functions for peak fitting.

### 2.1.3 Mechanical behavior

The mechanical behavior of the staghorn coral skeleton was studied using uniaxial compression, Vickers hardness, nanoindentation and uniaxial compression-compression cycling tests. The description of all mechanical tests performed on skeletons of *Acropora cervicornis* coral is provided in the sections below.
2.1.3.1 Uniaxial compression

The uniaxial compression tests on coral skeleton samples were performed using a universal testing machine (810 material testing system, MTS, Eden Prairie, MN, USA). The compressive load was applied using a 5 kN load cell. The applied compression stress, \( \sigma \) (in MPa), was calculated using Eq. (3)

\[
\sigma = \frac{F}{A}
\]  

(1)

where \( F \) is the applied load (N) and \( A \) is the cross-sectional area of the sample (mm\(^2\)) calculated as:

\[
A = \frac{\pi \times d^2}{4}
\]  

(2)

where \( d \) is the sample diameter.

During uniaxial compression testing, two methods of measuring the displacement were used to calculate the strain of the skeleton samples. In the first method, the crosshead displacement of the universal testing machine was measured for the strain calculation. In this first method, a displacement control mode was used during the loading with a displacement rate of 0.003 mm/s. In the displacement control mode, the displacement of the sample is controlled while the load is the variable measured parameter. In the second method, a clip-on extensometer (Axial, MTS, Eden Prairie, MN, USA) was directly attached to the skeleton sample and the experiment was performed in the load control mode with a 2.4 KN/s loading rate. In the load control mode, the load applied to the sample is controlled while the displacement is the variable measured parameter. In the second method, where the clip-on extensometer was used for displacement measurements, coral
samples were not loaded all the way until failure; instead, they were unloaded upon reaching a maximum stress at 3.12 MPa.

The strain of the skeleton samples was calculated, $\varepsilon$ (unitless), by dividing the measured displacement ($\delta = L_i - L_o$) by the original height of the sample ($L_o$) as shown in Eq. (5)

$$\varepsilon = \frac{\delta}{L_o}$$

(3)

Where $L_i$ is the current height of the sample.

Once the stress and strain values were obtained, Young’s modulus, $E$ (in GPa), was calculated as the slope of the initial, linear portion of the stress-strain deformation plot using Eq. (6)

$$E = \frac{\sigma}{\varepsilon}$$

(4)

2.1.3.2 Nanoindentation and atomic force microscopy (AFM)

The nanoindentation tests on the coral skeletons’ surface was performed using a Hysitron Nanoindenter (Bruker, Billerica, MA, USA) with a Berkovich diamond indenter, which has a three-sided, pyramidal shape. 25 indentations with a maximum load of 9500 $\mu$N were placed on the polished skeleton’s surface, taking special care to select indentation areas without visible defects. A 20 $\mu$m distance between adjacent impressions were maintained to avoid potential interaction between radial cracks growing from the corners. The loading rate was 400 $\mu$N/s, and the maximum load was held for 3 s before unloading began.

Young’s modulus and skeleton hardness were measured by analyzing the load-displacement curve for each indent. The hardness, $H$ (GPa), was determined using the equation:
\[ H = \frac{P_{\text{max}}}{A} \]  

(5)

where \( P_{\text{max}} \) is the maximum load and \( A \) is the resultant project contact area, which was estimated using (Oliver and Pharr 1992). The effective elastic modulus was obtained, \( E \) (GPa), as:

\[
\frac{1}{E_r} = \left( \frac{1-\nu^2}{E} \right)_{\text{sample}} + \left( \frac{1-\nu^2}{E} \right)_{\text{indenter}}
\]

(6)

where \( E_r \) is the reduced modulus of elasticity, \( \nu_{\text{sample}} = 0.25 \) (Juarez- de la Rosa et al. 2012), \( E_{\text{indenter}} = 1140 \) GPa, and \( \nu_{\text{indenter}} = 0.07 \). The reduced elastic modulus \( E_r \) was calculated from the unloading part of the load-displacement curve, as

\[
E_r = \frac{\sqrt{\frac{\pi S}{2}}}{\sqrt{A}}
\]

(7)

where \( S \) is the slope of the unloading curve at the beginning of unloading and \( A \) is the projected contact area. The projected area \( A \) was determined from the nanoindenter area function \( A(h_c) \), where \( h_c \) is the distance from the contour of contact to the maximum penetration depth, as

\[
h_c = h - \frac{3P}{4S}
\]

(8)

where \( h \) and \( P \) were the displacement and load from the nanoindentation diagram.

To evaluate the deformation behavior of coral skeletons during nanoindentation, load-displacement plots were recalculated into mean contact pressure – contact depth curves. The mean contact pressure was calculated as

\[
p_i = \frac{p_i}{A_i}
\]

(9)
where $P_i$ was the current indentation load on the load–indenter displacement diagram ($P_i$ was taken directly from indentation data points). The contact area $A_i$ was estimated using the area function:

$$A_i = C_0 (h_c)_i^2 + C_0 (h_c)_i + C_2 (h_c)_i^{1/2} + C_3 (h_c)_i^{1/4} + C_4 (h_c)_i^{1/8} + C_5 (h_c)_i^{1/16}$$

(10)

where $C_0$, $C_1$, $C_2$, $C_3$, $C_4$, and $C_5$ were the coefficients for a Berkovich indenter. For an ideal Berkovich probe, $C_0 = 24.5$; the remaining coefficients were determined from a series of indents at various contact depths in a sample of known elastic modulus (typically fused quartz).

The contact depth corresponding to $P_i$ and $(h_c)_i$, was calculated as

$$(h_c)_i = h_i - (h_e)_i$$

(11)

Where $h_i$ is the total measured indenter displacement corresponding to $P_i$ and $(h_c)_i$, is the corresponding elastic deflection.

$$(h_e)_i = (h_e)_{max} \sqrt{\frac{P_i}{P_{max}}} = \varepsilon \frac{P_{max}}{S} \sqrt{\frac{P_i}{P_{max}}}$$

(12)

where $(h_e)_{max}$ is the elastic deflection at maximum load $P_{max}$ of the indentation diagram; $S$ is the unloading stiffness or the slope at the beginning of the unloading portion of the indentation diagram; and $\varepsilon$ is a constant that depends on the indenter’s geometry ($\varepsilon = 0.75$ for a Berkovich indenter: Gao and Liu 2017).

The Atomic Force Microscopy (AFM) images of the indentations were obtained using a Digital Instruments D5000 AFM operating in amplitude-modulated tapping mode. Imaging was performed using a Si tip (NTESPA, Bruker, Camarillo, CA, USA). Image processing and depth profile extraction used Scanning Probe Image Processor software suite (Image Metrology, Lyngby, Denmark), with all collected images levelled via a plane fit correction before further processing.
The AFM images were used to estimate the indentation profile of the impression in vertical and horizontal directions.

2.1.3.3 Vickers hardness

Vickers hardness tester (Tukon 2100B, Wilson Instruments, Illinois, USA) was used to measure the ability of the coral skeleton samples to resist the plastic deformation by indentation. The Vickers hardness tester used a diamond oriented as a square-shaped pyramid to make consistent indentations without being deformed itself. Ten indentations in the coral skeleton were made using a 100 g load with 15 s dwell time under load.

2.1.3.4 Time dependent uniaxial compression-compression cyclic behavior

Uniaxial compression-compression cyclic test was performed using a servo-hydraulic universal testing machine (810 Material Testing System, MTS, Eden Prairie, MN, USA). A 5 kN load cell was used to apply load to the coral skeleton sample, which was preloaded to 1.3 MPa in uniaxial compression before cyclic loading began. The self-aligned compression platens were used to transfer load to the coral skeleton sample. The compressive stress was applied in sinewave form with a constant stress amplitude of 1.65 MPa, where the maximum and minimum compressive stresses were equal to 4.6 MPa and 1.3 MPa, respectively. The frequency of the cyclic compression test was equal to 1 Hz, which closely represents the frequency of the ocean waves that corals experience in their natural habitat. The strain was measured using clip – on extensometer with a gage length of 8 mm (Axial, MTS, Eden Prairie, MN, USA), which was attached to the coral
skeleton sample with two springs. All stress and strain data were recorded using Elite software (Multipurpose, MTS, Eden Prairie, MN, USA) at 0.05 – seconds intervals. It took 1548 minutes (25.8 hours) to collect all experimental data in this test. The skeleton’s Young’s modulus was calculated from the slope of the loading portions of four stress-strain deformation curves taken at 0.001 hours, 11.2 hours and 25.1 from the beginning of the experiment. These Young’s modulus values were further averaged for the four stress-strain deformation curves at each time interval. The temperature of the air near the coral skeleton sample was monitored using k-type thermocouples (Omega, Norwalk, CT, USA) for the whole duration of the experiment. The temperature data were recorded using DAQ-USB (Multifunction, National Instruments, Austin, TX, USA) at 1.1-seconds intervals. The collection of all data in the experiment such as a time, load, strain and temperature, was synchronized. The compression-compression cyclic test experimental setup is shown in Figure 53.

Figure 53 Image showing experimental setup for the air temperature measurements and the uniaxial compression-compression cyclic test on the staghorn coral skeleton
2.2 Experimental set-up for testing bones of the laboratory mouse, *Mus musculus*

The experimental methodology for testing the mouse bones to better understand the effect of dietary intake and estrogen depletion on their structural, spectral vibrational and mechanical properties are described in the sections below.

2.2.1 Animals

All procedures were approved by the Institutional Animal Care and Use Committee at the University of Central Florida and were performed in accordance with the American Veterinary Medical Associated guidelines. Male and female 8-week-old C57BL/6J “wild-type” mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and allowed to acclimatize for 2 weeks prior to dietary intervention. Mice were maintained on a 12:12-h light-dark schedule and given *ad libitum* access to chow and water. Mice were randomized into experimental groups, and following the introduction of the experimental diet, average food and water intake and body weight were quantified weekly over the 8 or 12-week study period.

2.2.1.1 Diet groups

Following acclimation, mice were challenged with either a (1) regular chow diet (RC), (2) high-fat diet (HFD), (3) high saturated fat diet (HSFD) or, (4) high polyunsaturated fat (PUFA) diet. Each of the high fat diets provided 60 kcal% energy from fat (Table 4). Fat ingredients included soybean oil, lard, hydrogenated coconut oil, safflower oil and cocoa butter, each introducing $\omega$-3, $\omega$-6 and $\omega$-9 unsaturated fatty acids as well as a range of saturated fatty acids including stearic
acid, palmitic acid, lauric acid and myristic acid. In terms of the essential polyunsaturated fatty acid contributions, the RC diet consisted of soybean oil (13 kcal% ω-3 and 55 kcal% ω-6), lard (0 kcal% ω-3 and 45 kcal% ω-6) and cocoa butter (0 kcal% ω-3 and ω-6). The HFD contained soybean oil and lard only, the HSFD soybean oil, lard and coconut oil and the PUFA diet contained soybean oil, lard and safflower oil (77 kcal% ω-9 oleic acid). In order to estimate the kcal% contribution of the essential ω-3 and ω-6 polyunsaturated fats within each diet, the total fat content was divided equally between each ingredient. The results are presented in Table 4. The undetermined %kcal in each group represents the saturated fat and unsaturated ω-9 contributions within each diet.

### Table 4 control diets and estimated contribution of the essential ω-3 and ω-6 fatty acids within each diet groups

<table>
<thead>
<tr>
<th>Diet Type</th>
<th>Diet #</th>
<th>Fat ratio: (Unsaturated to Saturated)</th>
<th>Total Fat (kcal%)</th>
<th>ω-3 and ω-6 content (of total) (kcal%)</th>
<th>Ratio ω-3:ω-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Chow Diet</td>
<td>D0702090 2</td>
<td>1:1</td>
<td>10</td>
<td>2.5</td>
<td>1:5</td>
</tr>
<tr>
<td>High Fat Diet</td>
<td>D12492</td>
<td>1:1</td>
<td>60</td>
<td>38.0</td>
<td>1:5</td>
</tr>
<tr>
<td>High Saturated Fat Diet</td>
<td>D0606230 2</td>
<td>1:10</td>
<td>60</td>
<td>15.2</td>
<td>1:5</td>
</tr>
<tr>
<td>High polyunsaturated Fat Diet</td>
<td>D0606230 3</td>
<td>3.3:1</td>
<td>60</td>
<td>29.0</td>
<td>1:10</td>
</tr>
</tbody>
</table>
2.2.1.2 Ovariectomized groups

Animals were anesthetized using 2% isoflurane and bilaterally ovariectomized (OVX). Mice received carprofen (20 mg/kg) before surgery and 12 h after surgery. Sham-OVX females received the analgesic and anesthesia, dorsal skin incision, and suturing similarly to that of the OVX animals without removing the ovaries. Mice recovered for 1 week prior to being used for further experimentation. To study the effect of estradiol (E2), 8-week-old female mice were treated with a hormonal regimen to mimic the physiological range of estradiol levels for a young adult female mouse. OVX mice received intrascapular s.c. injections of either 2 µg of 17β-estradiol benzoate (EB) (Sigma, St. Louis, MO) dissolved in 0.1mL sesame oil or the vehicle (sesame oil) alone. This hormone treatment was repeated every 4-days and lasted the duration of the study. All animals were fed the same high-fat diet described in Study 1 (D12492) beginning 2-weeks post ovariectomy and continuously over the 12-week study period. Mice were euthanized 8-weeks post diet in study 1 and 12-weeks post-diet in study 2. In both studies, the left and right tibia were retrieved. The right tibia was wrapped and stored frozen at -20°C in preparation for mechanical testing and analysis. The left tibia was immediately placed in 10% buffered formaldehyde before being processed for undecalcified histology.

2.2.2 Structure

The structure of the tibiae of laboratory mouse, Mus musculus was studied using the computed tomography (CT) scans and Raman spectroscopy. The description of these techniques is provided in the sections below.
2.2.2.1 CT scans

The micro-computed tomography (\(\mu\)CT) scans were performed using a cone beam scanner (GE Phoenix Nanotom-M\textsuperscript{TM}, Waygate Technologies). The tibiae were thawed and placed in 15 mL Eppendorf tubes were imaged at 90 kV source voltage, 110 \(\mu\)A source current (mode 0) using a tungsten-diamond target with a 500 ms exposure time at 7-9 \(\mu\)m isotropic voxel resolution (depending on tibia size). Data was collected for 1080 projections over \(360^\circ\) (0.33° steps) with three averaged images per rotation position. The volume reconstructions were performed with Phoenix Datos software. Visualization and production of DICOM images were performed with VG Studio Max (v 2.1) software.

2.2.2.2 Raman spectroscopy

Micro-Raman Spectroscopy was used to evaluate the vibration of atoms within the tibia of different mice groups. Raman spectra of the mice tibias were collected using a Renishaw\textsuperscript{®} inVia Raman microscope system (Renishaw, Gloucestershire, UK), which consisted of a 532 nm Si laser with maximum power of 300 mW, and a spectrograph with holographic notch filter attached to a Leica microscope (Leica Microsystems, Wetzlar, Germany) with a motorized XYZ-stage. Before data collection, we calibrated the spectrometer using a 520.3 cm\(^{-1}\) Raman peak of Si. Data were collected between two different spectrum ranges: 50-8000 cm\(^{-1}\) and 50-1800 cm\(^{-1}\) for each tibia. For the first 50-8000 cm\(^{-1}\) range, each spectrum was a result of 1 accumulation and 30 s exposure time, while for the second 50-1800 cm\(^{-1}\) range, 1 accumulation with 50 s exposure time were used to collect the data.
2.2.3 Mechanical behavior

The mechanical behavior of the tibia of laboratory mouse, *Mus musculus* was studied using 3-point-bending and dynamic mechanical analysis (DMA) techniques, which are described in the sections below.

2.2.3.1 3-point bending

Three-point-bending tests were performed on tibiae of different mice groups using a universal testing machine (Criterion® 43, MTS, Minnesota, USA). The tibiae were loaded until complete fracture in displacement control mode with 0.015 mm/s displacement rate. The distance between the support spans of the 3-point-bending fixture was 6 mm and the tibiae were positioned horizontally with the anterior surface facing upwards (Figure 54). The vertical force was applied to the tibias mid-shaft using a 3 mm in diameter loading roller until failure occurred. The resulting load-displacement curves were obtained, and the mechanical properties of the bones were calculated as follows:

\[
\sigma = \frac{FLc_o}{4I} \tag{13}
\]

\[
E = \frac{FL^3}{d^4l} \tag{14}
\]

Where \( \sigma \) is the stress (Pa), \( F \) is the applied load (N), \( L = 0.006 \) is the span distance between the supports (m), \( c_o \) is the outer radius of the tibia’s midshaft (m), \( E \) is the elastic modulus (Pa), \( d \) is displacement (m) and \( I \) is the moment of inertia (m\(^4\)) calculated as follows:

\[
I = \frac{\pi}{4} (c_o^4 - c_i^4) \tag{15}
\]
Where $c_i$ is the inner radius of the tibia’s midshaft (m). The inner radius of the tibia’s midshaft was obtained from the computed tomography (CT) scans of the bones. Four different cross-sections from the CT scans were selected for one bone from each experimental group, and both inner and outer diameter were calculated from each cross-section as shown in Figure 55. The average values of inner-to-outer diameters from the four different cross-sections was calculated to determine the inner radius of the remaining bones in each group.

Figure 54 Image showing the experimental set-up for 3-point-bending test on mouse tibia

The displacement was measured using as a crosshead displacement, thus, the displacement of the spring in the load cell as well as other attachments were also accounted along with the bone displacement itself. Therefore, providing not accurate measurements of the displacement.
Figure 55. Image showing CT scan of mouse tibia. AP represents the anterior-posterior plane, ML represents the medial-lateral plane, \(d_i\) represents the inner diameter and \(d_o\) represents the outer diameter.

2.2.3.2 Dynamic mechanical analysis (DMA)

The dynamic mechanical tests were performed using DMA 242E (Artemis, Netzsch, Selb, Germany). Prior to testing, each tibia was thawed and placed in PBS (Phosphate Buffer Solution) solution in a room temperature for 30 minutes. The maximum applied load which resulted in a stress of 1 MPa on the bone was calculated using (Eq.13). The experiments were performed isothermally, and the samples were loaded with frequencies of 0.05, 0.1, 1 and 10 Hz. The viscoelastic properties of the tibias (storage modulus \(E'\), loss modulus \(E''\) and loss tangent (\(\delta\))) were then obtained.

2.2.4 Statistical analysis

Analysis of the structural, mechanical and histological properties of both diet and ovariectomized groups was performed using SPSS software (v18; SPSS, Chicago, Illinois). The data obtained was
nonparametric and the Mann-Whitney U-test was used for statistical comparison between different experimental groups. The differences were considered to be significant at $p$-value $< 0.05$. 
3.1 Structural and mechanical properties of staghorn coral CaCO$_3$ aragonite skeletons, cleaned by chemical bleaching and biological processes

To study the mechanical behavior of coral skeletons in the laboratory, skeletons are cleaned of organic matter: mostly proteins from the living polyps, which reside in the skeleton’s corallites [55]. Different cleaning techniques can be used to remove the proteins; two typically processes are chemical bleaching and biological decomposition. Bleaching is performed by soaking corals in a 2 x 10$^{-1}$ M aqueous solution of sodium hydroxide (NaOH), which decreases coral pigmentation because it chemically digests protein from the skeleton [115]. In contrast, biological decomposition uses natural processes to remove protein; dead coral branches are simply buried on the sandy ocean floor and proteins are removed by microorganisms [116]. The effects of these alternate protein-removal techniques on structural and mechanical properties of the staghorn coral skeleton are discussed in the sections below.
3.1.1 Microstructure of the skeleton of *Acropora cervicornis* coral

The skeleton of *Acropora cervicornis* had typical perforate structure similar to *Acropora muricata*, which was described in detail by Marfenin [57]. Bleached skeleton of *Acropora cervicornis* coral along with an optical micrograph of the skeleton radial cross-section are shown in Figure 56. From figure 56, one can see that the optical micrograph of the staghorn coral skeleton revealed a highly porous structure with presence of the radial corallites (Figure 56).

![Figure 56](image)

Figure 56 (a) Bleached skeleton of *Acropora cervicornis* coral, (b) optical micrograph of the staghorn coral skeleton cross-section

The CT scans exposed more details, as major and minor cavities were different in size and non-homogenously distributed along the coral skeleton (Figures 57 and 58). From Figures 57 and 58, one can observe the presence of radial corallites and cavities in both the top cross-section and longitudinal cross-section close to the outer surface of the coral skeleton (Figures 57 and 58 A-D).
In addition, the longitudinal cross section in the center of the coral skeleton revealed the presence of the axial canal, along with radial corallites and both radial and circumaxial cavities (Figures 57 and 58 E,F). The wall thickness of both chemically bleached (Figure 56) and biologically cleaned (Figure 58) skeletons decreased from their outer wall toward the center of samples in a longitudinal direction and was coupled with increased porosity in the same direction [117] (Figure 57D, F) and (Figure 58 D, F). No differences were apparent in the structure of chemically bleached and biologically cleaned skeletons except for overall wall thickness.

Figure 57 (A,C,E) showing CT images, (B,D,F) showing wall thickness distribution of chemically bleached skeletons of *Acropora cervicornis* coral [117]
3.1.2 Vickers hardness

Vickers hardness values of CaCO₃ aragonite skeletons measured on polished sample surfaces were 3.67 ± 0.33 GPa (Mean ± SD) and 3.65 ± 0.24 GPa for the chemically bleached and the biological cleaned samples, respectively, which is close to the 3.31 GPa reported for Acropora palmata in [118]. Vickers hardness impressions of chemically bleached and biological cleaned coral
skeletons are shown in (Fig. 59). Crack propagation also was strongly retarded from the corners of Vickers impressions (Fig. 59A, B); the numerous pores reduced stress intensity at the tip of the indentation imprint. Thus, the indentation response of A. cervicornis reflect its perforate skeleton’s complex microstructure and ability to suppress crack propagation and growth. The skeleton’s ability to resist cracking is biologically important because branches do not separate easily from their parent structure even after small cracks develop. No difference was found in the hardness values of chemically bleached and biological cleaned skeletons of A. cervicornis.

Figure 59 Optical micrographs of Vickers hardness impressions of skeletons of (A) chemically bleached and (B) biologically cleaned Acropora cervicornis coral [117]

3.1.3 Uniaxial compressive strength

Stress-strain plots of the chemically bleached and biological cleaned coral skeletons are shown in (Fig. 60). The response of the skeletons under compressive load was linearly elastic with many pop-ins occurring upon loading. Each pop-in represented a crack propagation event, where the
crack propagated until it reached the next closest pore. This pore then causes the crack to stop growing because it reduces the stress concentration at the crack tip and the applied load must increase again until another cracking occurs. Crack accumulation eventually causes the specimens to fracture completely. The average maximum stress for chemically bleached samples was 9.5 ± 2.3 MPa, while for the biologically cleaned samples was 14.5 ± 6.4 MPa. These values were substantially less than Chamberlain [78] reported for A. palmata (47.7 ± 17.3 MPa), S. radians (32.0 ± 8.2 MPa) and M. annularis (22.4 ± 12.9 MPa). Despite their relatively low compressive strength, the unique and complex porous macrostructure of the skeleton of A. cervicornis contributed to “gracious” stress-strain deformation behavior, rather than the catastrophic failure that occurs in brittle materials with linear deformation response before fracture [119].

Figure 60 Stress-strain deformation plots of chemically bleached and biologically cleaned skeletons of Acropora cervicornis coral [117]

Due to natural variation in diameter, growth that was not always in one direction, and surface roughness (Figure 54A), skeleton samples were not perfect cylinders. Therefore, the strength
values reported here are nominal and have large variability. Accurately estimating the stress distribution and applied load during uniaxial compression experiments requires modeling to account for the bending moment resulting from eccentric loading.

Strength measurements at the point of failure and at the appearance of first pop-in were analyzed using Weibull statistics (Figure 61). From the distribution, the scale parameter $\sigma_o$ at first pop-in was equal to 7.31 MPa and its shape parameter $m$ was equal to 3.45. At skeletons failure, the scale parameter $\sigma_o$ was equal to 12.63 MPa and the shape parameter $m$ was equal to 3.10, which are quite similar. No difference was apparent between the compression behavior of chemically bleached and biological cleaned coral skeletons.

Figure 61 Weibull plots of chemically bleached and biologically cleaned skeletons of Acropora cervicornis coral [117]
3.1.4 Micro-Raman spectroscopy

The optical micrograph of coral skeleton surface taken using a Zeiss Axio Lab.A1 microscope (Carl Zeiss AG, Jena, Germany) (Figure 56) revealed the presence of light and grey colors in aragonite skeleton surface. Such variation in the colors in the skeleton surface can be attributed to difference in the porosity and non-homogenous CaCO$_3$ distribution. Therefore, Raman spectroscopy was used to study these structural properties within the coral skeleton. The Raman spectrum of aragonite CaCO$_3$ skeleton of *Acropora cervicornis* coral is shown in Figure 62. Selected $\nu_1$ 1083 cm$^{-1}$ peak was selected to create peak intensity and FWHM Raman maps in order to identify the area on the surface where the difference in structural properties of the aragonite material could be detected by Raman. The optical micrograph of the selected coral skeleton area along with the 2D Raman maps of peak intensity, full width at half maximum (FWHM) are shown in Figure 62. The optical micrograph (Figure 62B) taken by confocal microscope coupled with the Raman spectrum was used to photograph a surface, however, the quality of the surface was not such precise as with the photographs taken using Zeiss microscope, therefore, the inhomogeneous white and grey zones are not so visible in Figure 62. However, the 2D Raman map of $\nu_1$ 1083 cm$^{-1}$ peak intensity (Figure 62C) revealed the variation in intensity within the mapped area with the highest intensity appeared in the center of the photographed surface. Moreover, while intensity variation within the skeleton surface might be related to the topographic features of the surface, however, 2D map of full width at half maximum (Figure 62D) clearly showed that the peak broadened from (5.3 cm$^{-1}$) at the center to (5.6 cm$^{-1}$) outside of the center of the surface, thus also confirming the difference of vibrational structure of the coral skeleton surface between the different areas.
Figure 62 Raman spectrum of the skeleton of a staghorn coral (*Acropora cervicornis*) showing (A) Raman spectrum of the aragonite material, (B) optical micrograph and corresponding 2D Raman maps of (C) 1083 cm\(^{-1}\) Raman peak of peak position, and (D) peak full width at half maximum.

3.2 Porosity and the elastic behavior of staghorn coral (*Acropora cervicornis*) skeletons

In this section, porosity effects on Young’s modulus values of the skeleton of *Acropora cervicornis* coral measured from uniaxial compression and nanoindentation techniques will be discussed. As In the uniaxial compression test, the measured values of Young’s modulus are based on the structural response of the whole skeleton structure, while in the nanoindentation test, Young’s modulus can be measured locally, without consideration of the overall porosity of the skeleton. In addition, the high temperature stability and crystal structure of the CaCO\(_3\) aragonite coral skeleton are also discussed in the sections below.
3.2.1 Thermogravimetric (TG) analysis and Powder X-ray Diffraction (PXRD)

Thermogravimetric analysis revealed the temperature range of phase stability of the CaCO₃ aragonite structure, which was important for further BET measurements (Figure 63A). As one can see from Figure 63A, the CaCO₃ coral skeleton structure was stable and didn’t lose any wait until approximately 292 – 335° C, where the reported aragonite → calcite phase transition was reported in [80]. Upon further heating, CaCO₃ fully decomposed to CaO by the following chemical reaction CaCO₃(s) → CaO(s) + CO₂(g) at approximately 727° C. The decomposition process started at a temperature of 583 °C, and by the end of the process, CaCO₃ fully converted to CaO at 727° C with weight loss of 41 %. Similar decomposition of CaCO₃ aragonite ceramic derived from anadaragranosa shells to CaO with weight loss of 42 % studied by TGA was also reported in [81].

![Thermogravimetric curve of ground skeleton of Acropora cervicornis, broken pieces of the skeleton of A. cervicornis (Insert), and (B) powder x-ray diffraction pattern for both CaCO₃ before heating and CaO after heating.](image)

Figure 63 (A) Thermogravimetric curve of ground skeleton of Acropora cervicornis, broken pieces of the skeleton of A. cervicornis (Insert), and (B) powder x-ray diffraction pattern for both CaCO₃ before heating and CaO after heating.
The confirmation of the full conversion of CaCO$_3$ into simple CaO was obtained by XRD (Figure 63B). As one can see from Figure 63B, before heating, the coral skeleton indeed had aragonite crystal structure with orthorhombic unit cell (space group $Pmcn$ according to JCPDS (PDF 00-005-0453)), which corresponds very well with results presented in Behrens et al. [67] for aragonite structure as well as in Bao et al. [120] for aragonite microspheres. The following lattice parameters $a = 4.959$ Å, $b = 7.964$ Å and $c = 5.741$ Å of aragonite structure were reported in Ye et al. [121]. However, after heating above 727$^\circ$ C only peaks belonging to CaO cubic structure (space group $Fm-3m$) were present upon cooling, which were in a good correspondence with the JCPDS (PDF 77-2376) and the XRD pattern of the CaO powder reported in Abass et al. [122].

3.2.2 Surface area, pore size distribution and porosity

The specific surface area (SSA) of the staghorn coral skeleton samples measured by BET had very low values: $0.657 \pm 0.14 \frac{m^2}{g}$. Pore diameter estimated by the DFT method [114, 123] was very small: $3.87 \pm 0.043$ nm with the differential pore volume being equal to $0.0027 \pm 0.0004 \frac{cm^3}{g}$ (Figure 64). In addition, the majority of the pore sizes measured by B.E.T were in the range of 2-5 nm, which is indicative of the presence of mesopores in the coral skeleton sample [114, 123]. Such large discrepancies between nanopore size distribution and SSA occurred because large pores, such as radial corallites and the axial canal, cannot be measured by the BET method; it only measured nanopores presents in the coral skeleton. The specific surface area values of skeletons of Diplo...
Ethylene Glycol Monoethyl Ether (EGME) method [124] was reported to be in the range of 1.4-1.6 $\frac{m^2}{g}$ in Amiel et al. [125]. Although the methods used to measure the SSA of the skeletons of *Acropra cervicornis* coral in this study and *Diploria strigose*, *Diploria labyrinthiformis* and *Montastrea cavernosa* corals in [125] are different, one can notice the similarity of the SSA values between of *A. cervicornis* and the different coral skeletons studied in [125].

![Figure 64 Pore size distribution of the staghorn coral (*Acropora cervicornis*) skeleton](image)

In order to quantify the overall porosity of the staghorn coral skeleton sample, the computed tomography (CT) was used as one of the most appropriate technique for such measurements. The CT scans revealed a typical perforate skeleton structure of *Acropora cervicornis* coral (Figure 65).
Radial corallites were arranged on the skeleton’s outer surface (Figure 65A) and radial and axial cross-sections (Figure 65B and C, respectively) revealed a highly porous structure of the skeleton, with different pore sizes and non-homogenously distributed cavities inside. Pores varied in size and shape, and both radial and circumaxial cavities had major circle-like cavities ~1 mm in diameter. The corallites along with axial canal have substantial contribution to the overall porosity of the skeleton and is responsible for protecting the living tissue (polyps) within the coral skeleton [57].

Figure 65 CT images of (A) 3D model, (B) radial cross-section, and (C) longitudinal cross-section of a staghorn coral (*Acropora cervicornis*) skeleton

The CT radial and axial cross-sectional images showing the wall thickness of the coral skeleton, which was used to measure the porosity are shown in Figure 66A and B, respectively. As one can see from Figure 66, the major porosity elements present in skeleton that contribute the most to the
Overall porosity are the axial canal and the radial corallites, however, both radial and circumaxial cavities also have a substantial contribution to the overall porosity [117]. The overall porosity of the skeleton of *Acropora cervicornis* measured in this study was equal to 31 ± 5%. This overall porosity estimate was limited by the resolution (1 micron) of the CT machine; therefore, other techniques (e.g., BET) better estimate the fine porosity present in coral skeletons. Similar CT porosity analysis on skeleton of *Acropora pulchra* coral branch was performed in Roche et al. [126], it was determined that the overall porosity was measured to be equal to 30% at the base of the skeleton branch [126], which is in a good correspondence with porosity values measured in the current study.

Figure 66 CT images showing pore thickness of (A) radial cross-section, (B) longitudinal cross-section of a staghorn coral (*Acropora cervicornis*)
3.2.3 Strength, Young’s modulus and stress-strain behavior in uniaxial compression

The typical stress-strain deformation plots of the staghorn skeleton are shown in Figure 66. Stress-strain deformation behavior of coral skeleton in the displacement control mode loaded all the way until complete fracture is shown in Figure 67A. The photograph of broken skeletons of *Acropora cervicornis* coral after compressive test is shown in Figure 67B. The stress-strain deformation behavior of coral skeleton in the load control mode loaded till 3.2 MPa without causing failure of the sample is shown in Figure 67C, and the photograph of the uniaxial compression experimental set-up with the clip-on extensometer measuring the displacement of the coral skeleton sample is shown in Figure 67D.

![Stress-strain deformation plots and photographs](image)

Figure 67 Stress-strain deformation plot of the staghorn coral (*Acropora cervicornis*) skeleton after uniaxial compression using (A) displacement control mode, (C) load control mode, (B) image of the fractured skeleton after uniaxial compression, and (D) experimental set-up for the uniaxial compression test using clip-on extensometer
The skeleton of *Acropora cervicornis* responded very differently to uniaxial compression under displacement control mode compared to the load control mode. In displacement control mode, the mean compressive strength of the coral skeletons was measured to be equal to $10.7 \pm 2.2 \text{ GPa}$. The complex porous macrostructure exhibited “gracious” rather than catastrophic failure despite its relatively low compressive strength. Many “pop-ins” events, defined as “a sudden drop in an increasing applied stress” [6], occurred where either a new crack formed, or an existing crack propagated under compression. This “gracious” failure behavior is typical for brittle materials such as ceramics and rocks [119]. When crack density became very high at higher loads, all the cracks eventually coalesced into one big crack, which grew parallel to the applied stress and caused complete failure of the coral skeleton sample (Figure 67B). In contrast, under load control mode, the skeleton sample did not have visible “pop-in” events during compression, did not fail up to $3.12 \text{ MPa}$, and then was unloaded. Either the maximum applied load was insufficient to cause crack propagation and “pop-ins” or microcracking and “pop-in” events were not detected by measuring the skeleton displacement using clip-on extensometer. Moreover, corallites growth orientation also affects compressive strength of coral skeletons [78]. It was determined that the skeleton of branching corals such as *Acropora cervicornis* is strongest along the axial canal, where corallites grow parallel to the applied stress, and weakest across the axial canal, where polyps grow perpendicular to the applied stress [78]. The compressive strength of the coral skeleton was measured along the axial canal and expect compressive strength will be even lower if the skeleton is tested perpendicular to the axial canal.

Young’s modulus of skeletons of *A. cervicornis* was measured from compressive stress-strain deformation curves, where displacement was measured by two different methods. In one of the
methods, the displacement was measured from the movement of the crosshead of the universal testing machine, and, in the second method, the displacement of the coral skeleton was measured using clip-on extensometer attached directly to the coral skeleton surface. The mean Young’s modulus measured from the linear portion of the stress-strain deformation diagram at the beginning of loading, obtained by the first method, was 0.19 ± 0.16 GPa. Young’s modulus calculated from the loading portion of the stress-strain deformation curve, obtained by the second method, was much higher and equal to 7.34 GPa. This difference may arise from the strain measurement techniques that we used. Strain calculated from the crosshead displacement included both displacement of the coral skeleton sample and deformation of the load cell along with supporting columns, including the compression platen. Therefore, strain values might be overestimated and yield lower values of Young’s modulus. The strain that we measured using the clip-on extensometer included only displacement of the coral skeleton sample, and therefore is more accurate. However, the skeleton sample had a very uneven surface, and the extensometer could shift slightly during loading, thus introducing small uncertainties to the strain measurements. In any case, more research is needed to generate accurate Young’s modulus values for skeletons of *Acropora cervicornis*.

Elastic modulus values differ considerably among the different coral reef species (Table 5), which [78] attributed to differences in their porosity and skeletal growth form. Similarly, the Young’s modulus of thirteen gorgonians corals varied by more than an order of magnitude, from 0.2 – 9 GPa [127]. The elastic modulus values of both scleractinian and gorgonian corals was substantially lower than the value reported for the geogenic single crystal aragonite: 100.8 GPa in the {001} plane and 82.7 GPa in the {122} plane [75]. Unlike the dense geogenic single crystal aragonite
material, the coral skeletons contain high porosity, resulting in dramatic decrease of their Young’s modulus as compared to single crystal aragonite [78]. Thus, the entire skeleton of A. cervicornis is highly porous, not as hard and elastic as its parent dense aragonite crystals, is stronger along the axial canals than across them, and relieves compressive loads via gracious failure but effectively resists loading during compression.

Table 5 Compressive strength (MPa) and elastic modulus (GPa) of skeletons of the corals Acropora palmata, A. reticulata, A. cervicornis, Siderastrea radians, Montastraea (= Orbicella) annularis, and Porites lobata. Values compiled from [6, 78, 79, 117]. (hereinafter, mean ± SD)

<table>
<thead>
<tr>
<th>Species</th>
<th>Compressive strength (MPa)</th>
<th>Elastic modulus (GPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acropora palmata</td>
<td>47.7 ± 17.3</td>
<td>21.5 ± 8.3</td>
</tr>
<tr>
<td>Acropora reticulata</td>
<td>62.0 – 90.0</td>
<td>38.0 – 71.0</td>
</tr>
<tr>
<td>Acropora cervicornis</td>
<td>10.7 ± 2.2</td>
<td>0.19 – 0.29</td>
</tr>
<tr>
<td>Siderastrea radians</td>
<td>32.0 ± 8.2</td>
<td>15.1 ± 2.1</td>
</tr>
<tr>
<td>Montastraea (= Orbicella) annularis</td>
<td>22.4 ± 12.9</td>
<td>10.6 ± 7.0</td>
</tr>
<tr>
<td>Porites lobata</td>
<td>15.8 ± 4.7</td>
<td>-</td>
</tr>
</tbody>
</table>

3.2.4 Hardness, young’s modulus and stress-strain behavior by nanoindentation

Hardness and Young’s modulus calculated from a load-displacement nanoindentation plot shown in Fig. 68A were 3.8 GPa and 71.8 GPa, respectively. The mean hardness and Young’s modulus measured using 25 nanoindentation data points were 4.29 ± 0.46 GPa and 86 ± 7.26 GPa, respectively, which were similar the values reported for skeletons of the corals Balanophyllia.
europaea (76.3 – 77.0 GPa for Young’s modulus and 4.97-4.87 GPa for hardness) and Stylophora pistillata (76.0 – 76.6 GPa for Young’s modulus and 5.04 - 5.10 GPa for hardness) [75].

Figure 68 (A) indentation load vs. depth deformation plot, (B) corresponding mean contact pressure vs. contact depth, (C) Height, (D) amplitude AFM images showing the indent after the nanoindentation test on the staghorn coral (Acropora cervicornis) skeleton. (H-H) in (C) corresponds to horizontal depth profile as shown in (E), (V-V) in (C) corresponds to vertical depth profile as shown in (F).
The high Young’s modulus of the staghorn coral skeleton measured by nanoindentation as well as its hardness corresponded very well to the properties of dense CaCO$_3$ aragonite single crystals with reported values of elastic modulus equal to 100.8 GPa for \{001\} plane and 82.7 GPa for \{122\} plane [75] and hardness values equal to 4.60 ± 0.30 GPa, 4.36 ± 0.4 GPa and 6.20 ± 0.30 GPa for \{110\}, \{130\}, and \{001\} planes, respectively [128]. The load-displacement plot was relatively smooth during both loading and unloading portions with no ’pop-in’ events observed (Fig. 68A). The corresponding mean contact pressure – contact depth plot recalculated from load-displacement data using Eqs. 9-12 is shown in Figure 68B. The maximum mean contact pressure was 5.85 ± 0.86 GPa, which is close to the hardness values of 4.29 ± 0.46 GPa measured using the technique published in Oliver and Pharr 1992 [129].

To confirm that indentation surface was almost dense and also to visualize the indents the AFM images of one of the impressions were taken (Figure 68C and D). The AFM images revealed that no visible porosity would be seen on the surface and that the impression was placed in the dense material. In addition, there were no radial cracks grown from the corners of the impression detected after nanoindentation. In brittle ceramics, such radial cracks are usually used to evaluate indentation fracture toughness of the material [130, 131]. However, absence of such cracks after nanoindentation is indicative that aragonite coral skeleton’s structure has high ability to resist crack propagation and retard the crack growth during indentation.
Figure 69 (A,C) Indentation load vs. depth and (B,D) corresponding mean contact pressure vs. contact depth for two different indents on the staghorn coral (*Acropora cervicornis*) skeleton

Furthermore, the indentation profiles of the impression revealed that there are pile ups of the aragonite material on the left and the bottom sides of the indentation profiles indicated as (H₁) and (V₁), respectively (Figure 68E and F). Such pile ups appeared during nanoindentation because aragonite material is plastically squeezed out as a result of high compressive stress applied during nanoindentation. It was also detected that the staghorn coral skeleton can exhibit different load-displacement deformation behavior during nanoindentation (Figure 69), where a majority of load-displacement and mean contact pressure – contact depth plots were smooth and continuous during both loading and unloading (Figures 68A and B and 69A and B), some of the plots exhibited “‘pop-in’” events during the loading process (Figure 69C and D). Such presence of “‘pop-in’” events
during loading of the skeleton of *A. cervicornis* coral sample could be attributed to structural changes during nanoindentation, such as the onset of dislocation nucleation or microcracking leading to significant decrease of mean contact pressure under the indenter [128].

### 3.3 Comparison of pore distribution between young and mature staghorn coral (*Acopora cervicornis*) skeletons

Despite the fact that the porosity in most branching corals such as *Acropora* genus, decreases from the tip towards the base of the branch [126], the continues calcification, as the skeletons mature during their lifetime, prevents formation of regular pattern of skeletal density for branching coral species. To better understand such skeletal density pattern, the pore size distribution of young and mature skeletons of *Acropora cervicornis* corals are discussed in the sections below.

#### 3.3.1 Coral skeleton samples

The skeleton of *Acropora cervicornis* branch used in this study is shown in Figure 70. From Figure 70, one can notice the skeletal structural variation within the *Acropora cervicornis* subbranches, where the mature subbranch of the coral skeleton further extends into younger (thinner) subbranches. In this study, the porosity of two different skeleton samples was explored. The mature sample was cut near the base of the coral branch (Figure 70), while the young sample was cut near the tip of the coral branch. The CT images showing the outer surface of these two coral skeletons are included in Figure 70.
Figure 70 Photograph and CT images showing the branch of the staghorn coral (*Acropora cervicornis*) skeleton, along with outer surface of mature and young skeleton

3.3.2 Surface area and pore size distribution by B.E.T

As it was interesting to notice the difference between surface area and pore size distribution between younger and more mature coral samples, then the BET analysis was performed on both of those samples. The representative N$_2$ adsorption-desorption isotherms plots of mature and young skeletons of *A.cervicornis* coral are shown in Figures 71A and B, respectively.
Figure 71 Nitrogen adsorption-desorption isotherm of (A) mature, (B) young, skeletons of *Acropora cervicornis* coral samples

From this isotherm plots, one can observe that both isotherms showed Type *III* adsorptions, which occurs when N$_2$ adsorb on the surface gradually as the relative pressure increases [123]. As a result of the gradual surface adsorption of N$_2$ gas into the surfaces of the coral skeleton, there were no steep uptake in the adsorption (Type *I*), the monolayer-multilayer adsorption (Type *II*), capillary condensation (Type *IV*) and stepwise multilayer adsorption (Type *VI*) detected during the experiments [123, 132]. In addition, both mature and young skeletons exhibited Type H3 hysteresis loop, which is associated with the formation of slit-like pores within the structure of the
material [132, 133]. The specific surface area (SSA) of mature and young skeletons were measured to be equal to $0.657 \frac{m^2}{g}$ and $4.1 \frac{m^2}{g}$, respectively. From this SSA measurements, one can see that young coral skeleton has 6-fold higher SSA as the mature sample, which is indicative that porosity of younger sample might be higher. The pore size distributions of mature and young skeletons of *Acropora cervicornis* coral are shown in Figure 72.

![Figure 72 Pore size distribution of mature and young skeletons of *Acropora cervicornis* coral samples](image)

As one can see from Figure 72, the pore size distribution measured from the N$_2$ adsorption – desorption isotherms, revealed that the pores present within coral skeletons have a size in the range of 2-37 nanometers. The pore size, estimated by DFT model [114] as an average pore diameter for mature and young coral skeletons samples was measured to be equal to 3.87 nm and 3.2 nm, respectively. In addition, as one can see from Figure 3, most of the pore sizes for both coral
skeleton samples were between 2-5 nm, which indicates the presence of the mesoporosity in the coral skeleton [123]. However, larger pores with the diameters up to 37 nm also existed in both coral skeleton samples (Figure 4). Furthermore, one can also observe that mature skeleton sample contained much lower amount of pore volume ($0.0027 \text{ cm}^3 \text{ g}^{-1}$) in comparison with the young skeleton ($0.011 \text{ cm}^3 \text{ g}^{-1}$). These results confirmed that as coral skeleton mature during coral reef’s lifetime, its density increases and the amount of the porosity significantly decreases [56, 126]. The B.E.T method allowed the evaluation of the nanopores within the coral skeletons. However, the B.E.T method didn’t allow to estimate the overall porosity of the coral skeleton samples. Therefore, in order to determine the overall porosity and gain a more complete understanding of coral skeleton’s porosity characteristics, different techniques, such as computed tomography, have to be further explored.

3.3.3 Porosity from CT scans

The CT scan images showing the axial cross-sections and the pore thickness along the center of axial canal of mature and young coral skeletons are shown in Figure 73. As one can see from Figure 73A and B, the CT scans revealed the skeleton structure with axial canal and radial corallites along with circumaxial pores of *Acropora cervicornis* mature and young samples. Both mature and young skeletons of *Acropora cervicornis* coral were highly porous with the pores sizes and shapes vary significantly (Figure 73).
The calculated porosity of mature and young coral skeletons present in Figure 73, was equal to 31 ± 5% and 67 ± 5%, respectively, with the porosity of the young sample being more than twice as high as the porosity of the mature sample under study. In addition to variation in the porosity of mature and young skeletons, it was found that mature skeleton with lower porosity also exhibit lower pore diameters and higher wall thickness as compared with the young skeleton (Figure 73C and D). For example, the pore diameter of the axial canal highlighted by in green color in Figure 73C and D, was measured to be equal to 1.15 mm and 1.35 mm for the mature and young coral skeleton skeletons, respectively.
Figure 74 CT scans showing radial cross-sections (A,B) and wall thickness (C,D) of mature and young skeletons of Acropora cervicornis coral.

Similar CT analysis of the coral skeleton structures was performed on the radial cross-sections of the samples. The CT scan images of the radial cross-section of mature and young coral skeletons are shown in Figure 74. The radial cross-section images also revealed the presence of the axial canal, radial corallites and circumaxial cavities (Figure 74A and B). The pore diameter of the axial canal was also determined to be 1.3 mm and 2.1 mm for the mature and young skeletons, respectively, while the pore diameter of the radial corallites for mature and young skeleton were measured to be between 0.45 – 0.84 mm and 0.57 – 1.39 mm, respectively, therefore, confirming that mature skeleton has smaller pore diameters as compared to the young skeleton (Figure 74C.
and D). Therefore, both axial and radial cross-sections revealed that the major skeleton elements that contribute the most to the overall porosity of the coral skeleton samples are the axial canal and the radial corallites, however, circumaxial cavities also have substantial contribution to the overall porosity [117]. These results are in a good correspondence with the fact that, as the age of the coral skeleton increases, the calcification also increases, resulting in a denser skeleton [56].

3.4 **The effect of temperature on stress-strain behavior of staghorn coral (Acropora cervicornis) skeletons**

Elevated sea temperature is considered one of the main causes of mass bleaching events in coral reefs, and therefore, a major contributing factor to decline in coral reef population [134]. In this section, the effect of temperature on deformation behavior of the staghorn coral (Acropora cervicornis) skeletons under cyclic loading will be discussed. Such deformation behavior is important to better understand the physical response of coral skeletons to temperature rising and predict the corals response to changing ocean conditions.

3.4.1 **Uniaxial compression-compression cycling**

The typical stress vs. time and strain vs. time plots of the coral skeleton for four consecutive uniaxial compression-compression cycles collected within the first hour from the beginning of the experiment are shown in Figure 75. As the experiment was performed in the load control mode, all stress-time cycles looked symmetrical (Figure 75A), however, the strain-time cycles that were measured using clip-on extensometer didn’t maintain symmetry and showed both strain “plateau” at the maximum stress and “shoulders” upon unloading (Figure 75B).
Figure 75 plots showing (A) stress Vs. time (B) strain Vs. time, for four consecutive cycles at the beginning of the cyclic compression experiment on the staghorn coral (*Acropora cervicornis*) skeleton.

Using such stress vs. time and strain vs. time data, the stress – strain deformation plots of coral skeleton at the beginning, middle and end of the experiment were plotted in Figure 76. The stress-strain deformation plots collected during loading and unloading at 0.001 hours, 11.2 hours and 25.1 hours from the beginning of the experiments are shown in Figure 76A, B and C, respectively. Young’s modulus of the coral skeleton was determined from the loading portion of the stress – strain deformation plots, where the slopes of four consecutive loading cycles were averaged for the Young’s modulus calculation at each 0.001 hours, 11.2 hours and 25.1 hours, and no significant
differences were detected between the values measured at the beginning (7.27 ± 0.08 GPa), middle (7.37 ± 0.04 GPa) and at the end (7.52 ± 0.08 GPa) of the experiment.

Figure 76 Plots showing stress Vs. strain at the (A) beginning, (B) middle and (C) end of cyclic compression experiment on the staghorn coral (*Acropora cervicornis*) skeleton

The results of the uniaxial compression-compression cyclic experiment performed on the coral skeleton for the duration of 25.8 hours are shown in Figure 77, where stress, strain, and temperature collected during the experiment as a function of time are presented.
Figure 77 plots showing variation of (A) stress, (B) strain of the staghorn coral (*Acropora cervicornis*) skeleton under cyclic compression, (C) room temperature, over 25.8-hours period

As the experiment was performed in the load control mode, it is obvious that the loading amplitude was constant throughout the whole experiment, and thus, no deviation existed in the given 3.2 MPa stress range (Figure 77A). Unlike the stable stress vs. time measurements, the strain exhibited very different behavior as a function of time, where the periodic pattern of increase and decrease values
of strain could be clearly seen (Figure 77B). Such patterns of increased and decreased strain values correlated very well with the increase and decrease in the temperature of the air (Figure 77C), which was measured in the vicinity of the coral skeleton sample during loading (Figure 53). The temperature in the room was recorded to be equal to 23.8° C at the beginning of the experiment, but it varied from 22.2° C to the maximum 25.2° C in a periodic pattern for the duration of the experiment.

The details of the strain and temperature variations during cyclic loading of coral skeleton as a function of time are presented in Figure 78. As one can see from Figure 78A, there is a local increase in the strain at a certain period of time, where the strain reached its maximum point, followed by the decrease in strain to its minimum value. Such local increases followed by decreases in strain are observed both during maximum and minimum stresses upon cycling. In addition, the local increase in temperature at a certain period of time, where temperature reached its maximum point, followed by the decrease in temperature to its minimum value, were also recorded (Figure 78B). By comparing Figure 78A and B, one can see that a good correlation could be established between the appearance of maximum and minimum strains with an increase and decrease of air temperature recorded in the room as a function of time, however, it can be clearly observed that there is a time lag between the locations of maximum and minimum strains as compared to the maximum and minimum temperatures at the strain vs. time and temperature vs. time plots. Such lag can be attributed to the fact that the temperature of air was measured in the vicinity of the coral skeleton and thus it is not the temperature of the coral skeleton itself. Therefore, one can conclusively expect that the temperature of the coral skeleton will be increased with a certain time delay as compared to the temperature increase of the air nearby the skeleton.
Figure 78 Plots showing the variation of strain of the staghorn coral (*Acropora cervicornis*) skeleton and room temperature, over 2-hours period

3.4.1.1 Stress-strain deformation behavior for a single loading-unloading cycle

The changes in the stress-strain hysteresis response of the coral skeleton upon cycling at the beginning, middle and the end of the 25.8-hours experiment can be seen in Figure 76. As one can see from Figure 76, the largest stress-strain hysteresis loop occurred in the cycling at the very beginning of the experiment (Figure 76A). As experiment further extend in time, the hysteresis loops became much less pronounced (Figure 76B and C). The appearance of the hysteresis loops can be attributed to two features of the strain vs. time plots (Figure 75B). These two features are
“plateau” and “shoulders”, as highlighted in Figure 75B. These two features are much more visible in strain-time plots at 0.001 hours from the beginning of the experiment, but they became much less pronounced at 11.2 hours in the middle and, especially, at 25.1 hours by the end of the experiment. As the appearance of the “plateau” might be connected with some inconsistency in machine operation, when the direction changes from loading to unloading, thus, the data with “plateau” will not be considered and they will be further ignored in the discussion. However, appearance of non-symmetric “shoulders” deformation of the coral skeleton (Figure 75B) is indicative of appearance of some non-elastic component, such as microcracking during stress-strain deformation of porous CaCO$_3$ structure. Such microcracking introduced hysteresis loops during loading and unloading cycles, which, indeed, could be seen in all stress-strain diagrams of the coral skeleton (Figure 76). In addition, as one can see from Figure 76, the hysteresis area decreases as a function of time as the experiment proceeds. One of the explanations for the decrease of hysteresis loops area is that the coral skeleton sample used in the compression had a highly inhomogeneous outer surface with intricate porous structure, which is far less from being ideal for the proper attachment of clip-on extensometer edges to measure the strain of the coral skeleton. The shape of the strain vs. time loading-unloading plots had changed with the time leading to a decrease in the hysteresis area, which might be connected with the friction between surfaces of porous aragonite and the extensometer edges during cycling. Such friction might result in wear of aragonite, which would bring more stable and consistent stress-strain response by the end of the 25.8-hours long experiment.
3.4.1.2 Young’s modulus

The average Young’s modulus measured from the loading portion of stress-strain hysteresis curves was equal 7.39 ± 0.12 GPa (Figure 76). Young’s modulus measured at the beginning, in the middle and by the end of experiment didn’t neither remained constant nor decreased in the value, but, the opposite, a slight increase from 7.27 ± 0.08 GPa at the beginning, to 7.37 ± 0.04 GPa in the middle and to 7.52 ± 0.08 GPa at the end of the experiment was measured, which confirms that such changes might be attributed to the wear of porous coral skeleton surface during the 25.8 hours duration of experiment. The loading stress-strain deformation plots didn’t show the appearance of ‘’pop-in’’ events that were reported in [6, 117] because the loading was measured in the load control mode and the way how the strain was measured by the clip-on extensometer attached on the side of the coral skeleton was not sensitive enough to detect microcracking and ‘’pop-in’’ events occurring in the bulk of coral skeleton.

3.4.1.3 Strain variation as a function of time

The results of stress, strain and temperature variations as a function of time are presented in Figure 77. As the test was performed in the load control mode, the applied load remained constant for the whole 25.8 hours duration of the experiment (Figure 77A), however, significant changes in the deformation of the coral skeleton sample were recorded as a function of time (Figure 77B). The most important finding in this work is that the variation in compressive strain of the coral skeleton exhibited strong correlation with the variation in the measured air temperature in the vicinity of the coral skeleton (Figures 77C). The temperature of the coral skeleton was assumed to be equal
to the temperature of the air during the experiment. The changes in air temperature in the room occurred because of the operation of the A/C cooling unit with the temperature profile presented in Figure 77C recorded for 25.8 hours duration of the experiment. As one can see from Figure 77C that despite that the overall average temperature of the room was equal to 24.20 °C ± 0.46, however, this local changes in the temperature had a significant effect on the deformation of the coral skeleton upon cyclic loading.

At the beginning of the experiment, within the first 8 hours, one can see that the average measured temperature was increasing from a minimum average value of 23.02 °C at first 30 minutes of the experiment, to a maximum average value of 24.62 °C at 7.7 hours from the beginning of the experiment (Figure 77C). As the temperature is rising, the dimension of the coral skeleton sample should increase and the measured strain within this given interval of 7.7 hours should not exhibit such significant change as presented in Figure 77B. However, as temperature is rising, the distance measured by the clip-on extensometer edges decreased in the first 7.7 hours of the experiment. Such decrease in the axial dimension, shown by the increase in the negative compressive strain of the coral skeleton, can be explained by the friction during contacts between clip-on extensometer edges and non-even surface of the porous skeleton sample. The contact between extensometer edges and coral skeleton sample is far from being ideal, because of complex surface of the coral skeleton. Therefore, it is highly possible that at the beginning of the experiment, the friction brings the wear and possible decrease in the distance between clip-on extensometer edges, leading to a decrease in the measured axial dimension of the coral skeleton, despite that the overall average temperature increased during the first 8 hours of the experiment. Another factor that has to be taken into account is that the strain measurements by the extensometer might be affected by the
lateral strain normal to the loading direction. The lateral strain will be affected by the change in the volume of coral skeleton when the temperature increases, as the contact between coral skeleton surface and extensometer edges is not perfect. Besides the wear and lateral strain, we also need to consider that the shape of the coral skeleton sample is not ideally symmetric, and therefore, while we calculate the stress considering only centric loading, however, in reality, the loading is eccentric, thus, in addition to the normal uniaxial stress, coral skeleton sample also experiences a bending moment during loading. This bending moment can be affected and can increase when the volume of the sample is increased with an increase of the temperature. Therefore, at least three factors, such as friction, lateral strain and bending moment, can contribute to the increase in the negative compressive strain at the beginning of the experiment when the average temperature was raising. After 7.7 hours, no further significant decline of the overall strain was measured (Figure 77B and C).

The characteristics changes in the measured strain during cyclic loading between 9.3 and 11.3 hours from the beginning of the experiment as a function of temperature are shown in Figure 78. As a strong correlation between changes in temperature and strain can be immediately found. Considering the strain and temperature changes from Point 1 to Point 2 in Figure 78, one can see that a decrease in temperature from 25°C (Point 1) to 23.11°C (Point 2), corresponds to the change in the strain ($\Delta \varepsilon_{max}$) from (-1.33 * 10^{-3}) to (-1.39 * 10^{-3}). These maximum values were measured when the sample was at the maximum compressive stress of 4.6 MPa. Similar ($\Delta \varepsilon_{min}$) change was measured from ($\varepsilon_{min,2} - \varepsilon_{min,1}$) and equal to -5.6 * 10^{-5}. At the same time, an increase in temperature from 23.11°C (Point 2) to 24.65°C (Point 3), corresponds to the change in the strain
(\(\Delta \varepsilon_{\text{max}}\)) from (-1.39 \times 10^{-3}) to (-1.31 \times 10^{-3}). A schematic presentation of these changes of strain and temperature is presented in Figure 79.

As one can see from Figures 78 and 79, the decrease in the temperature (Point 1 to Point 2) resulted in an increase in the absolute value of compressive strain because temperature decrease would result in decrease in the measured length of the sample. While an increase in the temperature (Point 2 to Point 3) resulted in a decrease in the absolute value of compressive strain because the temperature increase would result in the increase of the measured length of the sample. However, both \(\Delta \varepsilon_{\text{max}}\) and \(\Delta \varepsilon_{\text{min}}\) values when the temperature decreases from Point 1 to Point 2, were 1.5 times smaller than the values resulting from the temperature increase from Point 2 to Point 3. Such differences can be explained that the length of the sample would increase when temperature increase bringing the \(\Delta \varepsilon\) to a higher value in comparison with the situation when the length of the sample would decrease bringing the \(\Delta \varepsilon\) to a lower value (Figure 79).
CHAPTER 4  THE EFFECT OF DIET and ESTROGEN DEPLETION ON THE MECHANICAL STRENGTH and ARCHETECTURE OF BONE

This chapter will discuss the impact of both different dietary consumption and estrogen on the geometrical, structural, spectral vibrational and mechanical properties of bone.

4.1 The effect of diet on strength and architecture of bone

Dietary intake plays a primary role in bone metabolism and has a significant impact on bone health and structural integrity; however, the consumption of the unhealthy Western diet is increasing. These highly processed convenience foods are high in saturated fats, sugars and salt and have been shown to contribute to osteoporosis, thus, causing the bone to become weaker and more likely to fracture. This bone loss and subsequent deterioration in microarchitecture is multifactorial, and is due in part, to the high levels of saturated fats causing bone cells to become senescent, impairing their ability to function correctly while also activating inflammation within the tissue. Chronic inflammation subsequently deactivates the bone forming osteoblasts, while activating the bone resorbing osteoclast, resulting in an overall loss in bone and a weakened structure. High saturated fat diets have also been shown to expand the levels of adipose tissue and recent data has shown that this is correlated with osteoporosis and increased fracture risk. This increase in adipose tissue alters the marrow composition and results in a long-term, persistent decline in bone quality. Western diets are relatively low in ω-3 polyunsaturated fatty acids (PUFA) and ω-6 polyunsaturated fatty acids. PUFAs are distinguishable from saturated and monosaturated fatty acids by the presence of two or more double bonds between carbons within the fatty acid chain.
However, polyunsaturated fatty acids are associated with improved health benefits due to the anti-inflammatory properties of ω-3 PUFAs. As the body is unable to synthesize both ω-3 and ω-6 PUFAs, they are considered essential fatty acids and require supplementation through our diet. Studies have shown that PUFAs are not only anti-inflammatory but also promote osteoblast activity while inhibiting the bone resorbing activity of the osteoclast. As both the proportion of older people and the length of life increases, paired with obesity and poor physical activity, a rise in age-related degenerating bone disease is forecast. This raises the question of the added negative impact on bone health through consumption of the Western diet. An additional question is whether a diet consisting of increased anti-inflammatory PUFAs combined with a low saturated fat intake, will aid in protecting against bone loss and improves fracture resistance? The effect of different dietary intake such as control, high saturated fat (HSF), high fat diet (HFD) and high polyunsaturated fat (HPUF) diet on the structural and mechanical properties of bone will be discussed in the sections below.

4.1.1 The effect of diet on structural and geometrical properties of bone

The mice body weight of control, HSF, HFD and HPUF groups over 8-weeks diet duration is shown in Figure 80. From figure 80, it can clearly be observed that the mice in all different groups gained weight as the diet duration increased, however, consumption of HSF, HFD and HPUF dietary induced obesity in mice, as the body weight of these diet groups were continuously higher compared to the regular control group. Such obese behavior in mice fed HSF and HFD was expected and can be explained by the increase in adipose tissue level in the mice body, thereby, increasing their overall body weight. However, the increase in ω-6 / ω-3 ratio of polyunsaturated
fatty acids revealed a significant increase of weight and obesity in the HPUF mice (HPUF and HSF, $p < 0.05$, HPUF and control, $p < 0.05$), as the body weight of HPUF mice was continuously higher compared to the other diet groups (Figure 80).

![Figure 80 Plot showing the mice body weight of control, HSF, HFD and HPUF groups over 8-weeks diet duration.](image)

The geometrical parameters of Control, HSF, HFD and HPUF mice tibia are shown in Table 6. From table 6, one can observe that tibias geometrical parameters for mice fed high saturated fat are higher compared to the control, high fat diet and high polyunsaturated fat groups, except for the overall length. Such increase in the tibias’ geometrical parameters of the high saturated fat group is expected and might be explained by the fact that high saturated fatty acids induced obesity and increased the level of the adipose tissue in mice, therefore consequently, increased the tibias’ geometrical parameters.
Table 6 Tibial geometrical parameters of control, HSF, HFD and HPUF mice groups

<table>
<thead>
<tr>
<th>Diet group</th>
<th>Outer diameter (m)</th>
<th>Inner diameter (m)</th>
<th>Length (m)</th>
<th>Moment of Inertia (m$^4$)</th>
<th>Total Area (m$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.00145 ± 0.00011</td>
<td>0.00089 ± 0.00007</td>
<td>0.0204 ± 0.0011</td>
<td>(1.97 ± 0.6) * 10$^{-13}$</td>
<td>(1.05 ± 0.16) * 10$^{-6}$</td>
</tr>
<tr>
<td>HSF</td>
<td>0.00161 ± 0.00008</td>
<td>0.00107 ± 0.00005</td>
<td>0.0176 ± 0.0013</td>
<td>(2.74 ± 0.52) * 10$^{-13}$</td>
<td>(1.16 ± 0.11) * 10$^{-6}$</td>
</tr>
<tr>
<td>HFD</td>
<td>0.00141 ± 0.00004</td>
<td>0.00091 ± 0.00003</td>
<td>0.0226 ± 0.001</td>
<td>(1.65 ± 0.21) * 10$^{-13}$</td>
<td>(0.92 ± 0.06) * 10$^{-6}$</td>
</tr>
<tr>
<td>HPUF</td>
<td>0.00145 ± 0.00020</td>
<td>0.00095 ± 0.00016</td>
<td>0.020 ± 0.0035</td>
<td>(2.07 ± 1.44) * 10$^{-13}$</td>
<td>(0.98 ± 0.33) * 10$^{-6}$</td>
</tr>
</tbody>
</table>

The intake of HFD, which is a combination between high saturated and high polyunsaturated fatty acids, was associated with lower tibial geometrical parameters compared to both HSF and HPUF groups, except for the overall length. Furthermore, it can also be observed that tibial outer diameter, moment of inertia and total area of the HFD mice were all lower when compared to the mice on the regular control diet. Similar femora’s geometrical analysis on mice fed either HFD or control diet was studied in [95], which showed contrary results as an increase in all geometrical parameters of HFD group as compared to the control group was observed. Such discrepancy in findings between this study and [95] might be attributed to the difference in kcal% fat content as well as the duration of the diet. The images showing tibiae of control, HSF, HFD and HPUF and sham mice groups are included in Figure 81.
Moreover, the CT scans of the tibial cancellous and cortical bones for control, HSF, HFD and HPUF groups are shown in Figure 82. As one can see from Figure 82, CT scans of the cancellous bone revealed a variation in bone density among the different diet groups (Figure 82 A-D). The regular control diet on mice resulted in higher cancellous bone density as compared to the other diet groups. The lower cancellous bone density in HSF and HFD might be explained by the higher bone marrow adiposity, which resulted in a decrease in the tibia’s cancellous bone density [135]. In contrast, Bartelt et al. [136] reported that the consumption of HFD didn’t affect the lumber vertebra bone density in mice.
The confliction in results between this study and [136] might be attributed to the component ratio of dietary fatty acids, as the relationship between high fat diet and cancellous bone density in mice depends on the fatty acid’s profiles of the diet [135]. For instance, it was determined that higher ratio of ω-6 (linoleic acid) to ω-3 (α-linolenic acid) in the HFD results in decreasing the bone density, while lower ratio of ω-6 (linoleic acid) to ω-3 (α-linolenic acid) is associated with increasing the bone density and enhancing the bone formation [135, 137]. Therefore, the component ratio of the fatty acids as well as the diet duration should be taken in consideration when comparing the bone density between different diet groups. Furthermore, one can observe from the CT images of the cortical bone (Figure 82 E-H) that there is not noticeable difference in tibial cortical bone thickness among different diet groups.
4.1.2 The effect of diet on spectral vibrational response of bone

The typical spectral response from mice tibiae of control, HSF, HFD and HPUF groups are shown in Figure 83. The spectral response at range of 50-8000 cm$^{-1}$ showed the presence of different bands with some of them corresponding to the mineral and matrix components of the cortical bone (Figure 83 A-D), while the Raman spectra at range of 50-1800 cm$^{-1}$ revealed more details of Raman bands (Figure 83 E-H).

![Spectral response of mouse tibia](image)

Figure 83. Spectral response of mouse tibia at ranges of 50-8000 cm$^{-1}$ and 50-1800 cm$^{-1}$ for (A,E) Control, (B,F) HFD, (C,G) HPUF and (D,H) HSF diet groups

According to [97, 100, 109] one can observe the appearance of Raman bands as follows: two phosphate bending bands $\nu_2$ PO$_4^{3-}$ and $\nu_4$ PO$_4^{3-}$ appear at 446-450 cm$^{-1}$ and 601-611 cm$^{-1}$, respectively, B-type carbonate band appear at 745-760 cm$^{-1}$, the collagen proline band appear at (826-830 cm$^{-1}$), the collagen hydroxyproline band appear at (865-868 cm$^{-1}$), while the strongest
mineral band $v_1 \text{PO}_4^3$ appear at (957-961 cm$^{-1}$). The phenylalanine amino acid band appear at (1007-1018 cm$^{-1}$), the band at (1078-1080 cm$^{-1}$) appeared due to the superposition of carbonate and phosphate $v_1 \text{CO}_3^{2-}/v_3 \text{PO}_4^3$. Raman spectrum also revealed the presence of the organic bands of Amide III at (1263-1271 cm$^{-1}$), CH$_2$ at (1463-1473 cm$^{-1}$) and Amide I at (1681-1700 cm$^{-1}$)

4.1.3 The effect of diet on the mechanical properties of bone

This section will discuss the effect of control, high saturated fat, high fat diet, high polyunsaturated fat dietary consumption on the mechanical strength of mouse tibia using dynamic mechanical analysis (DMA) and 3-point-bending techniques.

4.1.3.1 Dynamic mechanical analysis

The results of storage modulus $E'$, loss modulus $E''$ and loss tangent ($\delta$) of mice tibiae for control, HSF, HFD and HPUF at frequencies of 0.05, 0.1, 1 and 10 Hz are shown in Figure 84. From Figure 84, one can notice that all diet groups exhibited an increase in the storage modulus as the testing frequency increased (Figure 84A). In addition, high saturated fat group demonstrated higher storage modulus compared to the other groups. The variation in storage modulus between the different diet groups can be attributed to the change in bone chemical composition of each group [138]. Yerramshetty et al. [139] reported that lower values of mineral to matrix and carbonate to phosphate ratios in the human cortical bone are associated with higher elasticity. Therefore, the higher tibial elasticity of HSF group might be explained by lower values of bone mineral density and carbonate substitution. Furthermore, tibial loss moduli of different diet groups showed no
dependency on the testing frequency (Figure 84B). This finding also suggests that more energy is being dissipated by the tibia, except for the control and HSF groups, such pattern of energy dissipation might be a mechanism to maintain the elasticity and prevent bone fracture under high frequency loading [140]. (Figure 84B).

Figure 84 Plots showing (A) storage modulus (E’), (B) loss modulus (E’’) and (C) loss tangent (δ) of the mice tibiae for different diet groups
Fulcher et al. [141] performed similar DMA analysis on articular cartilage-on-bone, which also showed a pattern of increase of storage modulus as the frequency increases, while loss modulus didn’t depend on the testing frequency. Furthermore, tibial loss tangent (δ) of the different diet groups also showed no dependency on the testing frequency (Figure 84C), however, lower damping occurred at higher frequency of 10 Hz as compared to the lower frequency of 0.05 Hz for all diet groups. Moreover, different studies [142, 143] have been published addressing the bone chemical composition effect on the viscoelastic properties. It was determined that loss tangent depends on the interactions between bone composition (minerals, organic and water) rather than bone geometry [142, 143]. Such interactions might explain the difference in loss tangent between diet groups at each frequency, as different dietary intake results in variation of bone mineral density between diet groups, which indeed affected the bone damping behavior (Figure 84C).

4.1.3.2 3-point bending

The typical stress-displacement deformation plots for mouse tibia of control, HSF, HFD and HPUF groups are shown in Figure 85. From Figure 85, one can observe that the tibiae exhibited different deformation behavior under the 3-point-bending test. However, ‘‘pop-in’’ events were present in stress-displacement plots of all tibiae. Such presence of ‘‘pop-in’’ events is defined as a decrease in the applied stress as the load increases, which is attributed to either initiation of new microcrack or propagation of existing microcrack inside the bone [144]. The microcracks stopped growing once they reach the closest hydroxyapatite bone layer, and eventually the accumulation of the microcracks causes the complete fracture of the tibiae.
Figure 85 Typical stress-displacement diagram of mouse tibia for (A) control, (B) HSF, (C) HFD and (D) HPUF groups.

The mechanical properties of yield stress $\sigma_y$, ultimate stress $\sigma_u$, strength $\sigma_f$ and Young’s modulus $E$, varied between the tibiae of different diet groups (Figure 86). From figure 86, one can observe that the intake of high fat diet resulted in increasing the overall strength of the tibiae, as yield, ultimate and fracture stresses of the HFD groups are all higher compared to the other diet groups. Such increase in the strength of the HFD groups might be attributed to the increase in the bone marrow adipose tissue which impose an additional weight that acts as a supplementary mechanical load onto surrounding bone, as reported by [145]. However, other studies have also reported that the increase in bone marrow adipose tissue from the high fat dietary consumption is associated with osteoporosis, as it results in reduction in osteoblastogenesis from Mesenchymal Stem Cells (MSCs), which leads to a significant reduction in bone strength and increased incident of bone
fracture [146]. The confusion is likely arising from the difference in kcal% fat content, in addition to the duration of the diet.

![Figures showing stress and modulus across different groups](image)

**Figure 86** Plots showing (A) yield stress, (B) ultimate stress, (C) strength and (D) elastic modulus of HSF, HPUF, control and HFD mice tibiae

Moreover, from Figure 86, one can also notice that the intake of high saturated fatty acids reduced overall strength of the tibiae, therefore, resulting in the fracture of the tibiae at a lower stress compared to the other diet groups (*p*-value = 0.016 between HSF and HFD). Such reduction in the tibial strength of HSF group might be explained by the fact that a high saturated fat diet induced murine progenitor bone marrow stromal cells to undergo preferential adipogenic differentiation, reducing osteoblastogenesis, osteopontin and the formation of both trabecular and cortical bone [147]. Furthermore, the high saturated fat intake significantly increases the bone marrow adipose tissue, where the apoptosis of this elevated adipose levels increases the free fatty acids (FFAs) in the body [148]. Such presence of the FFAs has shown to decrease the bone
formation and enhance the bone resorption, thus, decreasing the bone strength and structural integrity [149]. Moreover, From Figure 84, one can also observe the higher mechanical properties of HPUF relative to the other diet groups, which suggests that diet high in ω-6 polyunsaturated fat would not be detrimental to bone strength, thereby protecting against bone loss, structural weakening and fracture [148].

4.2 The effect of estrogen depletion on strength and architecture of bone

Osteoporosis is a common chronic disease and although most common in postmenopausal women due to hormonal decline, age-related osteoporosis is inevitable in both men and women. Currently, approximately 12.3 million individuals over 50 years of age in the United States live with osteoporosis and given our aging population and due to fragility fracture, annual treatment costs are projected to total $130 billion by 2050. These statistics indicate a clear and urgent need for improved mitigation and treatment strategies. Decreased sex hormones appear to be one of the strongest associated risk factors, of which estrogen plays an important role in bone metabolism and indeed has a significant impact on maintaining bone strength and structural integrity. Estrogen depletion leads to osteoporosis as it increases inflammation and the number and activity of osteoclasts thereby enhancing bone resorption. As such, estrogen replacement therapy is often used to treat osteoporosis and has been shown to improve both bone mineral density (BMD) and reduce fracture risk. This chapter aimed to investigate the contribution that a high fat diet has on osteoporotic bone health (with and without estrogen therapy), and in particular, whether the positive effects of a balanced 50:50 saturated:polyunsaturated diet as detailed above, protected against osteoporotic bone loss, when compared with an HSF diet, over a 12-week period. The effect of the
high fat diet on mechanical and structural properties of the osteoporotic mouse tibia is discussed in the sections below.

4.2.1 The effect of estrogen depletion on the structural and geometrical properties of bone

The mice body weight of OVX+E2, OVX+oil and sham groups over 12-weeks diet duration is shown in Figure 87. From Figure 87, one can notice that OVX+oil group demonstrated higher body weight compared to OVX+E2 and sham groups ($p < 0.05$), which reflects the effect of ovariectomy and high fat diet on increasing the overall body weight. The results suggest that the obesogenic response of OVX+oil to a high fat diet was much higher than the other two groups.

Figure 87 Plot showing the mice body weight of OVX+E2, OVX+oil and sham groups over 12-weeks diet duration.

Similarly, Chen and Heiman [150] reported that ovariectomy on rats significantly increased both body mass and fat mass compared to the sham operated rats ($p < 0.05$). Furthermore, Riant et al. [151] reported that estradiol treatment on ovariectomized mice reduced the effect of high-fat diet
induced obesity, which might explain the lower body weight of OVX+E2 and sham groups compared to OVX+oil group. The tibial structural parameters of OVX+E2, OVX+oil and SHAM mice at the end of 12-weeks diet duration are shown in Table 7. From Table 7, one can notice that the oil treatment on the ovariectomized mice resulted in a decrease in tibial outer diameter and cross-sectional area with increase in tibial length relative to the other groups, which indicated that the estrogen reduction and obesity induced by the HFD diet did not improve the tibial structural parameters of mice. Furthermore, the presence of estrogen either in case of OVX+E2 and sham mice increased the tibial outer diameters and cross-sectional area. The images showing tibiae of OVX+E2, OVX+oil and sham mice are included in Figure 88.

### Table 7 Tibial geometrical parameters of OVX+E2, OVX+oil and Sham mice groups

<table>
<thead>
<tr>
<th>group</th>
<th>Outer diameter (m)</th>
<th>Inner diameter (m)</th>
<th>Length (m)</th>
<th>Moment of Inertia (m$^4$)</th>
<th>Total Area (m$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVX+E2</td>
<td>0.00139 ± 0.00016</td>
<td>0.00072 ± 0.00008</td>
<td>0.0199 ± 0.0015</td>
<td>(1.86 ± 0.8) * $10^{13}$</td>
<td>(1.13 ± 0.26) * $10^6$</td>
</tr>
<tr>
<td>OVX+oil</td>
<td>0.00137 ± 0.00003</td>
<td>0.00090 ± 0.00002</td>
<td>0.0210 ± 0.004</td>
<td>(1.43 ± 0.13) * $10^{13}$</td>
<td>(0.84 ± 0.4) * $10^6$</td>
</tr>
<tr>
<td>Sham</td>
<td>0.00146 ± 0.00008</td>
<td>0.00086 ± 0.00005</td>
<td>0.0188 ± 0.001</td>
<td>(2.03 ± 0.45) * $10^{13}$</td>
<td>(1.11 ± 0.12) * $10^6$</td>
</tr>
</tbody>
</table>
Figure 88 Images showing mouse tibia of (A) OVX+E2, (B) OVX+oil and (C) Sham groups. The CT scans of the tibial cancellous and cortical bones for OVX+E2, OVX+oil and Sham groups are shown in Figure 89. One can clearly observe that the estrogen loss in OVX+oil mice adversely affected the bone microstructure. Such effect can be clearly seen in the CT scans of the cancellous bone (Figure 89 A-C), where the cancellous bone density of OVX+oil is significantly lower compared to OVX+E2 and sham groups. Such lower cancellous bone density might be attributed to excessive bone resorption as a result of estrogen reduction and HFD intake. However, other studies [152, 153] reported that obesity on the ovariectomized mice did not induce any negative effect on the cancellous bone density. The discrepancy between this study and the results in [152, 153] is likely arising from the kcal% fat content in the HFD as well as the duration of diet.
Figure 89 CT images showing the cancellous and trabecular bone of mouse tibia of (A,D) OVX+E2, (B,E) OVX+oil and (C,F) SHAM groups

Similarly, Feresin et al. [154] reported that the estrogen loss in ovariectomized rats resulted in a decrease of cancellous bony density as compared to the sham group. Moreover, the CT scans also revealed that the estradiol treatment on the ovariectomized mice significantly increased both the cancellous and cortical bone thickness in OVX+E2 group (Figure 89 A and D). The findings suggest that treating the OVX mice with 2 μg of 17β-estradiol benzoate (EB) resulted in restoring the serum E₂ levels even higher than that of sham group, which as consequence, prevented cancellous and cortical bone loss (figure 89).
4.2.2 The effect of estrogen depletion on spectral vibrational response of bone

The typical spectral response of mice tibiae of OVX+E2, OVX+oil and sham groups are shown in Figure 90. The mineral and matrix Raman bands of the cortical tibia were present in the spectrum range of 50-8000 cm\(^{-1}\) (Figure 90 A-C), while the Raman spectra at range of 50-1800 cm\(^{-1}\) revealed the presence of such Raman bands in more details (Figure 90 D-F). According to [97, 100, 109], one can observe the appearance of Raman bands as follows: two phosphate bending bands \(\nu_2 \text{PO}_4^3\) and \(\nu_4 \text{PO}_4^3\) appear at (415-437 cm\(^{-1}\)) and (546-595 cm\(^{-1}\)), respectively, B-type carbonate bands appeared at (751-780 cm\(^{-1}\)), the collagen proline band appear at (826-830 cm\(^{-1}\)), the collagen hydroxyproline band appear at (864-867 cm\(^{-1}\)), while the strongest mineral band \(\nu_1 \text{PO}_4^3\) appear at (958-961 cm\(^{-1}\)). The phenylalanine amino acid band appear at (1007-1018 cm\(^{-1}\)), the band at (1072-1075 cm\(^{-1}\)) appeared due to the superposition of carbonate and phosphate \(\nu_1 \text{CO}_3^2/\nu_3 \text{PO}_4^3\). Raman spectrum also revealed the presence of the organic bands of Amide III at (1260-1261 cm\(^{-1}\)), \(\text{CH}_2\) at (1460-1463 cm\(^{-1}\)) and Amide I at (1680-1684 cm\(^{-1}\)).
4.2.3 The effect of estrogen loss on the mechanical properties of bone

4.2.3.1 Dynamic mechanical analysis

The results of storage moduli $E'$, loss moduli $E''$ and loss tangent ($\delta$) of mice tibiae for OVX+E2, OVX+oil and sham groups at frequencies of 0.05, 0.1, 1 and 10 Hz are shown in Figure 91. The storage moduli $E'$ values for all mice groups showed dependency on the testing frequency, and it increased as the frequency increased. However, oil treatment on the ovariectomized mice resulted in higher storage moduli values as compared to the other groups (Figure 91A). Such higher storage...
modulus in the ovariectomized group might be explained by the lower mineral to matrix and carbonate to phosphate ratios in the tibia compared to the other two groups [139]. The lower bone mineral density in the tibiae of OVX+oil group resulted in enhancing the bone resorption, and therefore, decreasing the bone density (Figure 91 B)

Figure 91 Plots showing (A) storage modulus ($E'$), (B) loss modulus ($E''$) and (C) loss tangent ($\delta$) of the mice tibiae for OVX+E2, OVX+oil and sham groups.
Furthermore, loss moduli $E''$ of the tibia of different groups were found to increase as the frequency increased, except for 1 Hz frequency (Figure 91B). Although the tibiae of different groups stored more energy at higher frequencies (Figure 91A), however, they also dissipated higher energy at such higher frequencies (Figure 91B). Such behavior of energy storage/energy dissipation might be a mechanism to prevent bone damage, as the bone will dissipate more energy under high frequency loading, therefore, maintain the elasticity and preventing bone failure [140]. Moreover, the loss tangent ($\delta$) for all groups decreased as the frequency increased (Figure 91C), with both OVX+E2 and sham groups showing higher loss tangent values compared to the OVX+oil group. Although the different mice groups were subjected to similar dietary intake, however, the difference in estrogen and serum $E_2$ in the body indeed altered the bone mineral composition, therefore, resulted in a variation in loss tangent between the three groups.

4.2.3.2 3-point-bending

The typical stress-displacement deformation plots of the tibiae of OVX+E2, OVX+oil and sham groups are shown in Figure 92. From Figure 92, it can be observed that the tibiae exhibited different deformation behavior under the 3-point-bending test with the presence ‘‘pop-in’’ events in stress-displacement plots of all tibiae. Such presence of ‘‘pop-in’’ events is again attributed to either initiation of new microcrack or propagation of existing microcrack inside the bone [144]. The microcracks stopped growing once they reach the closest hydroxyapatite bone layer, and eventually the accumulation of the microcracks causes the complete fracture of the tibiae.
The mechanical properties of yield stress $\sigma_y$, ultimate stress $\sigma_u$, strength $\sigma_f$ and Young’s modulus $E$, varied between the tibiae of different groups (Figure 93). From Figure 93, one can observe that the oil treatment on the ovariectomized mice resulted in a significant decrease of the overall bone
strength, as OVX+oil demonstrated lower yield, ultimate and fracture stresses and elasticity compared to the OVX+E2 and sham groups ($p = 0.021$). The results suggest that the HFD diet that improved the mechanical properties of bone in diet study did not prevent bone loss in the osteoporotic model despite the animals being fed with this diet for 12-weeks rather than 8-weeks. In addition, the effect of estradiol was evident in improving the strength and reducing stiffness of the tibiae. However, increased estrogen production in the obese OVX+E2 mice resulted in a higher tibial strength compared to the sham group ($p = 0.564$) (Figure 93).

![Figure 93](image)

**Figure 93** Plots showing (A) yield stress, (B) ultimate stress, (C) strength and (D) elastic modulus of OVX+E2, OVX+oil and sham tibiae

Previous studies [155, 156] reported that increased estrogen production in obesity would result in decrease of the bone resorption, thereby, increasing the bone strength. Such decrease in bone resorption might be attributed to the fact that adipocytes can synthesize estrogen from circulating androgens and therefore inhibiting the process of bone turnover [157]. However, the study suggests
that the estrogen reproduction by the adipocytes doesn’t compensate the effect of OVX on the diminishing the tibiae strength. Such effect can be explained by the reduction in the overall strength of the OVX+oil mice compared to the other groups (Figure 93). One can expect that the biosynthesized estrogen from the adipose tissue doesn’t have the same effect on preventing the bone resorption as the estrogen produced by the ovaries [157]. Overall, further research has to performed in order to better understand the effect of HFD obesity-induced on the bone health
CHAPTER 5 CONCLUSION

5.1 The staghorn coral (Acropora cervicornis) skeletons

The structural and mechanical properties of the critically endangered staghorn coral (Acropora cervicornis) skeletons were studied. Optical micrographs and CT scans of chemically bleached and biologically cleaned skeletons revealed the presence of a typical perforate skeleton structure, which was described for this species in detail by Gladfelter and Marfenin [56, 57]. The skeletons were highly porous and had a prominent axial canal and large circumaxial and radial cavities. Pores were non-homogenously distributed along the skeletons and varied in size and shape, with major circle-like cavities being approximately 1 mm in diameter [6]. Such unique and complex porous macrostructure of the staghorn coral skeletons contributed to “gracious” stress-strain deformation behavior, rather than the catastrophic failure that occurs in brittle materials. The porous structure of coral skeletons was responsible for appearance of several sharp “pop-in” events during loading in the displacement control mode, which occur when an increasing applied stress suddenly dropped due to the rapid crack growth and propagation. After the crack reaches the closest pore, it stops growing and the stress increases again upon continued loading. Eventually, the accumulation of the cracks caused the complete fracture of the skeleton. Such “gracious” failure of coral skeletons, detected by loading in the displacement control mode, contributes to the delayed fracture, increased toughness, and enhance ability of the skeleton to survive under the cyclic loading, which the corals experience in their natural habitat. In addition, the elongated grains of skeletons of Acropora cervicornis coral resisted the crack propagation and retarded the crack growth during indentation in almost dense part of aragonite microstructure,
which resulted in a few radial cracks originated from the corners of Vickers impression. Moreover, the two different protein removal techniques - chemical bleaching and biological decomposition - had no appreciable effect on mechanical properties of staghorn coral *Acropora cervicornis* skeletons. Coral skeletons cleaned using either process had very similar values for Vickers hardness (3.67 ± 0.33 GPa for chemically bleached and 3.65 ± 0.24 GPa for biologically cleaned) and uniaxial compressive strength (9.5 ± 2.3 MPa for chemically bleached and 14.5 ± 6.4 MPa for biologically cleaned) [117].

The porosity effect on the elastic behavior of the staghorn coral (*Acropora cervicornis*) skeletons was studied. The computed tomography was very instrumental tool to estimate the overall porosity of the coral skeleton samples within limitation by the resolution of this technique, however, it did not allow to evaluate nanoporosity. In contrast, the B.E.T analysis allowed to evaluate the nanoporosity within the coral skeletons, however, it didn’t provide allow to estimate the overall porosity of the whole samples. It was found that the overall porosity has a significant impact on the elastic property of aragonite coral skeleton material, which express itself with the fact that the elastic modulus measured in compression showed significantly lower values (0.19 ± 0.16 GPa or 7.3 GPa depending on strain measurements technique during the compression test) as compared to the elastic modulus of the coral skeleton measured by nanoindentation (86 ± 7.26 GPa). In uniaxial compression, significantly larger complex pore volume present in the material was involved, where at least a 31% of porosity were present during the loading process. However, only non-significant amount of nanoporosity was present during nanoindentation of aragonite material and overall porosity was low in the place where local nanoindent were placed. Therefore, much more dense material responded to the indentation resulting in higher Young’s modulus values that
corresponds very well to the values obtained by nanoindentation of the dense geogenic single crystal aragonite ceramics [75]. The overall porosity of the staghorn coral skeleton was measured to be equal to 31 ± 5% using CT scan. However, CT scan did not have sufficient resolution to measure the fine pore sizes, therefore, B.E.T technique was employed for this purpose. The CaCO$_3$ aragonite structure was stable at 200°C, as determined by TG analysis, therefore, this temperature was used for annealing of coral skeleton sample to prepare its surfaces for B.E.T measurements. The low SSA along with the presence of 2-5 nm (up to 37 nm) mesopores were measured by B.E.T. The obtained properties of skeletons of *Acropora cervicornis* coral corresponds very well with properties published on different perforate coral species such as: *Balanophyllia europaea, Stylophora pistillata, Acropora pulchra, Diploria strigose, Diploria labyrinthiformis* and *Montastrea cavernosa* [125, 75, 126]. The results established the relationship between the microstructure and elastic properties of the staghorn coral skeleton and how it contributed to better understanding of mechanics of coral survival in dynamic marine environments.

The porosity variation within mature and young skeletons of *Acropora cervicornis* coral has been studied using B.E.T and computed tomography techniques. The B.E.T measurements indicated that SSA (4.1 m$^2$ g$^{-1}$ and 0.657 m$^2$ g$^{-1}$) was 6-fold higher for the young skeleton sample as compared to the mature skeleton sample, respectively, with a majority of the pores being in the range of 2 nm to 5 nm with the largest pores reaching up to 37 nm. It was measured that the pore volume of mature coral skeleton was equal to 0.0027 cm$^3$ g$^{-1}$ which was much lower than the pore volume of the young coral skeleton, which was equal to 0.011 cm$^3$ g$^{-1}$. It was determined that indeed the overall porosity of the mature skeleton sample was more than twice low 31 ± 5% as compared to the overall
porosity of the young skeleton sample 67 ± 5% as determined by CT scans. Similar porosity analysis of the skeleton of *Acropora pulchra* coral using computed tomography was reported in Roche et al. [126]. It was determined that the porosity value at the tip of *Acropora pulchra* branch was equal to 70%, while only 30% porosity was measured at the base of the branch [126]. Indeed, the porosity analysis of skeleton of *Acropora cervicornis* coral were in a good correspondence with the fact that skeletal density increases as the coral mature during their lifetime [56, 126], however, the unique contribution of this study is represented in analyzing the different level of porosity within skeleton of *Acropora cervicornis* coral using both computed tomography techniques and B.E.T techniques. The results are important to better understand the calcification process of the staghorn coral (*Acropora cervicornis*) skeletons in their natural habitat.

The deformation response of the skeleton of *Acropora cervicornis* coral to temperature rising under cyclic compression-compression loading was studied. It was found that the deformation of coral skeleton was increased during cyclic loading when temperature of the environment was increased. Such expansion/contraction behavior of the coral skeleton might affect significantly the polyps habitat. The polyps reside inside of the skeleton’s pores, and when the pore volume, which is a polyp living space, shrink/expand rather dramatically as temperature of the sea water exceeds 32°C this might force polyps outside of the coral skeleton leading to the bleaching and death of the creatures. In addition to these finding, what was also determined that the technique used for displacement measurements of the coral skeleton during compressive loading is critical, as the surface of the coral skeleton is not even and rough and the contacts between clip-on extensometer edges and skeleton’s surface were not stable and consistent during cyclic compression. As a result, at the beginning of the cycling, the events, such as friction and volume expansion bringing wear
and lateral strain respectively, all contributed to the variation in strain at the beginning of the experiment. Such friction and possible microcracking contributed to the appearance of the hysteresis loops during single loading-unloading cycle, with the area of hysteresis loops decreased consistently as the time of the experiment increased. The 7.4 GPa Young’s modulus of coral skeleton was measured to be much higher value than previously reported 0.19 - 0.29 GPa [117]. Such difference in measured Young’s modulus values of the coral skeletons were explained by the difference in measurements of the displacement of the coral skeleton during loading, where the clip-on extensometer provided more accurate values of displacement, compared to the displacement values measured by the crosshead of the universal testing machine, which was utilized in Omer et al. [117]. The results are important to better understand the behavior of the staghorn coral (*Acropora cervicornis*) skeletons in their natural habitat and predict the corals response to changing ocean conditions.

5.2 Bones of the laboratory mouse, *Mus musculus*

The effect of different dietary intake of control, HSF, HPUF and HFD on the structural and mechanical properties of mouse bone was studied. The consumption of HSF, HFD and HPUF induced obesity in the mice, as compared to the control group, however, the higher $\omega-6/\omega-3$ ratio of polyunsaturated fatty acids resulted in increasing the overall body weight of the mice even higher than HSF and HFD groups. In addition, the tibial geometrical analysis revealed that the obesity in HSF groups resulted in a larger bone size compared to other diet groups. However, CT scans revealed that such obesity was associated in enhancing the bone resorption, as the cancellous bone density of HSF, HFD and HPUF groups were lower than the control group. Despite the
variation in the cancellous bone density between the different diet groups, CT scans showed that there is no noticeable difference in the cortical bone thickness between the groups. Such similarity in the cortical bone thickness might be attributed to the duration of the dietary. One can expect that as the diet duration increases more than 8 weeks, the increase in bone marrow tissue in mice as a result of the obesity would further enhance the cortical bone resorption, therefore, decreasing the bone density in the HSF, HFD and HPUF groups. Furthermore, the DMA analysis revealed that the storage moduli (E’) depended on the testing frequency and it increased as the frequency increased for all diet groups. HSF group demonstrated higher storage modulus as compared to the other diet groups, which one can attribute such higher values to bone chemical composition of the HSF group, thus, more research has to be conducted to better understand the pattern of storage moduli of the different diet groups. Moreover, the loss modulus (E’’) of the different diet groups showed no dependency on the testing frequency, however, HFD and HPUF groups showed higher loss modulus at higher frequencies, which might be a mechanism to maintain the bone elasticity and prevent fracture at higher frequency loading. The loss tangent (δ), which is indicative of the damping capacity of the bone, varied between the different diet groups and showed no dependency on the frequency. Such variation can be attributed to the difference in the bone chemical composition (e.g., minerals and organics) between the diet groups. Furthermore, it was determined that the intake of HSF diet diminished the tibial overall strength as the yield stress σy, ultimate stress σu, strength σf and Young’s modulus E of HSF group were all lower compared to the other diet groups. Such lower tibial strength in the HSF group was expected and might be explained by the fact that the increase in adipose marrow tissue in HSF groups increased the level of free fatty acids (FFAs), and as consequence, decreased the bone formation and enhanced the bone
resorption. The HFD groups demonstrated higher tibial strength compared to the other diet groups. Such higher tibial strength likely arises from the presence of marrow tissue in the bone, which acts as supplementary mechanical onto mechanical load.

Within this work, the effect of HFD on the structural and mechanical properties of the osteoporotic mice tibiae was also studied. It was determined that the obesogenic response of the OVX+oil group was higher than OVX+E2 and sham groups, which is reflected by the higher overall body weight of the OVX+oil group during the diet duration relative to the other groups. However, the oil treatment on the ovariectomized mice resulted in lower bone size compared to OVX+E2 and sham groups. Moreover, the CT scans of the cancellous bone revealed that OVX+oil group demonstrated a significant decrease in the bone density compared to the other groups, which indicated that intake of HFD induced obesity on the osteoporotic mice did not prevent or reduce the bone resorption. In addition, it was found that the estradiol treatment on the ovariectomized mice restored the serum E$_2$ level and increased density of the cancellous and cortical bones. Furthermore, the storage moduli (E’) of different groups exhibited an increase as the testing frequency increased, with OVX+ oil demonstrating higher storage moduli compared to the other groups. Indeed more research has to be conducted to better understand the pattern of storage moduli of the different diet groups. The loss moduli (E”’) also increased as the frequency increased for all three groups, which might be a mechanism to prevent bone fracture at high frequency loading. Loss tangent (δ) varied between the different groups. Such variation can be explained by the change in bone composition as a result of different estrogen levels within the three groups. In addition, it was determined that oil treatment on the ovariectomized mice resulted in decreasing the tibial strength, as the yield stress $\sigma_y$, ultimate stress $\sigma_u$, strength $\sigma_f$ and Young’s modulus $E$ of OVX+oil group were all lower.
compared to the other groups. Such decrease of the tibial strength was expected and can be attributed to the depletion of estrogen level, which significantly enhanced the bone resorption. In contrast, the OVX+E2 and sham groups demonstrated higher tibial strength which reflects the effect of estradiol in enhancing the bone strength and reducing the fracture risk. The results suggest that the consumption HFD diet that improved the mechanical properties of bone in diet study, did not increase bone strength and prevent bone loss in the osteoporotic model despite the animals being fed with this diet for 12-weeks rather than 8-weeks.
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