

**Comparing the Mineralization of Two Limpet Species *Lottia pelta* and *Lottia digitalis*  
Between Species and Intertidal Location**

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## **Abstract**

Intertidal gastropods have a variety of behavioral and physical adaptations to help them survive in the environment. One of the most obvious is the evolution of an outer shell for defense, which can protect organisms from dangers such as predators, as well as heat and wave force. The limpet species *Lottia pelta* and *Lottia digitalis* are two limpets with different external shell morphologies that live in overlapping habitats in the intertidal environment. In this study I aim to compare whether the visibly different shell morphological traits extend to the amount and distribution of mineralization within the shell and if this changes by tidal height. To do this I CT-scanned 31 limpet shells with phantoms of a known density to map the actual distribution of material densities. I found that while the overall density of each shell across species and intertidal height were not significantly different, the distribution of density differed for *L. digitalis* in low intertidal vs every other treatment. This difference that sets the low intertidal *L. digitalis* apart from the rest could be a result of the increased need for defense in a region that is often submerged and vulnerable to predation.

## **Introduction**

The study of gastropod morphology has found many causes of variation in the size and shape of shells that tie back to the phenotypic plasticity within certain species. Biotic factors such as predation, food quantity and quality, and population density can exert controls on the morphology of individuals (Williamson et al. 1976, DeWitt 1998, Dorgelo & Leonards 2001). Abiotic pressures such as thermal stress and wave exposure have also been noted to change shell morphology (Denny 2000, Harley et al. 2009). Specifically, the character of shell thickness

across species of limpets (clade Patellogastropoda) has been found to be influenced by a mix of these factors. A thicker shell generally offers greater resistance to strong wave forces and can better resist predation (Cabral & Natal Jorge 2006, Tyler et al. 2014). However, different limpet species do not all follow a uniform distribution of material across the shell. Some species, such as *Lottia pelta* have a smooth surface to their shell, while others, like *Lottia digitalis* have distinct radial ridges extending from the apex of the shell. Previous research has hypothesized that these two distinct shapes are to resist different environmental pressures. Smooth shelled species have been found to better resist predation from shell crushing crabs, while ridged shells have been found to be better adapted to resisting wave forces (Pennington & Currey 2009, Tyler et al. 2014).

The nonuniform spread of material in shell and the spread of each species throughout the intertidal leads me to ask (1) Does the distribution of minerals within the shell differ between those with a smooth surface and those with ridges? and (2) Does the distribution of minerals within the shell differ by tidal height?

My goal for this study was to utilize micro-computed tomography scanning ( $\mu$ CT) to map the distribution of mineral densities within the shells of the two intertidal limpet species, *L. pelta* and *L. digitalis* (Figure 1) in order to calculate the percentage of the total volume different densities took up in the shell and how different densities were distributed in the shell.



**Figure 1.** Photograph of representative shell morphologies for (a) *Lottia digitalis* and (b) *Lottia pelta*.

## Methods

### *Specimen Acquisition*

*Lottia pelta* and *Lottia digitalis* specimens were collected from the high and low intertidal zones at Cattle Point, Friday Harbor. The high intertidal was defined by the upper edge of the *Fucus distichus* zone and the low zone was the lowest accessible point during a tide of one foot below mean lower low water. I collected nine *L. pelta* and seven *L. digitalis* from the high intertidal, and seven *L. pelta* and eight *L. digitalis* from the low intertidal. I placed the limpets in the freezer to euthanize them. After several hours the limpets were thawed, and the body was separated from the shell. The shell was cleaned following the process outlined in Geiger et al., 2007, where the specimen was washed with freshwater to remove any salts, residual tissue,

and/or algae. The shells were then washed with 80% ethanol and left to dry. Once completely dry, the shells were weighed before being moved to the CT scanner.

### *Computed Tomography Imaging*

Shells were separately wrapped in cheesecloth and given a radio-opaque label for matching specimens to their scan. The shells were put in a 3D-printed PVA plastic cylinder to securely stack multiple specimens together. Included in the tube were two phantoms of known densities, 1.32 and 1.58 g/cm<sup>3</sup>. These were then imaged using micro-computed tomography scanning ( $\mu$ CT; Bruker Skyscan 1173; Bruker, Billerica, MA) at the University of Washington Friday Harbor Labs. Every CT scan used a 1mm aluminum filter to reduce beam-hardening artifacts. All of the scans had a voltage of 65kV and an amperage of 123 $\mu$ A. Scans ranged in resolution from 16.34 – 24.14  $\mu$ m, and following (Buser et al. 2020), were reconstructed as bmp image stacks, using the program NRecon, and then converted to .nrrd file format, using the program ImageJ/FIJI (Schindelin et al. 2012, Buser et al. 2020). Before being converted to a .nrrd file, specimens were downsampled by 50% to reduce file size.

### *CT Data Processing*

Specimens were loaded into the program 3D Slicer ([www.slicer.org](http://www.slicer.org)), an open source program for CT data analysis and visualization (Fedorov et al. 2012, Kikinis et al. 2014). The relationship of threshold value to density was estimated by calculating the average threshold of each phantom and then performing a linear regression to find the relationship between threshold and density. This calibration was then used to calculate the density of each shell from the threshold value.

Individual shells were selected using the “ThresholdEffect” tool and then made into separate volumes using the “MaskVolume” tool, both of which are found in the Segment Editor module. 3D Slicer’s ruler and fiducial tools were used to take linear measurements of the length, width, and height of each shell. The total volume and the average density were then calculated using the Segment Statistics module.

Density was binned into 10 groups and the associated threshold values were calculated using the previously calibrated relationship. Each threshold range was set using the “ThresholdEffect” tool and then the total volume of material in the bin was calculated with the Segment Statistics module.

### *Analysis*

The percent volume per bin over the total volume was calculated and then normalized using an arcsine transformation (Sokal & Rohlf 1981). Data were then grouped by species and then tidal height to perform a two-way ANOVA. Other variables that had been collected such as total volume, total density, and total investment in mineralization (volume \* average density) were also added at this time (Kruppert et al. 2020). The ANOVA test was done using the StatsModels package in the coding language Python. Groups with significant P values (<0.05) were then further analyzed using a Tukey post hoc test done with the same package.

### *Qualitative Analysis*

By colour coding the binned density data, I was able to visualize and compare where concentrations of high vs. low density minerals were occurring in the shell using 3D Slicer. This offered both a surface look at variation and a slice-by-slice view of the internal density pockets structures.

## Results

### *Analysis*

I found that the overall density, volume, and investment in mineralization were not significantly different between species, tidal heights, or the interaction of those two factors ( $p > 0.05$ ). While the overall density did not differ, the percentage of the overall volume different bins of density took up across the shell showed significant differences for the limpet *Lottia digitalis* from the low tidal height. In the bins 2.4-2.57 g/cm<sup>3</sup> and 2.57-2.73 g/cm<sup>3</sup>, *L. digitalis* from the low intertidal zone had a significantly higher concentration than any of the other groups. For the grouping of 2.067-2.23 g/cm<sup>3</sup> *L. digitalis* had a significantly lower concentration than either *Lottia pelta* group but was not different from the high zone *L. digitalis*. For the grouping of 1.567-1.73 g/cm<sup>3</sup> the only difference was that the low zone *L. digitalis* had a lower concentration than the low zone *L. pelta*. Finally, for the grouping of 1.40-1.567 g/cm<sup>3</sup>, *L. digitalis* from the low zone had a significantly lower concentration than either *L. digitalis* or *L. pelta* from the high intertidal (Figure 2).

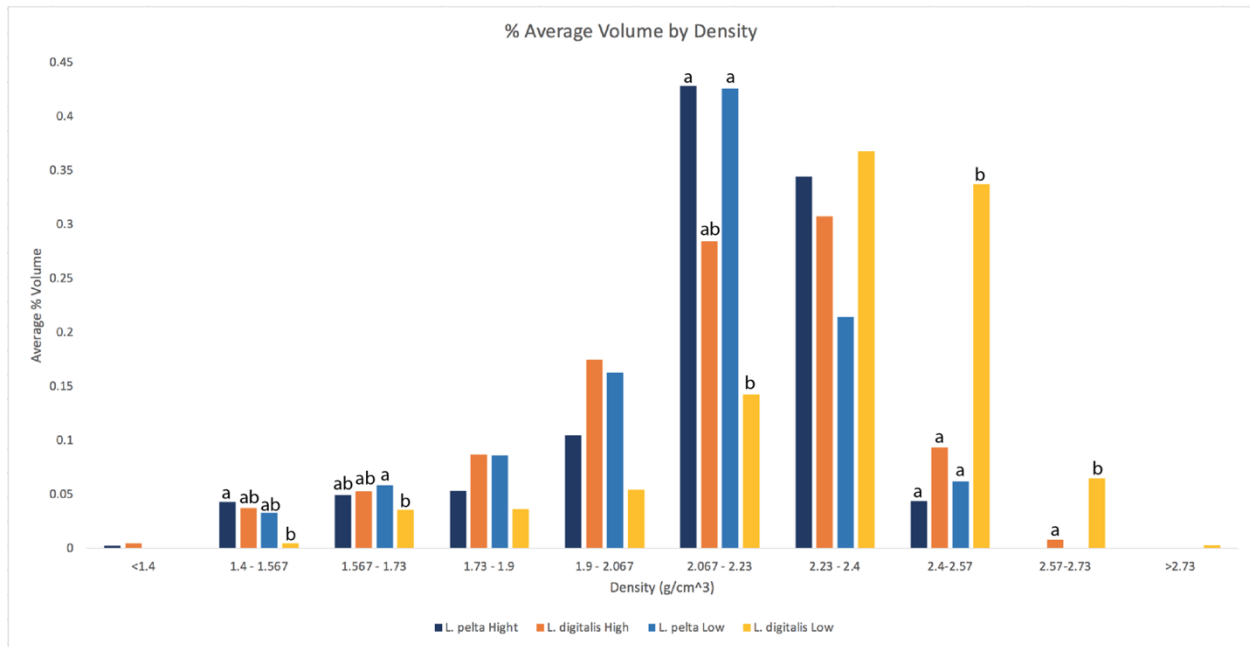


Figure 2. Average % volume of each treatment grouped by density bins. Letters show significance within a bin. Bins without letters had no significant differences among any of the treatment groups.

In addition, each group of limpets had a near-normal distribution of the binned densities in the shell, with a peak in the middle that trailed at the density extremes. However, the low intertidal *L. digitalis* had a majority of its mineralization between 2.23- 2.57 g/cm<sup>3</sup>. The other groups had the highest amounts of their mineralization between 2.067-2.4 g/cm<sup>3</sup> with a swift decrease for anything above 2.4 g/cm<sup>3</sup> (Figure 2).

Using measurements collected from each shell, the height to length ratio was calculated and a two-way ANOVA was used to compare the species and tidal height vs. the ratio measurement. This resulted in a significant difference between species ( $p < 0.001$ ) but not between the tide heights for the individual species ( $p > 0.5$ ).

### Qualitative Analysis

Across species and tidal heights there appeared to be a pattern of the highest-density material being around the apex of the shell (Figure 3a and Figure 3b). Furthermore, *L. digitalis* generally had pockets of the highest density material in the middle of the ribs as well as the apex (Figure 4).

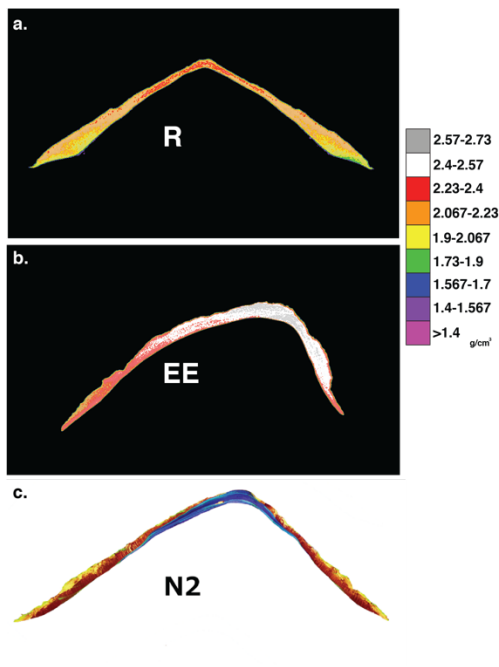


Figure 3. Lateral view of (a) *Lottia pelta* specimen R and (b) *Lottia digitalis* specimen EE with a colour map of the density bins. (c) Shows the distribution of aragonite in blue and calcite in yellow in a specimen of *Patella caerulea* (adapted from Langer et al. 2014)

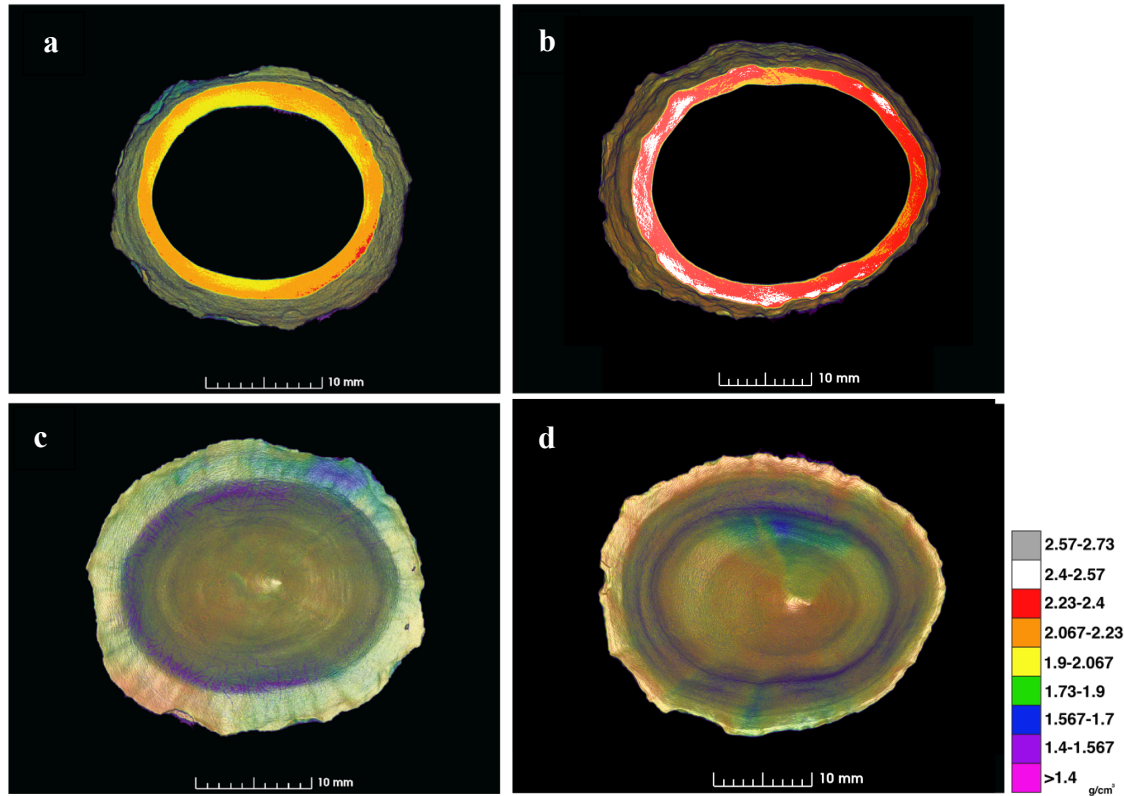


Figure 4. Comparison of *Lottia pelta* (a and c) and *Lottia digitalis* (b and d) mineralization. (a and b) show a cut off of the dorsal side of the shell exposing the distribution of minerals inside. (c and d) show the appearance and texture of the ventral side of the limpets.

## Discussion

The analysis done to compare the height to length ratio of limpets offers support that each was identified correctly, and the chance of mixed populations is unlikely.

One of the starkest differences between the groups was the greater percentage of denser mineralization of the low zone *Lottia digitalis* compared to the high zone *L. digitalis* group and both *Lottia pelta* groups. Tyler et al. (2014) found that *L. digitalis* had a significantly higher mortality rate than *L. pelta* when being attacked by shell crushing predatory crabs. This study took into account numerous variables, but the only significant correlation was shell shape: smooth vs. ridged. The more heavily mineralized shells in the lower intertidal *L. digitalis* are

consistent with a hypothesis of increasing vulnerability to crab predation in the low intertidal as shells with a higher density often able to better resist compression (Newell et al. 2007, Lombardi et al. 2013, Tyler et al. 2014). This could also explain the lack of change that is seen in *L. pelta* between the tidal heights, in that their smooth shell is already more adapted for surviving predator attacks, so they don't need to readjust the mineralization ratio. Previous research done on the sea snail *Trochus geversianus* found that the density of the shell increased from the intertidal to the subtidal, and also hypothesized this to be the result of pressures from predatory crabs (Márquez et al. 2015). While this was an overall density change instead of a change in the distribution of different densities within the shell, it lends support to the theory that mineral distribution changes in response to predation.

*L. digitalis* is a species well known for its morphological plasticity (Lindberg & Pearse 1990). It has previously been proposed that plasticity should only exist in a way that is beneficial to the fitness of an organism because otherwise it would not persist over evolutionary time (DeWitt 1998). The lack of significant difference in the overall density and investment in mineralization suggest that the different shell morphologies take about the same effort to produce, so this change in *L. digitalis* could be a less costly way to promote survivorship of predator attacks.

An interesting point of contrast between the different species could be seen when colour coding the density bins while visualizing the data in 3D Slicer. The highest levels of density in the shells of *L. digitalis* and *L. pelta* were often found at the apex of the shell (Figure 3a and Figure 3b). This is similar to a previous study that examined the distribution of the minerals aragonite (more dense) and calcite (less dense) throughout the shell of the limpet *Patella caerulea* through the use of confocal Raman microscopy (Langer et al. 2014)(Figure 3c).

The ridges in *L. digitalis* often had higher mineralization than the surrounding material, causing a distinct distribution when compared to *L. pelta* (Figure 4). It would be interesting to determine whether there is a correlation between ridge height and the amount of mineralization that is occurring to see if there is a connection to growth.

Some of the lowest areas of mineralization could be seen on the underside of the *L. pelta* shells from both tidal heights. These markings on the shell are irregular almost scratch mark like in appearance (Figure 4b). *L. digitalis* specimens are missing these marks. While this could be an artifact created during scanning, it appeared on multiple shells that were scanned with different resolutions on different days making it unlikely that this is the cause. It might have also been the result removing the limpet body from the shell, but the same process was used to remove the bodies of *L. digitalis*. It could be helpful to scan specimens with the soft body still inside to see if these 'scratch' marks remain.

In conclusion, I found variation found in the distribution of mineralized material in the shell of low intertidal *Lottia digitalis* when compared to high intertidal *L. digitalis* and low and high intertidal *Lottia pelta*. The exact reason for this could be related to factors like exposure to predatory crabs and the underlying phenotypic plasticity already known within *L. digitalis*.

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