Riders on a Shell:

The Effect of Algal Epibionts on Eagle Cove Limpets

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ABSTRACT

Limpets and algal epibionts develop mutually beneficial associations. This study examines the benefits of green algal epibionts on the limpet *Lottia pelta* at Eagle Cove. Density of algal cover was compared between populations of limpets at the middle and low regions of the intertidal zones. Temperature differences between limpet populations with varying degrees of epibiont cover and the adjacent rock were analyzed. Previous work *in vitro* suggested that a high epibiont density on a limpet shell acted as a shield against thermal stress. The results of this study showed that limpet shell surface temperature cannot be correlated with the observed epibiont density. These results supported the hypothesis that when the temperature of limpets is measured *in situ*, epibiont density does not necessarily provide heat tolerance adaptation by the host. This study showed no significant difference between the means of the limpet temperature at middle and low regions and no significant difference between the temperature of the limpet and its adjacent substrate, regardless of region or moisture of the substrate. This supports the hypothesis that epibiont density on a limpet does not reflect the immediate substratum and suggests that live limpets can manage heat stress. The green algae sampled from the limpets collected in this study were classified as *Ulva prolifera*, and other Ulva sp., Ulothrix. Sp., Acrosiphonia sp., and *Blidingia dawsonii*. This study highlights interspecies mutualistic associations and their possible role in limpet adaptations to heat stress. Further studies will include analyzing diverse limpet species, algal epibionts and sample sites.

INTRODUCTION

Intertidal zones are biotically stratified into low, middle, and high regions. These natural patterns are delineated by the biota and vegetation that distribute along this zonation pattern. For example, kelp distributes closest to the water's edge (low intertidal), mixed alga such as Ulva, sea stars and anemones at the middle-intertidal and barnacles, mussels, at the higher intertidal zones (Meadows & Campbell, 1988, pp. 144-145).

Interspersed within this array of biota are the limpets. Limpets are members of the phylum Mollusca, Class Gastropoda and Family Lottidae. These marine gastropod mollusks are categorized in the superfamily Lottioidea and fall within the clade Patellogastropoda (Bouchet et al. 2005). Most limpets reside intertidally and can be found on vertical rock faces in the low, middle and high intertidal zones. Limpets are biologically equipped with the ability to "excavate" rocky surfaces to feed on microscopic films of algae and diatoms that coat these surfaces. During low tide in the day limpets are motionless. They forage actively at night during low tide. Some species of limpets will return after grazing to their own unique resting spot also known as a "home scar ." This allows them to tightly cling to their rock surface home which is subjected to high velocity forces from ocean wave activity. Unlike the barnacles and mussels that reside at the intertidal zones, limpets possess locomotion, due to their "muscle foot." This allows the limpet to move between intertidal and zonation levels to scavenge for food or shelter from the sun, wind, waves, and predators (Cook et al. 1969).

Limpets have mutualistic relationships with the biota that reside in their habitat (McQuaid & Froneman, 1993). Algae native to the substratum in the limpet niche will become epibionts on limpet shells, with some observed preferences for basibiont (sessile host) species (Lindstrom et al., 2006; Pereira et al., 2022). The selection process between limpet and its epibiont may depend on the needs of both organisms. These associations between limpets and their algal epibionts have been shown to provide costs and benefits for both (Seaborn, 2014). In some cases, the presence of the algal epibionts Acrosiphonia and *Ulva lactuca* (now *Ulva fenestrata*; Kuba et al., 2022) assists the limpet with landing on its foot with a high frequency when dislodged, which is considered an advantage in the turbulent intertidal zone by the "parachute" hypothesis proposed by Seaborn (2014). Seaborn also tested the relationship between algae type and temperature on limpet shells by placing limpet shells with various degrees of algal growth in a wind tunnel and measuring their temperature. Seaborn showed that the presence of algal growth on limpet shells kept the shell cooler than shells that were bare, and that Acrosiphonia species on limpet shells correlated with lower shell temperature than limpet shells covered by *Ulva lactuca*. This study suggests that the presence of algal epibionts can provide protection from heat stress and that this protection may be species specific (Seaborn, 2014).

The algal epibionts may also protect hosts against predators by disguising them with the algal cover (Seaborn, 2014). McGowan and Iyengar (2017) observed that the algal epibionts, which are sessile, benefit from their limpet shell residence due to relocation. They state: "the basibiont can benefit sessile epibionts by escaping predators or harsh thermal environments." This means that sessile organisms such as algae may use the limpet host to assist in relocation away from herbivore-rich territories. This "choice" by the algae is deemed to be dictated by a natural selection process, rather than any active selection process by the epibiont itself.

In both the Seaborn and Lindstrom investigations, green algae of the order Ulvales was identified as a common epibiont on the limpets of the region studied. *Percursaria dawsonii*, genus Blidingia, was the frequent epibiont resident on the limpets of species *Tectura scutum* in

British Columbia (Lindstrom et al., 2006), while *Lottia pelta* shells on San Juan Island, WA were covered predominantly with *Ulva lactuca* and Acrosiphonia (Seaborn, 2014).

Different tidal Regions

Studies that have identified a range of algal epibionts on gastropods Pereira, F. et al. (2022), have shown that the type of epibiont is a function of the range of algae in a particular habitat. The middle region of the intertidal zone undergoes several hours of aerial exposure longer than the low region, and this region is defined by a range of biota that have adapted to these intermittent exposures. According to Pasparakis et al. (2016), the limpets analyzed in the middle intertidal zone were considered to have had a longer exposure to dry conditions and aerial temperature than those in the low intertidal region, which would have experienced longer immersion times in the water (Pasparakis et al., 2016). Pasparakis et al. suggest that contrasting the population of limpets in the low versus the middle regions of the intertidal allows for making a distinction between conditions experienced by these two populations. While Pasparakis et al. do point out that the boundaries between low and middle regions can define separate microhabitats, there are key differences between marine basibionts that occupy the intertidal zone. Expecting a sessile mollusk such as a barnacle, with a propensity for mutualism with an epizoic alga, to reflect the abundance of a particular alga on the substratum would make sense. However, given that limpets are mobile and cover a range that spans several feet in a radial direction from their home scar (Cook, A. 1969), this particular boundary defined by middle and low regions does not allow for a definitive separation of the limpet range. Therefore the algal epibiont species and their abundance on limpets can be representative of either region of the intertidal zone, both middle and low, depending on the limpets' foraging range.

This study documents the density of the green algal epibionts growing on the shells of the limpet *Lottia pelta*, a species ubiquitously found at the middle and low intertidal zones of Eagle Cove, San Juan Island, Washington (Seaborn, 2014). The hypothesis tested here was that the amounts of algae on shells will not be different in correspondence to different tidal heights. This is based on two observations. One, due to the locomotive abilities of the limpet (McGowan & Iyengar, 2017), expectations for the algal density to reflect the surrounding substratum is minimized. Foraging activity will take the limpet from low to middle regions, a difference of about 1.5 to 2 feet at minimum (Cook et al., 1969), and so the epibiont density will be representative of the two areas. Two, preliminary work at low tide at Friday Harbor Labs by this investigator did not reveal observable differences in density of epibiont correlated with location of the limpet in the intertidal zone. Consequently, the hypothesis tested in the current study is that the epibiont density will not differ between the low and middle intertidal regions.

Thermal Protection

Intertidal algae and limpets are subjected to the thermal stress and desiccation during low tide, as they are not covered by water during several hours and are exposed to air and wind. Studies of the limpet shell and its resistance to desiccation and thermal stress are documented by Denny et al. (2006). However, this study examined shell morphology and the relationship of thermal protection correlated with the shell shape and architecture in three distinct marine gastropod species (two of which were limpets). Limpet epibionts were not considered in this study by Denny et al. (2006). Harley et al. (2009) studied the upper limits of heat stress in limpets in relation to their angle on the substratum and their frequency of exposure to wetting.

But Harley et al. did not study the density of epibiont coverage in relationship to heat protection of their limpet hosts.

Previous work has suggested that the algal epibionts afford some protection to the limpet in from thermal stress (Seaborn, 2014) in experiments conducted with limpets and their specific epibionts in wind tunnels. Kagawa and Chiba (2022) measured the inner shell temperature of the intertidal gastropod *Lunella coreensis* with and without coverage by the algal epibiont *Pseudocladophora conchopheria*. The Kagawa and Chiba *in vitro* study (snail was removed from shell before temperature test) showed that the correlation between the area of the shell covered with the green algae and the temperature inside the shell (with lower temperature of shell corresponding to higher algal density) was due more to the presence of water retained by the algal epibiont, rather than the amount of cover provided by the algae itself. These investigators noted that the protection from heat by the algae cover was lost once the algae had dried out. However, in both studies, those of Seaborn (2014) and Kagawa and Chiba (2022), it is important to note that the temperature readings were performed after shells had been emptied of their host and the shells were tested under *in vitro* conditions in the laboratory.

Limpets have several ways to prevent desiccation which is the basis for their tendency to return to their home scar after foraging (Cook et al., 1969). Besides modifications in foraging behavior to avoid direct sunlight, limpets also sequester a droplet of water between their foot and home scar to endure hours of aerial exposure during low tide (Branch, 1985). According to thermal stress models by Denny and Harley (2006), it is assumed that the limpet is always at equilibrium with its thermal environment (Denny & Harley, 2006, Figure 1).

This study measures the surface temperature of the limpets in their natural environment. Given that live limpets are endowed with devices to manage heat stress, this current study tests the hypothesis that the limpet shell surface temperatures measured *in situ*, will not be affected by the density of their algal epibionts, suggesting that the specific epibionts found on the limpets of Eagle Cove do not grant their limpet hosts a thermal protective advantage.

Identification

This study includes the identification of epibionts on the regional limpet, *Lottia pelta*. These green algae are purported members of order Ulvales. In this study, 25 limpets were analyzed at Eagle Cove, San Juan Island, WA (Fig. 1).

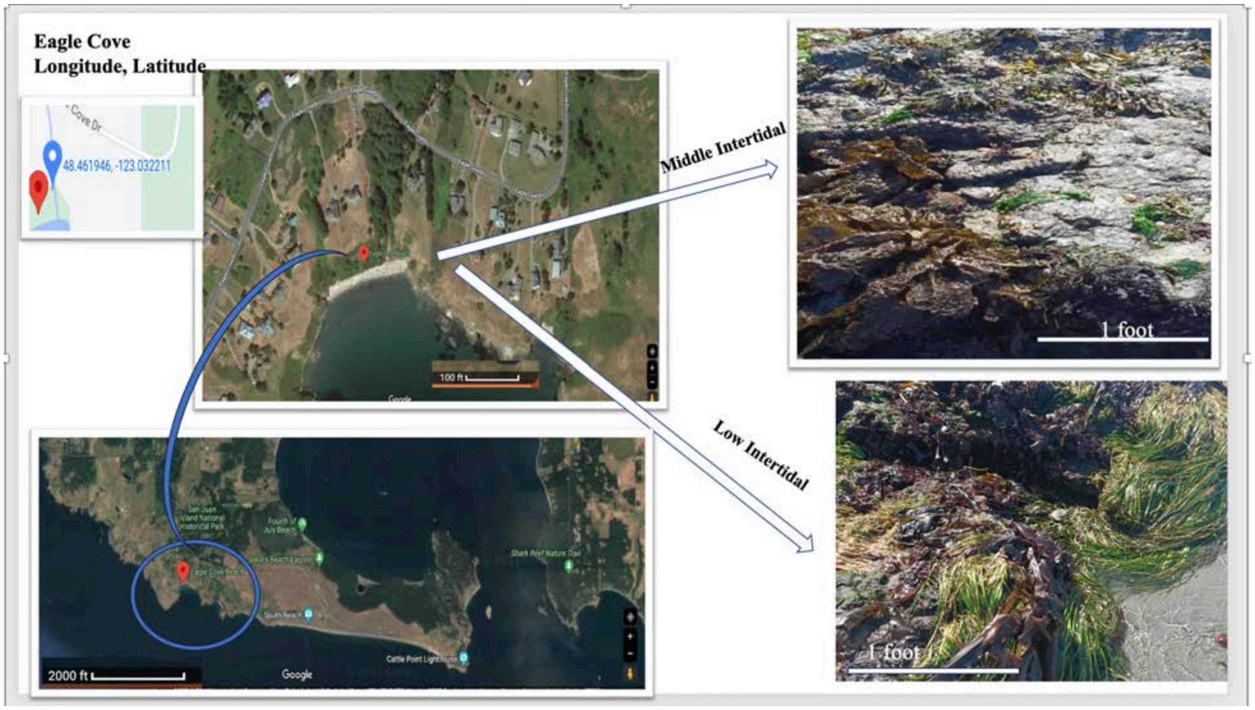


Figure 1. Map of Eagle Cove, San Juan Island, WA, with enlarged inset, and examples of the middle and low regions of the intertidal zone at Eagle Cove defined in this study. (Map credit: Google Maps; photos of Eagle Cove intertidal zones, credit: JM Calvillo)

This area is geographically close to the area studied by Seaborn (2014), who viewed limpet algal epibionts from Cattle Point, San Juan Island, WA (Fig. 2). The intertidal zone there is biologically similar to that of Eagle Cove.

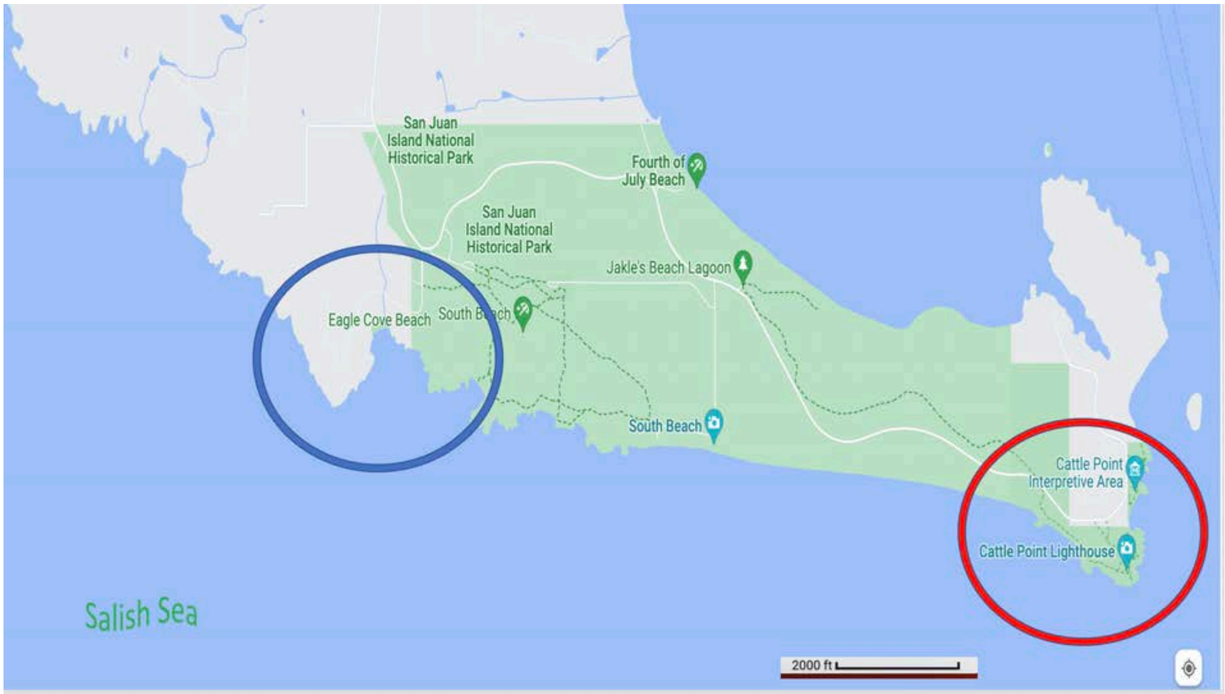


Figure 2. Map demonstrating the proximity of Cattle Point beach (red circle) and Eagle Cove Beach (blue circle) on San Juan Island, WA. (Map credit: Google Maps).

It was hypothesized that the green algal epibionts examined from the limpets collected at Eagle Cove, San Juan Island, WA would fall into similar taxonomic classifications as those in the Seaborn study at Cattle Point San Juan Island, WA. It was also hypothesized that the Ulva sp. identified in this study would be taxonomically close to those identified from San Juan Island, WA, in recent years. This hypothesis was based on barcoding studies of Ulva sp. in the San Juan Island region (Kuba et al., 2022). Kuba et al. (2022) identified at least four species of Ulva, purportedly dominant species in the San Juan Island area. To test the hypotheses on taxonomic similarity to the previously vouchered Ulva species, a sample of green algal filaments was taken from a subpopulation (12) of the limpets analyzed at Eagle Cove. Their genus was identified microscopically and, in one case, via molecular techniques that included polymerase chain reaction, genome sequencing and running the determined sequence via the Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI) database for comparison to other specimens collected in prior studies at San Juan Island.

At each location in the intertidal zone studied, the epibionts from the limpets were sampled, brought back to the laboratory, and identified using taxonomic dichotomous keys for algal identification.

MATERIALS AND METHODS

Limpets were identified as *Lottia Pelta* by visual inspection and comparison to the descriptions of Seaborn (2014) and Lowell, (1984) for its habitat and appearance. These limpets were visually inspected for the presence or absence of green algal epibionts. The target limpets were photographed, and temperature readings were taken of the limpet surface (shell) and the adjacent substrate. These analyses commenced at low tide at Eagle Cove at San Juan Island, WA (48.461946, -123.032211) on July 2, 2023 (Fig. 1). Low tide (- 3.1 feet) occurred at 9:37 AM PST. The air temperature was 68 °F (20 °C) and the day was cloudless and sunny.

Documentation of limpets was performed at low tide and at the middle intertidal from low tide and as the tide rose. The documentation included photography with an Android phone camera, and analysis of photos as JPGS using qualitative observation. The temperature data on limpets were collected using an infrared gun (Fluke 64 Max IR Thermometer, © 2017 Fluke Corporation) set at average (avg) scanning setting. Because the IR beam does not penetrate the limpet shell, the temperature measurements of limpets in this study are those of the limpet shell or surface (Fluke 64 Max IR thermometer Manual). A total of 25 limpets were documented at

Eagle Cove: nine from the low intertidal and 16 from the middle intertidal regions. Both bare and covered (with algal epibiont) limpets were included in the sample. The size of the limpets selected for *in situ* analysis ranged from 12 to 25 mm in length, 7 to 10 mm in width and 0.15 to 0.3 mm in height. Those outside this range were not analyzed in this study.

Density of the algae on the limpet shells was visually defined as follows:

Bare/Sparse: less than 20% coverage and no medium or large thallus growth on shell.

Medium: 20-70% coverage of shell, or medium thallus growth.

Dense: over 70% of limpet shell is covered by algae, or large thallus growth.

Fig. 3 shows examples of limpets placed in these three categories by visual inspection.

A. Examples for Bare/Sparse coverage of limpet shells by algae

B. Examples for Medium coverage of limpet shells by algae.

C. Examples for Dense coverage of limpet shells by algae.

Figure 3. The degree of algal coverage was determined by visual inspection of limpet shells. Two examples are shown for each category. **A.** Bare/Sparse coverage. **B.** Medium coverage. **C.** Dense coverage.

In addition to this documentation of location and temperature, the type of algal epibiont providing a dense cover over the limpet shell was analyzed. To collect these data, a small sample of the algal epibiont was pulled off the limpet shell with forceps and placed into a 15 ml centrifuge tube containing approximately 2 ml of seawater for most of the smaller amounts collected. One tube received 10 ml of seawater (Sample 12) as the biomass from a large thallus of green algae filled 1/2 the volume of the tube. These algal samples were analyzed microscopically (Olympus BH2 Microscope) and identified using the taxonomic keys developed by Gabrielson and Lindstrom (2018).

Genetic Sequencing

One Ulva sample taken from a limpet at Eagle Cove was collected on June 15, 2023, at low tide from a tide pool. A portion of this epibiont was pulled off the limpet shell, and the DNA was extracted with the Bioline Extract-PCR kit (Kuba et al., 2022) and purified through a spin column using the Zymo PCR inhibitor removal kit (Zymo Research Corp.). The chloroplast sequences were then amplified via PCR with *tuf*A primers (Fama et al., 2002). The amplicons were sequenced at a commercial laboratory (D. W. Freshwater, personal communication, July 19, 2023) via Sanger sequencing methods (Smith et al., 1986). This sequence was analyzed via BLAST in the NCBI database. The results from this program identified the epibiont taken from this Eagle Cove limpet as a close match to members of *Ulva prolifera*.

Statistical analyses were performed using the Microsoft Excel data analysis software add-in Analysis ToolPak and in one case via https://www.easycalculation.com/statistics/statistical-significance.php.

DATA

Epibiont Density in Low and Middle Intertidal

The hypothesis tested in the current study is that the epibiont density on the limpet shells will not be significantly different between the low and middle intertidal regions. Representative samples for the categories Dense, Medium, and Bare/Spare epibiont coverage were collected at Eagle Cove, at the middle and low regions of the intertidal zone.

As Table 1 shows, the majority of limpets analyzed were categorized as having bare/sparse coverage. The results on the table show the percentage of limpets in each epibiont density category that were observed in the low and middle regions of the intertidal zone.

Table 1. Distribution of limpets in low and middle regions of the intertidal zone by epibiont density.

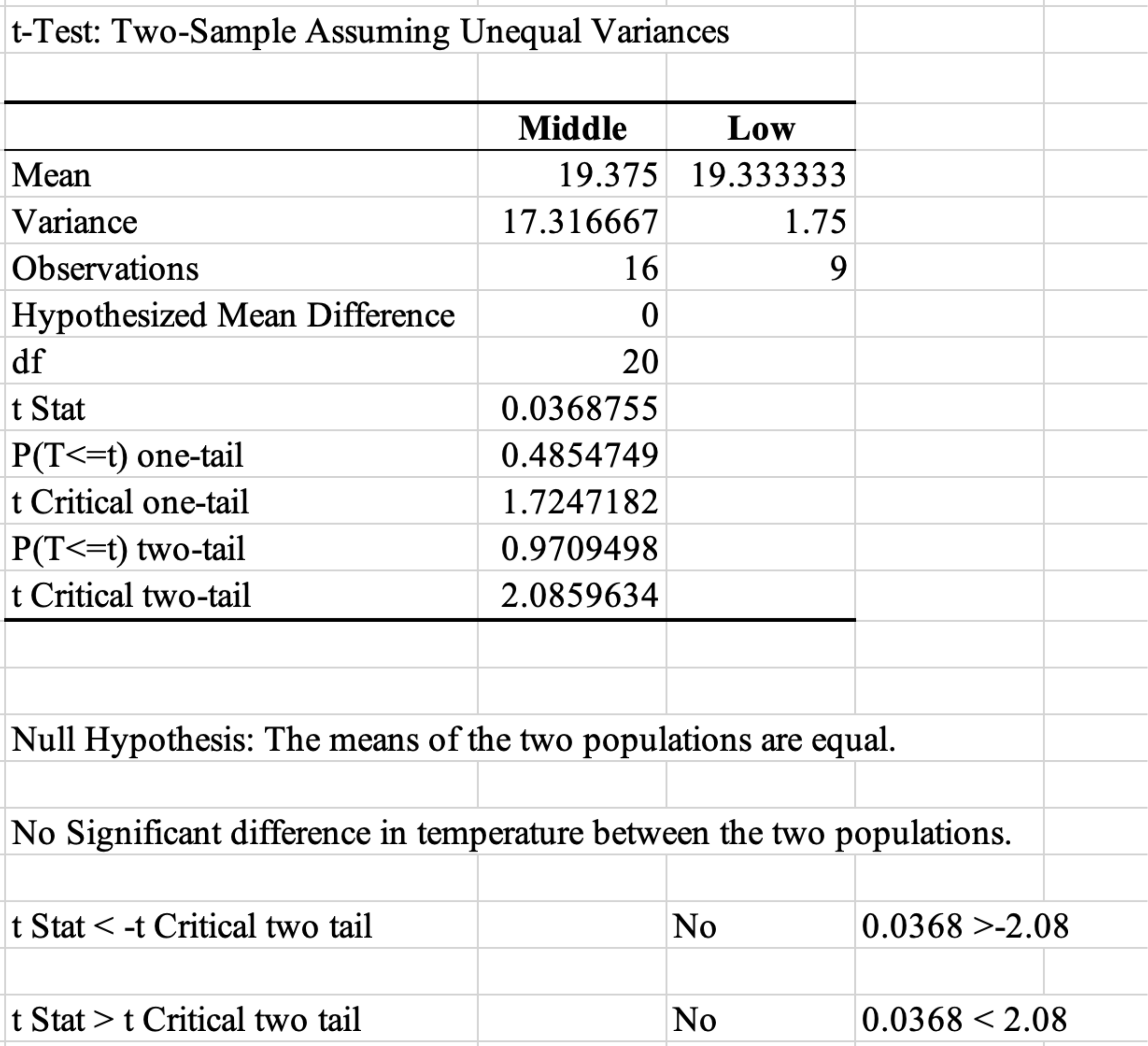
Intertidal Region	**Dense Epibiont Cover**	**Medium Epibiont Cover**	**Bare/Sparse Epibiont Cover**
	% Limpets	*% Limpets*	*% Limpets*
Low	11.1 (1/9)	22.2 (2/9)	66.6 (6/9)
Middle	11.1 (2/18)	27.7 (5/18)	61.1 (11/18)

There was no significant difference between the two populations of limpets at the low and middle regions of the intertidal zone at Eagle Cove in terms of epibiont algal coverage amounts as defined in this study (based on the value of the comparative error as shown here: https://www.easycalculation.com/statistics/statistical-significance.php).

From the total sample size of 25, the percentage of bare/sparse limpets was higher: 66.6 % (low), 61.1% (middle), than in either the medium or dense category for either region. Using comparative error analysis, the difference between the percentages within the Dense, Medium, and Bare/Sparse groups for low and middle regions was determined to not be significant, thus supporting the original hypothesis. The caveat here is that the sample size for the dense epibiont coverage analyzed for the low intertidal region was too small for the best statistical analysis.

The populations of the limpets from the low and middle regions of the intertidal zone were also analyzed for differences in mean temperature using the Student's t-test (Table 2). The results show no significant difference in the mean temperature taken from the two sets of limpets measured at the middle and low regions. Fig. 4 displays the comparison of the mean temperature readings taken from the limpets in the two populations tested, with a low standard deviation between these values. Notably the temperatures of the limpet shells fell within less than 1 degree of the air temperature, 20 °C on July 2, 2023, between 10 -11 am Pacific Standard Time (PST).

Table 2. Mean temperature of limpets at low and middle regions of intertidal zone.



t-Test: Two-Sample Assuming Unequal Variances

	Middle	Low
Mean	19.375	19.333333
Variance	17.316667	1.75
Observations	16	9
Hypothesized Mean Difference	0	
df	20	
t Stat	0.0368755	
P(T<=t) one-tail	0.4854749	
t Critical one-tail	1.7247182	
P(T<=t) two-tail	0.9709498	
t Critical two-tail	2.0859634	

Null Hypothesis: The means of the two populations are equal.

No Significant difference in temperature between the two populations.

t Stat < -t Critical two tail	No	0.0368 >-2.08
t Stat > t Critical two tail	No	0.0368 < 2.08

The means of the two populations, limpet temperature in the low and in the middle regions were also graphed and standard deviation of the means calculated. As Fig. 4 shows, there was no significant difference between these two populations for limpet shell temperature, supporting the original hypothesis.

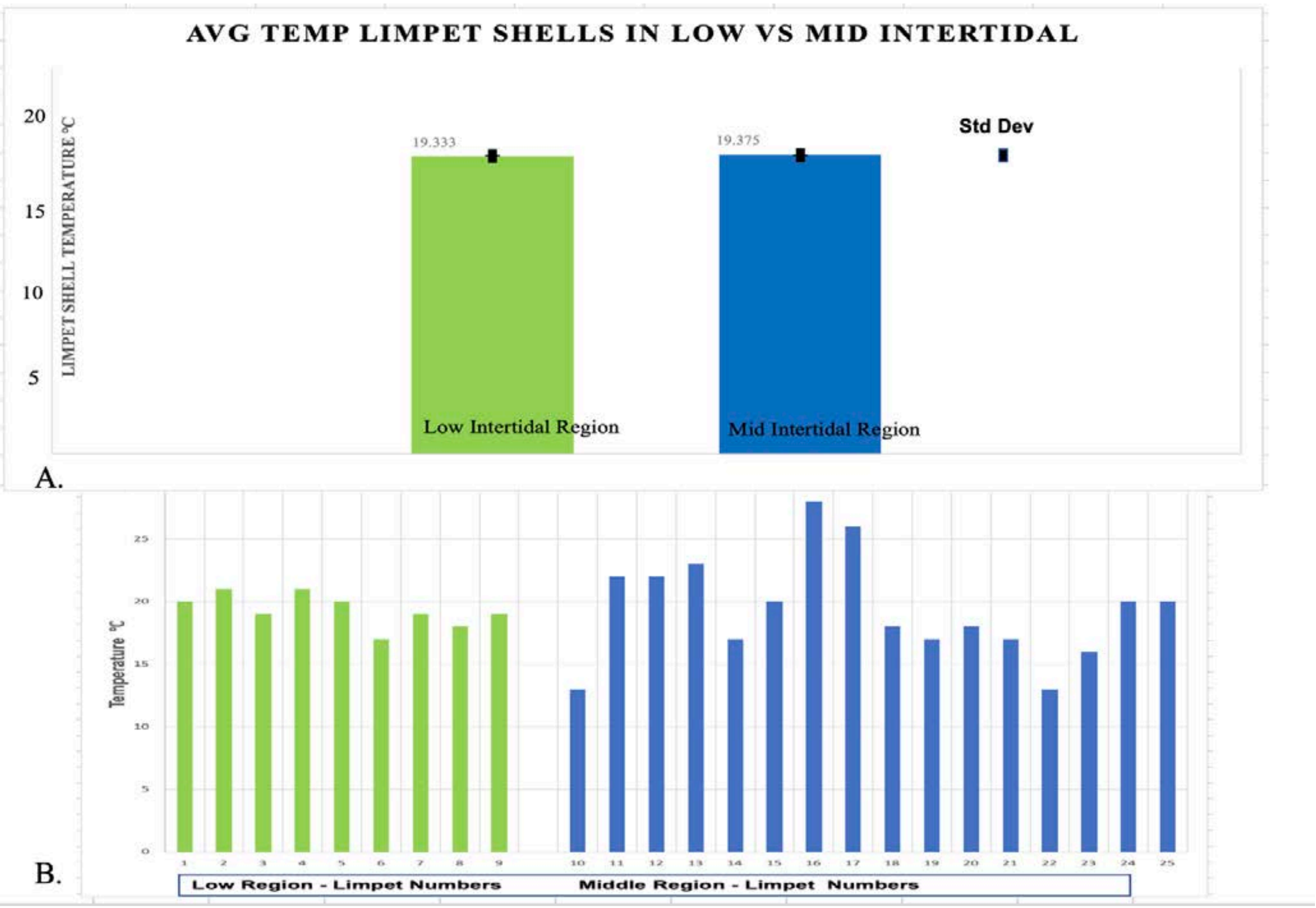


Figure 4. A. Mean limpet shell temperatures in two populations (low and middle) with standard deviation. **B.** Limpet shell temperature in the low and middle regions for all 25 samples.

Temperature of Limpet and Adjacent Substratum

The temperature differences between limpets with varying degrees of algae cover on shells and the adjacent rock were analyzed. Limpets were analyzed on both wet and dry substratum and the temperatures between these pairs were compared. Fig. 5 shows those results.

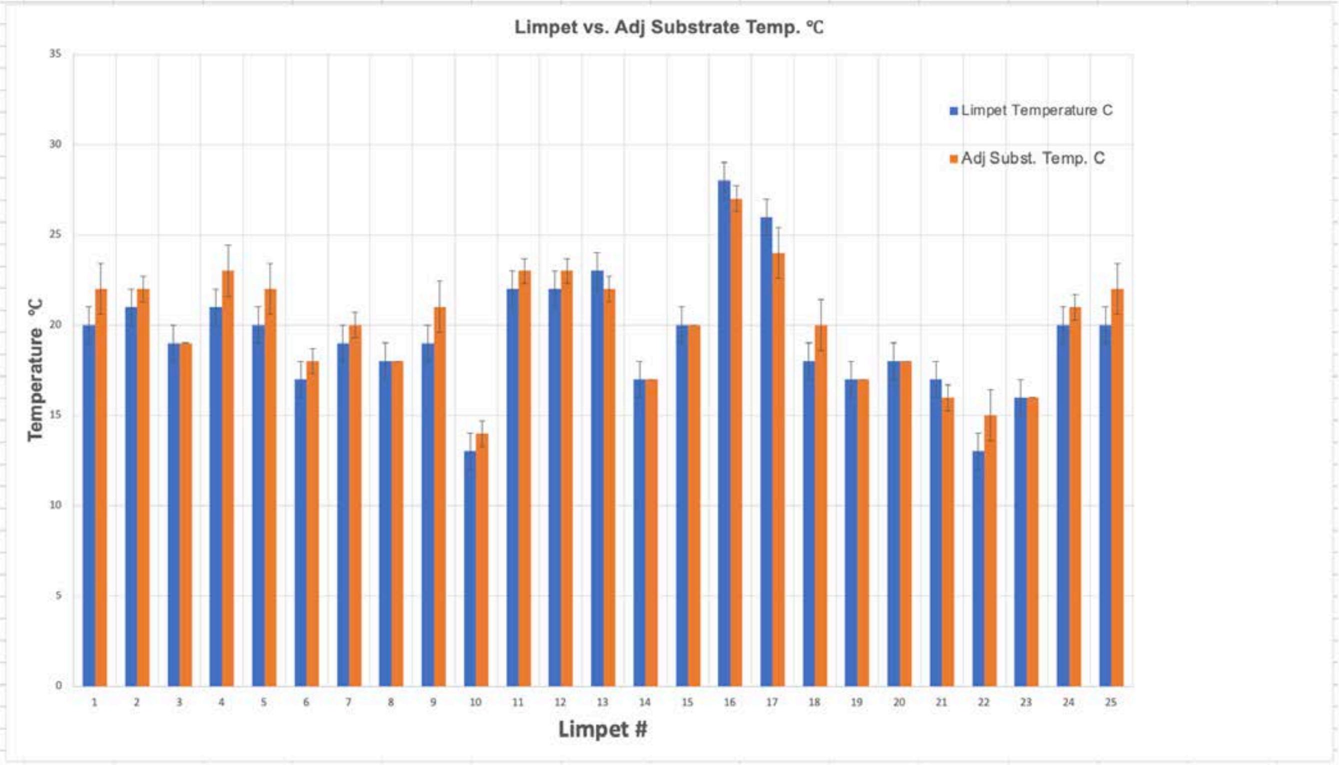


Figure 5. Comparison of limpet shell temperature with the adjacent substrate. (Bars show standard deviation [std dev] between each pair).

The results showed no significant difference between the temperature of the limpet and its adjacent substrate, regardless of location or moisture of the substrate. Comparative analysis of the temperature of the limpet and its adjacent substratum was also done for samples in the middle and the low regions of the intertidal zone. As shown in Fig. 6, there was no significant difference between limpets in the low or middle region when compared to their respective adjacent substratum. These results support the original hypothesis set forth in this study.

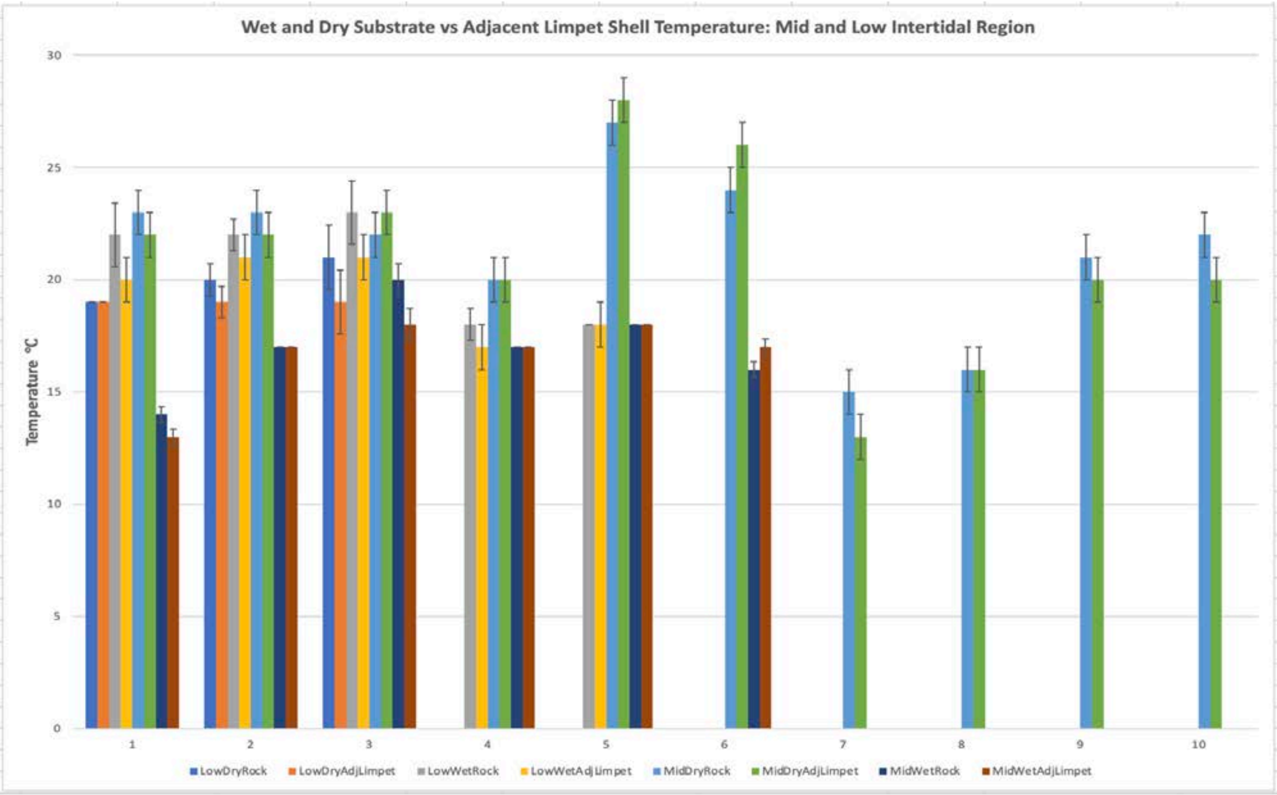


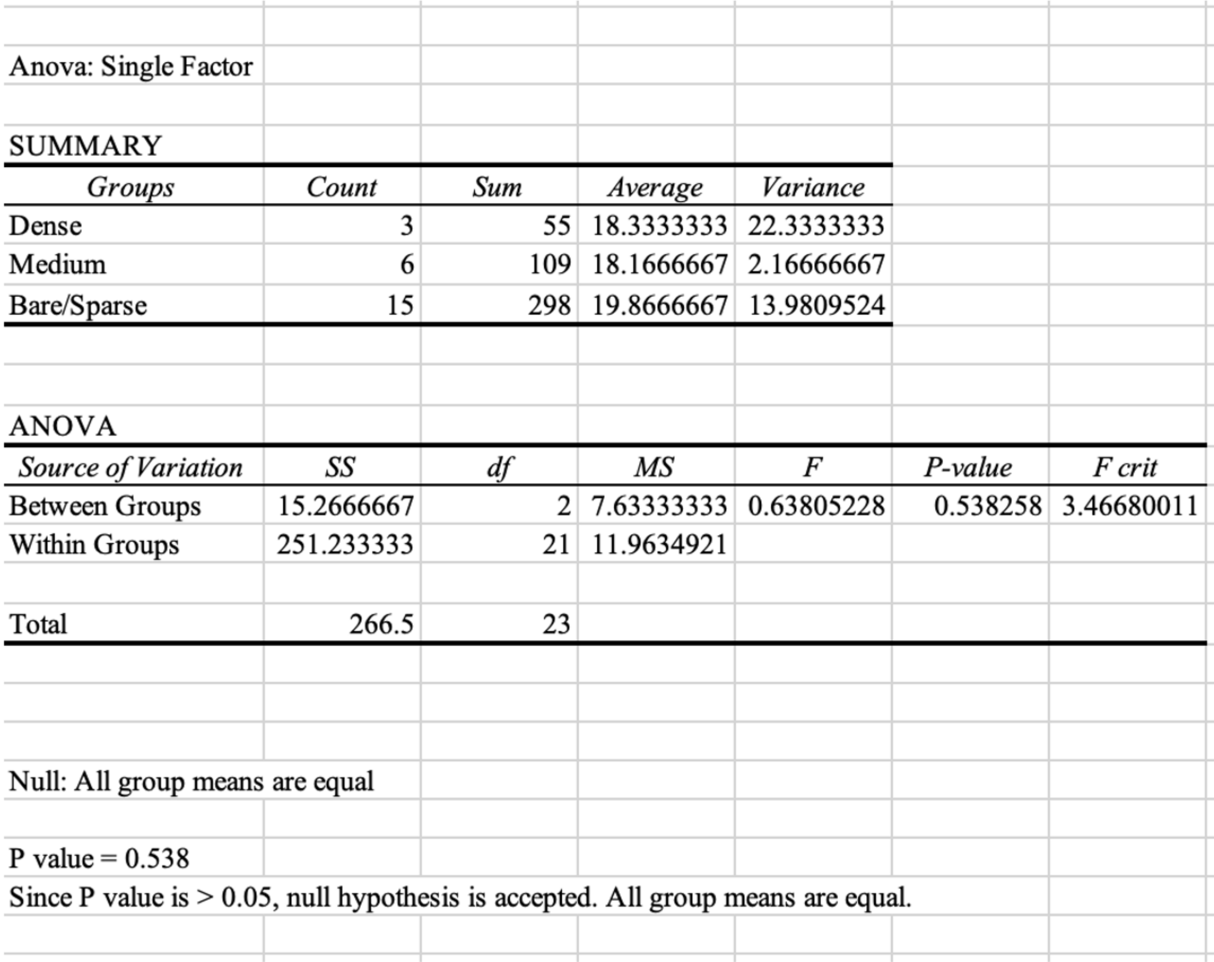
Figure 6. Comparison of limpet and adjacent substrate, color-coded to distinguish limpet populations from low and middle regions of the intertidal zone. (Bars represent std dev).

Thermal Protection

Limpet temperature was measured *in situ* and the limpets analyzed were categorized based on the density of the epibiont green algal population, as defined previously.

It had been hypothesized that the epibiont would not endow the limpets with a thermal protective advantage. Temperatures of the limpet surfaces were analyzed using ANOVA. The results are shown in Table 3.

Table 3. ANOVA of limpet surface temperatures with varying degrees of epibiont coverage.



Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Dense	3	55	18.3333333	22.3333333
Medium	6	109	18.1666667	2.16666667
Bare/Sparse	15	298	19.8666667	13.9809524

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	15.2666667	2	7.63333333	0.63805228	0.538258	3.46680011
Within Groups	251.233333	21	11.9634921			
Total	266.5	23				

Null: All group means are equal

P value = 0.538

Since P value is > 0.05, null hypothesis is accepted. All group means are equal.

The single-factor ANOVA, with temperature as the independent variable and the three categories of epibiont density, showed that the means between the three categories of epibiont coverage were equal. Therefore, the original hypothesis was accepted and there is no apparent correlation between limpet temperature and the amount of epibiont coverage on its shell.

The mean temperatures for the limpets in the three categories of epibiont density and the standard deviation between these groups were calculated. Those results are shown in Fig. 7.

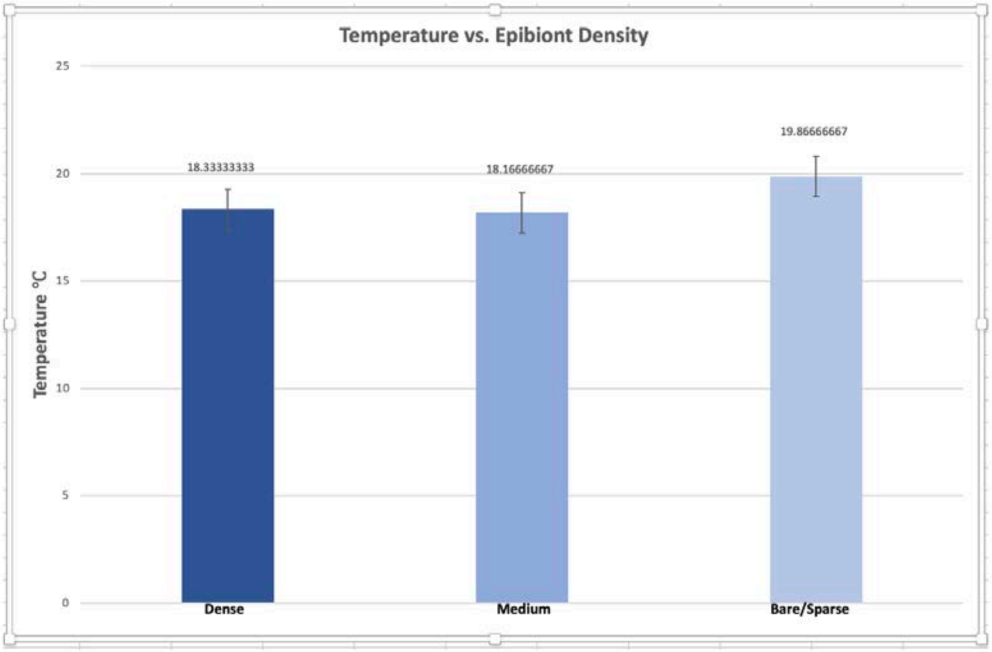


Figure 7. Mean temperature for three populations of limpets categorized by epibiont density as defined by this study. (bars = std dev).

As Fig. 7 shows, the temperature of limpets does not vary significantly in relation to epibiont density on their shells.

Identification

Algal samples from 11 limpets were removed from their shells and taken back to the laboratory where they were analyzed microscopically and identified using Gabrielson and Lindstrom (2018). The results of this analysis are displayed on Table 4. (For images of the samples shown, see Appendix S1.) With the exception of numbers 23 and 24, all the green algal species analyzed supported the hypothesis based on findings by Seaborn (2014) and Kuba et al. (2022). Algal sample number 12 is discussed in the next section on phylogeny.

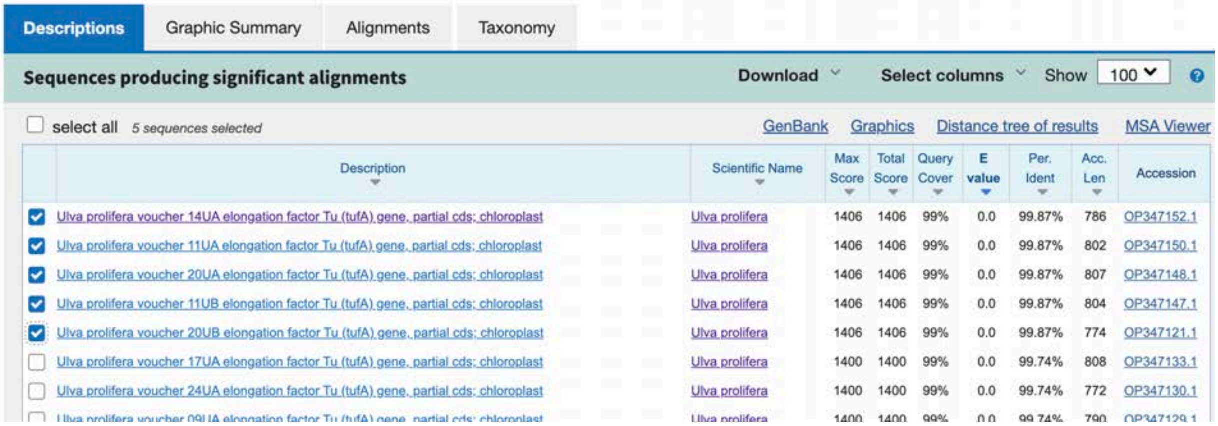
Table 4. Identification of green algae from 11 limpets of Eagle Cove. (Images in Appendix S1).

Limpet #	**Intertidal Region**	**Coverage**	**Identity of Algae**
7	Low	Medium	Ulva sp.
8	Low	Bare/Sparse	Ulva intestinalis (T. Mumford, personal communication, July 7, 2023)
10	Middle	Dense	Ulva sp.
11	Middle	Bare/Sparse	Ulva sp.
12	Middle	Dense	Ulva sp.
15	Middle	Medium	Ulothrix sp.
18	Middle	Medium	Ulva sp.
19	Middle	Medium	Acrosiphonia sp.
23	Middle	Medium	*Blidingia dawsonii*
24	Middle	Bare/Sparse	*Blidingia dawsonii*
25	Middle	Bare/Sparse	Ulva sp.

Phylogenetic analysis

One Ulva sp. sample was collected on June 15, 2023, at low tide from a tide pool at Eagle Cove. This sample was sequenced as described in Materials and Methods. The BLAST results for the sequenced Ulva sp. sample are shown in Table 4.

Table 4. BLAST results for FHL23-78 *tuf*A.



Sequences producing significant alignments

☐ select all *5 sequences selected*

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Ulva prolifera voucher 14UA elongation factor Tu (tufA) gene, partial cds; chloroplast	Ulva prolifera	1406	1406	99%	0.0	99.87%	786	OP347152.1
☑	Ulva prolifera voucher 11UA elongation factor Tu (tufA) gene, partial cds; chloroplast	Ulva prolifera	1406	1406	99%	0.0	99.87%	802	OP347150.1
☑	Ulva prolifera voucher 20UA elongation factor Tu (tufA) gene, partial cds; chloroplast	Ulva prolifera	1406	1406	99%	0.0	99.87%	807	OP347148.1
☑	Ulva prolifera voucher 11UB elongation factor Tu (tufA) gene, partial cds; chloroplast	Ulva prolifera	1406	1406	99%	0.0	99.87%	804	OP347147.1
☑	Ulva prolifera voucher 20UB elongation factor Tu (tufA) gene, partial cds; chloroplast	Ulva prolifera	1406	1406	99%	0.0	99.87%	774	OP347121.1
☐	Ulva prolifera voucher 17UA elongation factor Tu (tufA) gene, partial cds; chloroplast	Ulva prolifera	1400	1400	99%	0.0	99.74%	808	OP347133.1
☐	Ulva prolifera voucher 24UA elongation factor Tu (tufA) gene, partial cds; chloroplast	Ulva prolifera	1400	1400	99%	0.0	99.74%	772	OP347130.1
☐	Ulva prolifera voucher 09UA elongation factor Tu (tufA) gene, partial cds; chloroplast	Ulva prolifera	1400	1400	99%	0.0	99.74%	790	OP347129.1

The BLAST results identified the limpet epibiont collected from Eagle Cove as *Ulva prolifera*. The result is 99% comparable to voucher species identified by previous students for Marine Botany. The chloroplast primer *tuf*A was used in the amplification of this sequence marker.

Kuba et al. (2022) used *tuf*A sequences to identify the species *Ulva expansa* and *Ulva fenestrata* (previously classified as U. “lactuca”). Plastid-encoded rbcL sequences as well as the nuclear-encoded ribosomal ITS regions of representative specimens were used to identify the other two species as *Ulva prolifera* and *Ulva californica*. It is important to have additional types of specimen sequencing so that the number of Ulva species can be accurately identified (Kuba et al., 2022) to become more aware of this species diversity.

To this point, another barcoding technique developed by Liu et al. (2020) for *Ulva prolifera* and other Ulva sp. is based on mitochondrial DNA. Fig. 8 illustrates the Maximum likelihood (ML) phylogenetic tree for Ulva sp. based on their method.

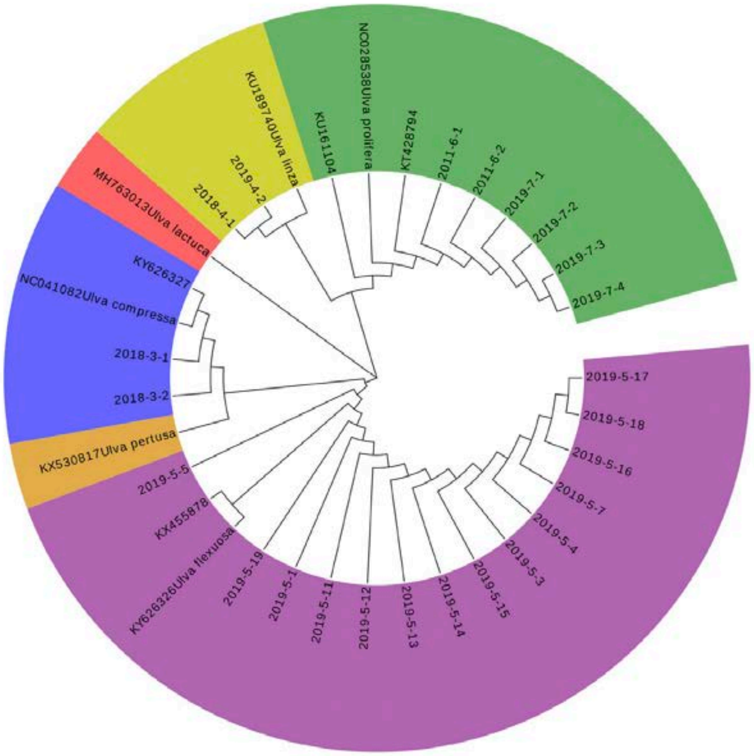


Figure 8. Maximum likelihood phylogenetic tree for *Ulva prolifera*, based on mitochondrial DNA barcoding technique originally published by Liu et al. (2020) and reprinted here under Springer Nature journals Creative Commons license.

Liu et al. (2020) performed bioinformatics analysis on the mitochondrial genome of *U. prolifera, U. flexuosa* and *U. linza*, *U. compressa, U. pertusa* and *U. lactuca* from which they built an ML tree. As Fig. 8 shows, there are close evolutionary lineages between *Ulva prolifera*, *Ulva compressa*, *Ulva lactuca* and *Ulva linza*. The sole sample identified by molecular analysis as *Ulva prolifera* is closely related to the other Ulva voucher species from Eagle Cove identified by Seaborn (2014), and from Cattle Point, Friday Harbor Labs beach front, and Iceberg Point by Kuba et al. (2022).

DISCUSSION

This study showed that there was no significant correlation between epibiont density and location of the limpet in the low versus middle regions of the intertidal zone. This supported the hypothesis tested in this study, based on the differences between a sessile basibiont (such as a mussel) and a mobile one, the limpet. Previous work on the proliferation of mollusk epibionts corresponding with their basibiont host mobility has addressed this aspect. According to Bell (2005), the degree of shell movement will differ between shells inhabited by gastropods and hermit crabs which may either enhance or inhibit settlement, depending on the species of algal epibiont being considered.

To demarcate the limpet-occupied regions within the intertidal zone more precisely, it will be useful to more thoroughly document limpet populations in terms of the distance taken by their foraging behavior, similar to the method of Hartnoll & Wright (1977), and to create definite boundaries in the particular site studied. The species of limpet and epibiont should also be more specifically documented.

The results of this study also showed that there was not a significant difference in limpet shell surface temperature that could be correlated with the observed epibiont density.

Limpets are equipped with tools for tenacity to withstand the unrelenting physical forces of the ocean's intertidal zones. The limpets' strong muscle foot keeps it glued to its "home scar" in this environment, which is subjected to wave forces up to 75 km/hour as well as predatory attacks by echinoderms and other carnivorous marine creatures. The powerful teeth of this gastropod, located on its radula, have been deemed to be "better than Kevlar" and this material, composed of goethite, is being developed for industrial applications to replace Kevlar (Webb, J. 2015). This powerful feeding apparatus allows the limpet to feast upon a garden of coralline algae and lichen, giving it a competitive advantage over those creatures who cannot survive on these encrusted food sources.

Within this challenging environment, it is no surprise that mutualistic relationships between marine flora and fauna have developed, to enhance each organisms' survival. For limpets, the physical assaults of desiccation, predation and dislodgement are countered by morphological and behavioral adaptations (Harley et al., 2009; Kagawa & Chiba, 2022; Lowell, 1984).

Mutualism involves a net increase in the fitness of participating individuals and populations and is thought to be an abundant and taxonomically diverse form of species interaction (Cushman & Beattie, 1991). Epizoic green algae that have developed a mutualistic partnership with limpets have been shown to provide costs in terms of increased drag forces (Seaborn, 2014), as well as benefits. The mutual benefits include protection from predators for both the epibiont and the basibiont (limpet). Riding on the backs of a creature with locomotion can allow these otherwise sessile algae to escape other algal grazers in the intertidal and subtidal regions (Pereira et al., 2022). And being covered by algal growth can provide a camouflage or crypsis, (Seaborn, 2014) which is an advantage for the limpet. Does the epizoic algae also provide a heat protective or desiccation-resistant benefit for the limpet as suggested by the Seaborn and Denny studies? This current work tested the hypothesis that there would be no differences in the surface temperature of limpets with or without a range of dense coverage by algal epibionts when limpet temperatures were documented in this marine gastropod's natural setting.

The lethal upper limit of temperature for a limpet is about 38 ° C for limpets (Denny & Harley, 2006). Denny and Harley have described morphological adaptations on the limpet shell to improve heat transfer away from the limpet body. Seaborn (2014) and Kagawa and Chiba (2022) have explored the heat protective benefits by epibionts on limpet and gastropod shells. However, these studies were not done on the organisms *in situ*. In both studies, the limpet body was removed and the tests were run only on the shells devoid of their resident, in one case, where the shells were filled with agar.

Within its natural setting, the limpet *Lottia pelta* can actively engage in several heat adaptation strategies if left alive to do so. This marine gastropod can duck under a rocky ledge (pers. observation, June 25, 2023), ostensibly to avoid direct sunlight. *Lottia pelta*, the species studied from Eagle Cove and the subject of this investigation, can create a mucus layer between its foot and home scar to prevent water loss according to Lowell, 1984 (written when *Lottia pelta* was also known as *Collisella pelta*). For this reason, it was not surprising that this current study did not show a difference between the temperature of the limpet and its adjacent substratum, or a difference in temperature that was dependent on the limpets' epibiont density. This study has presented some preliminary insight on the distribution of the algae, order Ulvales on limpets (specifically *Lottia pelta*) at two regions of the intertidal zone. Whether or not *Lottia pelta* or other limpet survives desiccation and thermal stress better with the presence of a particular algal epibiont species reminds to be studied *in situ*, perhaps by application of a more sophisticated technology that can track the movements of the creature and measure temperatures within the interior of this gastropod as it experiences real-time physical fluctuations while resting and foraging within its microhabitat.

This current study included the identification of green algal epibionts of the order Ulvales on the limpet *Lottia pelta*. In previous studies of the limpet epibionts of this San Juan Island habitat, *U. lactuca* and Acrosiphonia were identified by Seaborn, (2014). Kuba. et al. (2022)

provided vouchers for *U. linza* and *U. prolifera* while applying molecular identification methods to these algal species documented from islands within the San Juan Archipelago, including Eagle Cove Beach on San Juan Island. The microscopic identification of 7 of the 25 green algae sampled from the limpets collected in this study, classified them as Ulva sp. The one sample identified via molecular methods specified the Ulva sp. from an Eagle Cove limpet as *U. prolifera*. One sample of the green algal blades procured contained a mixture of Ulva. sp. and Ulothrix. Sp. Another sample was identified as Acrosiphonia sp. Additionally, two sampled algal epibionts from the Eagle Cove Limpets were classified as *Blidingia dawsonii* (Lindstrom et al., 2006).

The identity of all the Ulva species and even the Acrosiphonia sp. supported the original hypothesis that these would be some of the representative taxa present, based on Seaborn (2014) and Kuba et al. (2022). Ulothrix sp. was not mentioned in this original hypothesis, however this green algal species is distributed in the Pacific Ocean region and is found in marine habitats in the middle and low littoral zone of rocky shores and grows on hard substrata. Limpets with their hard shells, residing in the intertidal zone would qualify as a preferred residence for Ulothrix sp. (Guiry & Guiry, 2021).

Blidingia dawsonii was classified by microscopic observation using both Gabrielson and Lindstrom (2018), and Lindstrom et al. (2006) which based on the Lindstrom (2006) detailed life cycle, microscopic and molecular analysis, is more specifically named *Blidingia dawsonii* Hollenberg et I. A. Abbott S. C. Lindstrom, L. A. Hanic et L. Golden comb. nov. Basionym: *Percursaria dawsonii* Hollenberg et I. A. Abbott 1968 (Abbott, 1968, cited in Lindstrom et al. 2018).

While Lindstrom sampled the *Blidingia dawsonii* from the limpet *Tectura scutum* at Seppings Island, British Columbia, there is so far no evidence to suggest that *Blidingia dawsonii* is an obligate mutualistic epibiont on this specific limpet species (Thornber et al., 2017). Since San Juan Island is located in the Pacific Ocean region (The Salish Sea connects to the Pacific Ocean) and one of *Blidingia dawsonii*'s identifying features is that it is commonly found on limpets (Gabrielson & Lindstrom, 2018), finding this green algal species as an epibiont on a different limpet genus, such as the *Lottia pelta* in this study, is not outside the scope of expected results for this study.

SUMMARY

Mutualism between epibionts and limpets have been shown previously to provide costs and benefits to these marine gastropods. One of the benefits gleaned from the literature was that of protection for the gastropods from desiccation and heat stress. This study examined the presence and density of green algal epibionts on the limpet, *Lottia pelta* in regard to the location of the limpet in the intertidal region, as well as the abundance of the algae on the surface of the limpet shell.

Results from this study showed that there was not a significant effect of the epibiont on the limpet shell temperature, due to either location in the intertidal zone or qualitative density of the epibiont on the limpet shell surface. But this study highlights the importance of interspecies mutualistic associations and their possible role in adaptations to heat stress. In view of the observed and predicted effects on marine biota from ocean warming due to global climate change, exploring the implications of how epibionts can assist limpets and other marine gastropods with heat tolerance should continue. Further studies on the positive benefits of algal epibiont and limpet associations will include analyzing diverse limpet species and algal epibionts, more wide-ranging samples sites, and larger sample sizes.

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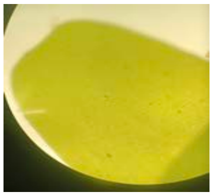
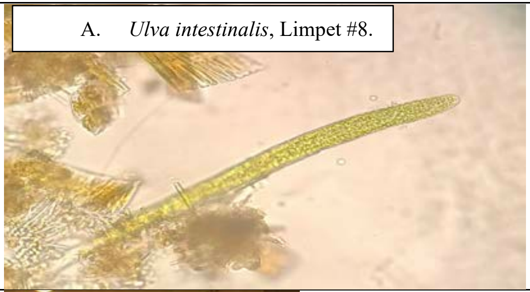
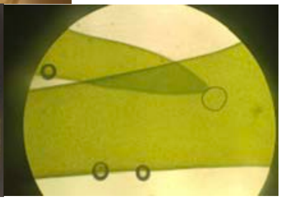
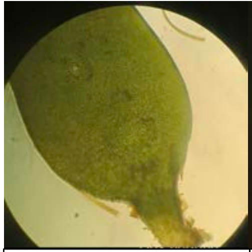
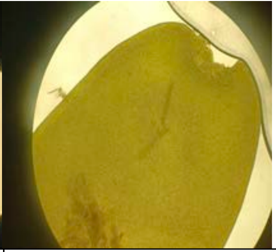
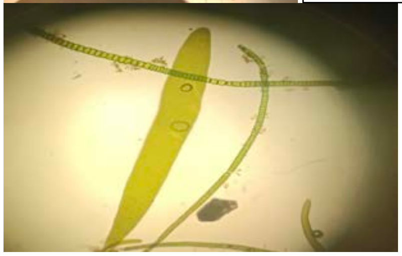
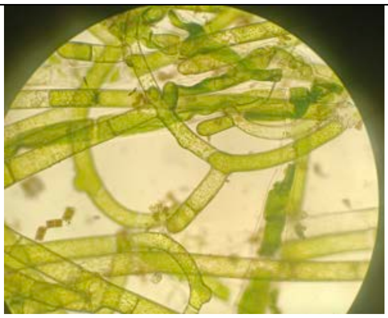
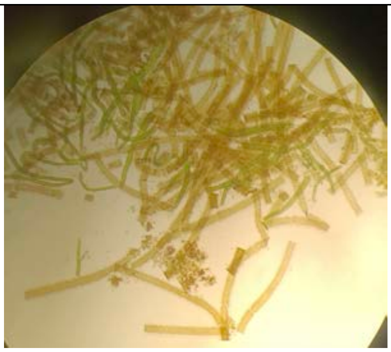
Appendix S1. Micrographs of green algae collected from 11 limpets from Eagle Cove, WA

Limpet #	Identity of Algae	Images
7	*Ulva* sp.	 A. Ulva, Limpet #7 dissecting microscope.  B. Ulva Limpet #7 at 10x, apical.  C. Ulva, Limpet #7 at 10x, basal.
8	*Ulva intestinalis* (provisional identification, T. Mumford, personal communication, July 7, 2023)	 A. *Ulva intestinalis*, Limpet #8.
10	Ulva sp.	 A. Ulva, Limpet #10, slide.  B. Limpet #10, dissecting microscope.  C. Ulva, Limpet #10 at 10x.

Limpet #	Identity of Algae	Images
11	Ulva sp.	 A. Ulva, Limpet #11, slide.  B. Ulva, Limpet #11 at 20x, basal.  C. Ulva, Limpet #11 at 20x, apical.
12	Ulva sp.	 A. Ulva, Limpet #12, slide.  B. Ulva, Limpet #12, at 4x.
15	Ulothrix sp.	 A. Ulothrix and Ulva, Limpet #15, slide.  B. Ulothrix and Ulva, Limpet #15, at 4x.
18	Ulva sp.	 A. Ulva, #18, dissecting microscope.  B. Ulva, Limpet #18, at 4x.

Limpet #	**Identity of Algae**	**Images**
19	Acrosiphonia sp.	 A. Acrosiphonia, Limpet #19, at 10x.
23	*Blidingia dawsonii*	 *A.* *Blidingia dawsonii,* Limpet # 23, at 10x.
24	*Blidingia dawsonii*	 A. *Blidingia dawsonii*, Limpet #24, at 20x.
25	Ulva sp.	No micrograph available.