DIFFERENCES IN THE MORPHOLOGY AND REPRODUCTION OF THE ASCIDIAN *BOLTENIA VILLOSA* ACROSS A LATITUDINAL GRADIENT

by

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A MARINE BIOLOGY HONORS THESIS

Presented to the Department of Biology
and the Robert D. Clark Honors College
in partial fulfillment of the requirements for the degree of
Bachelor of Science

May 2022

An Abstract of the Thesis of

Carmen Sanchez-Reddick for the degree of Bachelor of Science
in the Department of Biology to be taken December 2022.

Title: Differences in the Morphology and Reproduction of *Boltenia villosa* Across a Latitudinal Gradient

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While the larval and early juvenile stages of the stalked ascidian *Boltenia villosa* are well documented in the literature, little is known about the range of morphological variation in the adults. Anecdotal evidence suggests that larger individuals with short stalks make up the populations found in Washington, while Oregon populations consist of smaller individuals with longer stalks. The present study aimed to develop a qualitative understanding of the morphological and reproductive differences across the latitudinal gradient of *Boltenia villosa*. This was achieved by using a combination of morphometrics and scanning electron microscopy to determine any significant differences between different populations and the use of oocyte diameter and dissections to compare reproductive output. The results suggest a significant morphological and reproductive difference between the two populations in body proportions and spine character despite their genetic similarities. These preliminary results indicate the possible existence of an undescribed subspecies of *Boltenia villosa*, but more research into the morphological changes across the entire range of each morphotype's range are needed.

Acknowledgements

I would like to first thank Dr. Craig Young for his expertise and encouragements, and the late-night email exchanges necessary for the completion of this honors thesis. Craig has inspired me to pursue graduate school and a career in research and academia, and I can only hope to one day emulate his kindness, passion, and competence. I would also like to thank the other members of my Thesis Committee, Dr. Aaron Galloway and Dr. Lisa Munger for their edits to this manuscript and their flexibility in regard to my defense date. I want to thank Dr. Brian Bingham for introducing me to R and statistical analysis, sharing his knowledge of the local invertebrate communities in Anacortes, Washington, and helping Dexter Davis and I collect specimens. From my O.I.M.B. community, I want to thank Caitlin Plowman, Lauren Rice, Christina Ellison, Emma Burke, and Amanda Ludtke for their guidance in the areas of histology, scanning electron microscopy, and molecular analysis, and the entire Young Lab, including Dr. Sinja Rist and Avery Calhoun, for helping me collect specimens off Cape Arago, Oregon. I would like to extend a special thanks to Caitlin Plowman for providing invaluable intellectual and emotional support, even when out at sea on research cruises. I would like to thank the Robert. D. Clark Honors College for funding portions of my research through the Mentored Research Program and I appreciate the CHC for valuing my research. Finally, I want to thank my mother, Kathy Sanchez-Reddick, for always cheering me on, and my father, Bruce Reddick, who, although no longer with us, continues to strengthen and guide me.

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Introduction

Importance of the class Ascidiacea

Despite their unassuming nature, ascidians have proven their influence in various fields of study. As our closest invertebrate relatives (phylum Chordata, subphylum Tunicata), ascidians, or sea squirts, have a simplified genome that can be studied to provide insight on the regulatory pathways and functions of more complex vertebrate genomes (Monniot et al. 1991; Corbo et al. 2001). Ascidians are also demonstrating their usefulness to humanity as a source of chemical compounds useful to the pharmaceutical industry (Monniot et al. 1991). These compounds, often produced by an ascidian's symbionts, are being used to develop new antimalarials, antivirals, antibiotics, and cancer treatments (Shenkar and Swalla 2011; Watters 2018).

In the fields of ecology and marine environmental issues, sea squirts have been a valuable group to study. As sessile, benthic filter feeders, ascidians represent a key link in their food webs between the sea floor and open water communities (Monniot et al. 1991; Drgas and Całkiewicz1 2019), and therefore they have a powerful impact on local ecology. For environmental scientists, sea squirts can act as biomonitors of pollution and anthropogenetic stressors (Tzafriri-Milo et al. 2019). This is due to ability of ascidians to retain concentrated levels of toxins that impact overall water quality and their siphonal sensitivity to mechanical disruptions, such as port activities. Researchers have thus developed ways to record stress in sea squirts from contaminants and anthropogenetic disturbances (Monniot et al. 1991; Kuplik et al. 2019; Marques et al. 2022). While their use in biomonitoring is important, ascidians are also important to our

understanding of invasive species introduction (Shenkar and Swalla 2011; Tzafriri-Milo et al. 2019). The increasing number of invasive ascidian species, the need of native species for functioning ecosystems, and the value of sea squirts to evolutionary and medical breakthroughs underscores the need for researchers to advance existing research of ascidians

Life history of *Boltenia villosa*

Research centered around *Boltenia villosa*, a solitary ascidian found along the coast from southern Alaska to southern California, has historically focused on its settlement and metamorphosis. The larval stage of *B. villosa* is characterized by a free-swimming tadpole larva with orange pigmentation in the muscle and mesenchyme cells (Cloney et al. 2002). In this stage, the presence of a tail, notochord, and nerve cord classify ascidians in the phylum *Chordata* (Cloney et al. 2002; Davidson et al. 2003). The larvae use caudal musculature for swimming until an appropriate settlement site is chosen that will promote survivability and growth (Cloney et al. 2002; Roberts et al. 2007). *Boltenia villosa* larvae exhibit a strong preference for settlement on adult conspecifics or potential refuges, such as the ascidians *Pyura haustor* and *Halocynthia igaboia*, over rocky substratum to avoid predation by the Oregon hairy triton (*Fusitrition oregonensis*) (Young 1985; Young 1986; Young 1989). The mechanism for site selection may be a combination of physical cues and chemical cues from bacterial biofilm that cover potential substrata, which is likely how *Boltenia* larvae determine to delay settlement when refuge substrata are unavailable (Young 1989; Roberts et al. 2007). After settlement, the larvae undergo metamorphosis into their juvenile forms.

The metamorphosis of *Boltenia villosa*, which follows the typical order of ascidian development, occurs in two stages: a stage of rapid change completed within a few hours of settlement and a stage of sustained change across a 7-day period (Davidson et al. 2003). Minutes after settlement, the papillae used in adhesion retract, the tail is reabsorbed, and the notochord cells flow out of the notochordal sheath (Cloney et al. 2002). At the end of the first stage, the notochord, muscle, and nerve cord cells are consumed by phagocytes and the organism is considered a juvenile (Cloney et al. 2002). In the subsequent 7 days, differentiation of the gut and body wall musculature progress in tandem (Davidson et al. 2003). Once the second stage of metamorphosis is complete, the juvenile of *Boltenia villosa* can begin feeding (Cloney et al. 2002; Davidson et al. 2003).

While the larval and early juvenile stages of *Boltenia villosa* are well documented in the literature, little is known about the morphological variation in adults. Originally described by William Stimpson in 1864, *B. villosa* is identifiable by the short, fine-tipped spines that cover its tunic, the presence of a stalk used for attachment to the substratum, and the transverse stigmata within its branchial sac (Stimpson 1864; Van Name 1945; Berrill 1950). Recent studies have shown that *B. villosa* reproduces throughout the year (Cloney, 1987; Bingham, 1997) Reproductive timing is not dependent on light cycles (Cloney 1987; Bingham 1997).

Observed trends in biogeography and morphology

Outside of the current literature, scientists working in the eastern Pacific have noticed trends in the distribution of *Boltenia villosa*, but these observations remain mostly anecdotal. Equatorial submergence is a phenomenon in which individuals of a

species living along a latitudinal gradient are found at deeper waters as they approach the equator, probably to avoid warmer surface sea temperatures while receiving a similar level of illumination from the sun (Ekman 1953; Lüning and Liining 1990), and *B. villosa* is considered a species potentially subject this phenomenon. In the San Juan Islands of Washington, *Boltenia villosa* is easily found on docks and shallow waters (Young 1985), but are incredible rare on docks in southern Oregon, occurring instead on the continental shelf (Sanchez-Reddick 2021). Despite the observed trend, current literature does not reflect this. Another example is the variations in body size seen within the species. Early documentation of *B. villosa* describes a relationship between the body size and the stalk length as individuals with smaller bodies tend to have longer stalks (up to 3-4 times the length of the body) and individuals with larger bodies tend to possess very short stalks (Van Name 1945). Anecdotal evidence suggests that larger individuals with short stalks make up the populations found in Washington, while Oregon populations consist of smaller individuals with longer stalks (C. Young, personal communication). There remain many unknowns about *Boltenia villosa*, specifically the potential relationship between its size, depth distribution, and latitudinal gradient.

The present study aims to develop a qualitative understanding on how morphology, as well as reproductive output, changes across the latitudinal gradient of *Boltenia villosa*. This novel research also aims to uncover possible cryptic species through molecular analysis. I hypothesize that the *B. villosa* population of San Juan Islands, Washington will be significantly morphologically different from the population off the southern coast of Oregon and will have a higher reproductive output.

Materials and Methods

Specimen Collection

Boltenia villosa samples were collected from a shallow water site in the Salish Sea in Washington and a deep-water site off the southern coast of Oregon during the autumns. In Anacortes, Washington, 40 specimens were collected off floating boat docks (48°31.1439N, 122°37.3489W) in September 2021. Off the rocky reef of Cape Arago, Oregon (43°15.3521N - 43°16.8762N, 124°26.4627W - 124°27.7112W), 172 specimen were collected with a box dredge at depths of 44.19m to 54.86m in November 2021. Special care was taken to remove the ascidians from any hard substrate they were anchored on to preserve their stalks. All specimens were fixed in 10% formalin for at least 24 h and transferred into 70% ethanol for long-term preservation and storage at 20°C. Each ascidian was given an ID related to its collection location and date.

Morphology

Morphometrics

The external anatomy of all 212 *B. villosa* specimen was measured using a Vernier caliper. The lengths of the body and stalk were measured separately and the distinguishment point between the body and stalk was determined by a clear diameter change in the specimen. The width measurement of the body was collected at the widest point of each specimen and the stalk width measurement was collected at the base of the body (Fig.1).

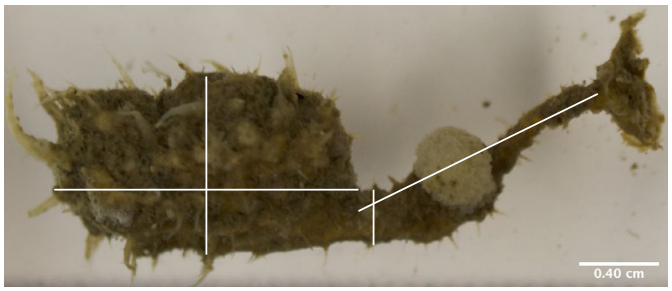

Figure 1: Diagram of morphometric measuring method. Individuals from the Oregon and Washington populations were measured using four morphometric variables: body length and width and stalk length and width. The end points for each measurement is outlined in white.

Scanning Electron Microscopy (SEM)

Five representative Oregon specimens and three representative specimens from Washington were chosen for SEM analysis of the spines covering their tunics. Six spines were cut at their bases from each individual and mounted onto a specimen stub using forceps and adhesive transfer tabs. Then, the spines were allowed to air-dry to evaporate any excess ethanol from sample preservation. The stubs were sputter coated with gold in a Cressington Sputter Coater 108auto. Photographs of the spines were taken with a Tescan Vega scanning electron microscope at an accelerating voltage of 10.00kV. The photographs were analyzed and any spiny protrusions with a visible attachment to the main spine were counted.

Reproduction

Gonadosomatic Index (GSI)

To compare the reproductive activity of the two populations, gonadosomatic indices were calculated for ten randomly selected Oregon and ten randomly selected

Washington specimens. Due to presence of fouling material and epibionts on the tunic of many individuals, the tunic was removed and not included in weighing of the body. Each specimen was weighted and dissected for their gonads so that both gonads could also be weighted. The following formula was used to compute each GSI: (gonad weight / total specimen weight) x 100.

Oocyte Diameter

Thirteen moderately to large sized specimens from Oregon were selected for histology. Both gonads were removed from each ascidian, dehydrated in an ethanol series from 50% to 100% ethanol, cleared with toluene, then submerged and embedded in molten paraffin. Gonads were semi-serially sectioned at a thickness of 7µm with an AO "820" microtome. Slices were stained using hematoxylin, ponceau de xylidine, phosphomolybdic acid, and fast green. Slide covers were adhered with Permount Mounting Medium. Six specimens collected during the autumns of 1979 and 1980 in Friday Harbor, WA (48°32.0813N, 123°1.1315W) were used to represent the Washington population. The Washington samples were dehydrated, sliced, and stained by Craig Young with assistance from an undergraduate intern at the time of collection, but analysis occurred in 2021 and 2022. The diameter of the first 100 oocytes from each specimen was measured using ImageJ on photographs captured with a ZEISS Axiocam 208 color microscope camera on a ZEISS Stemi508 microscope at a magnification of 5x.

Molecular Analysis

The same five representative Oregon specimens used for SEM were selected for molecular analysis and immediately transferred into 95% ethanol at 20°C. Using a DNeasy Blood and Tissue Kit (Qiagen), DNA was extracted from the internal tissues of the specimen. Before adding lysis buffer, the tissues were rinsed with RO water to wash away excess ethanol. For each 2x2x2 mm sample of tissue, tissue lysis was performed with 180 µl ATL butter and 20 µl proteinase K at 56°C for at least 24 h.

PCR-amplification was done for the cytochrome *c* oxidase subunit 1 DNA barcoding region (658-696 bp, COI). Two primers were used for amplification: universal LCO 1490 [5′ GGTCAACAAATCATAAAGATATTGG 3′] and HCO 2198 [5′ TAAACTTCAGGGTGACCAAAAAATCA 3′] (Folmer et al., 1994); tunicate-specific ASC_COI_F [5'-TCGACWAATCATAAAGATATTAG 3'] and ASC_COI_R [5' GTAAAATAAGCTCGAGAATC 3']. Tissue samples were diluted to 1 µl sample and 9 µl water. 2 µl of sample with water and 18 µl of master mix (nuclease free water, 5x buffer, dNTPS, Go Taq, forward and reverse primers) underwent a PCR cycling including initial denaturation for 2 min. at 95°C, 35 cycles of 40 sec. at 95°C, 40 sec. at 45°C, and 1 min. at 72°C, and final primer extension at 72°C for 2 min. Gel electrophoresis was used to check the quality of the DNA samples and the samples amplified with tunicate-specific primers were selected for sequencing. PCR purification was done with Promega SV Wizard Gel and PCR Cleanup kit (Promega) before the samples were sequenced (Sequetech Inc, Mountain View, CA) in the forward and reverse directions.

Data Analysis

Morphometric data from the two populations were compared with a multidimensional scaling (MDS) plot using untransformed data and Bray-Curtis similarity in R version 4.2.0 and RStudio. An ANOSIM (ANalysis of Similarities) statistical test was done to determine significance of the differences between the two groups. Box and whisker plots were created in Excel to visualize the gonadosomatic index calculations and the oocyte sizes of the Oregon and Washington specimens. Using 2-sample, 2-tailed t-tests, the differences in spine coverage, GSI, and oocyte sizes between populations were tested for significance. Analysis of molecular sequences was done using Geneious Prime 2022.1.1 and added to GenBank. Sequences from these Oregon specimens were compared to existing sequences in GenBank, including sequences from individuals in San Juan Island, WA and British Columbia.

Results

Morphology

During specimen collections, a similar pattern in *B. villosa* from Washington to Oregon was noted that corroborated previous anecdotal observations with larger, short-stalked individuals in northern waters and smaller, longer-stalked individuals in more southern waters (Fig. 2). This pattern was supported by morphometric data on quantitative differences in body length and width and stalk length and width. The frequency distribution of each morphometric variable showed that individuals found in Washington tended to have larger bodies in terms of length (mean = 22.923mm ± 6.003) and width (mean =17.4mm ± 5.3) with shorter (mean = 9.038mm ± 4.507), thicker stalks (mean = 8.132mm ± 2.626) while the Oregon population consisted of individuals with shorter (mean =5.9mm ± 2.519) and thinner (mean = 9.1mm ± 3.473) bodies and longer (mean =13.9mm ± 6.85) and thinner stalks (mean =1.4mm ± 0.439) (Fig. 3). A multidimensional scaling (MDS) plot, which was a good representation of the data (stress = 0.063), showed a clear separation between the two populations with very little crossover (Fig. 4). An ANOSIM test confirmed that there was a significant difference between the two groups, specifically as it compared to the similarities shared by individuals of the same population ($R = 0.7343$, $p = 0.001$).

There were also microscopic dissimilarities in the spines that cover the tunics of this species. Scanning electron microscopy revealed the presence of tiny spines protruding from the main spine on all by two of the spines collected for the Washington specimens, averaging 14.421 protrusions per spine (± 12.873) (Fig. 3). In the Oregon specimens, their spines were smooth, except in the case of one spine, averaging 0.37

protrusions per spine (± 1.925) (Fig. 5). These differences were shown to be statistically significant by way of a 2-sample, 2-tailed t-test ($t_{20} = -4.902$, $p = 8.603 \times 10^{-5}$).

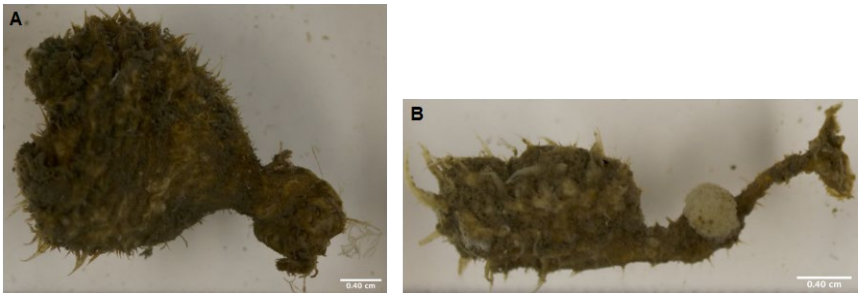

Figure 2: Photographs of representative specimens from Washington (A) and Oregon (B). Individuals collected from Washington were recognizable by their large bodies and short stalks and individuals collected from Oregon had small bodies and long stalks. The Oregon specimen has an epibiont attached to its stalk, which was common for individuals from that population.

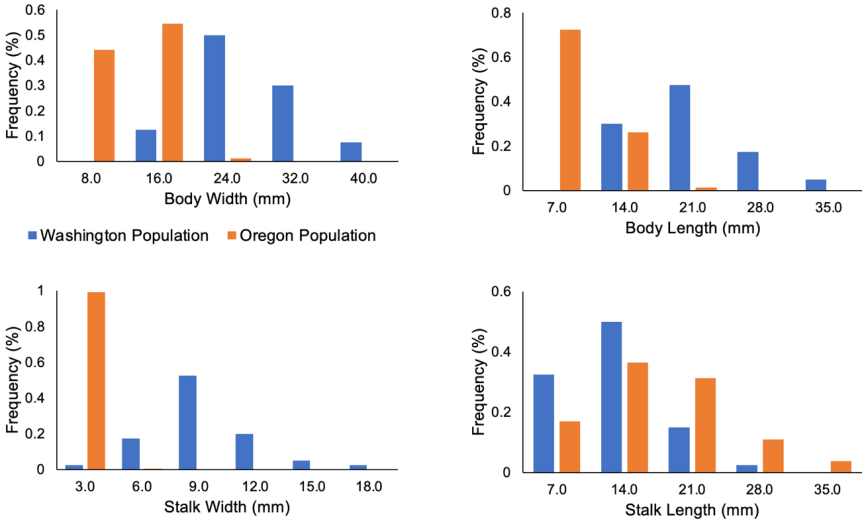

Figure 3: Frequency distribution of each of the four morphometric variables for the Washington (blue) and Oregon populations (orange). The x-axis is each individual variable and y-axis is the frequency of a measurement bin in a population. The Washington population usually had wider, taller bodies and wider, shorter stalks. Individuals in Oregon tended to have a thinner and shorter body and thinner and longer stalk.

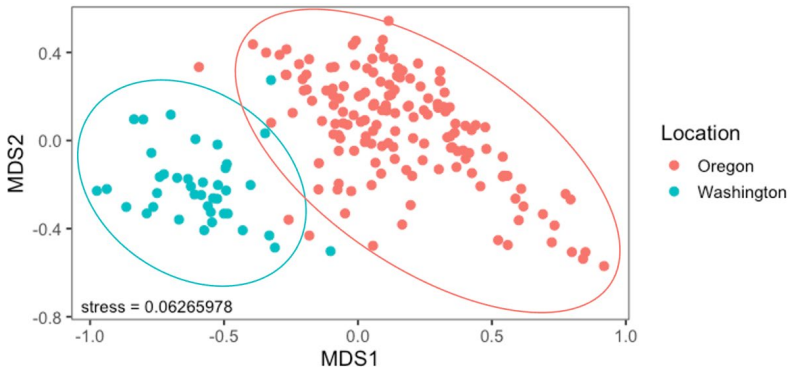

Figure 4: Multidimensional scaling (MDS) plot showing the morphological dissimilarity and similarity of all measured B. villosa specimens. The overall morphological differences due to body length, body width, stalk length, and stalk width between two specimens are visualized by the distances between their points. Individuals collected in Washington (blue) and individuals collected in Oregon (red) were distinguished using color to compare the two populations of *B. villosa*.

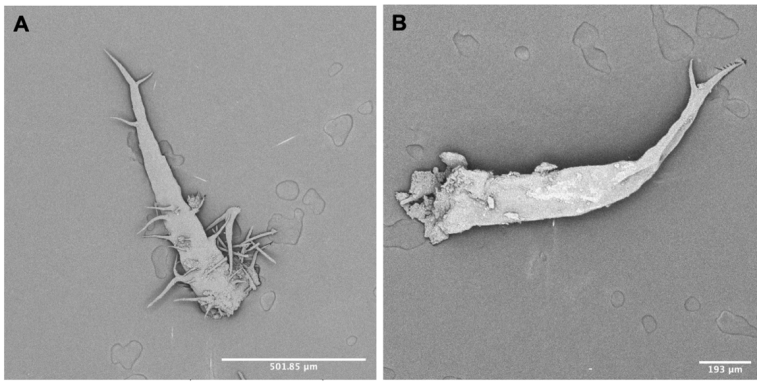

Figure 5: Scanning electron miscopy images of (A) a spine removed from a Washington specimen and (B) a spine removed from an Oregon specimen. There were distinct and significant differences between the spines of the two populations, with the spines from Washington having additional spiny protrusions and the Oregon spines being entirely smooth.

Reproduction

A box and whisker plot showed that the gonadosomatic indexes (GSI) of individuals in the two populations had similar means and large ranges of variance, with mean percent proportions of 30.656% (± 7.250) and 35.552% (± 9.439) for Oregon and Washington respectively (Fig. 6). Using a 2-sample, 2-tailed t-test, it was determined that there was no significant difference between populations in the percentage of body weight that can be accounted for by the two gonads of an individual ($t_{17} = 1.301$, $p = 0.211$).

Although, the GSI values did not differ greatly, the diameters of the first 100 oocytes in individuals from Oregon and Washington did differ. A 2-sample, 2-tailed t-test showed a significant difference between populations in the size of their oocytes during the same season ($t_{973} = 9.272$, $p = 1.151 \times 10\text{-}19$). On average, individuals from Washington had larger oocytes (mean = 0.377μm ± 0.166) than individuals found in Oregon (mean = 0.305μm ± 0.133) (Fig. 7).

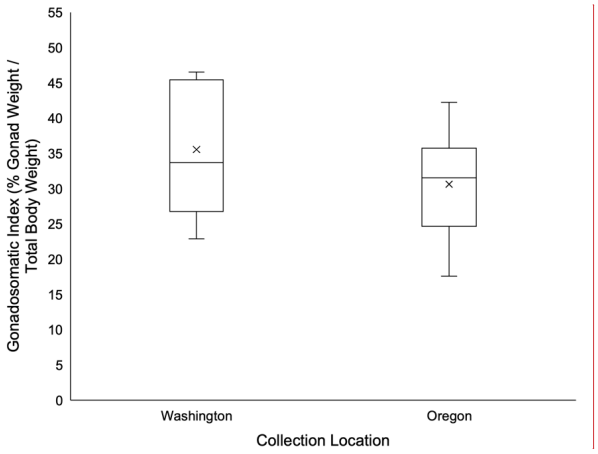

Figure 6: Box and whisker plot of the gonadosomatic indexes (GSI) of individuals collected in Washington and Oregon. The y-axis is the percentage of gonad weight to total body weight and the x-axis is collation site. The whiskers of the plot represent the upper and lower 25% of oocytes and the line is the medium for each population. There was no significant difference in the GSIs between the two populations.

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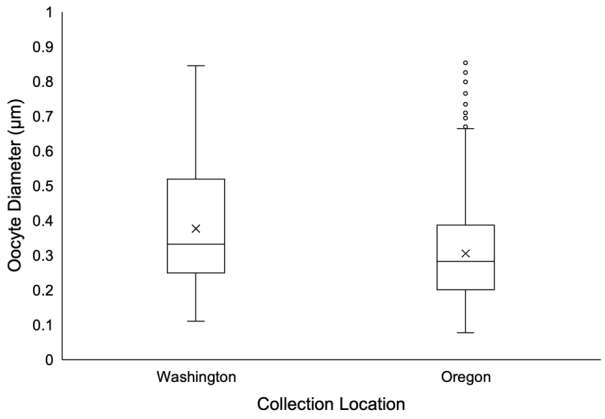

Figure 7: Box and whisker plot of the diameters of the first 100 oocytes from 19 individuals collected in Washington and Oregon. the 2-sample, 2-tailed t-test provided evidence to the contrary. Individuals from Washington tended to have larger oocytes than individuals found in Oregon. A 2-sample, 2-tailed t-test showed there was a significant difference between the two populations in their oocyte diameters.

Molecular Analysis

Four out of the five samples chosen for molecular sequencing were analyzed successfully. The four specimens were found to be the same species with less than 0.5% differences in their COI barcoding region. When compared to *Boltenia villosa* specimens barcoded from Washington and British Columbia, there was over 98% base similarity (Fig. 8). This indicates that the populations from Washington and Oregon are likely the same species, and that their morphological and reproductive differences are probably habitat-related phenotypic differences, not a result of interspecies differences.

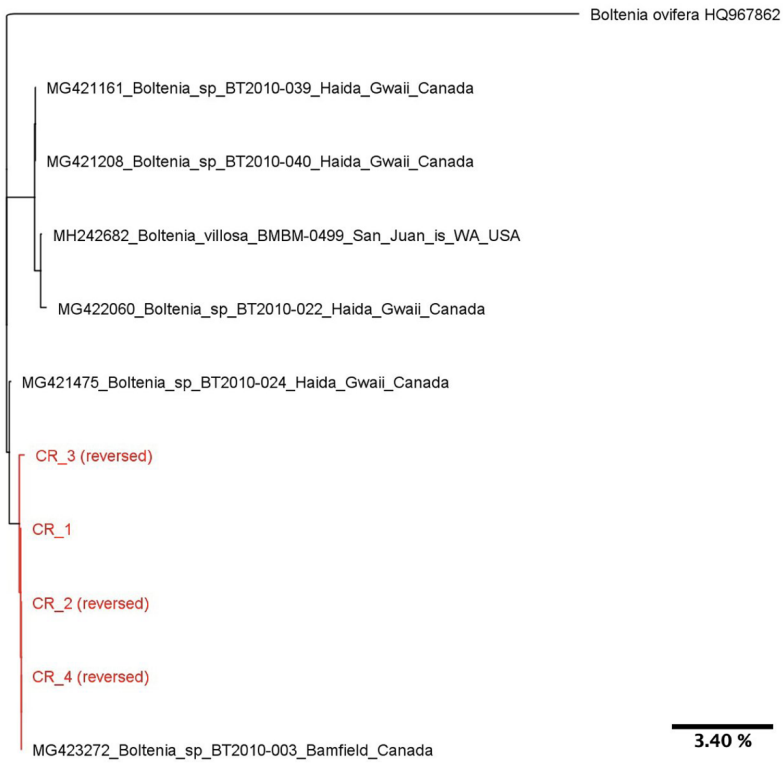

Figure 8: Barcoding region COI tree for *Boltenia villosa* specimens submitted to GenBank. Specimens from Oregon (red) were sequenced and compared to existing *B. villosa* entries from Washington and British Columbia. *B.villosa* from Oregon were genetically similar to members of *B.villosa* found elsewhere within its range. *Boltenia ovifera* is provided as reference point and has 17% similarity to *Boltenia villosa*.

Discussion

There are two distinct morphotypes with significantly different reproductive outputs found between the Anacortes and Cape Arago populations of *Boltenia villosa*. The quantifiable morphological differences between populations of *B. villosa* are consistent with previous anecdotal observations as individuals found in Anacortes, Washington could be identified by their large bodies, short stalks, while the Cape Arago, Oregon population consisted of individuals with small bodies and long stalks. Interestingly, their morphological differences also extended to the spine character of each morphotype as the Washington individuals had hairy spines and the Oregon population has smooth spines. In terms of their reproduction, the Washington population had larger oocytes than the Oregon population, but their gonadosomatic indices were similar. The difference in the proportion of gonad to total body weight was not significant, which may be explained by members of this species maximizing their fitness and reaching a similar GSI proportional limit.

Despite their genetic similarities, it is noteworthy that the query coverage of the molecular sequences was only 77%, meaning 25% of the sequences could not be compared to existing GenBank entries. With more query coverage could come slightly more dissimilarity between the populations. While comparing my sequences to sequences already deposited was a more convenient method of analysis, it would have been preferable to compare to individuals from Washington and British Columbia whose morphology had been quantified in a similar manner as those in the present study.

The distance between sampling sites and the lack of collection at varying depths from each site also prevents a more robust comparison of the distribution of *B. villosa* morphotypes. It is possible that the distinction of a new a subspecies of *Boltenia villosa* may be appropriate given the clear separation of its morphotypes across its range, but more sampling would be necessary to determine regions of sympatry in the species' latitudinal and depth ranges. This is especially important as *B. villosa* is assumed to be subject to equatorial submergence, but populations could exist at multiple depths at all latitudes. Despite limitations, these results create a strong foundation for the continued exploration into these populations of *B. villosa* and the possible explanations for their differences. Potential future research may examine the impacts of food availability and water temperature on oocyte diameter or the influence of water movement on a given habitat's morphotype (Koehl 1982; Gosselin et al. 2019). A high priority for future work would be to collect and analyze animals from multiple depths at each of several latitudes, to determine if the observed differences are latitudinal or bathymetric.

The present study successfully quantified the morphological and reproductive differences between populations of *Boltenia villosa*, but the need for future research highlights why understanding its biology and biogeographical range is important. Robust sampling of *B. villosa*'s distribution could provide necessary baselines for climate change, invasive species introduction, and the role *B. villosa* plays in its natural habitats. Further molecular and morphometric analysis to distinguish a new subspecies would add to our understanding of global biodiversity levels. Finally, understanding ascidian biology may provide researchers a better understanding of the possible ecological importance of *Boltenia villosa* in their communities.

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