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Differences in the Morphology and Reproduction of the Ascidian *Boltenia Villosa* Across a Latitudinal Gradient

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ABSTRACT

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 *B. villosa* adults. Anecdotal evidence suggests that larger individuals with shorter 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 *B. villosa*. Morphological differences between populations were studied through a combination of morphometrics and scanning electron microscopy. To compare reproductive outputs, the diameters of oocytes and the gonadosomatic indices of representative individuals were measured. The results suggest significant differences between the two populations in body proportions, spine character, and reproduction despite their genetic similarities. These preliminary results provide the basis for future research into the distribution of *B.villosa* and the possible existence of an undescribed subspecies of *B. villosa*.

1. INTRODUCTION

1.1. IMPORTANCE OF THE CLASS ASCIDIACEA

Despite their unassuming nature, ascidians have proven their usefulness across various fields of medicine. As the closest invertebrate relatives to humans (phylum Chordata, subphylum Tunicata), ascidians, commonly known as sea squirts, have a simplified genome that can be studied to provide insight into the regulatory pathways and functions of more complex vertebrate genomes (Monniot et al. 1991; Corbo et al. 2001). Ascidians are also a source of chemical compounds that are valuable 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, ascidians have been a valuable proxy in the study of food webs and changes to marine ecosystems. As sessile, benthic filter feeders, native 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 have a powerful impact on local ecology. Meanwhile, non-native and invasive ascidian species greatly influence local communities, as their arrival often threatens the survival of endemic species (Shenkar and Swalla 2011). Yet, invasive ascidians can be beneficial in the biomonitoring of pollution

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and anthropogenetic stressors (Tzafriri-Milo et al. 2019). This is because ascidians retain concentrated levels of toxins that impact overall water quality and have a siphonal sensitivity to mechanical disruptions such as port activities. Researchers have thus developed ways to record stress in ascidians and associate it with the presence of different contaminants and anthropogenetic disturbances (Monniot et al. 1991; Kuplik et al. 2019; Marques et al. 2022).

While other marine organisms may be more well-known or charismatic, there is value in advancing our existing understanding of ascidians. Without an adequate study of an ecosystem's ascidian populations, it is difficult to see the importance of a native ascidian species, the harm of an invasive species, or whether a specific species should be classified as native or non-native. In the fields of evolutionary and pharmaceutical medicine, undiscovered or cryptic ascidian species may remain overlooked and their possible benefits to humanity left untapped. Before additional advancements in medicine and ecology can occur, the first step to understanding more about ascidians is to study their biology—starting with their morphology and reproduction.

1.2. LIFE HISTORY OF *BOLTENIA VILLOSA*

Research centered around *Boltenia villosa*—a solitary ascidian found along the North American west coast, from southern Alaska to southern California—has historically focused on its settlement and metamorphosis. In its larval stage, *B. villosa* takes the shape of a free-swimming tadpole 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). *B. 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 covering 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 *B. 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 *B. villosa* can begin feeding (Cloney et al. 2002; Davidson et al. 2003).

While the larval and early juvenile stages of *B. 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 covering 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). More recent studies have shown that *B. villosa*

reproduces throughout the year (Cloney, 1987; Bingham, 1997), and reproductive timing is not dependent on light cycles (Cloney 1987; Bingham 1997).

1.3. OBSERVED TRENDS IN BIOGEOGRAPHY AND MORPHOLOGY

Outside of the current literature, scientists working in the eastern Pacific have noticed trends in the distribution of *B. 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 in 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 to this phenomenon. In the San Juan Islands of Washington, *B. villosa* is easily found on docks and in shallow waters (Young 1985), but it is incredibly rare on docks in southern Oregon, occurring instead on the continental shelf (Sanchez-Reddick 2021).

Another anecdotal pattern is the variation in body size seen within the species. Early documentation of *B. villosa* describes an inverse relationship between body size and 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 shorter stalks make up the populations found in Washington, while Oregon populations consist of smaller individuals with longer stalks (C. Young, personal communication). These patterns in *B. villosa*'s distribution and morphology have been observed by researchers, but they have not been formally studied or described in the literature. There remain many unknowns about *B. villosa*, specifically the potential relationship between its size, depth distribution, and latitudinal gradient.

The present study aims to develop a qualitative understanding of how morphology and reproductive output change across the latitudinal gradient of *B. villosa*. In this study, I test the hypothesis that the *B. villosa* population of the Salish Sea in Washington has significant morphological and reproductive differences from the population off the southern coast of Oregon. This novel research also aims to uncover possible cryptic species through molecular analysis.

2. MATERIALS AND METHODS

2.1. 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 autumn. 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 specimens were collected with a box dredge at depths from 44.19m to 54.86m in November 2021. Special care was taken to remove the ascidians from any hard substrate they were anchored onto to preserve their stalks. All specimens were fixed in 10% formalin for at least 24 hours 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.

2.2. MORPHOLOGY

2.2.1. MORPHOMETRICS

The external anatomy of all 212 *B. villosa* specimens was measured using a Vernier caliper. The stalk of *B. villosa* is identifiable as a protrusion found on the ventral side of a specimen's rounded body (Van Name 1945); as such, the lengths of the

body and stalk were measured separately (Fig.1). 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).

2.2.2. SCANNING ELECTRON MICROSCOPY (SEM)

To identify any minute morphological differences between the two populations, five representative specimens from Oregon 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 the sample preservation process. 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.

2.3. REPRODUCTION

2.3.1. GONADOSOMATIC INDICES (GSI)

To compare the reproductive activity of the two populations, gonadosomatic indices were calculated for a random selection of ten Oregonian and ten Washingtonian specimens. The tunics—which act as ascidians' protective outer layers—of many individuals were covered in fouling material and epibionts. The presence of fouling material and epibionts would have impacted the total weight of the specimens, so the tunics were removed and not included in body weight measurements. Each specimen was weighed and dissected for its gonads so that both gonads could also be weighted. The following formula was used to compute each GSI: (gonad weight / total specimen weight) · 100.

2.3.2. OOCYTE DIAMETERS

13 moderate-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, and 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, Washington (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 the analysis occurred in 2021 and 2022. These specimens were selected for analysis because quality histological slides were readily available, and it is unlikely the reproductive output of the *B. villosa* population of the Salish Sea has changed significantly over the last 40 years (C. Young, personal communication).

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.

2.4. MOLECULAR ANALYSIS

To identify any phenological differences between populations, 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 reverse

osmosis 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 hours.

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) and tunicate-specific ASC_COI_F [5'-TCGACWAATCATAAAGATATTAG 3'] and ASC_COI_R [5' GTAAAATAAGCTCGAGAATC 3']. 1-µl t issue samples were diluted in 9 µl water. 2 µl of diluted solution and 18 µl of master mix (nuclease-free water, 5x buffer, dNTPs, Go Taq, and forward and reverse primers) underwent a PCR cycling with the following framework: 2-minute initial denaturation at 95°C, 35 cycles of 40 seconds at 95°C, 40 seconds at 45°C, 1 minute at 72°C, and a final 2-minute primer extension at 72°C. 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 performed with the 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.

2.5. DATA ANALYSIS

Morphometric data from the two populations were compared with a multidimensional scaling (MDS) plot using untransformed data and a Bray-Curtis similarity test in R version 4.2.0 and RStudio. An analysis of similarities (ANOSIM) statistical test was done to determine the 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 performed 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 near San Juan Island, Washington, and British Columbia.

3. RESULTS

3.1. MORPHOLOGY

During specimen collections, a pattern in *Boltenia villosa* from Washington to Oregon emerged that corroborated previous anecdotal observations with larger, shorter-stalked individuals in northern waters and smaller, longer-stalked individuals in southern waters (Fig. 2). This pattern was further 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 ($\bar{x}$ = 22.923mm ± 6.003) and width ($\bar{x}$ =17.4mm ± 5.3), with shorter ($\bar{x}$ = 9.038mm ± 4.507), thicker ($\bar{x}$ = 8.132mm ± 2.626) stalks, while the Oregon population consisted of individuals with shorter ($\bar{x}$ =5.9mm ± 2.519), thinner ($\bar{x}$ = 9.1mm ± 3.473) bodies and longer ($\bar{x}$ =13.9mm ± 6.85), thinner ($\bar{x}$ =1.4mm ± 0.439) stalks (Fig. 3). A multidimensional scaling (MDS) plot, which provided 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 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 covering the tunics of this species.

Scanning electron microscopy revealed the presence of tiny spines protruding from the main spine on all but two of the spines collected from the Washington specimens, averaging 14.421 protrusions per spine (± 12.873) (Fig. 3). However, Oregon specimens tended to have smooth spines (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 x 10^{-5}).

3.2. 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 x 10-19). On average, individuals from Washington had larger oocytes ($\bar{x}$= 0.377µm ± 0.166) than individuals found in Oregon ($\bar{x}$ = 0.305µm ± 0.133) (Fig. 7).

3.3. 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% of the genetic sequence in their COI barcoding region being different. When compared to *B. villosa* specimens barcoded from Washington and British Columbia, there was over 98% base similarity in genetic sequencing (Fig. 8). This suggests that the populations from Washington and Oregon are of the same species and that their morphological and reproductive differences are habitat-related phenotypic differences, not a result of interspecies differences.

4. DISCUSSION

There are two distinct morphotypes of *Boltenia villosa* with significantly different reproductive outputs reflected by the populations found near Anacortes and Cape Arago. The quantifiable morphological differences between these populations of *B. villosa* are consistent with previous anecdotal observations: individuals found in Anacortes, Washington could be identified by their large bodies and short stalks, while the Cape Arago, Oregon population consisted of individuals with small bodies and long stalks. Interestingly, morphological differences also extended to the spine character of each morphotype, as the Washington individuals had hairy spines and the Oregon population had smooth spines. In terms of 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.

Limitations to sampling efforts are the primary source of ambiguity in the genetic analysis and the definitive outlining of morphotype ranges. 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 sequence entries and increased query coverage, the populations could have registered as having slightly more genetic dissimilarity. While comparing my sequences to

sequences already deposited was a more convenient method of analysis, it would have been preferable to compare my sequences to those of individuals from Washington and British Columbia whose morphology had been quantified similarly to 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. 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.

5. CONCLUSIONS AND FUTURE DIRECTION

The present study successfully quantified the morphological and reproductive differences between populations of *B. villosa*. These results are supported by the anecdotal evidence that suggested a relationship between *B. villosa*'s distribution and morphology yet had previously been undescribed in the literature. This study is the first to document the differences in spine character and reproductive output between populations of *B.villosa* in the literature. Despite their distinct morphological differences, no cryptic species were uncovered in the molecular analysis; therefore, these populations are not the result of the misidentification of two separate species. As members of the same species, researchers can freely use either or both *B. villosa* morphotypes in studies that may provide additional insights in various fields, including the areas of biomedicine or evolution. These results also create a strong foundation for the continued exploration into these populations of *B. villosa* and possible explanations for their differences. A robust sampling of *B. villosa*'s distribution could provide necessary baselines for the study of dynamic phenomena such as climate change, invasive species introduction, and the role *B. villosa* plays in its native habitats.

It is also possible that the distinction of a new subspecies of *B. villosa* may be appropriate given the clear separation of *B. villosa* morphotypes across its range. However, more sampling would be necessary to determine regions of sympatry in the species' latitudinal and depth ranges and identify any phenotypic variation that may explain the morphological differences. These efforts would add to our understanding of the global biodiversity level and contribute to studies on the potential environmental causes of *B. villosa* morphotypes.

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 and to distinguish any new subspecies.

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WORKS CITED

Berrill N. 1950. The Tunicata with an account of the british species. London: The Ray Society.

Bingham B. 1997. Light Cycles and Gametogenesis in Three Temperate Ascidian Species. Invertebr Biol. 116(1):61–70. doi:10.2307/3226925.

Cloney R. 1987. Phylum Urochordata, class Ascidiacea. In: Strathmann M, editor. Reproduction and Development of Marine Invertebrates of the Northern Pacific Coast. University of Washington Press. p. 607–639.

Cloney R, Young C, Svane I. 2002. Phylum Chordata: Urochordata. In: Young C, editor. Atlas of Marine Invertebrate Larvae. Academic Press. p. 565–570; 590.

Corbo JC, Di Gregorio A, Levine M. 2001. The Ascidian as a Model Organism in Developmental and Evolutionary Biology. Cell. 106(5):535–538. doi:10.1016/S0092-8674(01)00481-0.

Davidson B, Smith Wallace S, Howsmon R, Swalla B. 2003. A morphological and genetic characterization of metamorphosis in the ascidian Boltenia villosa. Dev Genes Evol. 213:601–611. doi:10.1007/s00427-003-0363-3.

Drgas A, Całkiewicz1 J. 2019. Assessing the role of benthic filter feeders in in the food web of the Gulf of Gdansk (Sothern Baltic) – an Ecopath with Ecosim exercise. Braga, Portugal: Frontiers in Marine Science.

Ekman S. 1953. Longitudinal Distribution and Bipolarity. In: Zoogeography of the Sea. p. 244–263.

Gosselin L, Gallego R, Peters-Didier J, Sewell M. 2019. Field evidence of interpopulation variation in oocyte size of a marine invertebrate under contrasting temperature and food availability. Mar Ecol Prog Ser. 619:69–84. doi:10.3354/meps12959.

Koehl M. 1982. The Interaction of Moving Water and Sessile Organisms. Sci Am. 247(6):124–135.

Kuplik Z, Novak L, Shenkar N. 2019. Proteomic profiling of ascidians as a tool for biomonitoring marine environments. PLoS One. 14(4):e0215005. doi:10.1371/journal.pone.0215005.

Lüning K, Liining K. 1990. Seaweeds: Their Environment, Biogeography, and Ecophysiology. Yarish C, Kirkman H, translators. Wiley.

Marques L, Calado R, Lillebø A. 2022. Potential of Ascidians as Extractive Species and Their Added Value in Marine Integrated Multitrophic Aquaculture Systems–From Pests to Valuable Blue Bioresources. Front Mar Sci. 9. doi:10.3389/fmars.2022.849870. https://www.frontiersin.org/articles/10.3389/fmars.2022.849870/full#B87.

Monniot C, Monniot F, Laboute P. 1991. Coral Reef

Ascidians of New Caledonia. France: Éditions de l'ORSTOM, Institut français de recherche scientifique pour le développement en coopération.

Roberts B, Davidson B, MacMaster G, Lockhard V, Ma E, Smith Wallace S, Swalla B. 2007. A complement response may activate metamorphosis in the ascidian Boltenia villosa. Development Genes and Evolution(217):449–458. doi:10.1007/s00427-007-0157-0.

Sanchez-Reddick C. 2021. Boltenia Villosa. In: Plowman C, Young C, editors. Oregon Shelf Invertebrates. Oregon Institute of Marine Biology.

Shenkar N, Swalla B. 2011. Global Diversity of Ascidiacea. PLoS One. 6(6):e20657. doi:10.1371/journal.pone.0020657.

Stimpson W. 1864. Descriptions of new marine invertebrata from Puget's Sound. In: Proceedings of the Academy of Natural Sciences of Philadelphia. p. 153–161. https://www.biodiversitylibrary.org/page/1942749#page/168/mode/1up.

Tzafriri-Milo R, Benaltabet T, Torfstein A, Noa Shenkar. 2019. The Potential Use of Invasive Ascidians for Biomonitoring Heavy Metal Pollution. Front Mar Sci. 6:611. doi:10.3389/fmars.2019.00611.

Van Name W. 1945. The North and South American Ascidians. Bull Am Mus Nat Hist. 84:11–19, 356–358.

Watters D. 2018. Ascidian Toxins with Potential for Drug Development. Mar Drugs. 16(5):162. doi:10.3390/md16050162.

Young C. 1985. Abundance patterns of subtidal solitary ascidians in the San Jaun Islands. Washington, as influenced by food preferences of the predatory snail Fusitriton oregonensis. Mar Biol. 84:309–321.

Young C. 1986. Defenses and refuges: alternative mechanisms of coexistence between a predatory gastropod and its ascidian prey. Mar Biol. 91:513–522.

Young C. 1989. Selection of predator-free settlement sites by larval ascidians. Ophelia. 30(2):131–140.

FIGURES

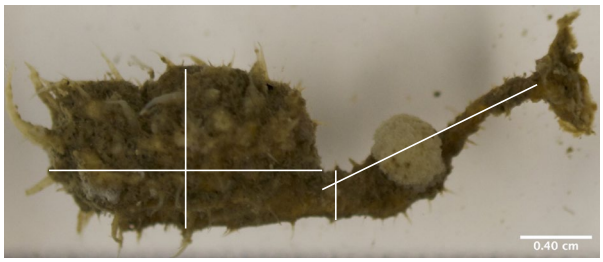

Figure 1: Diagram of morphometric measuring method. Individuals from the Oregon and Washington populations were measured using four morphometric variables: body length and width and stalk length and width. The endpoints for each measurement are outlined in white.

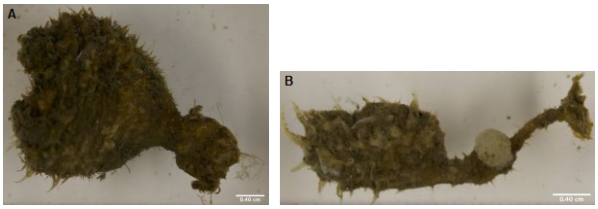

Figure 2: Photographs of representative specimens from Washington (A) and Oregon (B). Individuals collected from Washington were recognizable by their large bodies and shorter stalks, and individuals collected from Oregon had small bodies and longer stalks. The Oregon specimen has an epibiont attached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

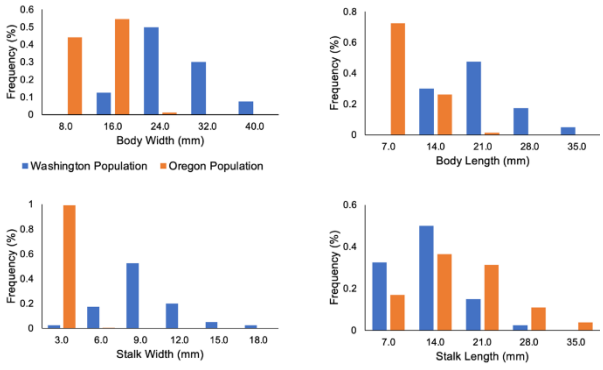

Figure 3: Frequency distribution of each of the four

morphometric variables for the Washington (blue) and Oregon populations (orange). The x-axis houses each variable and the y-axis depicts 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 thinner, shorter bodies and thinner, longer stalks.

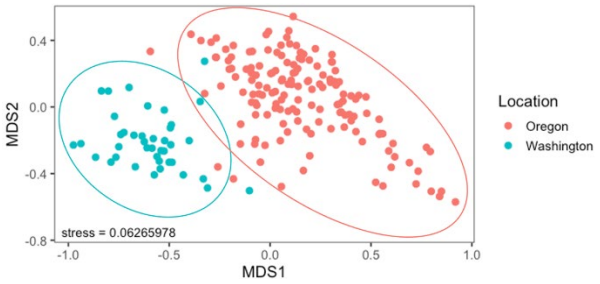

Figure 4: Multidimensional scaling (MDS) plot showing the morphological dissimilarity and similarity of all measured B. villosa specimens. The overall morphological differences due to body length, body width, stalk length, and stalk width between two specimens are visualized by the distances between 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. The low stress value indicates that the plot is a good representation of the data.

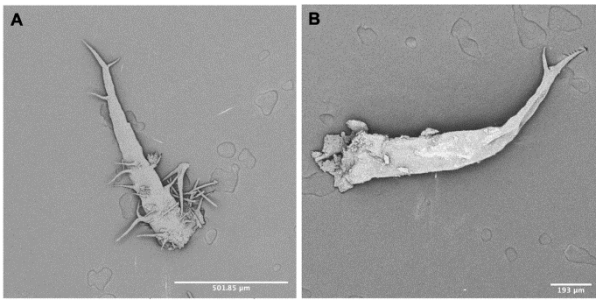

Figure 5: Scanning electron miscopy images of (A) a spine removed from a Washington specimen and (B) a spine removed from an Oregon specimen. There were distinct and significant differences between the spines of the two populations, with the spines from Washington having additional spiny protrusions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

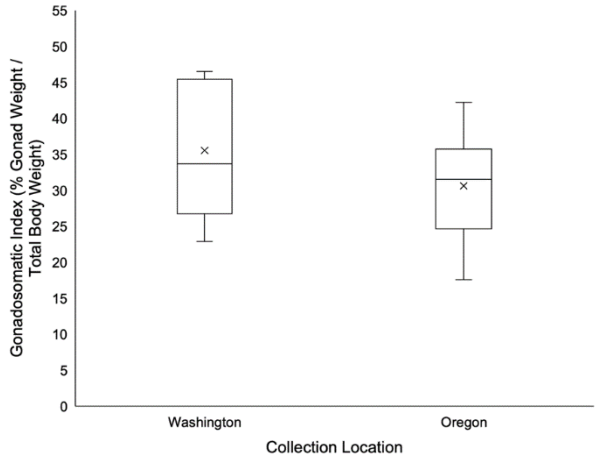

Figure 6: Box and whisker plot of the gonadosomatic indexes (GSI) of individuals collected in Washington and Oregon. The y-axis shows the percentage of gonad weight to total body weight and the x-axis displays the collection site. The whiskers of the plot represent the upper and lower 25% of oocytes,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

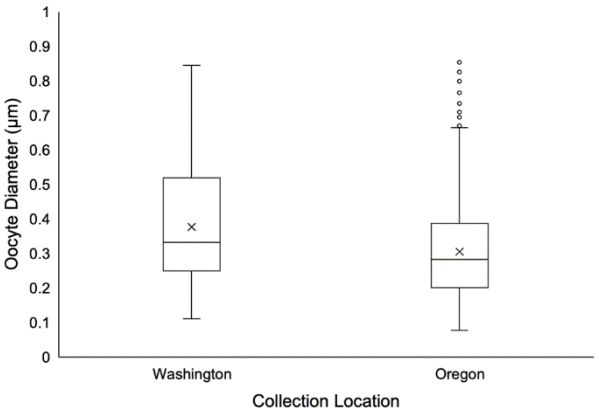

Figure 7: Box and whisker plot of the diameters of the first 100 oocytes from 19 individuals collected in Washington and Oregon. Individuals from Washington tended to have larger oocytes than individuals found in Oregon. A 2-sample, 2-tailed t-test showed there was a significant difference between the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

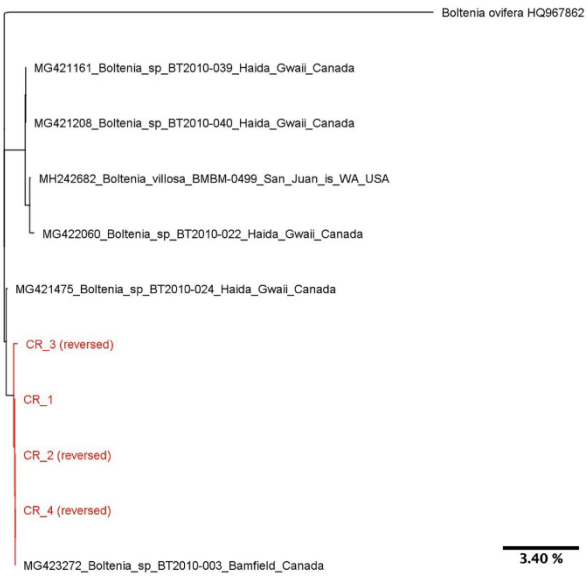

Figure 8: Barcoding region COI tree for *B. villosa* specimens submitted to GenBank. Specimens from Oregon (red) were sequenced and compared to existing B. villosa entries from Washington and British Columbia (black). B.villosa from Oregon were genetically similar to members of B.villosa found elsewhere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