The Prevalence of the Species: *Mytilus trossulus* and *M.edulis*, *M.galloprovincialis*, and Their Hybrids Advertised as 'Blue Mussels' in the Kitsilano Area

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The purpose of this study is to find the proportion of the three species of mussels, and their hybrids in a collection of thirty-two mussels from four stores around Kitsilano, British Columbia. The three species of mussels advertised as 'Blue Mussels' are: *Mytilus trossulus*, *M.edulis* and *M.galloprovincialis*. *M.galloprovincialis* is an invasive species that out-competes other mussels, potentially affecting British Columbia marine ecosystems. The three species of mussel are indistinguishable phenotypically; however, the species' and their hybrids show distinct banding patterns when a gene for a foot protein is examined. We used the processes of DNA Isolation, PCR, and Gel Electrophoresis to distinguish the proportions of the three mussels. The results of our study were that the mussels labeled as "Salt Spring Island" consisted of 86% invasive *M. galloprovincialis* , while the "P.E.I" mussels consisted of 23% invasive *M. galloprovincialis.*

Introduction

The pacific blue mussel found along the British Columbia coast, consists of three species of mussels: *Mytilus trossulus*, *M.edulis* and *M.galloprovincialis*. These three mussels often hybridize and are often considered a species complex rather than individual species. They are found in the intertidal zone attached to rocky substrate with their byssal threads (White et al., 2014).The *M.galloprovincialis* species is invasive, introduced from the Mediterranean and Atlantic coast of Southern Europe via ships.

These invaders are found worldwide and are considered a nuisance species outcompeting native mussels. *M.galloprovincialis* grows faster, is more tolerant of warmer temperatures, and has a reproductive output of 20%-200% greater than its native counterparts (Global Invasive Species Database, 2017).

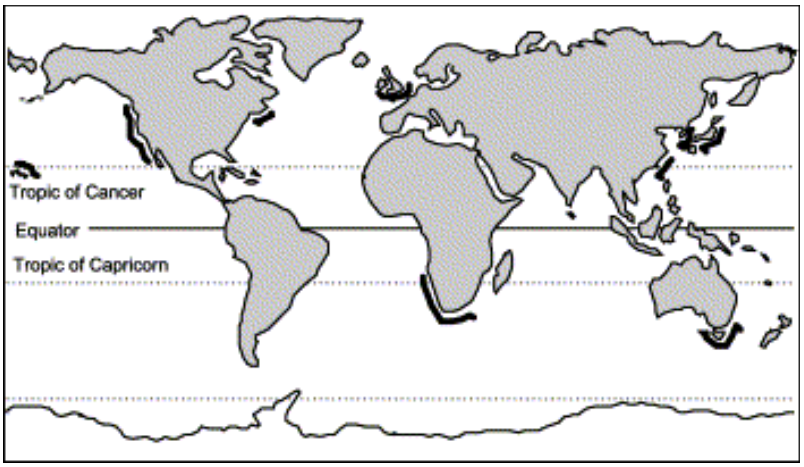


Figure 1: The global distribution of *M.galloprovincialis*. (Taken from Branch, 2004).

Little is known about the impact these non-native mussels have on British Columbia waters. Studies at Langebaan lagoon in South Africa, however, have found that the addition of the invaders result in significant changes to the naturally occurring habitat. The initial sandbank community of the lagoon was replaced with communities more typical of rocky shores (Robinson and Griffiths, 2002).The *M.galloprovincialis* shell is larger and harder than the *M.trossulus'* shells, possibly making it harder for predators to open them (Anderson et al., 2002).The invaders may also displace other benthic organisms by taking up the hard substrate, thus changing the composition of natural benthic communities (Branch et al. 2004). It is unclear how the invasion of *M.galloprovincialis* directly affects the salmon of British Columbia, however, some outcomes of their proliferation include: modification to food webs, reduction in native

biodiversity and a modification to the benthic communities of the intertidal zone (Global Invasive Species Database, 2017).

This experiment aims to find the proportion of the three species or their hybrids in a sample of thirty-two mussels from four different locations around Kitsilano, Vancouver. The three species *Mytilus trossulus*, *M.edulis* and *M.galloprovincialis* can only be distinguished through genetic testing as they are morphologically identical. The stores were unaware of the species of mussel they were carrying beyond 'Blue mussel' and the harvested location (either Salt spring or PEI).The mussel species' have different genes for an adhesive foot protein involved in making the byssal threads that allow the mussels to attach to substrate (Inoue et al., 1995).The invasive *M.galloprovincialis* has a deletions in its' gene creating a shorter fragment when amplified and run using 3% agarose gel (Wimberger & Rudensey). Using primers Me15 and Me16, *M. Trossulus* band shows up around 168bp, *M.galloprovincialis* band around 126bp and *M.edulis* band at 180bp.The hybrids show several bands depending on their gene composition (Inoue et al., 1995).

Methods

We collected 8 mussels from each of the following 4 locations: Granville Island Longliner Seafood, Granville Island Seafood City, Safeway, and 7seas Fish Market. After collecting our 32 mussels, we measured the length, width, and the height of each mussel as shown in *Figure 2*.

Using tweezers, we took a piece of each mussel about the size of a quarter of the pinky's fingernail, and placed it in a sterile 1.5mL Eppendorf tube. To reduce variation, we took each sample from the mantle of the 32 mussels. Once the mussel

samples were placed in the Eppendorf tubes, we used toothpicks to mash up the samples. We then added 300 µl of “Cell Lysis Solution with Proteinase K” and incubated at 65 °C for 15 minutes. During incubation we vortexed all samples every 5 mins until the solutions looked cloudy. The samples were then placed in ice for 5 minutes, and then we added 150 µl of “Protein Precipitate Reagent” to each. We vortexed them for another 10 seconds to make sure the samples were well-mixed with the reagent. Samples were then centrifuged at maximum speed for 10 minutes. Afterwards, we transferred the supernatants to Eppendorf tubes and discarded the old tubes along with the pellets and the fat layers. Before inverting the tube 30-40 times, we added 500 µl of ice cold isopropanol to each. The samples were then centrifuged for a second time at the maximum speed for 10 minutes. Then, we poured out the isopropanol without disrupting the pellet containing the DNA, and added 500 µl of ethanol to each to rinse out the leftover salts. We repeated the last step another time. Finally, the samples were left out for one week, without the cap on, at room temperature in order to evaporate any remaining ethanol.

For the PCR, we added 2.5 µl 10x PCR buffer to each sample and mixed them well by inverting the tubes. We then made the Master Mix (MM) in an Eppendorf tube. In our Master Mix we added 17µL of 10mM dNTP, 34 µl of 25mM $MgCl_2$, 34 µl of 5’ Primer 10 µM (Me15), 34 µl of 3’ Primer 10 µM (Me16), 170 µl of 50% Glycerol, 391 µl of dH_2O, and 17 µl of Taq polymerase. We added the larger volumes of the reacting reagents first, added Taq polymerase last, and mixed the contents of the tubes well. We added 23 µl of the MM into each labelled PCR tube, and added 2 µl of DNA to the corresponding PCR tube. We also had a control PCR tube, in which we added 2 µl of sterile distilled

water. Throughout the process, we kept the PCR tubes on ice. We then placed the tubes into the PCR machine to amplify our DNA samples for further analysis. The PCR machine was set to:

1. 95˚C for 2 minutes,
2. 95˚C for 30 seconds,
3. 54˚C for 40 seconds,
4. 72 ˚C for 90 seconds,
5. 72 ˚C for 5 minutes.

Steps two to four were repeated 35 times. The samples were then placed in a 4˚C freezer.

For the gel electrophoresis process, we added 10 µl of each PCR sample to a piece of parafilm. We added 2 µl of 6X loading dye to the dot of PCR samples and pipetted them up and down a few times to mix it well. We then pipetted the entire drop and loaded them into the gel, using a 3% agorose gel. Once each sample was loaded into the gel, we ran the gel at 120V for 120 minutes.

Results

Physical characterization:

Eight mussels from each source (Granville Island Longliner Seafood (GL), Granville Island Seafood City (GS), Safeway (S), and 7seas Fish Market (C)) were characterized by phenotype and genotype. S mussels displayed a gold tint with few byssal threads. GL mussels displayed a light beige color, with some a darker orange. Mean and standard deviation of each dimension are summarized in Table 1; dimension definitions are drawn in Figure 1. Collections, not easily differentiated phenotypically, showed variation among mussel replicates and differed significantly in width

(p=0.00015), length (p<0.0001), and height (p<0.0001) in one-way ANOVA tests (df = 3; F = 9.68, 16.66, 15.88).

Table 1: Average (standard deviation) reported in centimeters.

	Width	Length	Height
GL	3.07 (0.22)	6.37 (0.33)	2.17 (0.17)
GS	3.55 (0.31)	6.98 (0.32)	2.87 (0.39)
S	2.97 (0.41)	6.01 (0.62)	2.02 (0.24)
C	2.65 (0.38)	5.17 (0.70)	2.01 (0.30)

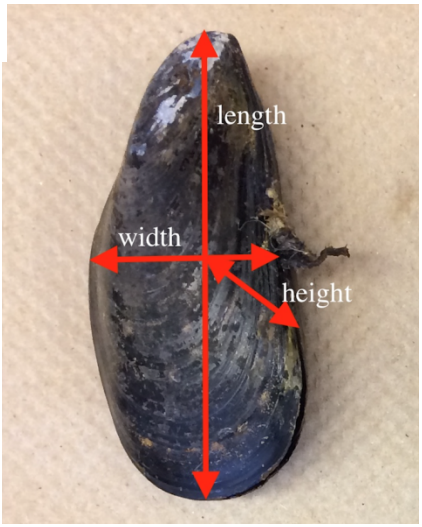
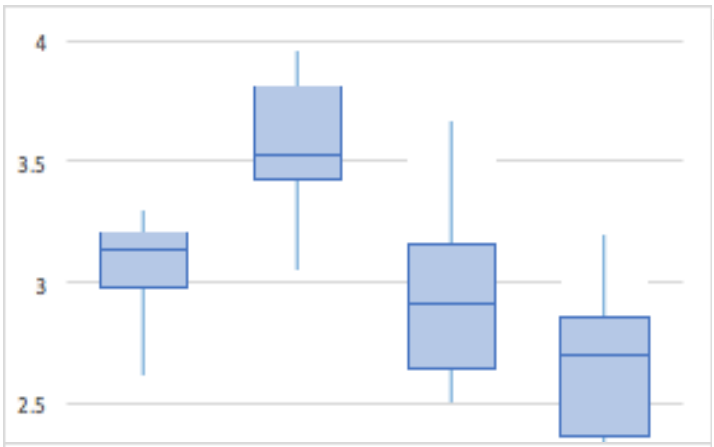


Figure 2: Mussel dimension measurement (shown on specimen sample S1) definitions.

a.)



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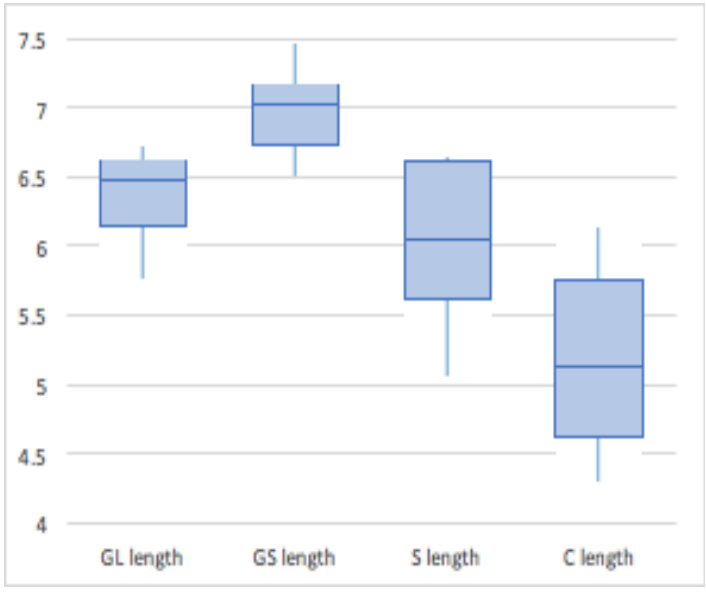

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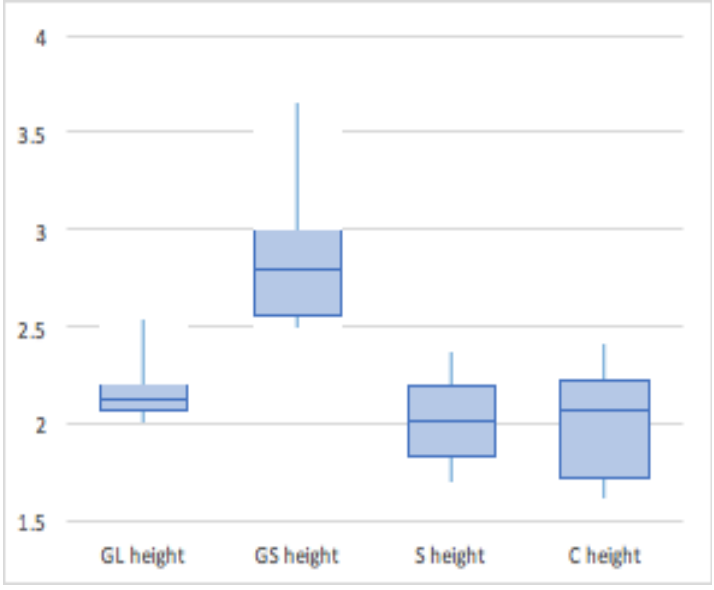

Figure 3: (a) Width (cm), (b) length (cm), and (c) height (cm) of mussels of GL, GS, S, and C sources. Box plots show first and third quartiles, median, with whiskers to largest and smallest measurements (no outliers). Pair-by-pair two-tail t-tests show significant difference (a=0.05) between means except in pair GL-S (all, a-c); in (a) there is also no difference in pair S-C, and in (c) none for S-C or GL-C.

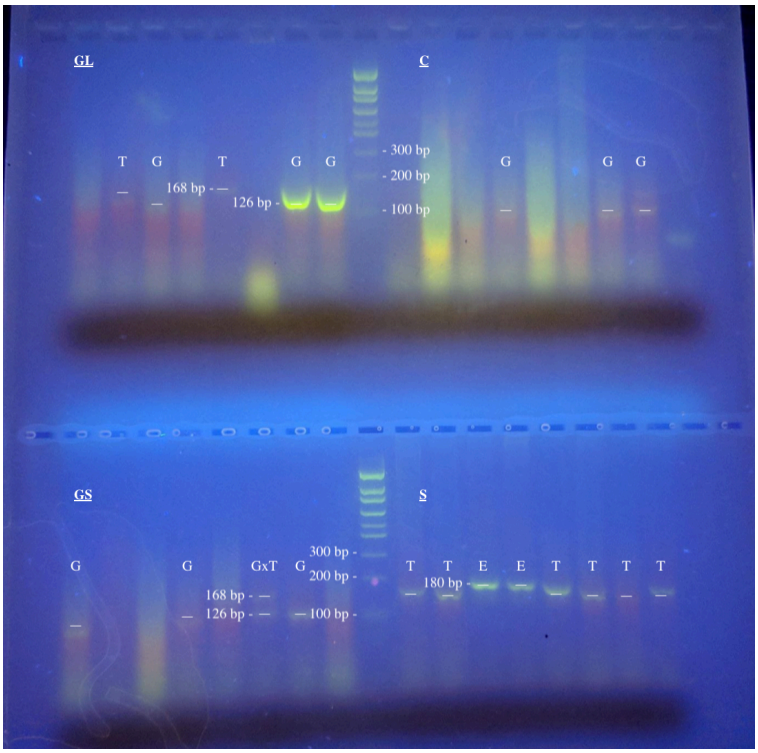

Figure 4: Amplification of the variable region of the adhesive protein gene. Amplified products were electrophoresized on 3% agarose gel. (T: *M. trossulus*, G: *M. galloprovincialis*, E: *M. edulis*)

Genetic characterization

Of the 32 mussels, 20 (62.5%) yielded gel electrophoresis bands classified as species shown in Table 2. Two GL mussels were *M. trossulus* and three *M. galloprovincialis*; in the GS collection, there were three *M. galloprovincialis* and one hybrid with *M. trossulus*; for S mussels, two were *M. edulis* and six *M. trossulus*; C mussels included three *M. galloprovincialis*. Replicates with absent bands, a result of incomplete DNA isolation, were not classified.

Table 2: Species characterization of sourced mussels.

	M. edulis	***M. trossulus***	***M. galloprovincialis***	***M. gallo x M. trossulus***
GL	0	2 (40%)	3 (60%)	0
GS	0	0	3 (75%)	1 (25%)
S	2 (25%)	6 (75%)	0	0
C	0	0	3 (100%)	0
Total:	2	8	9	1

For mussels sold as “PEI” (GL and S), three of thirteen (23.1%) were genetically characterized as the invasive *M. galloprovincialis*; most (eight, 61.5%) were *M. trossulus*, with two (15.4%) additional *M. edulis*. For those sold as Salt Spring Island mussels (GS and C), six of seven (85.7%) were classified as *M. galloprovincialis*, and one as its hybrid with *M. trossulus*. A Fisher’s exact test for association between mussel advertisement as “PEI” or “Salt Spring” and proportion of invasive *M. galloprovincialis* showed significant association (p=0.017).

Discussion

Our findings indicate that despite being labeled as “Blue Mussels”, there is variation in mussel species being sold in the public food markets. Our results from Table 2 may indicate that there is more variation in mussels harvested from PEI. Results also show a greater proportion of invasive *M. galloprovincialis* in mussels harvested from Salt Spring Island than in PEI mussels (p=0.017).

In a study by Tam *et al.* (2014) on two mussel species *M. edulis* and *M. trossulus* in Nova Scotia, Canada and Maine, USA, a similar genetic analysis was used to identify species and characterize their distribution. Similar to our findings of relative species prevalence for PEI mussels, the study found that *M. trossulus* predominated in Nova Scotia while *M. edulis* mussels were rare (Tam *et al*., 2014). Additionally, they found that hybrids were rare or nonexistent, which supports to our results of no hybrids in collected PEI mussels. The same study found that *M. edulis* and *M. trossulus* exhibited different distributions across natural gradients (Tam *et al.*, 2014). Additionally, their results matched a latitudinal survey which found that *M. edulis* are dominant in the south while rare in the north, whereas *M. trossulus* exhibited the opposite pattern (Tam *et al.*, 2014). Furthermore, while both species coexist in sheltered, exposed, and very exposed habitats, *M. edulis* is the only species in very sheltered habitats. This suggests habitats in PEI are exposed, supported by a study of similar habitats in central Nova Scotia where *M. trossulus* were predominant (Hunt & Scheibling, 1996).

A review by Wonham characterizes the population distribution in Chemainus, a community near Salt Spring Island, as consisting of *M. galloprovincialis* and *M. trossulus* mussels, also reporting that nearby Nanaimo and Victoria have hybrid mussels of the two species (Wonham, 2004). This lends support to our study of collected Salt Spring Island mussels, from which we identified one hybrid and six *M. galloprovincialis* mussels. However, our successes in gene isolation and amplification did not include any *M. trossulus* mussels. Furthermore, *Island Sea Farms Inc*. of Salt Spring Island reports that their grown mussels include all three species of pacific blue

mussels and their hybrids, which encompasses a greater diversity than that observed in our amplified products (*Our Mussels,* 2017).

In addition to variation in species between mussel samples, our results showed significant difference in one-way ANOVA for each of width, length, and height when comparing the dimensions of the four different sources of mussels (Figure 3). Statistical significance in pairwise comparisons varies in pair inclusion; all are significant except for GL-S measurements, S-C width and height, and GL-C height. The similarity of 'GL' and 'S' mussels may be attributed to their common advertisement as PEI mussels; however, our results show distinct distributions of genetic species classification. This shows the unreliability of phenotypic distinction between species and the importance of genetic analysis in classifying mussels.

Our experiment yielded twelve mussel samples with unsuccessful gene amplification that may have resulted from contamination, variation in mussel samples, presence of another mussel species and human error. Contamination could have occurred during isolation of DNA and subsequent, brief exposure to the open environment during tube transfer for PCR. Although efforts for consistency were made to enhance reproducibility of results, mussel tissue extraction was not identical across experimenters and between mussels; as a result, specimen contamination from handling prior to purchase may vary. Additionally, presence of the mussel species *M. californianus* within our samples could account for unsuccessful samples as the primers Me15 and Me16 are unable to amplify the genes of this species. Since they are also native in the BC coast, it is possible that they were present in our samples (White *et al., 2014)*. Finally, discrepancies in pipetting technique may have contributed to

experimental error. Future studies ensuring equal numbers of successful samples across those from the four mussel market sources and the two harvest locations would provide valuable evidence to assess the accuracy of our findings.

The distributions of mussel species obtained from public markets could give a picture of the distributions in the natural habitat which could help determine if invasive species are an issue on other species and their habitats.

Conclusion

Despite common labels in public market sales of mussels, there is variation in the species of mussels sold in Vancouver, British Columbia. There is, however, a significant association between labeling as “PEI” or “Salt Spring Island” mussels and the species observed. Of successful samples for labeled “PEI” mussels, 23% were invasive *M. galloprovincialis*; “Salt Spring Island mussels” had a higher proportion (86%) and included an additional hybrid with *M. trossulus*. Phenotypic characterization shows limited distinction between species, but genotypic characterization by amplification of the variable region of mussel adhesive protein gene can be used to differentiate *M. edulis, M. galloprovincialis, M. trossulus* species, and their hybrids.

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City (Granville Island Market), Longliner Seafoods (Granville Island Market) and 7seas Fish Market (2328 West 4th Ave). Lastly, we would like to mention Peter Wimberger and Lyle Rudensey from the Department of Biology at the University of Puget Sound for their work on the Invasive Mussel Project, which provided the inspiration of our experimental design.

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Appendix

ID #	Location bought	Location Harvested	Species found to be	Width (cm)	Length (cm)	Height (cm)
GL1	Longliner Seafood (Granville Island) Some mussels light beige colour, some a darker orange	PEI	NA	3.000	6.400	2.050
GL2			*M. trossulus*	3.250	6.720	2.000
GL3			*M. galloprovincialis*	3.200	6.560	2.530
GL4			NA	3.200	6.655	2.300
GL5			*M. trossulus*	2.620	5.770	2.150
GL6			NA	3.300	6.620	2.070
GL7			*M. galloprovincialis*	3.080	6.130	2.170
GL8			*M. galloprovincialis*	2.925	6.140	2.100
GS1	Seafood City (Granville Island)	Saltspring island	*M. galloprovincialis*	3.200	6.650	2.500
GS2			NA	3.500	6.750	3.150
GS3			NA	3.500	6.500	2.550
GS4			*M. galloprovincialis*	3.050	7.050	3.650
GS5			NA	3.950	7.150	2.650
GS6			*M. galloprovincialis* x *M. trossulus*	3.855	7.455	2.950
GS7			*M. galloprovincialis*	3.555	7.250	2.950
GS8			NA	3.800	7.000	2.550
S1	Safeway (2315 West 4th Ave) Comments:	PEI	*M. trossulus*	3.430	6.640	1.880
S2			*M. trossulus*	2.610	5.060	2.210

S3	Gold tint, few threads		*M. edulis*	3.070	6.645	2.375
S4			*M. edulis*	3.030	6.265	2.145
S5			*M. trossulus*	2.505	5.710	1.795
S6			*M. trossulus*	2.660	5.840	1.840
S7			*M. trossulus*	3.665	6.615	2.195
S8			*M. trossulus*	2.795	5.335	1.700
C1	7seas Fish Market (2328 West 4th Ave)	Saltspring island	NA	3.200	6.125	2.410
C2			NA	2.700	5.125	2.040
C3			NA	2.250	4.300	1.720
C4			*M. galloprovincialis*	2.400	5.150	2.100
C5			NA	3.040	5.700	2.200
C6			NA	2.100	4.300	1.700
C7			*M. galloprovincialis*	2.800	5.920	2.300
C8			*M. galloprovincialis*	2.700	4.740	1.620

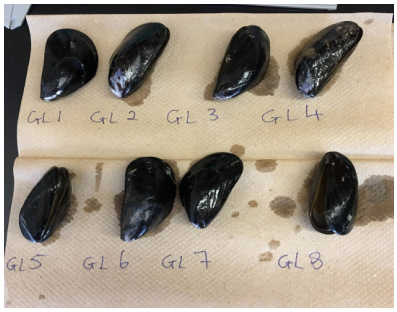

Figure A: Clockwise from top left: GL, GS, S (showing dimension definitions), and C mussels.