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OCCURRENCE AND MECHANISMS OF ONTOGENETIC CHANGES IN BODY COLOUR LIGHTNESS IN INTERTIDAL MARINE INVERTEBRATES

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OCCURRENCE AND MECHANISMS OF ONTOGENETIC CHANGES IN BODY COLOUR LIGHTNESS IN INTERTIDAL MARINE INVERTEBRATES

by

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ABSTRACT

Ontogenetic changes in body colouration are known to occur in some benthic marine invertebrates and have been associated with ecological shifts in diet and physiological tolerance. It is not known, however, whether ontogenetic colour changes are common among benthic marine invertebrate species or which mechanisms control these changes in body colour. This study therefore examines the degree of lightness in body coloration (converted to greyscale); the specific goals were to determine (1) the proportion of species that undergo a change in body colour lightness from a sample of 15 intertidal species, (2) if changes in lightness during ontogeny are associated with changes in microhabitat use, and (3) if diet or exposure to light affects the production of shell pigmentation in the snail *Nucella ostrina*, which are known to hatch white but later add coloured pigments to new shell growth. For each of the 15 species that I examined, 60-140 individuals of varying sizes were collected, weighed, and their body lightness index was quantified using digital imaging. Six motile species were then analyzed for a microhabitat shift during ontogeny. Of the 15 species studied, 11 underwent some degree of change in body colour lightness during ontogeny. Six of those 11 species had a change in body colour lightness of at least 10% between the 10 smallest and 10 largest individuals. The study of microhabitat use by six motile species revealed only one (a hermit crab) that substantially changed microhabitat use during ontogeny; two species partially changed microhabitat use during ontogeny, one other species showed no change, and results were inconclusive for the last two species. The last experiment revealed that *N. ostrina* hatchlings raised in the dark remained significantly lighter in colour than those exposed to light and that diet did not appear to impact shell colour, indicating that the production of dark shell pigmentation in this species is stimulated by exposure to bright sunlight.

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INTRODUCTION

Adaptive significance of body colour in marine invertebrates

Colouration plays a key role in survival for many invertebrate species. It can aid in camouflage, warning colouration, and displaying the reproductive readiness, and temperature regulation for many species (Wichsten, 1990; Bandaranayake, 2006). In particular, colouration and its role in predator avoidance is a strong selective force placed on marine invertebrates such as hermit crabs, snails, and shore crabs (Booth, 1990; Gosselin, 1997; Bandaranayake, 2006). If individuals, particularly juvenile individuals, are not well hidden, then they are at a higher risk of being eaten. This pressure likely selects for the most successful body colouration that allows for the most protection, resulting in adaptations such as aposematic, cryptic, camouflage, or disruptive colouration. Aposematic colouration is when the individual has bright colouration such as red, green, and yellow, which indicate to their predators that they are toxic. Disruptive colouration is body patterns that break up the outline of the body. Overall, colour affects many aspects of an invertebrate's life, and can either assist in its survival and reproduction, or impede it. Below I give some examples of the significance and ecological importance of colouration in marine invertebrates. Furthermore, I mention past examples of ontogenetic colour change found in marine invertebrates, and why a study such as this one is important.

Ontogenetic colour change is a change in body colouration during the lifetime of an individual (Booth, 1990; Bandaranayake, 2006). As individuals grow in size, their vulnerability to predation changes and generally decreases (Booth, 1990). To avoid predation, many juvenile animals utilize mechanisms such as mimicry, camouflage, disruptive, and aposomatic colouration to survive (Booth, 1990; Palma & Steneck, 2001; Bandaranayake, 2006; Krause-Nehring et al., 2010). Mimicry is often found among marine vertebrate species such as Red Sea Blenny and snapper fish, rather than invertebrates (Wickler, 1968; Dafni & Diamant, 1984; Booth, 1990). However, mimicry has also been seen in some shrimp species (Hacker, 1991). Camouflage and disruptive colouration is common among shrimp, lobsters, and crabs, and has been studied extensively (Palma & Steneck, 2001; Tlusty, 2009; Manriquez, 2009; Krause-Nehring et al., 2010). Aposomatic colouration is seen in opisthobranchs, also known as sea slugs; this warning colouration advertises to predators that the organism produces secondary metabolites which are highly

toxic (Cortesi & Cheney, 2010). These uses of body colouration for protection against predation are generally heritable; however, environmental factors can influence the onset and intensity of body colouration (Booth, 1990; Palmer, 1984; Bandaranayake, 2006).

The effects of body colour on temperature regulation may be more profound in terrestrial animals which are exposed to extreme variations in temperature on a daily and seasonal basis, as opposed to marine species (Booth, 1990). Due to the greater thermal inertia of water, the ocean tends to maintain a consistent temperature range, such that colour likely has no significant effect on temperature regulation in subtidal marine invertebrates. However, in intertidal species, such as the snail *Littorina keenae,* which can be exposed to high and low tide and therefore marine and terrestrial conditions, its shell colour is found to change the internal body temperature by 0.5-2.5˚C (Miller & Denny, 2011). This is due to body colouration influencing the amount of solar radiation that is absorbed (Kettlewell, 1973; Burtt, 1981). Moreover, as body size increases the surface area to volume ratio decreases; larger individuals may therefore be able to maintain their body temperature more effectively than small individuals, and smaller individuals may rely on colouration to help them avoid extreme temperatures or desiccation (Booth, 1990). The changes in body size or location in the intertidal zone (and exposure to extreme temperatures) could therefore be cues triggering body colour change in some marine invertebrate species.

Using colouration for mate attraction is common in many marine and terrestrial species, particularly fish and birds (Booth, 1990; Bandaranayake, 2006). For example, gravid females of the three-spine stickleback (*Gasterosteus aculeatus*) consistently chose males with redder nuptial colour to mate with when given a choice (Braithwaite & Barber, 2000). It is hypothesized that this red colouration is an indicator of male quality: the redder they are the better they are (Braithwaite & Barber, 2000). Colouration has also been found to affect how female fiddler crabs, *Uca mjoebergi,* identify potential conspecific male mates (Detto, 2007). Therefore, body colouration can provide important information on a potential mate's species identity, reproductive status, and health condition (Booth, 1990).

Colour change in marine invertebrates

During ontogeny, a marine invertebrate may change its habitat and exposure to the elements and predators and will need to adapt to survive; one adaptation is to undergo ontogenetic

colour change (Booth, 1990; Bandaranayake, 2006). Throughout ontogeny, other changes may occur that have an effect on colouration of a species such as changes in diet, exposure to environmental conditions, predators, and reproductive status (Booth, 1990; Bandaranayake, 2006). Although some marine invertebrates are known to undergo ontogenetic colour change, the majority of research on ontogenetic colour change has focused on terrestrial species. Some examples of marine invertebrate species known to undergo ontogenetic colour change include the red rock crab (Krause-Nehring, 2010) and European green crab (Palma & Steneck, 2001). Early juveniles of these species have a variety of colour morphs, which are eventually replaced by a monochromatic adult carapace (Todd et al., 2009). This ontogenetic colour change is correlated with a change in predators and habitat (Todd et al., 2009). Ontogenetic colour change has also been reported in snails (Hacker, 1991; Gosselin, 1997), lobsters (Tlusty, 2009), and shrimp (Hacker, 1991; Manriquez, 2009). The frequency of occurrence of ontogenetic colour change across marine invertebrate species has never been documented. Understanding the role of body colouration for marine invertebrates, as well as the cues and mechanisms behind colour change, is important because it gives insight into the ecology of the species, their life cycle stages, and evolutionary history.

Mechanisms of colour change

Colouration in hard bodied animals can be obtained in three ways: (1) metabolic formation or ingestion of pigments that are stored in the body structures, (2) formation of structural colours through ridges, bumps and striations, and (3) a combination of the first two (Kennedy, 1979). It is important to realize that the formation of structural colour does not involve pigmentation; rather, it involves the reflectance and absorption of different wavelengths of light in ways that cause a perception of colour (Kennedy, 1979). Pigments produced through metabolic pathways may either be directly responsible for colouration or indirectly by being produced as secondary metabolites and excreted into outer body structures (Bandaranayake, 2006). The type of pigments and how these pigments are incorporated into body tissues and shells of marine invertebrates varies tremendously. For example, colouration in lobsters is determined by the amount and location of deposition of the carotenoid pigment astaxanthin (Tlusty et al., 2009). The changing deposition pattern allows for variation in colour among lobsters but also over the lifetime of one individual.

Background colour and exposure to light affects this deposition of pigments and therefore the colour (Tlusty et al., 2009). Crustaceans have specialized cells called chromatophores, which contain pigments, and control their body colouration through blood-borne substances such as hormones (Fingerman & Couch, 1967). Furthermore, crustaceans moult regularly when they outgrow their exoskeletons, and thus changes in colour can occur rapidly (Mellville-Smith et al., 2003). Snails and mussels, on the other hand, incorporate polyenes, carotenoids, and porphyrin pigments into their shells gradually and ontogenetic colour changes can be seen in the areas of early shell growth (Comfort, 1951; Hedegaard et al., 2005; Furuhashi et al., 2009).

The physiological mechanisms controlling color change are not well understood, but it is known that colouration can be influenced by diet, environmental cues such as light and background colour, and can also be influenced by gene expression (Brake et al., 2004; Bandaranayke, 2006). Exposure to light and UV radiation has been found to influence body colouration in species of lobsters (Tlusty, 2009) and shrimp (Manriques, 2009; Hacker, 1991). Exposure to waves also affects body colouration, particularly in snails (Etter, 1988). To date, little evidence indicates that diet influences colouration of hard outer body structures such as exoskeletons and shells. Understanding why a species undergoes a colour change and the mechanisms behind these changes helps us to understand the underlying significance of ontogenetic colour change (Booth, 1990).

A correspondence between ontogenetic habitat changes and colour changes has been found in snails (Gosselin, 1997), lobsters (Anderson et al., 2013), crabs (Krause-Nehring, 2010), and shrimp (Hacker, 1991). These studies reveal that species change both their habitats and body colour during ontogeny; however, they do not reveal any common cue or mechanism between the species that could be responsible for initiating colour change. For the snail species *Nucella ostrina*, the ontogenetic changes in habitat and colour occur at approximately the same body size (Gosselin, 1997), suggesting that during the transition between habitats, a cue initiates a colour change.

Objectives of study

This study aims to examine the occurrence of ontogenetic changes in lightness of body colouration among benthic marine invertebrates and to determine the cues and mechanisms responsible for initiating these changes. Understanding the differences and similarities

among species that undergo an ontogenetic colour change will provide insight into their ecology and to the functions of colour change for marine invertebrates. Specifically, this study aims to (1) determine the proportion of species that undergo ontogenetic changes in body colour lightness by examining 15 species of intertidal invertebrates from three different phyla, (2) determine if colour lightness change is associated with habitat change in motile species, and (3) determine if colour lightness change can be influenced by environmental factors such as diet and exposure to light in the intertidal snail *Nucella ostrina.*

MATERIALS AND METHODS

The collections and the experiment described below were carried out at the Bamfield Marine Science Centre near the town of Bamfield, BC. Collections were carried out at nearby field sites within Barkley Sound on the west coast of Vancouver Island, and occurred during the months of June, July and August 2012. See Appendix B for latitude and longitude coordinates of sampling sites. For this study, body colour was assessed as a grey-scale value from 0-255 (0 being black and 255 being white), these values are referred to as body colour lightness index values and are not a direct measure of colour. Ontogenetic colour measurements mentioned in the methods, results and discussion refer to body colour lightness index values as determined by this study.

Proportion of species that undergo ontogenetic changes in colour intensity

Fifteen species of intertidal benthic invertebrates were collected to examine the proportion of species that undergo an ontogenetic colour change (Table 1). These 15 species were chosen based on their abundance and because they represented species from three separate phyla. The study organisms included six snail species *(Lirabuccinum dirum, Littorina scutulata, Littorina sitkana, Nucella lamellosa, N. canaliculata, Tegula funebralis),* one vermetid gastropod species (*Petaloconchus compactus*), one bivalve species (*Mytilus trossulus*), two barnacle species (*Balanus glandula and Chthamalus dalli),* two hermit crab species (*Pagurus granosimanus, P. hirsutiusculus),* one crab species (*Petrolisthes cinctipes*), and two tube worm species (*Serpula columbiana, Spirorbis bifurcates)*(Table 3). It is important to note that although care was taken to properly identify each of these species, there is a species of hermit crab that is nearly identical to *P. hirsutiusculus* (Kee Ng & McLaughlin, 2009).

To determine if body colour lightness changes as individuals grow, animals of a broad range of sizes were collected for each species, from the smallest to the largest available individuals, over a period of two months (Table 1). All animals were collected by hand, and each animal was then placed in a plastic container; small animals in small containers (approximately 10 x 10 x 5 cm), and large individuals in larger containers. Each individual was then brought back to the laboratory and placed in a tank with flowing seawater until processing could occur. Each container had at least two cut-out sides covered by mesh to ensure sufficient water flow when placed in a seawater tank.

Table 1: Location and number of individuals collected from each species for size vs colour analysis

Animal	**Species Name**	**Number Collected**	**Location of Collections**
Snail	*Lirabuccinum dirum*	134	Scott's Bay
	Littorina scutulata	112	Scott's Bay
	Littorina sitkana	139	Scott's Bay, Robber's Pass, Fleming Island
	Nucella lamellosa	111	Ross Islets, Grappler Inlet
	Nucella canaliculata	87	Prasiola Point
	Tegula funebralis	126	Scott's Bay
Vermetid gastropod	*Petaloconchus compactus*	80	Grappler Inlet, Dixon Island
Mussel	*Mytilus trossulus*	97	Scott's Bay
Barnacle	*Balanus glandula*	101	Scott's Bay
	Chthamalus dalli	92	Scott's Bay
Hermit crab	*Pagurus hirsutiusculus*	95	Scott's Bay, Grappler Inlet
	Pagurus granosimanus	92	Scott's Bay, Grappler Inlet
Crab	*Petrolisthes cinctipes*	93	Scott's Bay
Tube worm	*Serpula columbiana*	59	Dixon Island, Entrance to Grappler Inlet
	Spirorbis bifurcates	83	Dixon Island

Within 48 h of collection, individuals were removed from the seawater tank, blotted dry, and weighed using a digital scale to the nearest 0.001 mg for small animals (between 0.001-1000 mg) and to the nearest 0.01 mg for larger animals (>1000 mg). Hermit crabs were removed from their shells before being weighed; to accomplish this, all hermit crabs were euthanized by freezing, then carefully removed from their shells, rehydrated in salt water for two

minutes, blotted dry, and weighed. Following weight measurements, the body length of each individual was also measured; these measurements were then used to determine the relationship between body length and weight by linear regression analysis. These regression analysis were used to determine body mass of individuals from each species that may have been referred to in other reports, for example, if a report stated that a snail species undergoes ontogenetic colour change at approximately 3 mm shell length (SL), I could use my regression analysis to determine the approximate body weight. These regression equations are shown in the Appendix C. The following size dimensions were measured: shell lengths of snails from front of aperture to tip of apex; shell diameter of barnacles; length of right claw of hermit crabs; the carapace width for crabs; diameter of tube opening for tube worms; and shell length of mussels.

Each individual animal was then photographed under a dissecting microscope with a top-mounted digital camera (Olympus Model QCOLOR5). One photograph per animal was taken along the area of the body with the newest shell or body growth, and colour intensity from these areas was quantified. Photographs of snails were taken near the lip of the aperture; photographs of barnacles were taken at the base of their shell; photographs of mussels were taken at the margin of their shells; and photographs of tube worms were taken at the margin of the aperture. For crab and hermit crab species, the colour of their entire carapace and right claw, respectively, were analyzed. For individuals of the crab species *Petrolisthes cinctipes* that were too large to photograph in totality under the dissection microscope, various regions of the carapace were captured in numerous photographs (4-7 photographs depending on size), analyzed separately, and then averaged.

All photographs were analyzed for body colour lightness values using Adobe Photoshop Elements 2.0 similar to the methods used by Hultgren and Stachowicz (2008) and Tlusty (2005); however, instead of analyzing red, blue, and green values I used lightness index values on a grey-scale. Each photograph was converted to grey-scale to enable comparisons among species and to reduce the effects of light reflection that could alter color intensity readings. The area of newest shell or body growth in each photograph was then selected in Adobe Photoshop Elements and analyzed. This software then creates a histogram of average lightness index of the pixels in the selected area, with values ranging from 0-255, with 0 representing black and 255 being white. The body colour lightness index value was used to

represent the individual's body colour. Finally, the lightness value of each individual was plotted as a function of body mass to visualize ontogenetic trends in body lightness. Finally, a comparison of the body colour lightness index values between the 10 smallest and 10 largest individuals for each species was done to determine the percent change in lightness or darkness during ontogeny. The species were divided into three categories; (1) no change in body colour lightness, (2) change <10%, and (3) change ≥10%.

Published reports of the smallest size at settlement or hatching were found for 11 of the 15 species. For 9 of these 11 species I was able to find and collect the smallest individuals available. However, for two species (*N. canaliculata and T. funebralis*) the smallest known individuals were not available for collection at the time of this study. To my knowledge, the size at hatching or settling is not known for the other four species (*L. scutulata, P. compactus, S. columbiana,* and *S. bifurcates*); it is therefore not clear if my samples include the full range of sizes for these species.

Relationship between microhabitat change and changes in body colour lightness

Six species from the first part of the study (see above) were further investigated to determine if body colour lightness shifts are specific to species that undergo ontogenetic shifts in microhabitat. Species were chosen on the basis of being motile, and therefore capable of changing their distribution during ontogeny. These included: four snail species (*L. dirum, T. funebralis, L. scutulata, L. sitkana*), and two hermit crab species (*P. granosimanus* and *P. hirsutiusculus*). These species were collected from Scott's Bay and Grappler Inlet. Habitat use by other motile species investigated in the first objective, including *N. lamellosa, N. canaliculata,* and *P. cinctipes* was not examined due to time constraints.

Habitat use was assessed differently for snails and hermit crabs. For snails, a 10 m transect line was haphazardly positioned in the intertidal zone at low tide, parallel to the water line within the range of intertidal heights occupied by each species. The methods used to determine habitat use are similar to those used by Gosselin (1997). Animals were collected within 25 x 25 cm quadrats that were placed at 2 m intervals along the transect line; all individuals of the given snail species that could be found within the quadrat without disturbing the substratum or algae were collected and placed in a container (Gosselin, 1997). These individuals were considered "exposed". Once all exposed individuals were collected,

debris, rocks, and seaweed were then slowly removed and any individuals that were hidden by these objects were collected; individuals buried in the sand were also collected. These were considered "cryptic" individuals. For each 10 m transect line, a total of five quadrats were analyzed. For the snail species *L. dirum* and *L. scutulata,* three transects and a total of 15 quadrats were analyzed for each species. For each of *T. funebralis* and *L. sitkana,* only one transect with five quadrats were analyzed due to high densities of the species. The above method was modified for hermit crab species. During low tide, almost all hermit crabs of both species were found to be hidden, probably because crabs are much more motile than snails and can therefore travel over relatively large distances and reach shelter within each tide cycle. All microhabitats were therefore examined within each quadrat at low tide and the hermit crabs that were collected were used to document the size frequency distribution of the population. The same habitat was then revisited at high tide and all individuals found crawling on exposed surfaces were collected by standing in one location for 10 min and capturing all individuals that became visible. These two hermit crab species coexist in the same intertidal area. In this way, six different locations were sampled for 10 min each, during which both species of hermit crabs were collected.

All the animals collected as described above were then returned to the laboratory and weighed within the next five hours. Data for each species was then organized according to weight classes (Table 2). The weight classes were determined by the maximum size of the largest adult collected for each species.

Table 2: Weight classes used to determine ontogenetic microhabitat shifts

Species	**Size Classes (mg)**
P. granosimanus	0-20, 21-50, 51-200, 201-500, 501-1000, and 1001+
P. hirsutiusculus	0-20, 21-50, 51-200, 201-500, 501+
L. dirum	0-50, 51-200, 201-400, 401-1000, 10001-2500, and 2501+
T. funebralis	50-200, 201-400, 401-600, 601-1000, 1001-2000, and 2001+
L. scutulata	0-5, 5.1-10, 10.1-15, 15.1-30, 30.1-100, 100.1-200
L. sitkana	0-2, 2.1-5, 5.1-10, 10.1-15

Effect of diet and light exposure on shell lightness index values in *Nucella ostrina*

To determine if the timing of ontogenetic changes in body colour lightness is controlled by external factors, I examined the lightness index values of new shell growth in *Nucella ostrina* in response to different light exposure and diet treatments. First, I collected *N. ostrina* egg capsules from Scott's Bay and Grappler Inlet. Egg capsules were collected only when the young snails appeared close to hatching; that is, when the capsule's membrane plug was dissolved and hatchlings were clearly visible through the opening. These capsules were carefully removed from the rocks to which they were attached using needle-nose forceps. They were then brought back to the laboratory and placed in small plastic containers which had mesh siding to allow water flow while in the seawater tray in the laboratory. Containers were checked every day for newly emerged hatchlings.

All hatchling snails that emerged from the egg capsules over a period of three days were used in the following experiment, carried out in an outdoor seawater tray. The hatchlings were placed in one of four treatments: (1) exposed to light and fed barnacles, (2) exposed to light and fed mussels, (3) kept in the dark and fed barnacles, and (4) kept in the dark and fed mussels. In the barnacle-fed treatments, hatchlings were provided with small rocks colonized by *Balanus glandula* and *Chthamalus dalli*. In the mussel-fed treatments, the hatchlings were fed small (1-3 mm SL) *Mytilus trossulus*, although these may have included some *Mytilus californianus* since the small juvenile stages are difficult to differentiate. These prey species were selected because they are known to be important food sources for newly hatched *N. ostrina* (Gosselin & Chia, 1994, 1996).

The design of this experiment was as follows: 4 treatments X 7 replicate cages per treatment X 10 hatchlings per cage, for a total of 70 hatchlings per treatment and 280 hatchling snails for the entire experiment. The cages consisted of small plastic containers with mesh siding; in addition, all cages for a given treatment were placed in a larger 30 x 20 x 15 cm container which also had mesh siding to allow water flow. The larger containers were then placed in a seawater tank of which half was covered by a sheet of opaque black cloth and the other half was exposed to full sunlight. The hatchlings in the dark treatments were placed in the shaded side of the tank. At the start of the experiment an additional 10 hatchlings from the same group of egg capsules were weighed and photographed to document the initial average size and body colour lightness of newly hatched individuals.

Hatchlings were reared in the above four treatments for 21 d. Every two or three days, each cage was checked and more food was added as necessary. The temperature of the seawater tank was checked daily, and once a week the larger containers were removed for approximately 20 minutes to clean algal growth in the seawater tank. Given that these are intertidal snails, this brief removal from the seawater tank was not expected to negatively affect their health.

At the end of the 21 d study period, all snails were removed from the cages and the number of live individuals was determined. Live individuals were individually weighed and photographed using the same methods as described earlier. The average shell colour lightness was then compared among treatments using a 2 factor ANOVA.

RESULTS

Proportion of species that undergo ontogenetic changes in body colour lightness

An examination of body colour lightness throughout ontogeny for 15 species of benthic marine invertebrates revealed that a number of these species do indeed undergo a colour lightness change during their lifetime. Comparisons of average body colour lightness index values between the 10 smallest and 10 largest individuals that were collected for a given species revealed that 11 species (73%) underwent statistically significant changes during ontogeny (Table 3). These included two barnacles (*B. glandula, C. dalli,*), a hermit crab (*P. granosimanus*), a crab (*P. cinctipes*), four snails (*L. scutulata, N. lamellosa T. funebralis,* and *P. compactus),* a bivalve (*M. trossulus*), and two tubeworms (*S. columbiana, S. bifurcates*). The ontogenetic changes in lightness index values in these species range from 2-29% (Table 3). Four species (*P. hirsutiusculus, L. dirum, L. sitkana,* and *N. canaliculata*) did not undergo a significant change in lightness index values (Table 3).

Among the 11 species that did change in lightness, species varied in whether they were becoming lighter or darker during ontogeny. In four species, adult body colour was darker than in the smallest juveniles: *P. granosimanus, N. lamellosa, T. funebralis,* and *M. trossulus,* whereas adult colouring was lighter than the smallest juveniles in seven species: *B. glandula, C. dalli, P. cinctipes, L. scutulata, P. compactus, S. columbiana,* and *S. bifurcates* (Table 3).

Table 3: Comparisons of the body colour lightness index of the 10 smallest and 10 largest individuals per species. MI=Mean intensity (body colour lightness index value); S=Sessile; M=Motile (during early juvenile life); *=Significant; Bolded species have >10% change in mean colour intensity index

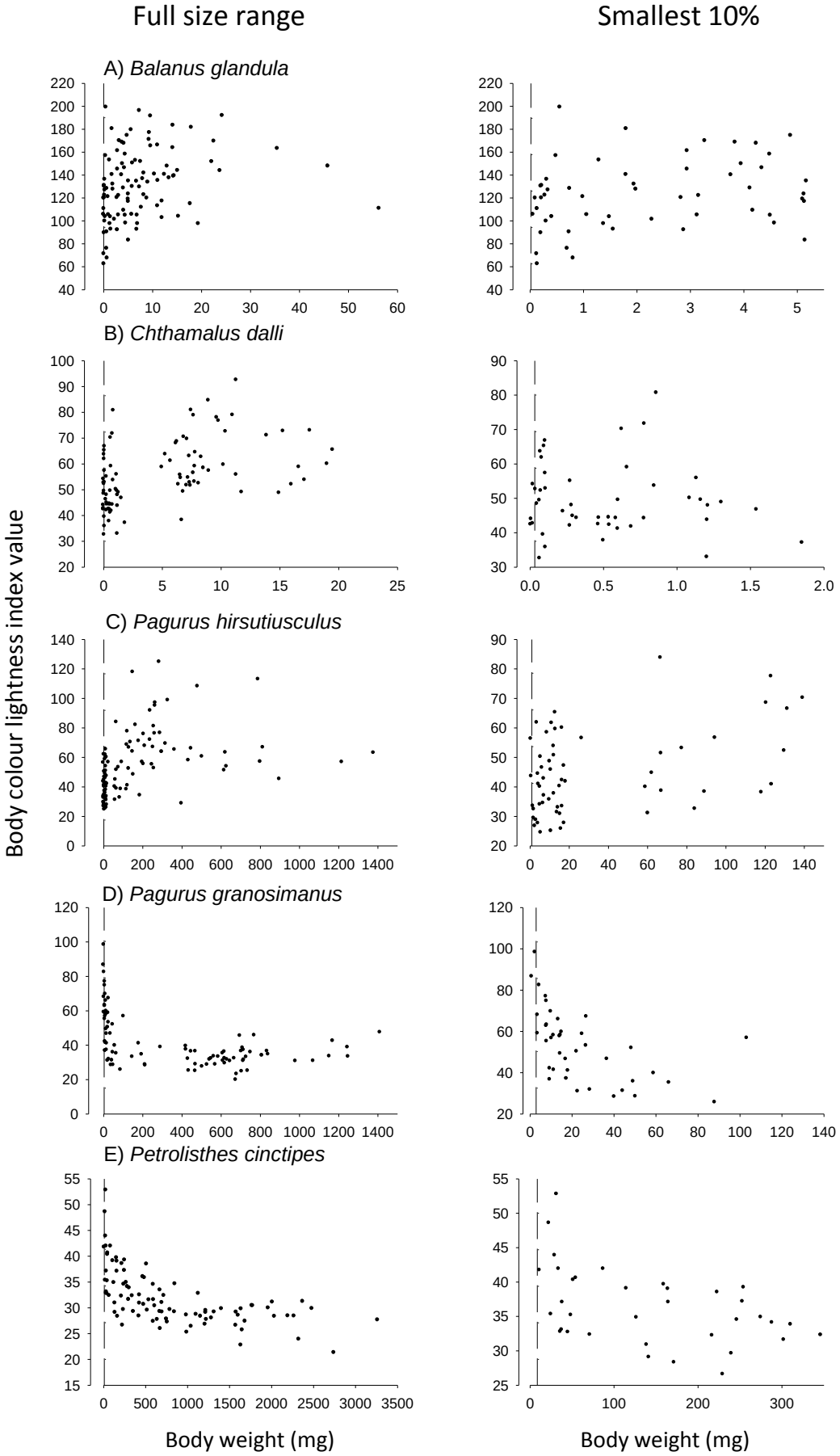
Phylum, Subphylum/Class, Species	Motility	MI of 10 Smallest (± STD)	MI of 10 Largest (± STD)	P-value (t-test)	Difference
Arthropoda					
Maxillopoda					
Balanus glandula	S	106 ± 8	155 ± 10	0.001*	19% lighter
Chthamalus dalli	S	48 ± 3	59 ± 4	0.022*	4% lighter
Malacostraca					
Pagurus hirsutiusculus	M	38 ± 4	63 ± 6	0.171	
Pagurus granosimanus	M	73 ± 4	37 ± 2	<0.001*	14% darker
Petrolisthes cinctipes	M	42 ± 2	28 ± 1	<0.001*	5% lighter
Mollusca					
Gastropoda					
Lirabuccinum dirum	M	45 ± 5	45 ± 3	0.153	
Littorina scutulata	M	26 ± 3	40 ± 3	0.005*	5% lighter
Littorina sitkana	M	36 ± 5	42 ± 6	0.394	
Nucella lamellosa	M	153 ± 6	81 ± 14	0.030*	29%darker
Nucella canaliculata	M	86 ± 10	89 ± 8	0.832	
Tegula funebralis	M	34 ± 2	29 ± 1	0.007*	2% darker
Petaloconchus compactus	S	56 ± 6	118 ± 13	<0.001*	24% lighter
Bivalvia					
Mytilus trossulus	M	62 ± 6	22 ± 4	<0.001*	16% darker
Annelida					
Polychaeta					
Serpula columbiana	S	159 ± 11	182 ± 8	0.005*	9% lighter
Spirorbis bifurcates	S	148 ± 11	196 ± 10	0.004*	19% lighter

Of the 11 species that did have a significant difference in lightness index values, changes greater than 10% were observed in only six species (Table 3). A 10% change (i.e. 25 points on the intensity index) was considered a biologically significant threshold because this amount of change was detectable to the human eye. Changes of less than 10% (less than 25 points) could be detected by the digital imaging equipment, but were too subtle to be apparent to the naked eye.

The most pronounced changes in body lightness occurred in three species: *P. granosimanus* (Fig. 1D), *P. cinctipes* (Fig. 1E), and *M. trossulus* (Fig. 1M). These species exhibit a rapid,

almost exponential, change in colour lightness early in juvenile life and all became darker with increasing body size. However, comparisons between the 10 smallest and 10 largest individuals for *P. cinctipes* showed only a 5% difference in body colour lightness index value (Table 3). The 4 other species which had a change in body colour lightness index value greater than 10%, *B. glandula* (Fig. 1A), *N. lamellosa* (Fig. 1I), *P. compactus* (Fig. 1L) and *S. bifurcates* (Fig. 1O) exhibited a more gradual change in colour lightness. In addition, the three sessile species became lighter in colour with increasing body size, whereas the three motile species became darker with increasing body size.

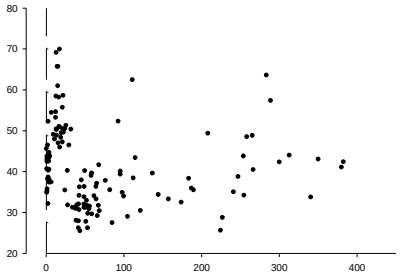
Ontogenetic changes in body colour lightness were not restricted to a single phylogenetic lineage. Each of the 3 phyla included some species that exhibited ontogenetic changes in lightness as well as species that did not change colour lightness (Table 3). Significant colour lightness changes were also recorded in motile as well as in sessile species.

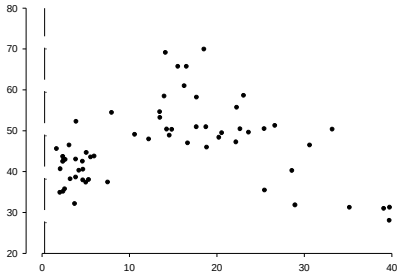

Full size range
Smallest 10%
A) Balanus glandula
B) Chthamalus dalli
C) Pagurus hirsutiusculus
D) Pagurus granosimanus
E) Petrolisthes cinctipes
Body colour lightness index value
Body weight (mg)
Body weight (mg)

Full size range

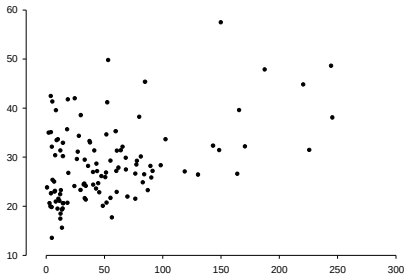
Smallest 10%

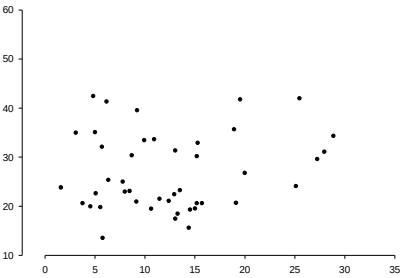
F) *Lirabuccinum dirum*



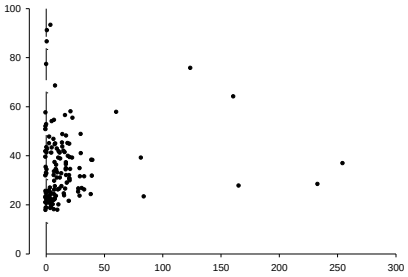


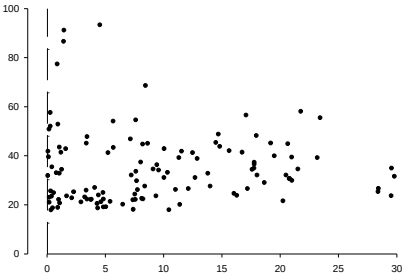
G) *Littorina scutulata*



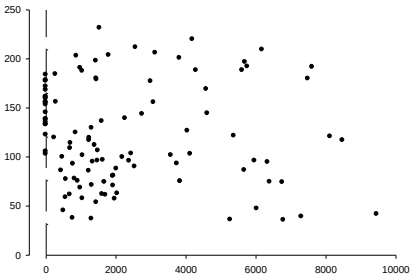


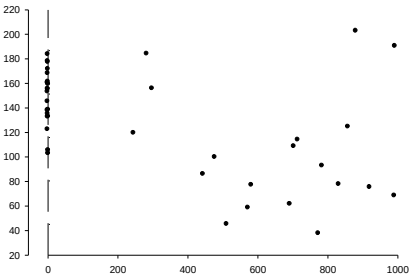
H) *Littorina sitkana*



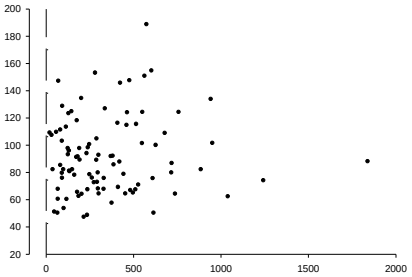


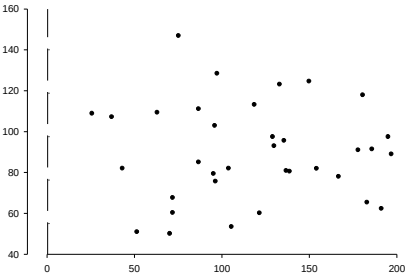
I) *Nucella lamellosa*





J) *Nucella canaliculata*





Body colour lightness index value

Body Weight (mg)

Body weight (mg)

Body Wieght (mg)

Body weight (mg)

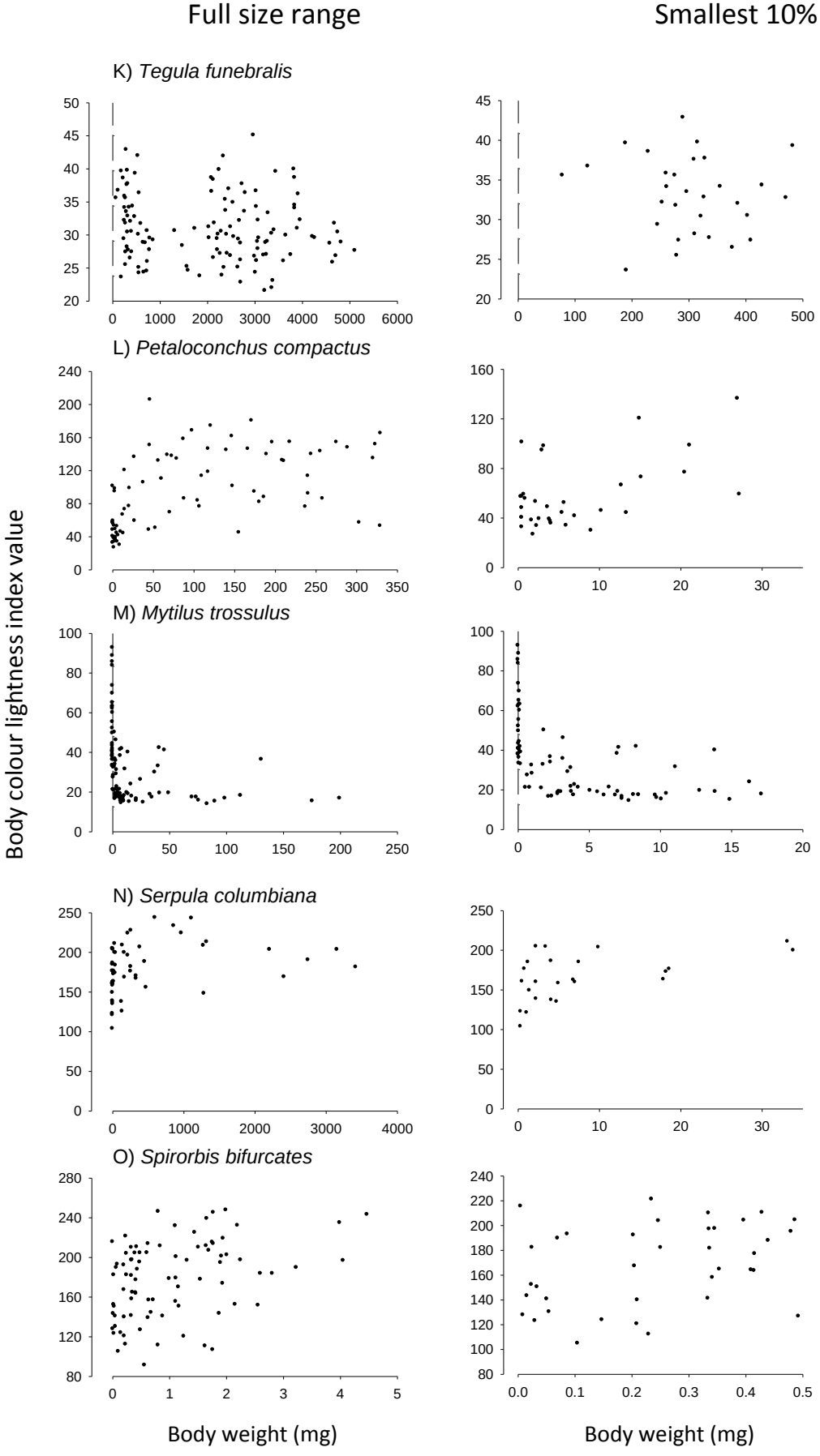

Full size range
Smallest 10%
K) Tegula funebralis
L) Petaloconchus compactus
M) Mytilus trossulus
N) Serpula columbiana
O) Spirorbis bifurcates
Body colour lightness index value
Body weight (mg)
Body weight (mg)

Figure 1: Body colour lightness index value of external body covering as a function as body weight (mg) for each of the 15 species studied. Each species is represented in two graphs; the one on the left shows the full size range of the species (smallest juvenile to the largest adult collected) and the one on the right is an expanded view of the juvenile body weights (first 10% of the entire body weight range) for each species. The vertical dashed line indicates literature values of the recorded hatching or settling size for that species.

Relationship between microhabitat change and changes in colour lightness

For each of the six motile species for which microhabitat use was examined, I compared the size frequencies of cryptic individuals with the size frequencies of individuals in exposed microhabitats. This analysis revealed that only one of these six species, the hermit crab *P. granosimanus*, undergoes a clear microhabitat shift (Figure 2A) during ontogeny. The frequency of cryptic *P. granosimanus* individuals shifted from 100% for the smallest individuals to less than 20% for the largest individuals (Figure 2A). Two other species, *P. hirsutiusculus* and *L. dirum* exhibit partial microhabitat shifts (Figure 2B,C). In these two species, the frequency of cryptic individuals does not change substantially during ontogeny; however, changes between the percent of small individuals that are cryptic to the large individuals that are cryptic ranged from 60% to <20% for *P. hirsutiusculus*, and from 100% to <60% for *L. dirum* (Figure 2B, C).The two other motile species, *T. funebralis* and *L. scutulata*, did not exhibit an ontogenetic change in microhabitat use (Figure 2D, F). In these two species the proportion of cryptic individuals did not increase or decrease substantially with increasing body weight (Figure 2D, F). Finally, for *L. sitkana*, too few individuals could be sampled to determine whether microhabitat use changed during ontogeny (Figure 2E). It is interesting to note that in my microhabitat use surveys, which for gastropod species were carried out at low tide, all size classes of *T. funebralis* were primarily cryptic, whereas all size classes of *L. sitkana* were primarily found in exposed microhabitats.

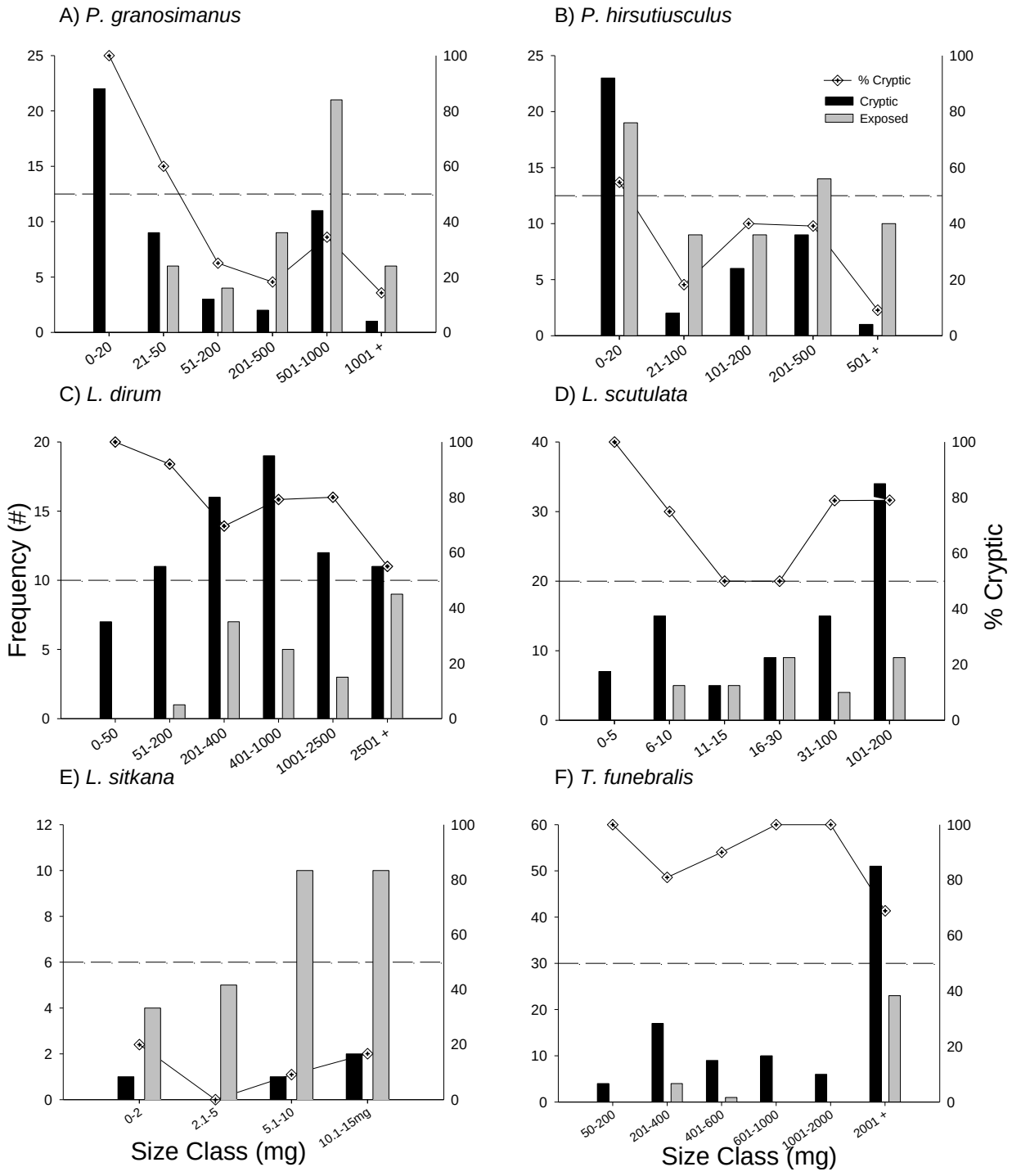


Figure 2: Analysis of microhabitat changes for six motile species showing the number of individuals collected from each category of body size (mg) that were in cryptic or exposed microhabitats. The percent of individuals that were cryptic in each size class is represented by the diamond points connected with the line. The dashed line indicates the threshold of 50% of individuals that are cryptic.

Effect of diet and light exposure on shell colour lightness in *Nucella ostrina*

On day 1 of the experiment 10 additional hatchlings were weighed and photographed. The average weight of those initial hatchlings was 0.422 mg and the average colour lightness index value was 178.63. Over the rearing period of 21 days, there was 34.7% mortality in the experiment and the surviving hatchlings had increased in body weight by 62% on average. Experimental rearing of *Nucella ostrina* hatchlings in two diet and two light exposure treatments for 21 days revealed significant differences in body colour lightness between light treatments but not between diet treatments (Table 4).

Table 4: Two-way ANOVA of body colour lightness index values for *Nucella ostrina* hatchlings placed in light and diet treatments.

Source	***df***	**MS**	**F**	**P**
Light	1	152180	190.73	0.001*
Diet	1	822	1.03	0.312
Light x Diet	1	320	0.40	0.528
Residual	118	798		

The shells of the 21 day old juvenile snails had become much darker in the "exposed to light" treatment than in the "dark" treatment. Body colour lightness index values were 28% lower (71±4 points on the body colour lightness index scale), among snails reared in the "exposed to light" than in the "dark" treatments, regardless of diet (Figure 3,4).

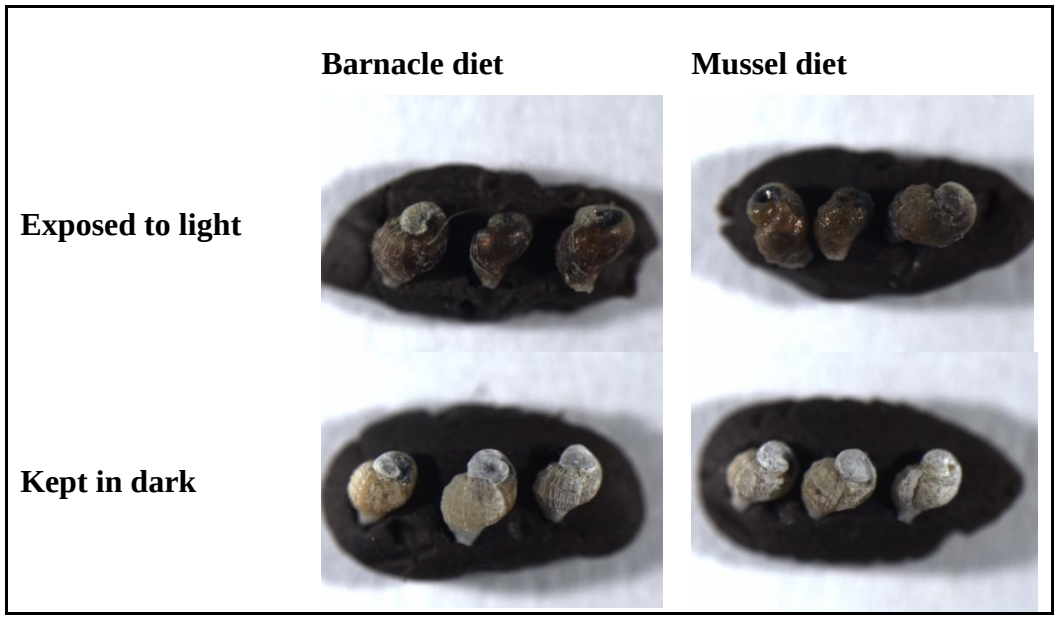


Figure 3: Photographs revealing colouration assumed by snails from each treatment after 21 d.

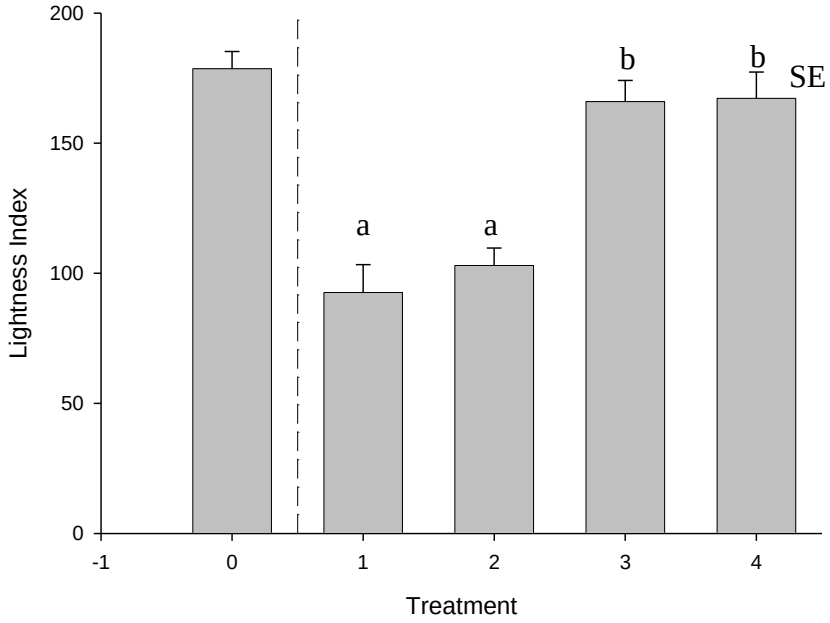


Figure 4: Body colour lightness of new shell growth in *N. ostrina* for each treatment. (0) sample of 10 hatchlings on day 0, (1) exposed to light fed barnacles, (2) exposed to light fed mussels, (3) in dark fed barnacles, (4) in dark fed mussels. ($F=20.37$, $p<0.001$, $N=7$ per treatment). Letters above each bar identify groups of values that are not significantly different based on ANOVA.

DISCUSSION

Proportion of species that undergo ontogenetic changes in body colour lightness

This study has revealed that a large proportion of benthic marine invertebrate species undergo some degree of ontogenetic change in colour lightness. Of the 15 species that were examined in this study, comparisons of the smallest and largest individuals revealed that only four species (27%) do not significantly changes body colour lightness. However, among the 11 species that did change significantly, the degree of colour lightness change between the smallest and largest individuals varied from small changes of only 2% (*Tegula funebralis*) to substantial changes of 29% (*Nucella lamellosa*). Significant colour lightness changes <10% occurred in 5 species (33%) and colour lightness changes ≥10% occurred in 6 species (40%).

In the five species with colour changes <10%, there did not appear to be any common trait among them that might explain the changes in colour lightness. These included motile and sessile species, and four of the five became lighter during ontogeny, but one became darker. Based on my observations of the shells or exoskeletons of these animals, changes in colour lightness that were <10% appear to result primarily from shell erosion or algal growth rather than changes in pigmentation, and therefore may not have adaptive implications (Bandaranayake, 2006).

Previously, casual observations (Gosselin, 1997) had suggested that ontogenetic colour changes might be most common among motile species, and less so in sessile or sedentary species. This hypothesis was based on the fact that motile species are more likely to change their diet, exposure to light and other environmental elements, as well as exposure to predators, than sessile species. My study however, revealed that even sessile species undergo ontogenetic colour change. Of the six species in which mean colour lightness changed by >10%, three were sessile species and three were motile. However, the type of colour lightness change differed between sessile and motile species. All three motile species became significantly darker during ontogeny, whereas all three sessile species became lighter. Sessile species might become lighter during ontogeny because of the process of shell formation and growth. From my observations, the calcareous plates or tubes of newly metamorphosed barnacles, tubeworms, and vermetid snails are not yet fully calcified and so the tissues and rock surface beneath the thin, translucent shell material are thus visible, causing their body colour lightness index value to be lower (i.e. darker) than in their adult form when full

calcification has occurred and the shells (white in the barnacle and tubeworm species, and purple in the vermetid snail) have become opaque. This hypothesis would further explain why the colour lightness changes seem to occur gradually in these species. On the other hand, in the three motile species, it was apparent that the incorporation of dark pigments to their shell or exoskeleton began not long after metamorphosis. In these species the change in colour lightness occurred rapidly over a very short body size interval.

This study purposely included species from three distantly-related phyla (Mollusca, Annelida, and Arthropoda), with the goal of determining if ontogenetic colour changes are phylogenetically constrained. This study revealed that each phylum included species in at least two of the three categories (no change, moderate change, and extensive changes in colour intensity), demonstrating that ontogenetic colour lightness changes are not phylogenetically constrained, and suggesting that ontogenetic colour change may have evolved separately in each phylum.

Relationship between microhabitat change and changes in colour lightness

Ontogenetic changes in colour lightness did not occur exclusively in species that are motile or only in species that change microhabitats. Of the 10 motile species that I studied, six exhibited significant ontogenetic changes in colour lightness, two becoming lighter in colour and four becoming darker. On the other hand, all five sessile species exhibited some significant degree of ontogenetic change in colour lightness, all becoming lighter in colour. Consequently, it appears that ontogenetic colour lightness change occurs independently of motility.

Similarly, not all motile species change microhabitat during ontogeny. This study investigated ontogenetic changes in microhabitat use in six motile species, and only one (*P. granosimanus*) displayed an extensive change, from 100% of small juveniles to less that 20% of large adults occupying cryptic habitats. Two other motile species (*P. hirsutiusculus* and *L. dirum*) displayed partial changes in microhabitat use, from approx. 60% of small juveniles occupying in cryptic environments to less than 10% for *P. hirsutiusculus*, and from 100% of juveniles to less than 60% of adults occupying a cryptic environment for *L. dirum*. One other species (*T. funebralis*) did not undergo any ontogenetic change in microhabitat use and, finally, for two species (*L. scutulata and L. sitkana*) an ontogenetic change in microhabitat

use was unclear. Although not all motile species undergo ontogenetic changes in microhabitat use, it appears that those species that do change microhabitat use also change colour lightness during ontogeny.

The hermit crab species, *P. granosimanus*, had ontogenetic changes in both colour lightness and microhabitat use. These changes in microhabitat use observed in *P. granosimanus* occurred over approximately the same body size range (1-50 mg) as the changes in body colour lightness. This suggests the two changes may be synchronized in this species. As the hermit crab increased in body size it became darker in colour and moved from a cryptic habitat to an exposed habitat.

There is also evidence of a correspondence between ontogenetic change in colour lightness and microhabitat use in other species. Past studies have found that changes in microhabitat use also occur early in juvenile life in *N. ostrina* and *M. trossulus* (Gosselin, 1997; Jenewein & Gosselin, 2013). *Nucella ostrina* undergoes an ontogenetic colour change at approximately 2 mg in body size (Gosselin, 1997) and *M. trossulus* undergoes an ontogenetic colour lightness change between 0 and 3 mg (this study). In both species the size at which these changes in colour (or colour lightness) occur corresponds with the sizes at which microhabitat change occur.

It is possible that being lighter in colour makes small juveniles more inconspicuous among the sand, shells, and rocks among which they live in their cryptic habitat, similar to the white hatchlings of *Nucella ostrina* (Gosselin, 1997). As they increase in body size, however, individuals become less susceptible to predation and therefore colour crypsis may not be as imperative (Palma & Steneck, 2001; Krause-Nehring et al., 2010).

Effect of diet and light exposure on shell colour lightness in *Nucella ostrina*

Shell colour lightness in *N. ostrina* changed in response to light exposure, but not in response to changes in diet. These results suggest that light exposure can act as a cue that initiates ontogenetic colour change, but do not support the hypothesis that diet plays a role in ontogenetic colour change. Light exposure could be responsible for the colour lightness change seen in *N. ostrina* because this species of snail undergoes an ontogenetic shift in microhabitat use, from cryptic to exposed microhabitats, which corresponds to a change in

exposure to light (Gosselin, 1997). In this study, hatchling snails were exposed to broad spectrum natural light (from the sun), and thus to visible, infrared and ultraviolet light.

Exposure to broad spectrum light has also been associated with colour change in other species. Tlusty et al. (2009) discovered that the American lobster, *Homarus americanus*, changes its pigmentation density (becomes darker) when exposed to broad-spectrum light; when specifically exposed to UV light, this colour change was even more more drastic, suggesting that UV light has more of an impact on body colouration than broad-spectrum light. Vividness of body colour in the shrimp, *Litopenaeus vannamei*, also increases with an increased exposure to light (You et al., 2006). Colouration changes in response to light exposure also occur in terrestrial vertebrates such as the Moorish gecko (Vroonen et al., 2012). Etter (1988) found that snails (*Nucella lapillus*) that were located in an area with low wave exposure and high exposure to heat and UV were white, whereas snails at sites with high wave exposure and low exposure to heat and UV were predominantly brown, although, this study did not mention a colour change during ontogeny. These past studies each found that exposure to light (broad spectrum or UV) affected each species differently, in some species high exposure increased pigmentation in their outer body layers, and in some their colour remained white, as seen in the snail *Nucella lapillus* (Etter, 1988). For some species, such as the lobsters and shrimp, increased pigmentation results in increased photo-protection against harmful UV rays (You et al., 2006; Tlusty et al., 2009); however, in snail species, which are protected from the harmful effects of UV by their shells, pigmentation may be more important for temperature regulation (Etter, 1988) or predator avoidance.

Diet did not appear to have any effect on the colour lightness of *N. ostrina* hatchling shells. Past studies examining the effect of diet on colouration of body tissues in other species have found that internal or living tissues are more susceptible to colour changes associated with diet than shell material (Tume et al., 2009). The three sessile species in which shell colour lightness changed by ≥10%, *B. glandula, P. compactus,* and *S. bifurcates,* are filter feeders and feed on plankton. On the other hand, the three motile species exhibiting ≥10% change in colour lightness, *M. trossulus, N. lamellosa,* and *P. granosimanus,* have very different diets: *M. trossulus* is a filter feeder and consumes mostly plankton (Gofas et al., 2001), *N. lamellosa* feeds mainly on barnacles and mussels (Kowalewski, 2004), and *P. granosimanus*

feeds on detritus (Hazlett, 1981). In addition, although several of these species have varied diets, it is unclear whether their diet changes during ontogeny.

Ecological and evolutionary implications

Adult body colour in at least some marine invertebrates is genetically determined (Palmer, 1984). For example, the snail *Nucella ostrina,* exhibits vast variation in colour, banding, and sculpture of its shell. A study done by Palmer (1984) found three distinct colour morphs of this species: black, orange and white, with variation in banding patterns. He found that these colour morphs were determined by an autosomal gene and that black colouration was dominant to orange, orange was dominant to white, and white was recessive to all. He also discovered that the banding pattern was controlled by an autosomal gene, but that it was separate from the colour gene (Palmer, 1984; Palmer, 1985). While Palmer discovered that overall body colouration is determined by genes, the role of genetic control during ontogenetic changes in colour remains uncertain. The occurrence of ontogenetic colour change in virtually all individuals of a given species, as seen in *P. granosimanus* studied here for example, suggests that ontogenetic colour change itself is genetically determined; however, the timing and intensity of colour change within an individual could be controlled by environmental cues. These environmental cues could involve changes in predators, habitats, or background colours during ontogeny.

When an individual relocates to a different habitat, it is likely to be exposed to a different set of predators. Past studies suggest that ontogenetic colour changes may allow for the growing organism to reduce predation risks by adapting its body colour to its surrounding habitat (Bandaranayake, 2006). If an individual undergoes a habitat change during ontogeny, from cryptic to more exposed habitats, it may change its colour from matching its background (camouflage) to a patterned (disruptive colouration) adult form which makes it more inconspicuous to visual predators. For example, in the shrimp *Hippolyte coerulescens,* small juveniles have a solid green or yellow colour whereas adults have translucent banding (Hacker, 1991). The juvenile body colouration effectively blends into the leaves and stems of the algae on which they live, whereas the banded adults are inconspicuous to predators by adopting a disruptive colouration (Hacker, 1991). In both the juvenile and adult colourations, the shrimp is attempting to best match its background colour; as juveniles they are matching

the fronds of the algae, and as adults they are attempting to match the stems of the algae. As well, Todd et al. (2006) discovered that juvenile shore crabs, *Carcinus maenas*, living in macroalgal environments consisting of one colour (monochromatic) had monochromatic carapaces, whereas crabs living in mussel beds (polychromatic backgrounds) had exoskeletons that were polychromatic. Based on these findings, the authors suggested that carapace colour matches background colour; they suggested that disruptive colouration may be beneficial to juvenile crabs to avoid detection by predators. Also, as individuals grow in body size, their ability to blend into their surroundings will change, as observed in some snail and shrimp species (Hacker, 1991; Gosselin, 1997). For example, all juvenile *N. ostrina* hatch from egg capsules with white shells, and small size and white colouration makes them nearly impossible to distinguish from small shell fragments and sand grains, but as they increase in size a solid white colouration could cause them to be more obvious to predators (Gosselin, 1997). Therefore, a change in body colouration likely helps them avoid predation risks (Gosselin, 1997).

Some crab species, such as the red rock crab (*Cancer productus*) and European green crab (*Carcinus maenus*), lose variation in colour pattern when they reach adult size, presumably because adults have fewer predators than juveniles and thus adults no longer benefit from the cryptic patterning of the juveniles (Hannaford Ellis, 1984; Palma & Steneck, 2001). Certain invertebrate species, such as crabs and lobsters, also have a negative relationship between size and predation risk (Hannaford Ellis, 1984; Palma & Steneck, 2001; Anderson et al., 2013). This explains why small individuals often have cryptic and disruptive colouration, which aids in avoiding predation; however, as they grow larger the risk of predation decreases and thus they can afford to forgo cryptic colouration (Hannaford Ellis, 1984; Palma & Steneck, 2001; Anderson et al., 2013).

Changes in microhabitat may also lead to changes in exposure to environmental conditions, such as wave exposure and light exposure, which have been found to affect colouration in some species (Etter, 1988; this study). The effects of UV exposure on body colouration have already been discussed, and thus seasonal colour changes for some species may also occur due to the variation in exposure to UV and broad spectrum light (Auerswald et al., 2008). Although seasonal changes may affect colour in other marine invertebrate species, all of the

species examined in this study were collected during a two month period and thus seasonal changes in colouration are unlikely to have affected our findings.

Future studies

Based on the findings of this study, I suggest that future research examine (A) the occurrence of ontogenetic colour change in subtidal marine invertebrate species, and (B) the occurrence of ontogenetic changes in pigment types in marine invertebrate species. The first suggestion (A) may be addressed by the collection of subtidal species and analysis of their colouration using digital imaging analysis similar to this study. If light is the primary cue triggering colour lightness changes in invertebrate species, then collection of species at depths >10 m should reveal that they do not undergo ontogenetic colour changes because at these depths even exposed habitats only receive very low light intensities. The second suggestion (B) could be addressed by performing similar collections and image analysis as used here, but analysing red, blue, and green hue values to determine if species undergo ontogenetic shifts in primary colours (e.g. from red to blue). Furthermore, elemental and composition analysis of shell materials could provide more information on the types of pigments in the shell or exoskeleton, and comparisons between juvenile and adult shells may show that incorporation of different pigment types may be the physiological process by which colour changes in some species.

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APPENDIX A

Collection site names and latitudinal and longitudinal coordinates

Collection Site	Latitude, Longitude Coordinates
Scott's Bay	48°50.206'N, 125°8.596'W
Robbers Pass	48°53.823'N, 125°7.251'W
Fleming Island	48°52.699'N, 125°9.653'W
Ross Islets	48°52.414'N, 125°9.641'W
Grappler Inlet	48°49.922'N, 125°7.103'W
Prasiola Point	48°49.077'N, 125°10.096'W
Dixon Island	48°51.153'N, 125°7.054'W
Entrance to Grappler	48°50.254'N, 125°8.011'W

APPENDIX B

Regression equations for each species collected. Equations were determined by plotting the cube body length in mm^3 (y-axis) and the body weight in mg (x-axis) for each individual of a species. N=number of individuals; P-value= significance.

Species Name	N	Regression Equation	R^2 Value	P-value
Lirabuccinum dirum	83	y=67.25x	0.9813	<0.001
Littorina scutulata	112	y=3.89x	0.9520	<0.001
Littorina sitkana	71	y=2.41x	0.9809	<0.001
Nucella lamellosa	88	y=7.10x	0.9628	<0.001
Nucella canaliculata	93	y=6.66x	0.9769	<0.001
Tegula funebralis	125	y=1.82x	0.9432	<0.001
Petaloconchus compactus	80	y=0.03x	0.6713	<0.001
Mytilus trossulus	75	y=10.36x	0.9755	<0.001
Balanus glandula	102	y=3.17x	0.9284	<0.001
Chthamalus dalli	94	y=3.72x	0.8926	<0.001
Pagurus hirsutiusculus	47	y=0.51x	0.9451	<0.001
Pagurus granosimanus	78	y=0.18x	0.8665	<0.001
Petrolisthes cinctipes	93	y=0.91x	0.9562	<0.001
Serpula columbiana	56	y=0.05x	0.7512	<0.001
Spirorbis bifurcates	83	y=0.09x	0.1524	<0.001