Selective cnida sequestration in the aeolid nudibranch *Hermissenda crassicornis*: ecology and mechanism of defense acquisition

by

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Abstract

The aeolid nudibranch *Hermissenda crassicornis* (Eschscholtz, 1831) (Mollusca, Gastropoda, Opisthobranchia, Cladobranchia) belongs to a special group of shell-less gastropods that sequester cnidae stolen from cnidarian prey in their cerata (dorsal papillae). Cnidarians produce over 30 morphological types of cnidae (harpoon-like subcellular capsules), a particular subset of which are present in a given species. Cnidae are used by cnidarians for prey capture, defense, and substrate adhesion. The aeolid *Flabellina verrucosa* (M. Sars, 1829) sequesters different cnidae in the presence of seastar and fish predators *vs* no predator, by prey switching, thereby potentially gaining cnida types more apt at combatting that particular predator. I repeated such an experiment with *H. crassicornis*, and found that this species does not switch prey in the presence of predators. I also found that *H. crassicornis* from various locations in Barkley Sound, BC have similar cnida complements. This indicates that prey abundance and predator pressure are either similar at each site, or have no influence on sequestered cnidae. Prior to the discovery of prey switching in *F. verrucosa*, aeolids were assumed to selectively sequester cnidae most useful to them by the dissolution of unwanted cnidae. This hypothesis was based upon observations that aeolids do not sequester all cnida types produced by their prey; but some of these observations were based on the examination of only a single ceras. By collecting several cerata from different locations on the body, I found that rare cnida types – those produced in low numbers by the cnidarian prey – were present in only a few cerata, and may have been missed in previous studies due to small sample sizes. Sequestered cnidae can be switched over to newly collected cnidae within two weeks, but cnida retention time without replacement is unknown. Cnidae are stored

in a functional state within cells (cnidophages) at the tips of the cerata. When attacked by a predator, the aeolids forcibly eject the cells through a small pore, rupturing the cell membrane, and releasing the cnidae. I found that without attack, or ability to replace cnidae from prey, the cells containing cnidae degraded and diminished over time, leaving *H. crassicornis* bare of cnidae after 44 days. With an unlimited supply of cnidarian prey, I found that *H. crassicornis* maintained a constant supply of cnidae in their cnidosacs. These experiments show that *H. crassicornis* does not selectively sequester cnidae under the conditions I exposed them to, and that previous observations of cnida selectivity may have had flawed sampling methods. In *H. crassicornis*, cnidae are replaced to maintain a constant complement of cnidae in the cnidosac, but are not retained indefinitely.

Acknowledgements

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Chapter 1: General introduction to aeolids and their defenses

1.1 General introduction

Nudibranchs of the superfamily Aeolidida (Mollusca, Gastropoda, Opisthobranchia, Cladobranchia), commonly referred to as aeolids, are shell-less marine carnivores that acquire defenses in a remarkable way: by stealing them from their prey. Aeolids feed upon cnidarians (such as anemones, corals, and hydroids), and isolate and store the stinging capsules (=cnidae) (Figure 1.1) produced by these prey. The cnidae are transported within the body to the most distal ends of their dorsal papillae (=cerata, *singular*= ceras) (Figure 1.2) and stored in a functional state in special cavities (=cnidosacs) (Figure 1.3). The cnidosacs are surrounded by muscles, which contract when the nudibranch is disturbed by a predator, forcibly ejecting the cnidae from the ceratal tips (Figure 1.3). This process of cnida sequestration has fascinated biologists since its discovery in the mid-19th century.

The research contained within this thesis adds significantly to previous research, and will hopefully inspire further exploration of this extraordinary process of defense stealing by aeolid nudibranchs.

1.2 History

Stinging capsules within cavities at the tips of the cerata in aeolid nudibranchs were first identified by Hancock and Embleton (1852), and assumed to be produced by the aeolid itself. Based on their description and detailed sketches, Hancock and Embleton (1845)

were likely describing cnidae. Four years later, Wright (1858) commented on the similarity between the cnidae ("thread-cells" (p.38)) in the aeolid *Eolis* (=*Cuthona*) *nana* (Alder & Hancock, 1842) and the cnidae in their hydroid prey, *Hydractinia* sp. Of the more than 30 different types of cnidae produced within the phylum Cnidaria (Mariscal, 1974; Östman, 2000) (Plate 1.1), Wright (1858) found the cnida types to be identical between the aeolids and hydrozoans. He therefore concluded that the cnidae in *C. nana* were somehow acquired from the cnidarian prey. To test this, Wright (1858) switched the prey of aeolid *Eolis drummondii* (now *Facelina bostoniensis,* Couthouy, 1838), and the cnida types produced by the newly available prey species soon appeared in the cnidosacs of the aeolid. However, this conclusive evidence of sequestration of cnidarian stinging bodies was still considered improbable, prompting Wright (1858) to write:

"it was certainly a strange fact...that one animal should be furnished with an apparatus for storing up and voluntarily ejecting organic bodies derived from the tissues of another animal devoured by it, and that these should still retain their destructive functions unimpaired" (p.40)

At the time, Joshua Adler, a world renowned expert on nudibranchs and friend of Wright's, was not convinced, and considered the idea "improbable" (Glaser, 1910, p.120). The idea of cnida sequestering was dismissed for over 40 years, while references to the production of cnidae by aeolids continued (Bergh, 1862; Herdman, 1890). Then in 1903, the research into "thread cells" in aeolids was rekindled by Grosvenor (1903). Grosvenor (1903) also switched the cnidarian prey of two aeolid species (*Rizzolia* (=*Cratena*) *peregrina* (Gmelin, 1791) and *Spurilla neapolitana* (Delle Chiaje, 1841)), and after one month, 98% of the cnidae in the cnidosac were from the new prey species. This pivotal study settled the debate, and all subsequent research on aeolids has included their extraordinary ability to sequester cnidae from their prey. Current aeolid research on

this topic can be grouped into two categories: a) the mechanism of how cnidae are separated, sequestered, and released by aeolids, and b) the evolutionary pressures favoring such an adaptation.

1.3 Cnidae in cnidarians

In cnidarians (*e.g.*, jellyfish, anemones, and hydroids) the sting for which they are well known is caused by subcellular capsules (cnidae) formed in cnidocytes nested among other epithelial cells (Slautterback and Fawcett, 1959). Cnidae are formed by the synthesis of proteins, and strengthened by disulphide linkages in post-Golgi vesicles (David et al., 2008; Engel et al., 2001; Mariscal and Lenhoff, 1969). Most cnidae are rounded structures with spine-covered, inverted tubules (David et al., 2008; Engel et al., 2001; Mariscal and Lenhoff, 1969; review by Ozbek, 2011) (Figure 1.1, Plate 1.1). When triggered to fire, a proton ion transport channel opens, and protons flow out of the capsule (Berking and Herrmann, 2005; Graham, 1938). The resulting negatively charged molecular domains in the capsule wall molecules repel each other, and the pressure within the capsule increases to 7.7GPa, causing the rapid "firing" or eversion of the tubule (Ozbek et al., 2009). The process is exceptionally fast (700 ns) and the firing occurs with an acceleration of greater than $5x10^6$ the acceleration of gravity (Nüchter et al., 2006; Ozbek et al., 2009). These fired cnidae produce physical harm to predators and prey in contact with the cnidae, via the spines that cover the tubule and the venom contained within (David et al., 2008; Kepner, 1943; Mariscal, 1974; Martin and Walther, 2002). Spent or fired cnidae are then shed.

Cnidae evolved in Cnidarians prior to the split between the Anthozoa and Medusozoa, more than 500 million years ago (Collins et al., 2006; David et al., 2008), and they have diverged into over 30 different morphologies among extant taxa (Östman, 2000). The forms are suited for different functions: large spines occur on the tubule of defensive cnidae, and long filamentous tubules aid in prey capture (Edmunds, 1966; Kepner, 1943; Mariscal, 1974). Each cnidarian species produces a particular subset of cnida types (Mariscal, 1974; Östman, 2000). In general, cnidae for prey capture are in the tentacles, defensive cnidae are in defensive structures like acontia and acrorhagi, and adhesive cnidae are on the pedal disc (Mariscal, 1974).

Cnidae are separated into two morphological categories: nematocysts (28 or more varieties) and non-nematocysts (spirocysts and ptychocysts). Nematocysts are the most widely known types, and for this reason the terms "cnida" and "nematocyst" are often used interchangeably. Nematocysts have a double-walled structure and are acidophilic (David et al., 2008; Ozbek, 2011), whereas spirocysts and ptychocysts differ structurally from nematocysts. Spirocysts are produced in the tentacles of hexacorallians (Mariscal et al., 1976). The capsule walls are not strengthened by disulphide bonds (Mariscal and Lenhoff 1969), and they are neither acidophilic, nor basophilic (Weill, 1929, 1934). Ptychocysts are used by burrowing anemones (Cerianthidae) for burrow building. They are basophilic capsules that were only identified as being non-nematocysts in 1977 (Mariscal et al., 1977).

Many researchers identify cnidae following Weill (1934), who characterized 16 cnida types, or Mariscal (1974), who identified 26 different cnidae types including spirocysts (*e.g.*: Frick, 2005; Stachowicz and Lindquist, 2000). Recent investigations have revealed

that the number of cnida types may be over 30 (Östman, 2000). Currently, there is no consensus on the nomenclature of cnidae, so the total number of cnida types is only an estimate (Östman, 2000).

1.4 Evolution of shell-loss

Sea slug is the common name for marine, shell-less gastropods, and although they are superficially similar, shell loss and reduction is believed to have occurred several times in marine gastropods (Medina et al., 2011) (Figure 1.4). Due to their poor fossil record, the adaptive radiation of sea slugs can only be inferred using molecular data and the morphology of extant species (*e.g.*: Klussmann-Kolb et al., 2005; Vonnemann, 2005). This thesis follows a species classification system based upon a combination of molecular trees from Klussmann-Kolb et al. (2005), Medina et al. (2011), Pola and Gosliner (2010), and Vonnemann (2005), and morphological data from Wägele and Klussmann-Kolb (2005). The marine shell-less gastropods are within the Opisthobranchia which is split into five groups. Of these groups, two are comprised of shell-less members: Sacoglossa, the photosynthetic sea slugs, and Nudibranchia (Figure 1.4).

Nudibranchia is a monophyletic taxonomic classification, and the most speciose group of opisthobranchs (Vonnemann, 2005; Wägele and Klussmann-Kolb, 2005). The Nudibranchia have diverged into the Doridaea and Cladobranchia, where Cladobranchia is further split into nine clades, one of which is the Aeolidida (Vonnemann, 2005; Wägele and Klussmann-Kolb, 2005).

The Aeolidida, and its closely related clades Dendrontoidae and Hancockiidae, are similar in form and ecology: they all have dorsal cerata and many feed upon cnidarians (Wägele and Klussmann-Kolb, 2005). But whereas Aeolidida store cnidae, Hancockiidae do not (Wägele and Klussmann-Kolb, 2005). The hancockiidids have glands in the epidermis that may be for the storage of repugnant chemicals, like the defensive dermal pockets seen in other opisthobranchs (Putz et al., 2010). There is some evidence of repugnant chemicals in aeolids, but this has not been studied extensively (Aguado and Marin, 2007; Glaser, 1910).

In both marine and terrestrial lineages of shell-less gastropods, the evolution of alternative defenses must be of comparable effectiveness to the defense provided by a shell, but with presumably lower costs (Medina et al., 2011). Costs associated with producing and maintaining a shell include the acquisition of materials and fabrication of shell matrix and its calcification (Palmer, 1992), and reduced movement or gas exchange over the epidermis (Putz et al., 2010). Shell costs may yield lower metabolic activity (Putz et al., 2010) and reduced fecundity (Geller, 1990), and therefore the reduction or loss of the shell would be favoured, if other defenses have evolved (Faulkner and Ghiselin, 1983; Wägele and Klussmann-Kolb, 2005). Faulkner and Ghiselin (1983) and Wägele and Klussmann-Kolb (2005) concluded that the reduction or loss of shells in the Opisthobranchia occurred in conjunction with the evolution of alternative defenses. Shell-less gastropods have defenses absent in shelled relatives, such as repugnant chemicals, acid glands, and sequestered cnidae (Wägele and Klussmann-Kolb, 2005).

1.5 Morphology of aeolid nudibranchs

The exterior of the aeolid sea slug has four anterior tentacles, a pair of rhinophores, and many cerata (Figure 1.2). The tentacles are smooth, unbranched and extend parallel to the substrate, whereas the rhinophores are rough-looking and extend perpendicular to the substrate at the anterior end of the body. The tentacles are for tactile sensing, and the rhinophores are likely chemosensory structures (Avila, 1998; Kjerschow Agersborg, 1922; Tyndale et al., 1994). The cerata extend posteriorly from the rhinophores along the dorsum to the tail (Figure 1.2). The number of cerata varies by species and recent contact with predators, as cerata can be autotomized when the animal is attacked (Miller and Byrne, 2000; Glaser 1910). The cerata have many functions in aeolids in addition to storing cnidae: they are also the site of digestion and possibly respiration (Hancock and Embleton, 1845; Herdman, 1890).

1.6 Mechanism of cnida sequestration

In aeolids, alimentary diverculae (digestive glands) branch off from the stomach and extend into the cerata (Figure 1.3). The cellular lining of the digestive glands produce enzymes for dissolving and digesting prey tissue, and here the cnidae are isolated from the tissue and transported into the cnidosacs (Cockburn and Reid, 1980). Food enters and leaves the digestive gland by the same passage, and digested tissue continues through to the intestine (Graham, 1938; Martin, 2003).

Aeolids are protected from the firing of cnidae that travel with the food, by a chitinous layer covering the mouth and esophagous (Graham, 1938). At the areas of absorption and secretion, such as the stomach, cells filled with granular chitin line the organs and absorb

the impact of firing cnidae (Martin and Walther, 2002; Martin et al., 2007). Fired cnidae are then egested in the faeces (Martin and Walther, 2002). The cnidae that have not fired, possibly because they are "immature" and are unable to fire (Greenwood and Mariscal, 1984a), are carried through a ciliated canal at the distal end of the digestive gland, and engulfed by phagocytes into the cnidosac (Greenwood and Mariscal, 1984b). The nucleus and other organelles of the phagocyte eventually disintegrate, and the cnidae and surrounding membrane (the cnidophage) remain (Graham, 1938) (Figure 1.30).

It is unclear how long cnidophages remain in the cnidosac, but Glaser (1910) stated that cnidae persisted in the cnidosac of an unnamed aeolid for at least one month. Graham (1938) hypothesized that the cnidophage provides a pH environment similar to the *in situ* cnidocytes of cnidarians, and this prevents cnidae from firing while stored in the cnidosac. As new cnidae enter the cnidosac, older cnidophages are forced to the distal end of the cnidosac, and show evidence of degradation (Martin, 2003).

Cnidae are forced out through the tips of the cerata when the aeolid is attacked by a predator (Edmunds, 1966; Graham, 1938; Grosvenor, 1903). Muscles surrounding the cnidosac contract, and as the cnidophages are squeezed out through a small cnidopore (Figure 1.3), the cnidophage membrane rips to release the cnidae (Edmunds, 1966). The cnidae fire on contact with seawater (Glaser and Sparrow, 1909; Grosvenor, 1903).

Although the long-term storage of cnidae from prey is unique to aeolid nudibranchs, the process of isolating cnidae in the cerata also occurs in at least two species of the genus *Hancockia* (Hancockiidae, sister group to the Dendronotidae) (Martin et al., 2008; Pola and Gosliner, 2010). *Hancockia* spp. have several cnidosacs per ceras, unlike the single

cnidosac per ceras in aeolids, and many of these cnidosacs open to the exterior; closed cnidosacs were completely devoid of cnidae (Martin et al., 2008). The structures associated with cnidae isolation in the *Hancockia* spp. allow cnidae to be egested via the cerata, however the storage of cnida is unique to aeolids.

1.7 Selective cnida sequestration in aeolids

Beginning with Graham (1938) and continuing through the late 20th century, researchers investigating cnida sequestration in aeolids have found that the complement of cnidae in aeolid cerata do not include all the cnida types produced by the cnidarian prey (Conklin and Mariscal, 1977; Day and Harris, 1978; Graham, 1938; Greenwood and Mariscal, 1984b; Kepner, 1943; Thompson and Bennett, 1969). The cause for the difference is unknown, but the two prevailing hypotheses are: 1) some cnidae are too large (Conklin and Mariscal, 1977) or require a different chemical environment (Grosvenor, 1903) to be sequestered; and 2) unwanted cnidae are dissolved or digested (Greenwood and Mariscal, 1984b; Kepner, 1943). The first invokes a mechanical explanation, whereas the second implies selection by aeolids.

Differential sequestration may provide a partial explanation of the observed patterns. Spirocysts, due perhaps to their structural or chemical difference, are not sequestered as often as nematocysts (Grosvenor, 1903; Reft and Daly, 2012; pers. obs.). Spirocysts may be less likely to be sequestered because of their delicate structure and different chemical nature (Weill, 1929, 1934). I can find no record of sequestered ptychocysts in aeolid nudibranchs, or any species that feeds upon cerianthid anemones, the producers of ptychocysts. Cnidae selection is thought to arise because ineffective cnidae are identified

and dissolved or digested (Graham, 1938; Kepner, 1943). This mechanism of cnidae selection has not been confirmed, but is widely accepted (Edmunds, 1966; Greenwood and Mariscal, 1984b; Martin et al., 2008; Thompson and Bennett, 1969).

What would make particular cnidae unnecessary or ineffective in aeolids? Mariscal (1974) characterized cnidae based upon their function in cnidarians, such as defensive, adhesive, and offensive. Frick (2003) suggested that cnidae may be sequestered based upon the predator pressures experienced by the aeolid at that time. Because cnidae are assumed to be sequestered as a defense, selective sequestration of particular cnidae might be a response to the presence of specific predators. Frick (2003) found that the cnidae sequestered in the aeolid *Flabellina verrucosa* (M. Sars, 1829) were different when exposed to fish (compared to no predators) and seastars (compared to no predators). Although Frick (2003) found no evidence of cnida selectivity within the aeolid, she did find that the aeolids switched prey, and thereby acquired different cnidae in the presence of the predators. This is the first evidence of an induced defense in an aeolid nudibranch.

1.8 Induced defenses

Induced defenses are adaptations where an organism alters or augments a defense in the presence of predators. Tollrian and Harvell (1999) concluded that four prerequisites favour the evolution of induced defenses: 1) predators, or predation pressure, must fluctuate, otherwise the defense would be constitutive (fixed); 2) predator cues, whether from sight, sound, scent, *etc*., need to be detectable at an appreciable distance, giving enough time for a defensive change to occur; 3) the defense must be effective; 4) the

defense must be costly, otherwise it again would be constitutive. Induced defenses in plants, animals, and protists all fit within these parameters (Tollrian and Harvell 1999).

Selective uptake of cnidae seems to occur in some aeolids (Conklin and Mariscal, 1977; Day and Harris, 1978; Graham, 1938; Greenwood and Mariscal, 1984b; Kepner, 1943; Thompson and Bennett, 1969), although not in all aeolids examined (Martin and Walther, 2002; Martin, 2003). However, these apparent differences may have been caused by different methodologies. For instance Day and Harris (1978) state that only one ceras per individual nudibranch was sampled when comparing cnidae complements between aeolids and their prey. Based on the description of cnida sequestration in aeolids (Graham, 1938; Greenwood and Mariscal, 1984b; Grosvenor, 1903; Martin, 2003), there is no known mechanism that would equally distribute cnidae to each ceras. Therefore, each ceras could have a different complement of cnidae due to chance, and rare cnidae – those produced in low numbers by the cnidarian – may not appear in all cnidosacs.

1.9 Efficacy of sequestered cnidae as a defense

Given a) the controversy surrounding selective cnidae sequestration in aeolids, b) the discovery of cnidae egestion in the Hancockiidae, and c) the observation that the aeolid *H. crassicornis* is readily eaten by *Crossaster papposus* (Miller and Byrne 2000, pers. obs., 2013), the function of sequestered cnidae is still debated (Edmunds, 2009). The evidence for a defensive function includes: 1) the extrusion and subsequent firing of cnidae when an aeolid is accosted (Edmunds, 1966; Grosvenor, 1903), and 2) the retention of cnidae for over a month (Glaser, 1910). When cerata are pinched off, they flail and contract muscles surrounding the cnidosacs, which causes cnidae to be extruded

(Edmunds, 1966; Grosvenor, 1903). The movement of the cerata is thought to attract the attention of predators or attackers to the dispensable structures, and cnidae release is thought to deter subsequent attacks (Grosvenor, 1903).

Glaser (1910) stated that cnidae are retained in aeolids for over a month. Unfortunately, he did not provide a description of the methods he used, and whether the cnidae were evacuated all at once or the cnidophages degraded over the month. Whatever the process, the retention of cnidae separates the cnidae sequestration in aeolids from the cnidae egestion in Hancockiidae (Martin et al., 2008). As egestion is the only other hypothesis suggested for the function of cnidae within aeolids (Edmunds, 2009; Miller and Byrne, 2000), the retention of cnidae strongly implies a functional benefit.

1.10 Research conducted in this thesis

For my thesis research, I explored cnidae as a defense in the aeolid nudibranch *Hermissenda crassicornis* (Eschscholtz, 1831). This aeolid is common subtidally in Barkley Sound, British Columbia, near the Bamfield Marine Sciences Centre (BMSC). Inspired by the discovery of predator-induced changes in sequestered cnidae in *Flabellina verrucosa*, I performed similar experiments with *H. crassicornis*. I hypothesized that given the similarities between *F. verrucosa* and *H. crassicornis*, including predators and prey, *H. crassicornis* would also exhibit a similar induced defense. I also tested whether variation in cnida storage occurs in the wild, and if *H. crassicornis* collected from the same location share the same cnida complements given that they would likely have experienced the same predator pressure and access to prey.

I was also curious about the discrepancy between the cnida sequestered in some aeolids and the cnidae available in their prey, especially since it is not universally observed (e.g., Day and Harris, 1978; Martin and Walther, 2003). Given that there is no obvious mechanism for equally partitioning cnidae to each ceras I tested whether cnida assemblages in cerata from different areas along the dorsum of *H. crassicornis* were the same.

Finally, I tested how long cnidae are retained in cnidosacs, and calculated the rate of cnidophage loss in *H. crassicornis*. The retention of cnidae by aeolids is a key feature that distinguishes a defensive function of cnidae from cnida egestion in the closely related Hancockiidae.

The results from these studies will increase our understanding of processes involved in prey choice in aeolids, where prey provide energy and nutrients, as well as a possible defense that results from cnida sequestration. The behavioural ecology and mechanism of cnida sequestration in aeolid nudibranchs are difficult to investigate in isolation, and therefore this research addresses on both to better understand the influences underlying this remarkable system.

Figures

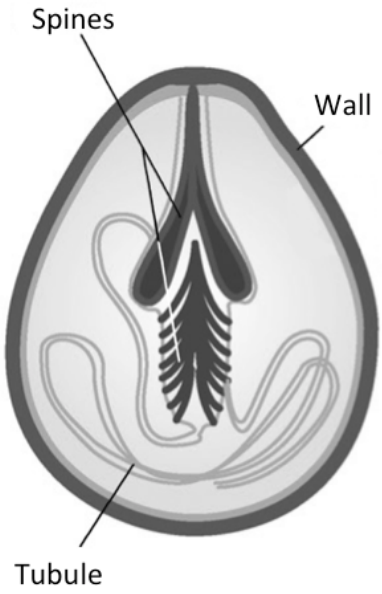

Figure 1.1: Schematic drawing of an unfired cnida. Modified from David et al., (2008). The inverted tubule covered in spines creates identifying shaft and coiling patterns.

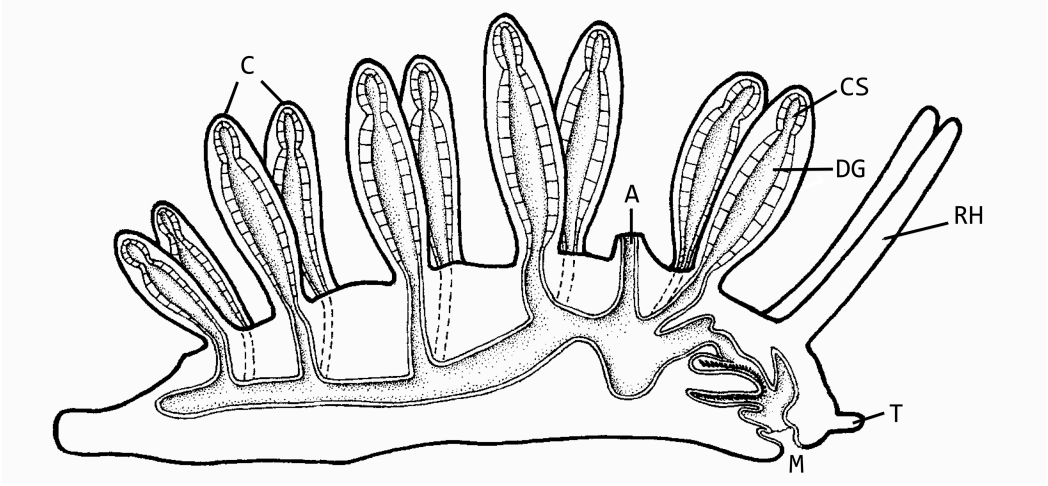

Figure 1.2: Schematic drawing of the alimentary system of a generic aeolid nudibranch. Modified from Kalker and Schmekel (1976). C=cerata, A=anus, CS=cnidosac, RH=rhinophore, T=tentacle, M=mouth, DG=digestive gland. The anus is located at the right anterior region of the body. Intestines originating from the end of the digestive system and the second set of anterior tentacles are missing from the diagram.

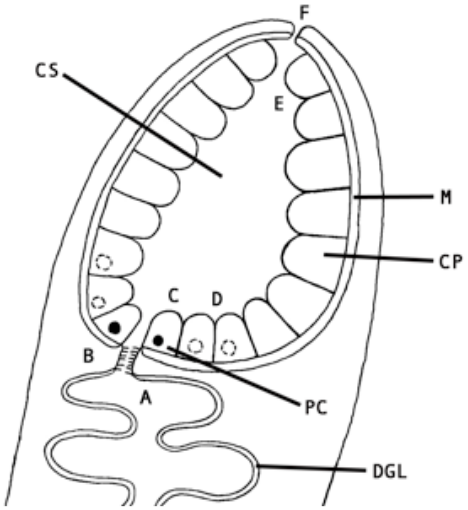

Figure 1.3: Schematic longitudinal section of a generalized cnidosac from an aeolid nudibranch. The progression of cnidae begins in the digestive gland (A) where the cnidarian tissue is dissolved with enzymes released from the digestive gland lining (DGL). Isolated cnidae are transported along a ciliated canal (B), and are engulfed by phagocyte cells (PC) lining the cnidosac (CS) at the most proximal end of the cnidosac (C). The phagocyte organelles, including nucleus, degrade (D). The cnidae remain in an unfired state surrounded by a membrane, in a structure called a cnidophage (CP). As new cnidae are phagocytized, the older cnidophages move towards the distal end of the cnidosac (E). Cnidophages are forced out of the cnidopore (F) when the muscles surrounding the cnidosac (M) contract.

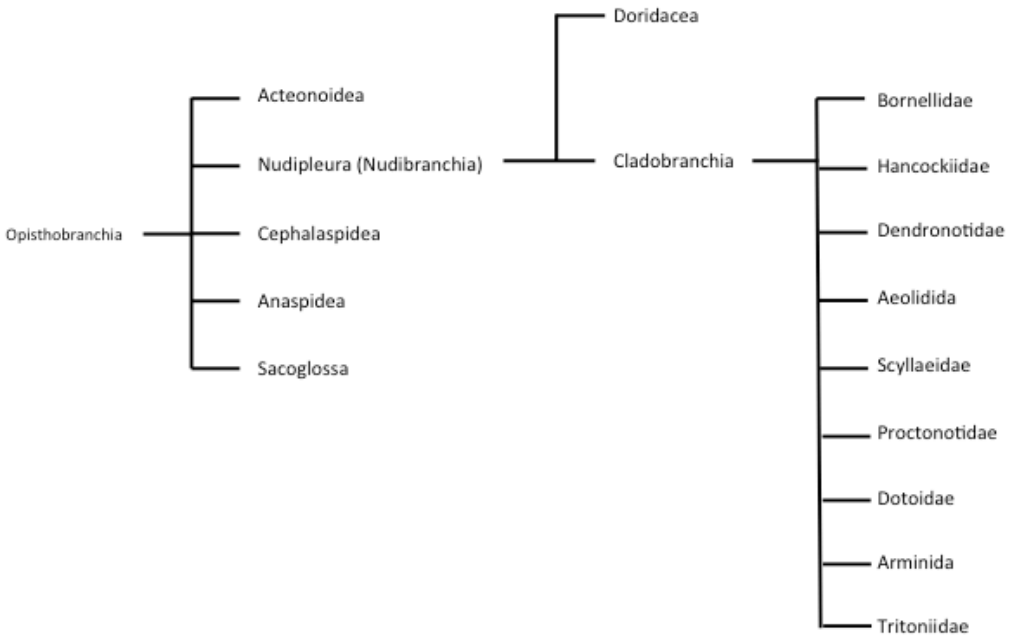

Figure 1.4: Graphical classification of the Opisthobranchia. Composite of phylogenetic information from Medina et al. (2011) and Pola and Gosliner (2010).

Plates

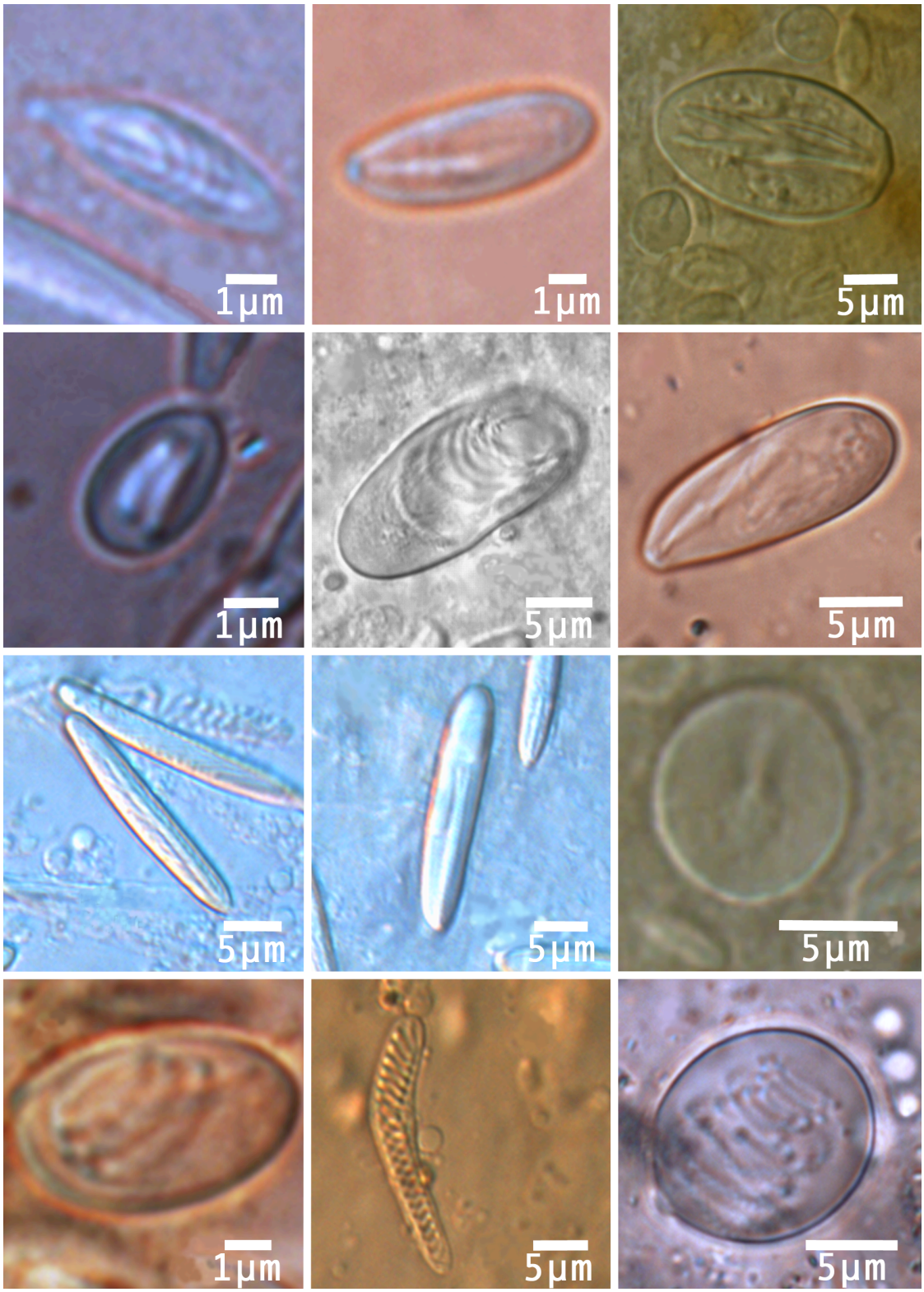

Plate 1.1: Cnida types found in the cnidosacs of *Hermissenda crassicornis*. Numbers 1 through 12 correspond to cnida types 1 through 12. Cnidae were assigned numbers based upon order of first observation. Cnidae 1, 2, 4, 5, and 12 were viewed with an Olympus BX51 light microscope with DIC filter, images captured with an Olympus Q-Color 5 and QCapture imaging software. Cnidae 3, and 6 through 11 were viewed with a Leica DMRXA compound light microscope with DIC filter, images captured with a QIClick and QCapture imaging software. Scale bars are as marked, all images were captured at 100x magnification.

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Chapter 2: The effects of predator effluent, scent of eaten conspecifics, and growth environment on the cnida complement of *Hermissenda crassicornis*

2.1 Introduction

Many organisms have the ability to adjust defenses according to the type and abundance of predators around them, a phenomenon called induced defense (Tollrian and Harvell, 1999). Among the many examples of reversible induced defenses, those that change multiple times and with the identity of the predator, is the pond snail *Physella gyrina* (Say, 1821) that adjusts its vertical position in the water column depending on whether the predator approaches from the benthos or the surface (Turner et al., 1999). Aeolid nudibranchs (Mollusca, Gastropoda) have been added to the list of animals with reversible induced defenses (Frick, 2003). This is a surprise because aeolids do not produce their defenses, but steal them from their prey.

Aeolids are a group of shell-less gastropods well known for their ability to steal and store cnidae (pressurized, harpoon-like capsules) produced by their cnidarian prey (Grosvenor, 1903; Wright, 1858). Each species of cnidarian prey produces a subset of the over 30 varieties of cnidae (Östman, 2000), and the aeolids sequester their prey's cnidae at the distal end of their cerata (dorsal papillae) (Figure 1.2). Cnida functions in cnidarians are reflected in their form: defensive cnidae have piercing spines, whereas those for prey capture have long filamentous tubules, for "lassoing" prey (Edmunds, 1966; Mariscal, 1974).

Remarkably, the aeolid *Flabellina verrucosa* preferentially sequesters different cnidae types by switching prey depending on the predator to which it is exposed (the seastar *Crossaster papposus* and the fish *Tautogolabrus adspersus*) (Frick, 2003). The suggestion is that they did so to acquire the cnidae that were most effective against that particular predator (Frick, 2003; Mariscal, 1974). The results from Frick (2003) were surprising because, unlike many defenses, defense switching requires that the aeolids adjust their feeding activities. The transport of cnidae from the mouth to the cnidosac takes only a few hours, but a complete replacement of cnidae may take up to 4 days (Day and Harris, 1978). This is also a rather slow response compared to *Physella gyrina*, and would be beneficial only if the predators were predictable for long periods, and if predator cues were detectable well in advance of predator contact. Whether these conditions are met for *F. verrucosa* remains unknown.

Like many other means of defense, cnida sequestration has costs, especially for a generalist carnivore like *Flabellina verrucosa*. According to the optimal foraging theory, organisms choose prey to maximize energy intake per unit time (Macarthur and Pianka, 1966). However, for aeolids food is not only a source of energy but of defenses as well. Therefore, to predict the optimal prey choice for aeolids, the handling time, search time, defense provided, as well as energy gained needs to be weighed. Although *F. verrucosa* switch prey in response to predators in the laboratory, cnidarian prey choice may not be as simple in the field. Thus, prey switching to the most beneficial cnidarians for defense may result in a greater search time and therefore a greater cost to the aeolid.

To test the generality of the induced-defense response reported by Frick (2003), I replicated these experiments with another generalist aeolid *Hermissenda crassicornis*. In

addition, I also tested whether exposure to the scent of conspecifics being eaten by a predator altered the cnida complement and whether holding environment (laboratory versus field) had an effect on cnida complement.

Hermissenda crassicornis is an aeolid found in the North Pacific, ranging from Japan to California, and is particularly abundant in Barkley Sound, British Columbia (McDonald and Willard, 1980). Like *Flabellina verrucosa*, *H. crassicornis* is a generalist predator, and feeds on many different species of cnidarians in the wild and in the laboratory, including the anemones *Anthopleura elegantissima* (Hoover et al., 2012)*, Metridium senile*, and *M. farcimen* (Avila and Kuzirian, 1995; Avila, 1998), the cup coral *Balanophyllia elegans* (pers. obs.), various hydroids including *Obelia* spp. (Hoover et al., 2012), *Tubularia* spp. (Avila and Kuzirian, 1995; Avila et al., 1994; Tyndale et al., 1994), and *Aurelia labiata* (Hoover et al., 2012). *Hermissenda crassicornis* also eats both living and dead polychaetes (pers. obs.), mussels (Avila and Kuzirian, 1995), and conspecifics (Zack, 1975). The predators of *H. crassicornis* include *Pugettia producta* (a kelp crab), *Crossaster papposus* (the rose seastar), as well as other seastar species and fish, as determined by underwater field observation and gut content analysis (Greenwood, 2009; Mauzey et al., 1968; Miller and Byrne, 2000). *Pugettia producta* and *C. papposus* were chosen as the predators for this experiment because the defenses that would protect aeolids from these species were thought to be quite different (Frick, 2003; Mariscal, 1974): long and filamentous cnidae might interfere with the crustaceans' mouthparts, whereas spiky cnidae (referred to as "penetrants") pierce the soft tubefeet of the seastar (Edmunds, 1966; Frick, 2003; Mariscal, 1974; Miller and Byrne, 2000). Thus, if

inducible defenses are present in *H. crassicornis*, the cnida complements should differ depending on the predator to which they are exposed.

2.2 Methods

2.2.1 Predator effects on cnida complements

Forty-one *Hermissenda crassicornis* were collected by SCUBA in July, 2013 off Sanford Island in Barkley Sound, British Columbia, Canada (48° 51' 57", -125° 10' 28") (Figure 2.1). Ten *H. crassicornis* were used for each of the following treatments: those exposed to the scent of *Crossaster papposus* feeding upon crushed mussels (*Mytilus trossulus*), those exposed to the scent of *C. papposus* eating *H. crassicornis*, and those exposed to no predators. Eleven *H. crassicornis* were exposed to the scent of *Pugettia producta* fed mussels. Only 17 survived to the end of the two-week treatment, two individuals from the control group, four from the *C. papposus* group, six from the *C. papposus* fed *H. crassicornis* group, and five from the *P. producta* group. The cause for the high mortalities is unknown. The animals were housed in a w:12.5 x l:12.5 x h:5.0cm mesh "basket" (for the aeolids and their prey) within a larger 35.6 x 20.3 x 12.4 cm, 5.7 L plastic container (for the predators). Running seawater flowed at a rate of 50 mL/s, into one end of the larger container, and dye tests confirmed that the water that passed through the predator container also passed through the "baskets". The aeolids were all given five prey species *ad libitum*: four cnidarians (*Balanophyllia elegans, Metridium senile*, *Anthopleura elegantissima*, and *Obelia* sp.) and crushed mussels. Since all *H. crassicornis* were fed crushed mussels, the scent of crushed mussels was present in each treatment, regardless of whether mussels were fed to the predators. Prior to the treatment,

three to eight cerata were removed from each *H. crassicornis* from the right anterior region, to ensure that at least one cnidosac was complete, as the removal process can often cause cnidae to be ejected by the aeolid. *Hermissenda crassicornis* were allowed to acclimate in the experiment set-up with food for twenty-four hours before predators were added to the larger holding container. The predators were housed in outdoor aquaria for two weeks prior to the start of the experiment, and fed crushed mussels to control for previous prey scents influencing the *H. crassicornis* cnidae selection. The predators were given a blade of *Macrocystis pyrifera* for shelter and crushed mussels for food, except for the one treatment group where *C. papposus* was fed *H. crassicornis*. The predators were fed every 2-3 days, and prey for the *H. crassicornis* were replaced as needed. The two-week treatment time corresponded to the length of time required for *H. crassicornis* to replace the cnidae in the cnidosac as determined by preliminary studies (Anthony, unpublished data). After two weeks in the experiment set-up, cerata samples were again collected form the right anterior region of the surviving animals.

2.2.2 Laboratory effects on cnida complements

Sixteen *Hermissenda crassicornis* were collected from ropes hanging off the BMSC docks. Eight individuals were housed in the laboratory under the same conditions as the control group from the previous experiment, and eight individuals were housed in mesh containers suspended from the docks at BMSC at a depth of between 0.5m and 2m below the sea surface. Both treatments were given the same five prey species (*B. elegans, M. senile*, *A. elegantissima*, *Obelia* sp. and crushed mussels) *ad libitum*; the animals were

checked every 2 – 3 days, and prey were replaced as needed. Samples of cerata from the right anterior region of the dorsum were collected after two weeks.

2.2.3 Among-population variation in cnida complements

Hermissenda crassicornis were collected from four sites in Barkley Sound, BC near BMSC: six from Hosie Island (48° 54' 27", -125° 2' 20"), nine from Sanford Island (48° 51' 57", -125° 10' 28"), four from Satellite Passage (48° 51' 29", -125° 10' 32"), and six from the docks at BMSC (48° 50' 6", -125° 8' 13") (Figure 2.1). Cerata samples were taken from the right anterior region and sealed in glycerol on glass slides.

2.2.4 Cerata removal and sample preservation

Cnidosacs were sampled by pinching the cerata with forceps and transferring them to a glass slide, taking care to avoid transferring seawater with them. As pinching causes the autotomy of the cerata, injury to the animal is reduced. However, this process may also cause the cnidosacs to extrude the cnidae, therefore it was important to take more than one ceras per sample. Ciliary action on the exterior surface of the cerata caused loose cnidae to flow around in low-viscosity liquid media, such as seawater, thus making them difficult to count. Half a drop of glycerol was added to the glass slide to prevent cnidae firing and ciliary action. A glass coverslip was placed on top of the sample and sealed with clear nail polish. After 24 hours, the slides were examined for the presence of cnidae. If cnidae were present in at least one ceras, the slide was stored for future counting; during the short storage time, the tissue did not degrade and the cnidae

remained intact. If no cnidae were present in any of the cerata, the animal was re-sampled.

2.2.5 Sample collecting and cnidae counting

Slides containing samples of 3 – 10 cerata were examined and the cnidosac that was easiest to view was selected for counting. The identification label of the slides were covered with opaque labeling tape, and the slides were counted in a random order to avoid bias. The samples were viewed on 100x oil immersion light microscope with a DIC filter (Olympus BX51), and all unfired cnidae inside the cnidosac, plus those immediately outside, were identified and counted. The cnidae were categorized with an identification key (Plate 2.1), as there is no current consensus on cnida nomenclature (Ostman, 1997; Penney, pers. comm., 2012; Francis, pers. comm., 2012). In total, the number of cnidae present in the cnidosacs ranged from 26 to 2864, and all sampled cnidae were counted and identified from each of the 33 individuals.

2.2.6 Data Analysis

For all of the experiments, the count data were log transformed [log(count+1)] to better describe cnida counts by reducing the effects of oversaturation of one cnida type. I ran a 2-way ANOVA on the log-abundance of cnida (cnida type by treatment category) in R (R Core Team, 2013), to test the interaction of treatment or location and cnida type on cnida counts. This analysis tested for differences in the cnida complement in the cnidosac, and even though there may be slight differences between the counts of each cnida type, the

main statistical question being tested was: Did the distribution of cnidae among individuals depend on treatment (i.e., was the interaction term from the ANOVA significant)? The proportion of the most common cnida type was also compared before and after the treatment using a paired t-test in R (R Core Team, 2013).

2.3 Results

2.3.1 Predator- and laboratory-effects treatments

Of the 30 or more types of cnida made by cnidarians, 14 were found in the cnidosacs of *Hermissenda crassicornis* in the predator and laboratory-effects experiments (Plate 2.1). In the three predator treatments - *H. crassicornis* exposed to *Pugettia producta* (crab), to *Crossaster papposus* (seastar), and to the seastar feeding upon *H. crassicornis* – a different number of cnida types were found in their cnidoms (ten, eight, and nine, respectively) (Table 2.1). For the laboratory-effects treatment, the control group had 11 and the *H. crassicornis* hung off the dock had nine (Table 2.1).

The cnida complements of *H. crassicornis* in the laboratory-effects experiment were not significantly different (2-way ANOVA, df=1, f=0.09, interaction of location*cnida type $p>0.75$; Figure 2.2). These cnida complements also did not differ significantly from the control group for the predator-effects experiment (2-way ANOVA, df=2, F=0.51, interaction of location/control*cnida type $p>0.60$; Figure 2.2). The three treatment groups – those hung off the dock, and the laboratory control groups from the two experiments – were then pooled into a single group labeled “Pooled Control” and used in subsequent analysis of predator treatments.

There were no significant differences in cnida complements among treatment groups – pooled control, crab treatment, seastar treatment, and *H. crassicornis* fed seastar (2-way ANOVA, df=3, F=0.67, interaction of treatment*cnida type $p>0.57$; Figure 2.3) – nor any difference between each treatment group and the pooled control. Although several cnida types were present in the cnidosacs, cnida type 1 formed the highest proportion in all treatments, including the laboratory controls and those off the dock: an average of 0.84 ±0.04 for all individuals in the predator-effect experiment (Figure 2.3), and an average of 0.97 ±0.01 for all individuals in the laboratory-effects experiment (Figure 2.2).

If cnidae type 1 was removed, the laboratory controls still did not differ between the aeolids hung off the dock (2-way ANOVA, df=2, F=2.7, interaction of treatment/location*cnida type $p>0.07$), but there was a difference between the pooled control and the predator treatment groups (2-way ANOVA, df=3, F=3.1, interaction treatment*cnida type $p<0.03$). The significant difference is explained by the difference between the *H. crassicornis*-fed seastar and pooled control group (2-way ANOVA, df=1, F=9.1, interaction treatment*cnida type $p<0.003$). There were still no differences between the pooled control group and the seastar group (2-way ANOVA, df=1, F=0.80, interaction seastar/pooled control*cnida type $p>0.37$), or the crab group (2-way ANOVA, df=1, F=0.09, interaction crab/pooled control *cnida type $p>0.76$).

The abundance of cnida type 1 increased significantly during the experiment for all predator-exposed groups (Figure 2.3; t-test, t=2.23, df=14, $p<0.05$) (Figure 2.4).

2.3.2 Variation in cnida complements among natural populations

Two locations (Hosie Island and the docks of BMSC) were sampled on two separate occasions, and the effect of time was tested with a 2-way ANOVA (Hosie Island Time 1 and Time 2: df=1, F=0.79, interaction time*cnida type p>0.37; BMSC Docks Time 1 and Time 2: df=1, F=0.02, interaction time*cnida type p>0.87). The two collection times were therefore pooled and the analysis was repeated to test for differences between sample sites only, and these were not statistically significant (2-way ANOVA, df=3, F=0.52, interaction sites*cnida type p>0.68) (Figure 2.5). No single cnida type was consistently more common than the others, although cnida types 1 and 2 made up over 50% of the sample from all sites except Hosie Island. These results contrast with the predator-control and laboratory-effects experiments where cnida type 1 clearly predominated by the end of the experiments (Figure 2.2 and 2.3).

2.4 Discussion

The proportion of each cnida type found in *Hermissenda crassicornis* did not differ after two weeks of controlled conditions, when exposed to predator scent (crab *Pugettia producta* and seastar *Crossaster papposus*), when exposed to predator scent and the scent of conspecifics being eaten, or when raised on lines in the wild, even though *H. crassicornis* had access to four cnidarian prey with different cnidoms. At the end of the experiments, cnida type 1 was by far the commonest cnida type in all cnidosacs for all treatment groups. It is abundant in the hydroid *Obelia* sp., and is not known to occur in the other cnidarian species provided in the experiments (Hand, 1955a, 1955b; pers. obs.). Even though *H. crassicornis* feed upon all species that were offered to them (Conxita

Avila et al., 1994; Conxita Avila & Kuzirian, 1995; Conxita Avila, 1998; Hoover et al., 2012; Tyndale et al., 1994; pers. obs.), *Obelia* sp. and mussels were the only prey species that were replaced with regularity during the experiment. These results strongly suggest that *H. crassicornis* fed preferentially on these two prey species during this experiment, but that this prey choice was not influenced by predator abundance or type. The significant difference between the proportion of cnida type 1 before and after the treatments (Figure 2.4) clearly indicates that sufficient time was available for *H. crassicornis* to alter its cnidom.

Prey choice by *H. crassicornis* and other aeolids has been investigated by many researchers and *H. crassicornis* does show some prey preference, although that preference may be the result of ingestive conditioning, where prey preference is the result of previous feeding on a particular species (Avila, 1998b). *Obelia* sp., the preferred prey in the predator and laboratory effects, is common at the BMSC docks. However, *Obelia* is uncommon at the field site where *H. crassicornis* were collected for the predator treatment experiment (near Sanford Island), where *Balanophyllia elegans* was more common. *Metridium senile* are abundant on the BMSC docks, but uncommon at Sanford Island. So, even though different cnidarian prey would have been previously encountered by the aeolids collected for these experiments, all *H. crassicornis* preferred the same prey, indicating no strong influence of ingestive conditioning. It has been suggested that *Obelia* sp. might release similar chemical cues to *Plumularia* sp., a hydroid species found at Sanford Island, and may therefore be indistinguishable to *H. crassicornis* (Avila, 1998b). If this was the case, then choice of cnidarian prey by *H. crassicornis* may have

been due to prey familiarity (Avila, 1998b). Nonetheless, the selection of prey by the animals in these experiments was not influenced by exposure to predator scent.

Among the *H. crassicornis* collected at different field sites, cnida complements differed as much among individuals within a site as they did between sites. Given that *H. crassicornis* appears to prey preferentially on hydroids, the variation of the sea slugs' cnida complements within sampling locations may reflect microscale variation in the abundance of cnidarian species within each location. Similar cnida complements would therefore be expected if the prey were homogeneously distributed.

The abundance and availability of prey within a site could have a greater influence than innate prey preference on benthic predators. Future field investigations of prey choice by *Hermissenda crassicornis* should examine the abundance and accessibility of prey, and compare the cnidae sequestered to characterize prey choice in the wild.

Investigations into the role of prey choice in aeolids can have ecological and pharmacological benefit. In their natural habitat, aeolid nudibranchs could be used as a means of determining ecological functioning if the cnida complements in aeolids reflect the abundance of cnidarians in the surrounding habitat. Their cnida complements could provide a catalogue of cnidarians present where the aeolid nudibranchs live, and in the case of *Flabellina verrucosa* the cnida complements can also provide information on local predator pressures. Unfortunately, the influences behind prey choice and the cnidae sequestered by aeolids is still not clear, and until they have been resolved, this is not a viable option for determining ecological functioning or biodiversity.

Some cnidae are being examined for their pharmacological properties, but the process of isolating cnidae from cnidarian tissue can be difficult. The delicate mechanism of cnidae sequestration by aeolids has been used to isolate cnidae (Schlesinger et al., 2009). Through further research into the conditions that result in the sequestrations of particular cnidae – predator pressure, certain prey species, etc. – aeolids could be manipulated to extract particular cnidae types under investigation.

2.5 Conclusions

Hermissenda crassicornis shows innate prey preference affecting the cnida complement in this species, but that preference is not influenced by the presence of the predators *Pugettia producta* or *Crossaster papposus*. Therefore the predator-induced prey change in *Flabellina verrucosa* is not seen in all aeolids (Frick, 2003). At present, it is difficult to determine whether induced defenses are the rule in aeolids, or if *F. verrucosa* is an exception.

The variation in the cnida complements of *H. crassicornis* within sampling locations points to a potential conflict between prey choice and availability in the wild. Although *H. crassicornis* chooses a given prey species when available *ad libitum*, evidence indicates that they may not forgo more accessible prey for a preferred one. *Hermissenda crassicornis* could be a valuable tool for studying how prey choice may be influenced by energetic versus defensive considerations in a species that gains defenses from its food. Benefits to particular prey include energetic gains, defenses, and other nutrients whereas costs include searching and handling (Greenwood et al., 2004).

Tables and Figures

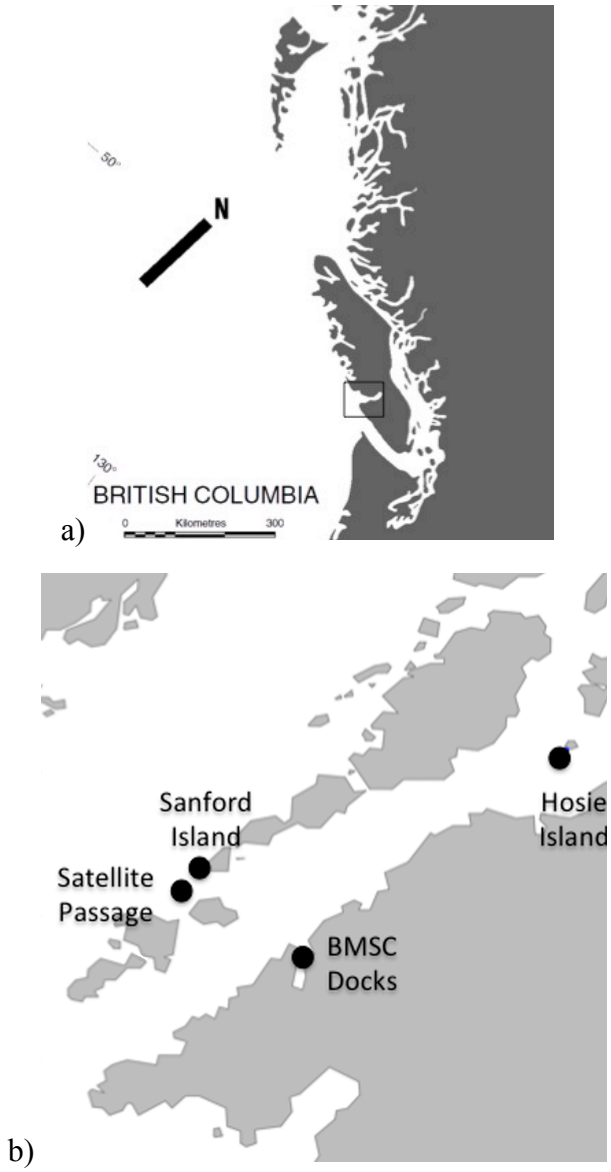


Figure 2.1: Locations of *Hermissenda crassicornis* collected in Barkley Sound, British Columbia, Canada. a) Map of the West Coast of British Columbia, Canada. Square indicates Barkley Sound. Map courtesy of Weller Cartography Ltd. b) Close up map of Barkley Sound with collection sites indicated. Hosie Island (48° 54' 27", -125° 2' 20"), Sanford Island (48° 51' 57", -125° 10' 28"), Satellite Passage (48° 51' 29", -125° 10' 32"), and the docks at BMSC (48° 50' 6", -125° 8' 13"). Map created with software R (R Core Team, 2013).

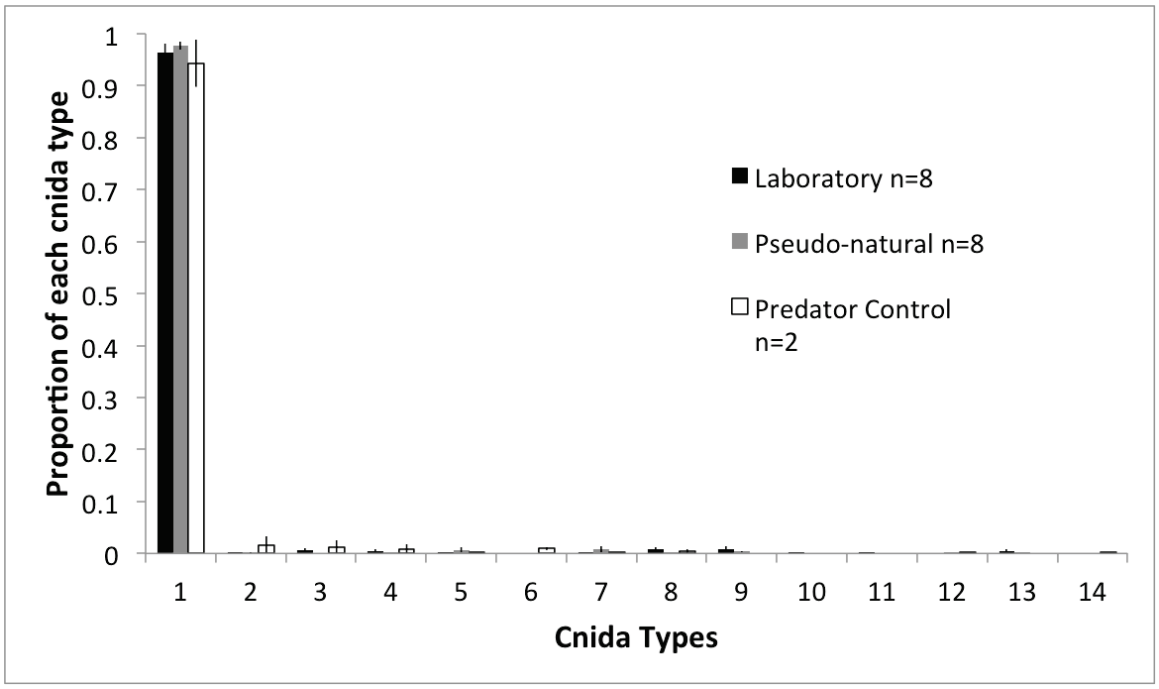


Figure 2.2: Average proportion of each cnida type in the cnidosacs of *Hermissenda crassicornis* in the absence of predators from the laboratory-effects experiment. Error bars indicate standard errors. The relative numbers of cnida of each cnida type did not depend on treatment category (2-way ANOVA of log(n+1) transformed counts, df=1, f=0.09, p>0.75 for interaction term of treatment*cnida type (independent variables) on log(n+1) transformed cnida counts (dependent variable)).

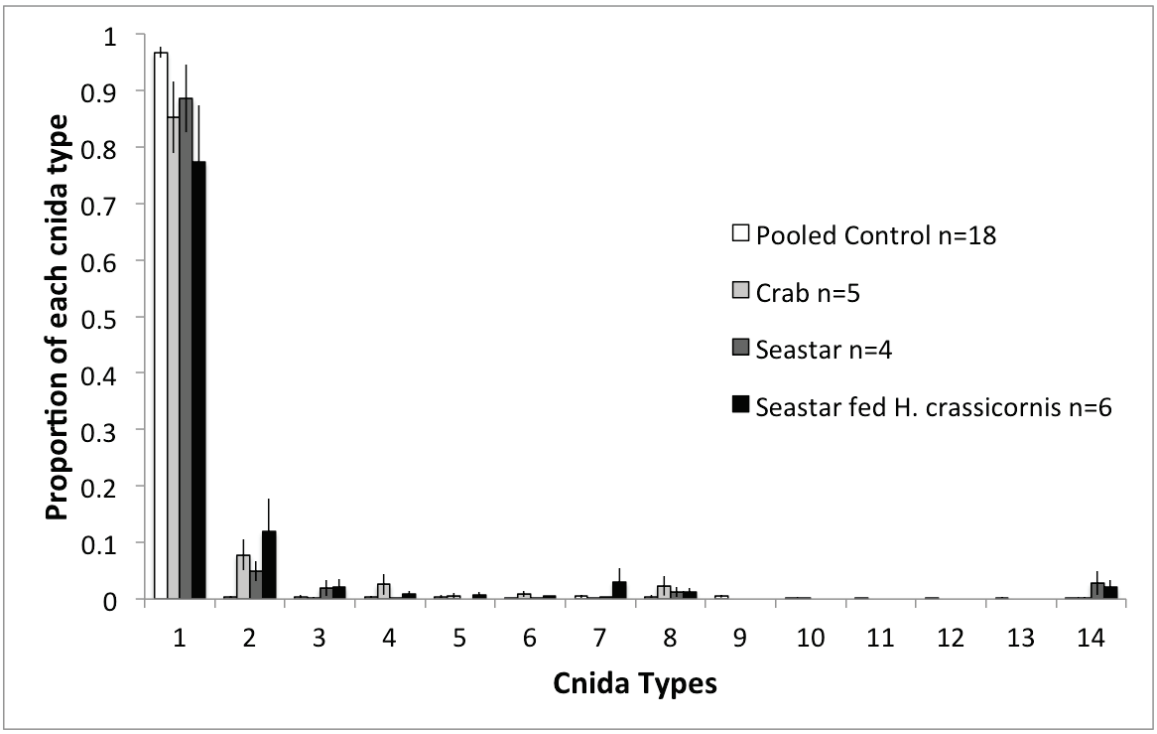


Figure 2.3: Cnida complements of *Hermissenda crassicornis* from the predator-effects experiment. The average proportions of each cnida type are presented for each treatment group. Error bars indicate standard errors. The relative numbers of cnida of each cnida type did not depend on treatment category (2-way ANOVA of log(n+1) transformed counts, df=3, F=0.67, p>0.57 for interaction term of treatment*cnida type (independent variables) on log(n+1) transformed cnida counts (dependent variable)).

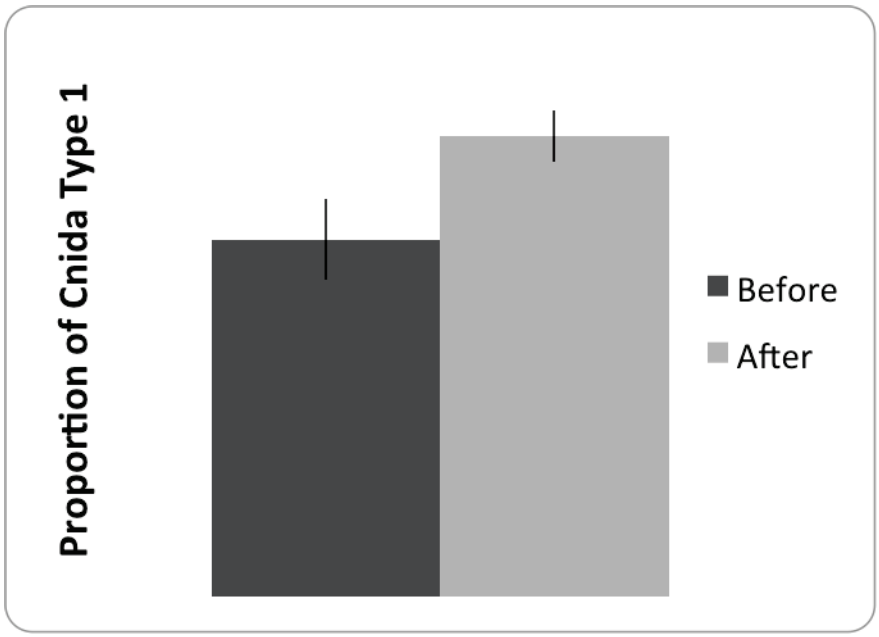


Figure 2.4: The average proportion of cnida type 1 in *Hermissenda crassicornis* pooled for all treatments before and after the predator effects treatments. There was a significant difference between the proportion of this cnida type before and after the two-week treatments (Paired t-test, $t=2.23$, $df= 14$, $p<0.05$). Error bars indicate standard errors.

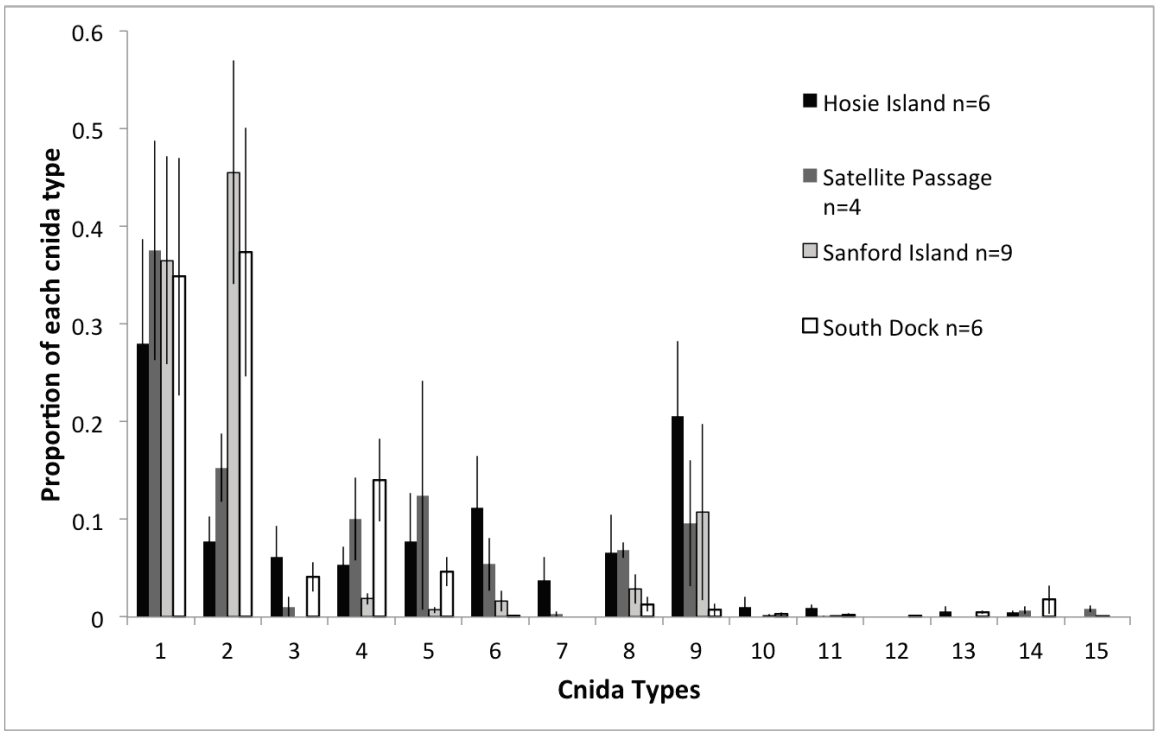
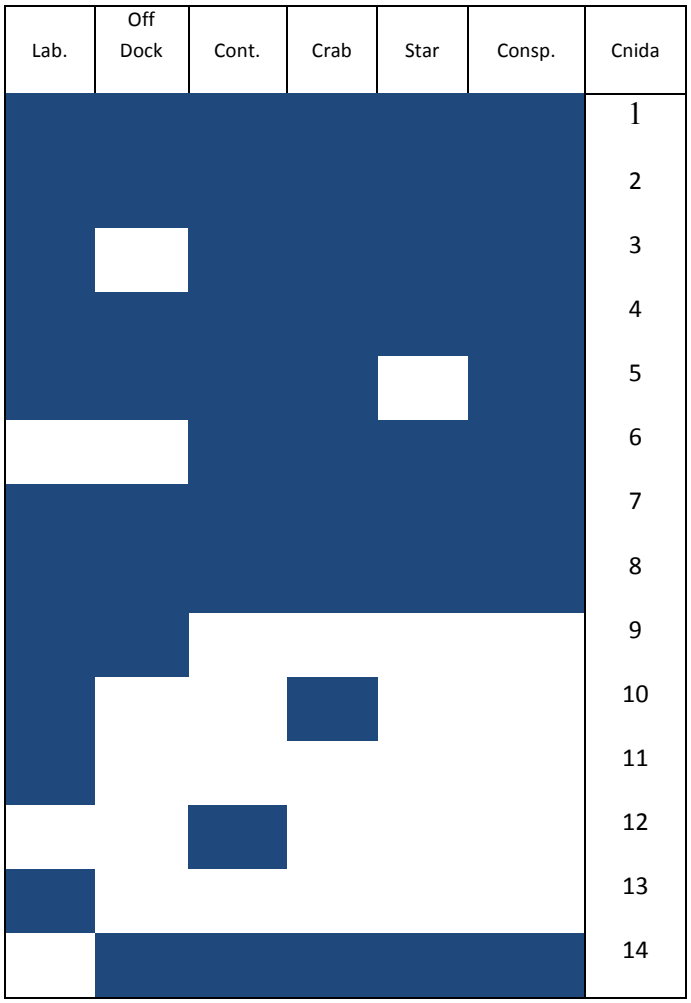


Figure 2.5: The proportion of each cnida type in the cnidosac of *Hermissenda crassicornis* collected from various locations in Barkley Sound, BC. Error bars indicate standard errors. The relative numbers of cnidae of each cnida type did not depend on treatment category (2-way ANOVA of log(n+1) transformed counts, df=3, F=0.52, $p>0.68$ for interaction term of location*cnida type (independent variables) on log(n+1) transformed cnida counts (dependent variable)).

Table 2.1: Presence (blue) and absence (white) of each cnida type in the cnidosacs of *Hermissenda crassicornis* from all treatment groups. Lab= Laboratory group, Off Dock= Group suspended off the dock, Cont.= Control from the predator effects experiment, Crab= Those exposed to the scent of *Pugettia producta,* Star= Those exposed to the scent of *Crossaster papposus*, Consp.= Those exposed to the scent of *H. crassicornis* fed to *C. papposus*. Presence indicates that at least one individual had the cnida type, where absence indicates that none of the individuals in that treatment group had the cnida type.



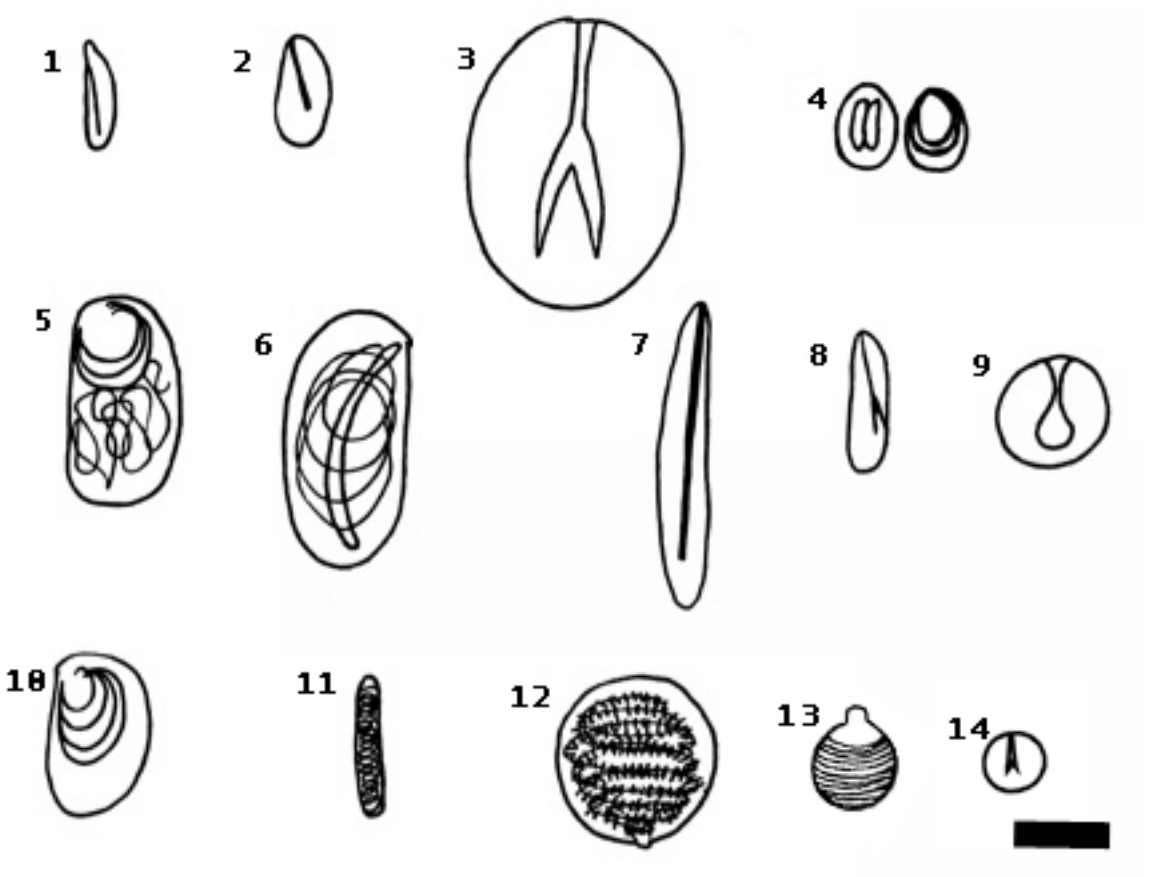


Plate 2.1: Identification key for cnida found in the cnidosacs of *Hermissenda crassicornis*. Numbers correspond to cnida identification numbers. Scale bar is an estimate and indicates approximately 5 μm. See Plate 1.1 for photographs.

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Chapter 3: Stolen cnidae in space and time: Variable sequestration along the body and retention time in an aeolid nudibranch

3.1 Introduction

Aeolid sea slugs were first discovered to steal cnidae (stinging capsules produced by cnidarians) from their prey over 150 years ago (Wright, 1858). Since then, the mechanism of this fascinating sequestration process has been investigated, but many questions still remain. Most importantly: is sequestration of cnidae primarily for defense, as usually presumed? The evidence is conflicting. For instance, one species of aeolid (*Hermissenda crassicornis*) is readily consumed by predators, whether or not the aeolid has cnidae (Miller & Byrne, 2000; pers. obs.). On the other hand, evidence for a defensive use of cnidae includes: a) the apparent selective uptake of cnidae compared to what is present in their cnidarian prey (Conklin and Mariscal, 1977; Day and Harris, 1978; Kepner, 1943), and b) the retention of cnidae rather than their egestion. To validate some of the methods used to test for selective uptake of cnidae, and to determine how long cnidae are retained, I examined variability of cnidae in space (different body regions) and in time (following addition or removal of cnidarian prey) in the aeolid nudibranch, *H. crassicornis*.

Cnidae are produced by cnidarians in cnidocyte cells nested within epidermal cells of the animal's exterior, in the gastrovascular cavity and defensive structures like acrorhagi and acontia in anthozoans. They are produced for prey capture, adhesion and defense (Mariscal, 1974; Weill, 1929). Each species produces only a subset of over 30 types of cnidae, and the subset varies with body region and function (David et al., 2008; Ewer,

1947; Mariscal, 1974; Östman, 2000; Weill, 1929). Of the over 30 cnida types, 28 are classified as nematocysts, and the non-nematocyst types are of two morphs: ptychocysts and spirocysts. The nematocysts are double-walled, acidophilic structures, whereas the non-nematocyst cnidae are acidophilic (spirocysts), acidophilic and basophilic for ptychocysts, and have a single capsule wall for both spirocysts and ptychocysts (Mariscal et al., 1977, 1976; Östman, 2000).

The maturation process of cnidae involves the inversion of the tubule followed by an increase in pressure within the capsule (Berking and Herrmann, 2005). Upon triggering – caused by physical contact, pH change, or in some cases, neuronal control (Mariscal, 1974; Ozbek et al., 2009) – the highly pressurized capsule will evert its tubule at an acceleration that exceeds $5.4x10^6$ the acceleration of gravity, and taking onlt 700 ns to fire (Nüchter et al., 2006; Ozbek et al., 2009).

Aeolid nudibranchs are one of the few predators of cnidarians, that include coral eating fish and turtles that feed upon jellyfish, and they have developed many adaptations to protect themselves from the cnidae sting, as well as a means of stealing and storing cnidae. The prey tissue is ingested with chitinous jaws and radula, and passes through a chitin-coated buccal mass and esophagus, which protects the aeolid's own tissue from the effects of firing cnidae (Graham, 1938; Martin and Walther, 2002). The immature cnidae, those that have the tubule inverted, but without increased pressure, pass with the tissue into one of the many digestive glands that extend into the cerata (dorsal papillae) (Greenwood and Mariscal, 1984a) (Figure 1.2). The tissue is digested by enzymes released from the lining of the digestive gland (Cockburn and Reid, 1980; Graham, 1938). Any undigested material, including fired cnidae, exit the aeolid as faeces (Graham,

1938; Martin and Walther, 2002; Martin, 2003). The cnidae that are stripped of their cnidocyte cells but remain unfired, are transported through a ciliated canal at the distal end of the cerata, and are engulfed by phagocytes in storage cavities (=cnidosacs) at the tips of the cerata (Graham, 1938). Many cnidae are phagocytized per phagocyte (Greenwood and Mariscal, 1984b; pers. obs.), and over time, the nucleus and other organelles in the phagocyte degrade, leaving the cnidae surrounded by a membrane (the cnidophage) (Graham, 1938). The cnidophages remain in the cnidosac and provide the cnidae with an environment in which they remain in a functional state and mature by unknown mechanisms (Greenwood and Mariscal, 1984; Obermann et al., 2012), but do not fire. When the animal is accosted physically, muscles surrounding the cnidosac contract, and the cnidophages are forced out the cerata tip through the cnidopore, breaking the cnidophage membrane in the process (Edmunds, 1966; Graham, 1938; Greenwood and Mariscal, 1984b). The cnidae then fire upon contact with seawater (Glaser and Sparrow, 1909; Graham, 1938; Grosvenor, 1903; Kalker and Schmekel, 1976).

The purpose of cnidae sequestration is still debated, although sequestered cnidae are almost always assumed to be for defense. No studies have conclusively shown that cnidae alone are effective as a defense, as opposed to other possible means of defense, including autotomy and repugnant chemicals (Aguado and Marin, 2007; Edmunds, 2009, 1966; Miller and Byrne, 2000; Penney, 2009). The blenny *Fundulus heteroclitus* will reject an aeolid when in its mouth (Glaser, 1910) when the aeolid has no cerata, but it is difficult to isolate the effectiveness of one defense from another, as experienced by Aguado and Marin (2007) (see response by Penney, 2009). Two pieces of evidence supporting a

defensive function of cnida sequestration in aeolids are: 1) cnidae sequestered are not a random sample of all cnidae produced by the cnidarian prey upon which the aeolid fed; 2) aeolids retain the cnidae in specialized structures (cnidosacs) rather than egesting them in the faeces.

Grosvenor (1903) first noted that the cnidae produced by the cnidarian *Pennaria cavolini* (Ehrenberg, 1834) did not match the cnidae in the cnidosacs of two aeolids, *Rizzolia* (=*Cratena*) *peregrina* (Gmelin, 1791) and *Spurilla neapolitana* (Delle Chiaje, 1841), that had fed upon it. Others found a similar discrepancy (Conklin and Mariscal, 1977; Day and Harris, 1978; Glaser, 1910; Graham, 1938; Kepner, 1943; Thompson and Bennett, 1969). This was advanced as further evidence for sequestered cnidae as a defense: presumably these particular cnidae must have a function if they are selected. How and where cnidae are taken up selectively is a puzzle. Cnidae are generally thought to be taken up selectively by phagocytes in the cnidosac, with the unwanted cnidae left to be digested (Greenwood and Mariscal, 1984b; Kepner, 1943). In previous studies comparing the cnidoms of cnidarians to the cnidae sequestered by aeolids assumed that sequestration was the same in each ceras (Day and Harris, 1978). I tested this assumption by comparing the cnidae within the cnidosacs from different locations along the dorsum of *Hermissenda crassicornis*. If the cnida complements differ in different locations along the body, the selectivity, if any, is not at the level of individual cnidosacs.

The second line of evidence supporting a defensive function to cnidae in aeolids involves retention of cnidae within cnidosacs. An alternate hypothesis is that cnidae are egested via the cerata to rid the body of the dangerous structures (Edmunds, 2009; Glaser, 1910; Miller and Byrne, 2000), as seen in non-aeolid cnidarian-eating nudibranchs of the clade

Hancockiidae (Martin et al., 2008). Like the aeolids, the closely related hancockiidids (Martin et al., 2008; Figure 1.4), also transport cnidae from their food to cnidosacs, but they are egested from the ceratal tips, rather than stored (Martin et al., 2008). This similarity in form may have influenced the hypothesis of similarity in function. I therefore attempted to quantify the retention time of cnidae within the aeolid nudibranch *Hermissenda crassicornis* when transferred to a cnidarian-free diet. Cnidophages most likely have a finite lifespan, as evidenced by the poor condition of the cnidophages at the most distal end of the cnidosac (Martin, 2003). I hypothesize that without cnidarian prey, *H. crassicornis* will retain cnidae for extended periods compared to what would be expected for an egestion process. Glaser (1910) observed cnida retention after one month of starvation, but did not mention if the cnidae were all lost at once, or if there was a slow decrease of cnidae over time. I hypothesized that the cnidae-containing cnidophages would degrade and the cnidae would be lost within one month, as reported by Glaser (1910).

3.2 Methods

3.2.1 Study species

Hermissenda crassicornis are abundant in Barkley Sound near the Bamfield Marine Sciences Centre (BMSC) in Bamfield, British Columbia, Canada. They are generalist predators, and will feed upon many cnidarians and living and dead tissue (Avila and Kuzirian, 1995; Avila et al., 1997; Tyndale et al., 1994; pers. obs.). In the laboratory at BMSC, *H. crassicornis* feed most readily on the hydroid *Obelia* sp. and crushed mussels, *Mytilus trossulus*.

3.2.2 Cnida complements by body region

Ten *Hermissenda crassicornis* were collected from two sites near BMSC in Barkley Sound, British Columbia, Canada: four individuals from ropes off the BMSC dock (1 – 3 m deep, by hand), and six from the west side of Sanford Island (48° 51' 57", -125° 10' 28") (Figure 2.1) from 7m deep, collected by SCUBA. The animals were brought to the laboratories at BMSC, and each housed individually in mesh containers (w:12.5 x l:12.5 x h:9.0cm) in a tank with approximately 5 cm of continuous flowing sea water.

Cerata were sampled within two days of collection from four regions along the dorsum, each of which was separated by a naturally occurring gap in the cerata: a midline separating the left and right regions, and a gap in cerata where the anus and gonopore are located, separating the anterior and posterior regions (Figure 3.1). The cerata were squashed under a coverslip with glycerol, and sealed with clear nail polish (as in Chapter 2). The cnidae were identified under 100x oil immersion light microscope with DIC filter (Olympus BX51), and the slide label was covered to avoid scoring bias. The cnidae inside and just outside of the cnidosac were classified and counted by me using my own identification key (Plate 2.1). Cnidae types were not given a formal name because there is no firm consensus on cnidae nomenclature (Östman, 2000).

3.2.3 Data analysis

Cnidae counts were log transformed [log(count+1)], to homogenize variances and normalize the count distributions, and the values were analyzed with a two-way ANOVA

using the statistics software R (R Core Team, 2013) to test the difference within an individual compared to the difference between individuals. The log-transformed counts of all cnidae types were the response variables.

The count data were transformed to proportional data for the three commonest cnida types. Each common cnida type was entered into a mixed linear model in the software R (R Core Team, 2013) to test for cnida variation among regions and individuals.

3.2.4 Cnidae retention

Sixteen *Hermissenda crassicornis* were collected between 1 – 3 m deep off the docks at BMSC. The animals were individually housed at BMSC in mesh containers (w:12.5 x l:12.5 x h:9.0cm), resting in a shallow tank with approximately 5 cm of flowing seawater. The animals were split into two treatment groups: those fed only the mussel *Mytilus trossulus*, and those fed *Obelia* sp. (a hydrozoan cnidarian) *ad libitum* as well as mussels, replaced every 2-3 days, or as needed. Cerata samples were collected sporadically for 44 days after starting the experimental diet. At each sampling point in time, the largest eight cerata were selected from each individual, to avoid removing any cerata that had regenerated from a previous sampling event (regeneration of cerata takes approximately 43 days (Miller and Byrne, 2000)); the larger cerata also have more cnidophages (pers. obs.). The cerata were placed under a coverslip in glycerol and sealed with clear nail polish, and viewed under 100x oil immersion light microscopy with DIC filter (Olympus BX51). The label of each slide was covered to prevent scoring bias. Each cnidophage with cnidae, both inside and just outside the cnidosac, was counted for each cerata per individual. The two highest counts per individual at each time period – the cnidosacs with

the largest number of cnidophages filled with cnidae – were averaged. This average was then used as the cnidophage count for that sample.

3.2.5 Data analysis

The number of cnidae containing cnidophages at each sampling time for all individuals were counted. The two groups – those that fed upon mussels, and those that fed upon mussels and *Obelia* sp. – were each analyzed by linear regression. Comparisons between the cnidophage counts over time for the two groups were analyzed by ANOVA, a t-test was performed to test whether the slope of the regression lines for each of the groups differed significantly from zero, and an ANCOVA to compare the regression lines between treatment groups. All analyses were conducted in R (R Core Team, 2013).

3.3 Results

3.3.1 Along the body

The variation in cnidae complements in *Hermissenda crassicornis* was lower within individuals, than among individuals (2-way ANOVA, df=9, F=0.91, interaction of body areas*cnida type p>0.50; Table 3.1); and there was no difference between the right and left regions, nor the anterior and posterior regions (2-way ANOVA: Right *vs* Left: df=8, F=0.37, interaction Right/Left*cnida type p>0.93; Anterior *vs* Posterior: df=8, F=0.41, interaction Anterior/Posterior*cnida type p>0.91; Table 3.2). Within each individual, the proportions of the most common cnida types, cnida types 1, 2, and 3, were not significantly different (ANOVA df=3, F=0.05, p>0.68; df=3, F=1.27, p>0.30; df=3, F=2.73; p>0.06, respectively; Table 3.3). The assumptions of normality were validated.

Although the proportions of the most common cnidae types were similar, some cnida types were not present in all samples collected from the same individual aeolid (Table 3.4). In all individuals, at least one cnida type was present in only one out of three or four samples.

3.3.2 Cnidae retention

The number of cnidophages containing cnidae were counted for two treatment groups: those fed mussels only and those fed mussels and the cnidarian *Obelia* sp. Cnidophages containing cnidae decreased significantly over 44 days in the mussel-only group (r^2= 0.16, df=63, slope=-0.23, t=-3.45, f=11.88, $p<0.002$). No significant change was observed in the group fed mussels and *Obelia* sp. over the same time period (r^2=0.01, df=53, slope=0.04, t=0.73, f=0.53, $p>0.45$) (Figure 3.2). There was a significant difference between the cnidophage counts over time in the two treatment groups (ANCOVA, df=106, F=4.8, $p <0.035$).

3.4 Discussion

The sampling of one ceras from an individual sea slug does not appear to be sufficient to capture the full list of cnida types sequestered by the individual. Although the full cnida complement of cnidosacs did not differ significantly along the body, and the proportions of the most common cnida types also did not differ, rare cnida types were not consistently present or absent in all samples. Previous reports that some cnida types sequestered did not match the cnidoms of prey species, should therefore be reexamined. Unfortunately, earlier reports that cnidae complements differed from their prey either did not mention

the number of cerata examined (Graham, 1938; Grosvenor, 1903; Kepner, 1943; Thompson and Bennett, 1969) or only examined a single ceras per individual (Day and Harris, 1978). Possible reasons for the discrepancy between cnidae counts in the cnidarian and the cnidosac include: a) unnecessary cnidae were dissolved by lysosomal activity (Kepner, 1943), b) some cnida sizes are not sequestered (Mariscal et al., 1977), c) only immature cnidae can be sequestered (Greenwood and Mariscal, 1984a), and d) only useful cnidae are sequestered and the rest are egested (Day and Harris, 1978). Although the results reported here for *Hermissenda crassicornis* cannot distinguish among these possible explanations, they nonetheless call into question previous reports of selective uptake. Some studies report no selectivity (Martin and Walther, 2002; Martin, 2003) whereas others infer prey choice based on the cnidae stored in cnidosacs (Frick, 2003; Garese et al., 2012). Multiple cerata samples from along the body might have shown less of a difference between the cnidarian cnidom and sequestered cnidae than has been claimed.

The presence and absence of rare cnidae types along the body of aeolids (Table 3.4) might be attributed to: a) the particular cnidarian tissue consumed, b) the level of "maturation" of the cnidae ingested, c) the cnida type or d) random sampling variation.

Of the many known cnida types, only a subset occurs in each cnidarian species, and different cnidae may occur in different parts of the body. For example, defensive structures have different cnidae than feeding structures (Hand, 1955a, 1955b; Weill, 1929). By switching prey, or by switching the part of the prey upon which they are consuming, an aeolid would sequester different cnidae. Given that the cnidarian tissue is not digested until it reaches the digestive gland in the ceras, one morsel of cnidarian

flesh, along with its cnidae, could be transported to a single ceras; therefore, each ceras might receive cnidae from a single piece of cnidarian flesh, until the next piece of cnidarian tissue enters the digestive gland. As described in Grosvenor (1903), tissue fragments are most likely assigned to a digestive gland haphazardly, so it is not surprising that each ceras would have a different selection of cnidae.

At the same time, mature cnidae are not sequestered, but fire while being transported through the digestive system. Only the immature (unpressurized) cnidae are sequestered in the cerata, where they mature (Greenwood and Mariscal, 1984a; Obermann et al., 2012). Different proportions of cnidae in the cerata could be the result of the different state of maturation of the cnidae at the time of consumption by the aeolid.

Finally, cnida structure may affect the ability to stay intact in the aeolid. Most cnidae are nematocysts which are are double-walled, basophilic capsules. Spirocysts and ptychocysts are two non-nematocyst types of cnidae that are single-walled and acidophilic (or basophilic and acidophilic for ptychocysts) (Mariscal et al., 1977, 1976; Weill, 1929, 1934). Of the two non-nematocyst cnidae, there is no evidence of ptychocyst sequestration, but spirocysts have been observed in the cnidosacs of *Hermissenda crassicornis*, although in low quantities (maximum 1 per cnidosac) (pers. obs., 2013). The structure of these non-nematocyst cnidae may make them less likely to "survive" ingestion, transport, and sequestration in an aeolid nudibranch.

The discrepancies between the cnidarian cnidom and the cnidae sequestered by aeolids observed by Grosvenor (1903), Kepner (1943), Tompson and Bennett (1969), and Day and Harris (1978) might be partially explained by the results reported here. Although I

can not clarify the cause of the variation of cnida types among cerata along the dorsum of *H. crassicornis*, rare cnida types are clearly not distributed uniformly among cerata. Future studies will need to be done with more care to permit adequate sampling of sequestered cnidae in aeolids. If the complete list of cnidae sequestered is desired, then more than one ceras must be sampled. For comparisons of the cnida complement of multiple individuals between treatment groups, such as with Frick (2003) and Chapter 2, one ceras per individual may be adequate, so long as multiple individuals were sampled.

The results reported here show quite clearly that cnidae sequestered by aeolid *Hermissenda crassicornis* do not reside in the cnidosac indefinitely, and must be maintained by consuming additional cnidarian prey. Over 44 days, the number of cnidae containing cnidophages decreased significantly in *H. crassicornis* fed only crushed mussels (Figure 3.2). Other *H. crassicornis* fed both mussels and the cnidarian hydroid *Obelia* sp., retained a relatively constant number of cnidophages containing cnidae in the laboratory over the same time period. Previous studies reported that cnidae are replaced by new cnida within 4 days (Day and Harris, 1978), but that cnidae are retained by aeolids when fed non-cnidarian prey (Conklin and Mariscal, 1977; Glaser, 1910). Martin (2003) was the first to suggest that sequestered cnidae are not retained indefinitely. He observed that cnidophages – the cells that surround the cnidae during their storage in the cnidosac – appeared to degrade at the most distal end of the cnidosac (Martin, 2003). Since newer cnidae make up the most proximal area of the cnidosac, and the older cnidae, and by extension the older cnidophages, are at the most distal end of the cnidosac (Greenwood and Mariscal, 1984b; Martin, 2003), Martin (2003) observed the possible degradation of the older cnidophages. My results confirm that cnidophages have a finite

lifespan and that aeolids will continue to feed upon cnidarians even when other prey is available.

Given that sequestered cnidae are lost over time (Figure 3.2), aeolids must continuously replenish the cnidae in their cnidosacs. The maintenance of cnidae when other prey are available further supports the view that sequestered cnidae are of value to aeolids, most likely for defense, rather than resulting from an elaborate egestion process (Edmunds, 2009; Glaser, 1910; Miller and Byrne, 2000). This study reveals that in the presence of adequate food (mussel tissue), *H. crassicornis* will still choose to feed upon the cnidarian *Obelia* sp. It is unknown whether *H. crassicornis* prefer cnidarians because they provide higher nutritive or caloric value compared to mussel flesh, or require less handling time than mussel flesh. However, at the end of the experiment, those fed mussels only did not appear to be in poor shape and continued to grow during the course of the treatment time (pers. obs., 2013).

3.5 Conclusion

The cnida complements in the cerata of the aeolid nudibranch *Hermissenda crassicornis* appear to be the same along the dorsum, although not every ceras will yield a complete catalogue of the cnida types sequestered. Future studies must be careful when sampling the cnidae complements of aeolids as they may be misleading if multiple samples are not taken. This is not to say selectivity does not occur in aeolids such as *H. crassicornis*, only that in sampling one cnidosac will not be adequate to make such a conclusion. Future work on cnidae sequestration should compare the cnidom of the cnidarian prey to the cnidae sequestered by the whole aeolid body before concluding selective uptake.

Hermissenda crassicornis must continue to eat cnidarians to maintain cnidae within their cnidosacs. Without cnidarian food, aeolids slowly lose their cnidae. Whether *H. crassicornis* eats the cnidarians primarily for the cnidae they provide, or whether cnidarians provide this aeolid with greater nutritional or caloric value than non-cnidarians should be the focus of future work.

Tables and Figures

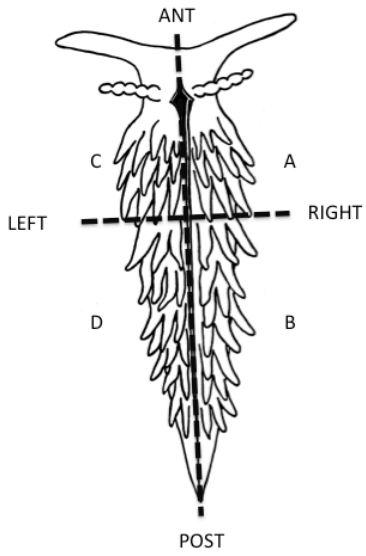


Figure 3.1: Schematic drawing of the aeolid nudibranch *Hermissenda crassicornis* showing regions of cerata sampling. ANT= anterior, POST= posterior. The two natural gaps in cerata are marked with dashed lines, and the regions along the dorsum are named A-D.

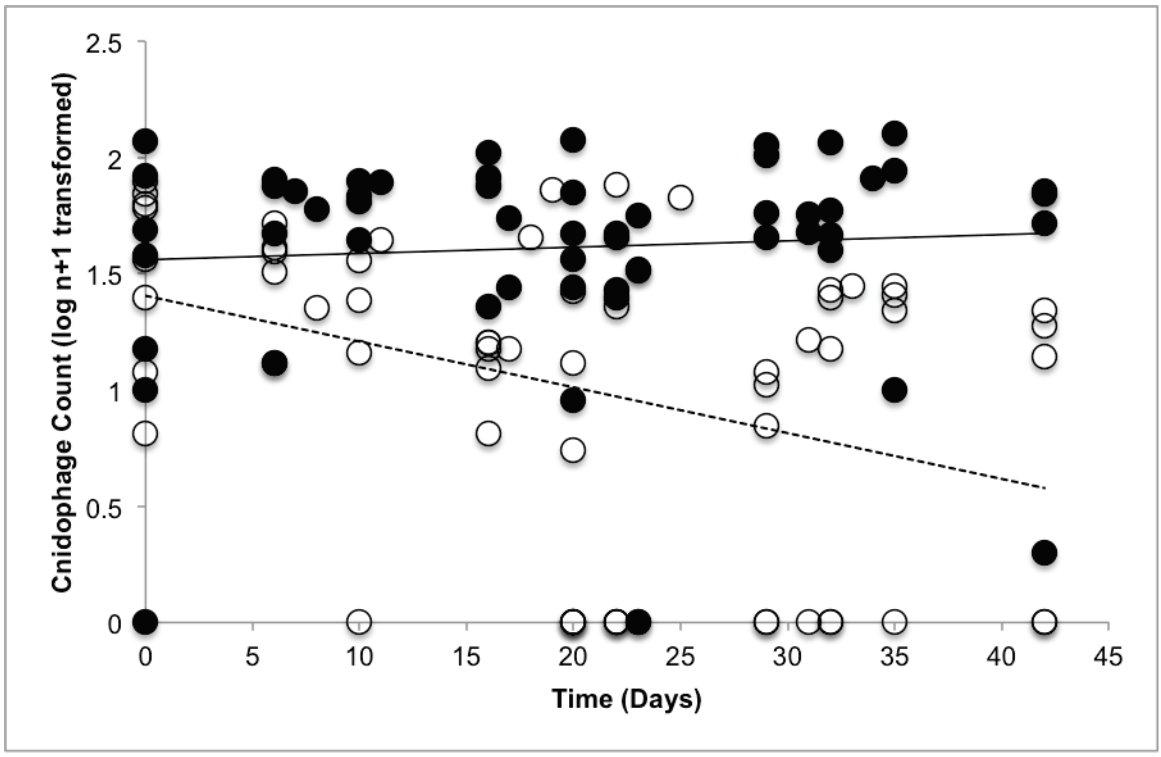


Figure 3.2: The number of cnidophages containing cnidae within the cnidosacs of *Hermissenda crassicornis* fed cnidarian or non-cnidarian prey. Solid dots: *H. crassicornis* fed both mussels (*Mytilis trossulus*) and hydroid *Obelia* sp. Open dots: *H. crassicornis* fed mussels only. The regression slope for the mussel + hydroid food does not differ significantly from zero (solid line, slope=0.0027). The group fed mussels only showed a significant decline in cnidophages containing cnidae over time (dashed line, slope= -0.0197). The slopes of the two groups were significantly different (ANCOVA, df=106, F=4.813, $p<0.035$). All count data has been log n+1 transformed for graphical purposes, and slopes described here are for the transformed data.

Table 3.1: ANOVA results of difference in cnida complements between body regions in *Hermissenda crassicornis.* The results of the ANOVA indicate a difference between individuals (*Individual*), between cnida types (*Cnida type*) but no significant differences in the interaction between individuals and cnida types (*Animal*Cnida type*). Asterisk indicate significant values.

	F	*df*	*P*
Individual	2.1082	9	0.02783*
Cnida type	224.6241	1	<2e-16*
*Animal*Cnida type*	0.9097	9	0.51652
Residual		409	

Table 3.2: ANOVA results comparing the cnida complements between cerata from different body regions: a) right and left, b) anterior and posterior. The right and left regions are separated by a naturally occurring gap in the cerata down the midline of the dorsum; the anterior and posterior regions are separated by another naturally occurring gap in the cerata partially down the dorsum, where the anus and gonopore are located.

a)

Right *vs* Left	*F*	*df*	*P*
Body Region	0.0402	1	0.8413
Cnida type	220.76	1	<2e-16*
*Body region*Cnida type*	0.2644	1	0.6074
Residual		370	

b)

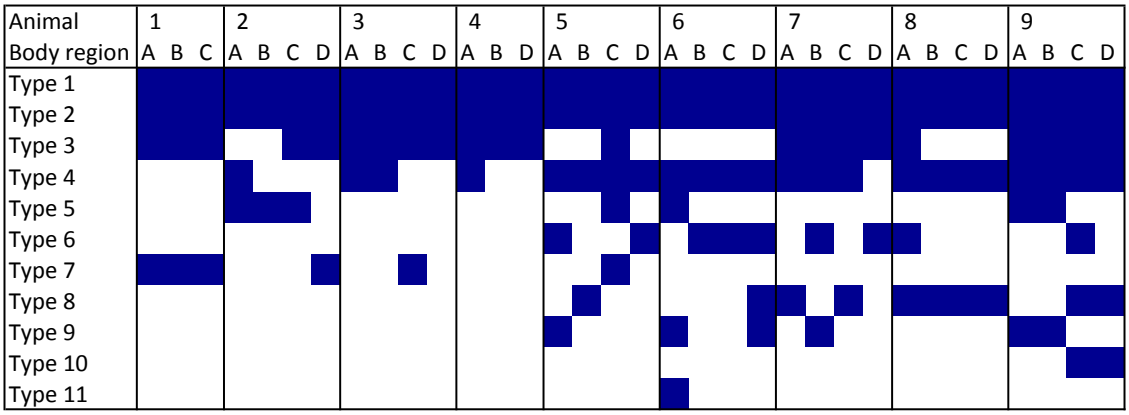
Anterior *vs* Posterior	*F*	*df*	*P*
Body Region	0.2010	1	0.6544
Cnida type	132.9737	1	<2e-16*
*Body region*Cnida type*	0.0147	1	0.9035
Residual		194	

Table 3.3: Difference in the proportions of the most abundant cnidae types along the body of each individual *Hermissenda crassicornis* analyzed with a generalized linear model. The proportion of each cnida type in each body region is compared to the other body regions within the same individual.

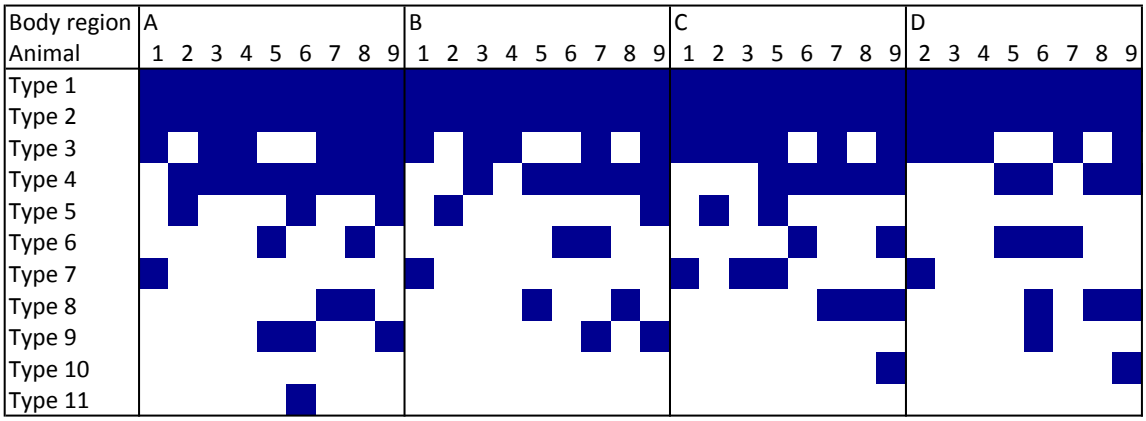
	df	*F*	*P*
Cnida type 1	3	0.05	0.68
Cnida type 2	3	1.27	>0.30
Cnida type 3	3	2.73	>0.06

Table 3.4: Presence and absence of cnida types in body locations in the aeolid nudibranch *Hermissenda crassicornis*. Cnida types per identification in Chapter 2, Plate 2.1.

a) Presence (blue) and absence (white) of cnida types at various locations along the body separated by individual. Several cnida types were not present in all body locations within individuals.



b) Presence (blue) and absence (white) of cnida types grouped by body location. There is no obvious pattern in the presence or absence of cnida types in the region among the individuals sampled.



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Chapter 4: General discussion of results

4.1 Background and results

This work has yielded insights into the processes involved in cnida sequestration and storage in aeolids, a topic that has intrigued biologists since its discovery (Wright, 1858). The experiments described in the previous chapters can be categorized into two groups: 1) prey choice in the presence of different predators and in different wild locations in the aeolid *Hermissenda crassicornis*, and 2) the mechanism of *H. crassicornis* cnida sequestration.

Edmunds (1966) first suggested that cnidae may be selected by aeolid nudibranchs based on their efficacy against particular predators. This suggestion inspired Frick (2003) to study cnida selectivity by the aeolid *Flabellina verrucosa* in the presence of different predators. This aeolid sequesters different cnidae in the presence of a fish and a seastar compared to no predator, although no test was done for differences between the two predator groups (Frick, 2003). Thus, although these results do not provide evidence for selective cnidae sequestration in response to particular predators, they do indicate that *F. verrucosa* switches prey in the face of elevated predation risk (Frick, 2003).

I replicated this experiment with the generalist aeolid *Hermissenda crassicornis*. I found that with equal access to five prey species (the cnidarians *Obelia* sp., *Balanophyllia elegans*, *Metridium senile*, and *Anthopleura elegantissima*; and non-cnidarian *Mytilus trossulus*) and scent from potential predators (*Pugettia producta*, *Crossaster papposus*, or

C. papposus consuming *H. crassicornis*), *H. crassicornis* did feed selectively on different prey. However, this selectivity was independent of predator presence or identity: all treatment groups fed mostly on *Obelia* sp. There was no evidence of cnida selection within cnidosacs, nor of prey switching (Chapter 2). In each treatment group – including the laboratory-effect treatment group that were suspended in mesh containers from the docks at BMSC – cnida type 1 was sequestered in much greater numbers than all other cnidae types combined, and made up 80-100% of all cnidae counted for these treatments (Chapter 2). This cnida type is produced by *Obelia* sp. Like cnida types 2 and 4, cnida type 1 (Plates 1.1, 2.1) is more likely to be present in greater numbers due to its small size, although the large difference between the counts of cnida type 1 and the rest outweighs any size effects. The cnidae complements in aeolid nudibranch therefore seem to result primarily from prey choice and not predator presence. In other words, the induced defenses observed in *Flabellina verrucosa* do not appear to occur in *Hermissenda crassicornis*. Whether the difference between the results presented here for *H. crassicornis* and those presented by Frick (2003) for *F. verrucosa* are the result of different influences in prey choice – a result of predator pressure in *F. verrucosa* or prey preference in *H. crassicornis* – or whether they differ due to differences in experimental set-up and data analysis is unclear. The offer of four prey species to *H. crassicornis*, compared to the two choices given to *F. verrucosa* (Frick, 2003), was selected to mimic the various prey species found in the wild. And a 2-way ANOVA for the entire cnida complements, as opposed to individual 1-way ANOVAs as per Frick (2003) was selected because cnida counts for each cnida type were not independent of one another. Each

cnidosac is a confined space that can hold only a finite number of cnidae, so the increase in one cnida type must translate to a reduced number of all other cnida types.

4.2 Induced defenses in aeolids

Induced defenses occur in many organisms from protists to vertebrates (Tollrian and Harvell, 1999). However, the induced defense in the sea slug *Flabellina verrucosa* (Frick, 2003) is unique because its defense is not produced, but stolen from its prey (Wright, 1858). There was no evidence of induced defenses in *Hermissenda crassicornis*.

Even with differing conclusions, the results of the laboratory experiments reported here and those by Frick (2003) may not reflect what occurs in nature. Aeolids are not surrounded by an unlimited supply of only a small number of prey species, and they are likely exposed to several predators at one time. Aeolids from the same collection site would most likely experience the same predator cues, and have access to the same prey sources; and therefore similar cnida complements within sites. But I found that the cnida complements generally did not differ significantly between *H. crassicornis* collected from different locations (Chapter 2). This was surprising because the *H. crassicornis* collected from the BMSC docks had similar cnida complements to those from other locations, even though the prey species at the BMSC docks were quite different than the other locations (pers. obs., 2012, 2013). The dock community is composed of animals that attach to human-made structures, and the animals at the other locations live in a more natural environment.

4.3 Selective cnida sequestration

Despite several accounts of cnida selectivity in aeolids (Conklin and Mariscal, 1977; Graham, 1938; Greenwood and Mariscal, 1984; Kepner, 1943), the mechanism behind such apparent selectivity is unknown. Earlier work is difficult to judge because some authors only sampled one ceras per individual, and others did not make note of their sampling methods (Edmunds, 1966; Graham, 1938; Martin, 2003; Day and Harris 1978). To assess the validity of this approach, I compared cnidae complements from different cerata along the body of *Hermissenda crassicornis* and found that not all cnida types are represented in every ceras. Cnida complements did not differ between the left and right sides, between the anterior and posterior regions; or in the proportion of the most common cnida types (types 1, 2, or 3) within each individual. However, significant differences were observed in the presence or absence of rare cnida types. Future studies comparing the complements of cnidae in cerata to that in food should look at multiple cerata. It would be particularly interesting to repeat the experiment by Day and Harris (1978) with more than one sample per species.

4.4 Cnida retention in aeolids

The fate of the cnidae in aeolids has been traced from nematogenesis (the production of cnidae in the cells of cnidarians) to the cnidosac and finally the extrusion by force through the cnidopore in the aeolid. New cnidae stolen from prey will replace previously sequestered cnidae between four days and two weeks (Day and Harris, 1978; Frick, 2003, 2005). But the retention time of cnidae without replacement or exterior forces instigating their release has not been investigated thoroughly. Under starvation conditions, cnidae

remain in the cnidosac for over a month (Glaser, 1910), but the methods used were not described in detail, and no mention of whether the cnidae were lost all at once or diminished slowly over time. Observations that older cnidophages appeared degraded in the cnidosacs (Martin, 2003) suggest that cnidophages have a finite life. Without the cnidophage membrane, the cnidae are free and could potentially fire. As the cnidophages are not produced at the same time, the number of cnidophages containing cnidae will decrease over time.

I found that *Hermissenda crassicornis* fed only mussels (*Mytilus trossulus*) gradually lost cnidophages over 44 days, and *H. crassicornis* fed mussels and *Obelia* sp. maintained their cnidophage number over the same period. These results can be interpreted two ways: 1) the retention of cnidae, and the replacement of cnidae when cnidarian prey are available, provide evidence that aeolids intentionally sequester cnidae, and retain them rather than egest them directly; 2) cnidae are a byproduct of feeding upon cnidarians that provide more nutritive and caloric benefit than the mussel flesh. An analysis of cnidarian and non-cnidarian food quality could help distinguish between these hypotheses.

4.5 Conclusions

The results from the experiments described here reveal that: a) there is no evidence that *Hermissenda crassicornis* displays induced defenses, b) the cnida complements from animals from different locations usually do not differ, c) more than one ceras sample may be required to assess the full cnida complement of an individual, and d) cnidae are not

retained indefinitely in the cnidosac, but instead must be constantly replaced through ingestion of cnidarian prey.

These results do not resolve the debate over whether cnida sequestration by aeolids is a form of defense, but they do provide new information into the mechanism of cnida sequestration. The retention of cnidae within the cnidosac, and the replacement of cnidae if available, indicate that the cnidosac and cnidophages did not evolve as a means of egestion, as seen in the Hancockiidae (Martin et al., 2008).

Sequestered cnidae likely reduce the risk of predation, but this defense may not be effective against all predators. For example, cnidae sequestered by *H. crassicornis* did not deter the sea stars *Crossaster paposus* or *Pycnopodia helianthoides*. However, predation by the crab *Pugettia producta* was deterred, not by release of cnidae but by autotomy of the cerata, (Miller and Byrne, 2000). In my own observations, *C. papposus* will feed upon *H. crassicornis* with or without cnidae. An unnamed aeolid without cerata, and therefore without cnidae, was spit out by the fish *Fundulus heteroclitus* (Glaser, 1910), suggesting that these aeolids may posses defenses against fish other than cnidae. However, separating the effectiveness of cnidae from other defenses is a challenge, and the methods for doing so have not yet been perfected (Aguado and Marin, 2007; with critique by Penney, 2009).

4.6 Future inquiries

The knowledge of the ecology of the aeolid *Hermissenda crassicornis* and the mechanism of cnidae sequestration by this species has been increased with the results

presented here. Cnida sequestration may be influenced by predators in *Flabellina verrucosa*, but not in *H. crassicornis*. The retention and replacement of cnidae in *H. crassicornis* strongly suggests that cnidae have a function within the cnidosacs of the aeolid, but a defensive function remains conjecture. Induced defenses are therefore not a universal feature in aeolids. The effect of predator type, and of prey availability and preferences, on cnida sequestration in other aeolids still requires further investigation.

Given the observed variation in cnida complements among *H. crassicornis* collected from the same field sites, it would be interesting to assess other influences on prey choice, such as caloric or nutritive value of cnidarians over other prey, and prey choice in the wild. With the increased availability of underwater cameras with large memories and long battery life, future researchers can make *in situ* observations of subtidal predator-prey interactions, like those of aeolids and cnidarians.

Given that rare cnidae types are not equally represented in cnidosacs along the body, a re-examination of the assumptions of cnidae sequestration are in order. Future research that seeks to understand the relationship between cnidae produced by cnidarian species and the cnidae sequestered by aeolids should be guided by these results, and care should be taken to look at multiple samples of cnidosacs from various regions along the body.

I began this exploration into cnida sequestration in aeolids nudibranchs with the assumption that the cnidae were stolen and stored as a defense. Through research and inquiry, I am now aware that the evidence of the efficacy of cnidae as a defense is hardly conclusive. Nonetheless, a defensive function remains the most likely explanation. Future inquiries into the function of cnidae sequestered by aeolids should investigate alternative

defenses, such as repugnant chemicals, and explore the effectiveness of all their defenses in the wild.

4.7 Complexities

4.7.1 Cnida identification

My research was greatly complicated by the lack of consistent nomenclature for cnidae by different cnidarian researchers. The seminal work was by Weill (1934), but under closer observation with more sophisticated light microscopy, many differences between the cnidae are now visible (Östman, 2000). In 1974, Mariscal (1974) provided a cnidae identification key to replace Weill's (1934). Mariscal (1974) descriptions were the original source of identification for my thesis, but I soon discovered through communication with B. Penney (pers. comm., 2012) and L. Francis (pers. comm. 2012) that new discoveries using more sophisticated techniques revealed Mariscal's nomenclature to be outdated; over 30 types of cnidae were recognized by Östman (2000).

Lastly cnida size is not a reliable criterion for identification because the size of cnidae relate to the size of the cnidarian (Francis, 2004; Kramer and Francis, 2004). Even though documented cases of identification error are lacking, it is worth mentioning for future identification work.

Östman (2000) suggests that a consensus of cnidae nomenclature will be needed prior to using cnidae as a tool for systematic classification. For the research reported in this thesis, naming the cnidae types was not necessary, so long as the cnida types could be distinguished reliably. I was consistent in my identification of cnidae throughout the study (Plate 2.1).

4.7.2 Cerata and empty cnidosacs

These experiments required that I find the best ways to visualize cnidae from cnidosacs. I found that cerata samples were easy to attain by pinching them midway along their length with forceps, but that this act often caused the aeolid to extrude the cnidae. Many cerata samples yielded no cnidae. For the experiments comparing cnidae complements before and after exposure to predators, and the samples of cerata along the body or from the different sites, I collected 5-8 cerata per sample; I collected 8-10 for the cnidophage degradation experiment to ensure I had at least two complete cnidosacs. As a possible defensive action, cerata continued to contact and move after autotomizing. To stop the movement, the cerata were pinched again midway along their length. The cilia covering the distal external surface of the cerata continued to beat after the cerata were sealed under a coverslip. The cilia caused extruded cnidae and cnidophages to flow, which impaired accurate cnidae counting. Fortunately, the addition of glycerol stopped the ciliary beating.

4.7.3 Deaths

The predator-effects experiment (Chapter 2) was marred by mortality of many *Hermissenda crassicornis.* Of 41 individuals that began the treatments, only 17 survived to the end. None of the other experiments experienced the same level of mortality, including the laboratory-effects experiment that used the same laboratory set-up. The mortalities were most severe in the control group, although all treatment groups had fatalities. The cause of these deaths is unclear. Most deaths occurred in the sea tables located in the same room (the other sea tables were located in an room adjacent). The

water temperature was similar between the two rooms (11.2 ± 0.2°C (most fatalities) and 10.2 ± 0.1°C (less fatalities)), although the air temperature was greater in the room with the most fatalities (20.4 ± 0.1 °C, 18.2 ± 0.1 °C, respectively). I suspect the deaths were related to increased air temperature or an infestation of an unidentified copepod (found in several cerata samples). The laboratory effects treatment group were held in the sea tables that did not experience the majority of the fatalities.

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