A QUANTITATIVE EVALUATION OF MORPHOLOGICAL CHANGES DURING FUSION EVENTS IN A COLONY OF DIDEMNUM VEXILLUM AND OF POLYCUNUM CONSTELLATUM

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A QUANTITATIVE EVALUATION OF MORPHOLOGICAL CHANGES DURING FUSION EVENTS IN A COLONY OF DIDEMNUM VEXILLUM AND OF POLYCLINUM CONSTELLATUM

BY

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THESIS

Submitted to the University of New Hampshire in Partial Fulfillment of the Requirements for the Degree of Master of Science in Natural Resources: Environmental Conservation

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This thesis has been examined and approved.

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May 2nd, 2012

Date
Dedicated to my family for their endless support through this process.
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A QUANTITATIVE EVALUATION OF CELLULAR CHANGES DURING FUSION EVENTS IN THE COLONIAL ASCIDIANS POLYCLINUM CONSTELLATUM AND DIDEMNUM VEXILLUM (TUNICATA)

BY

ANDREA FREY

University of New Hampshire, May, 2012

We documented changes in the abundance and distribution patterns of cells involved in the allore cognition response in two species of colonial aplousobranch tunicates lacking a common vascular system. A histological examination of the fusion junction of isogeneic (CIAs) and allogeneic (CAAs) colony assays revealed that tunic cuticles were rapidly regenerated. The underlying tunic matrix fused readily in Didemnum vexillum in all assay and control types, however it did not fuse in CIAs in Polyclinum constellatum. We identified four and five different cell types in P. constellatum and D. vexillum, respectively. Phagocytic cells represented the most abundant cell type in both species and were most abundant in CAAs, followed by morula (or morula-like) cells. Additionally, the two cell types were more abundant at the fusion junction than at 120 µm or 240 µm from the junction (p<0.05 for P. constellatum; p=0.000 for D. vexillum). Hence, we concluded that phagocytes and morula cells are likely involved in the allore cognition reaction. Filopodial and granular cells, were present, although only at very low abundances. A layer of bladder cells was located immediately below the cuticle in D. vexillum. No bladder cells were found in P. constellatum.
BACKGROUND INFORMATION ON COLONIAL TUNCATA (UROCHORDATA)

INTRODUCTION

Tunicates, commonly known as sea squirts, are exclusively marine, and are the only chordates in which some species form colonies. The subphylum Tunicata (Urochordata) consists of three classes. Members of the classes Appendicularia and Thaliacea are pelagic, whereas Asciacea are sessile. Ascidians a have worldwide distribution and are comprised of the orders Aplousobranchia, Phlebobranchia, and Stolidobranchia, containing with over 17 families and 2,300 described species (Perez-Portela et al., 2009; Satoh, 1994). Most ascidians live as fouling organisms or as epibionts on algal species, sea grasses, hard-bodied marine invertebrates and artificial substrate. Although most ascidians are found in shallow water, they tend to avoid direct light. A few species are interstitial and some are found at abyssal depths (Ruppert et al., 2004).

Ascidians are represented both by solitary and colonial forms. Most colonial tunicates grow as flat sheets of irregular outline that can attain diameters of up to a third of a meter (Cohen, 2005). Individuals composing a colony are called zooids and are arranged into clusters known as systems (Ruppert et al., 2004).
ANATOMY AND TUNIC STRUCTURE

Generally, a zooid possesses a thorax, an abdomen, and reproductive organs. Zooids can be divided into one to three regions and these body divisions are of taxonomic importance. Zooids of *Polyclinum constellatum* are composed of three regions, namely the thorax which houses a branchial basket (pharynx), the abdomen which contains the digestive tract, and the post-abdomen which includes the heart and reproductive organs (Fig. 1a). Zooids of *Didemnum vexillum* are divided into a thorax and an abdomen (Fig. 1b).

![Figure 1. Anatomy of zooids of (a) *Didemnum vexillum* (modified from Kott, 2002) (b) and of *Polyclinum constellatum* (modified from Van Name, 1945). BS, branchial siphon; BB, branchial basket; AS, atrial siphon; E, endostyle; T, testis; S, stomach; G, gonads.](image)

Each zooid has two siphons. The oral or branchial siphon actively pumps water containing food and organic compounds into the branchial basket, where mucus strands trap food particles and move them by ciliary action to the esophagus, while excess water is forced out through pharyngeal gill slits. Food travels through a U-shaped intestine.
where it is digested and absorbed. The rectum opens into the atrial cavity and waste is expelled through the atrial (cloacal) siphon.

All ascidians, solitary or colonial, are enveloped in a tunic that is secreted by the epidermis. Physiological characteristics of the tunic, such as color, thickness, firmness, types of inclusions and interdependency of zooids within the tunic are species-specific (Burighel and Cloney, 1997). The outermost layer of the tunic is very dense and composed of stacked sheets of tunicin (cellulose) fibers running at different angles. Their arrangement is reminiscent of arthropod cuticles (Ruppert et al., 2004). Below this fibrous cuticle are layers containing various tunic cell types, sensory receptors and in some species, a vascular network and/or calcareous spicules. The tunic lacks muscles and nerves. The main functions of the tunic are to attach zooids to the substrate and to form a protective external skeleton.

In colonial ascidians, zooids are embedded in a common tunic. The zooids are arranged into systems with each zooid maintaining a branchial siphon independently from the colony. However, the zooids of each system in a colony share an exhaust or cloacal siphon (Fig. 2).

Figure 2. Schematic of a botryllid ascidian, showing arrangement of zooids in a common tunic. (Left) Dorsal view of colony showing one system. (Right) Section through two individual zooids connected by a common blood vascular system and sharing a cloacal siphon (modified from Hirose, 2003).
Stolidobranch ascidians possess a common vascular system that connects the zooids of a colony (Fig. 2). At the periphery of the colony the vascular network ends in sausage like ampullae. The vascular system including the ampullae, is responsible for assisting in asexual propagation, substrate adhesion, and blood flow regulation (Gasparini et al., 2007).

Most importantly, hemocytes in the vascular system have been identified as the effector cells of the allorecognition response, i.e., the ability of an organism to distinguish self from non-self tissues (Hirose et al., 2003).

Aplousobranch ascidians on the other hand, lack a common vascular system (Fig. 3). Again, zooids are embedded in a common tunic and share a cloacal siphon.

![Figure 3](image.png)

**Figure 3.** Schematic of a vertical section through the colonial ascidian *Diplosoma listerianum*, showing arrangement of zooids in a common tunic. Didemnids lack vascular systems. Each zooid hangs from the upper tunic by the oral siphon and is attached to the basal tunic layer with a strand of tunic that originates from the lower layer. The entire colony is attached to the substrate via “crampons”. Arrows indicate water flow (modified after Mackie and Singla, 1987).

Unlike stolidobranchs, species in this order possess several different tunic cell types located within as well as on the outside of the tunic (Hirose, 2009). Phagocytes are present in the tunic of all ascidians and are responsible for engulfing foreign particles or discarded cells. Tunic bladder cells are present in Didemnidae. These highly vacuolated cells contain sulfuric acid and possibly provide chemical defense against predation.
Tunic net cells or myocytes are present in Didemnidae and Polyclinidae and form a network consisting of long, connecting filopodia (Hirose, 2009). The network is responsible for contracting the tunic around wounds and for slight colony movement (Mackie and Singla, 1987). Morula-like cells are responsible for biological defense; these cells are major contributors to the allorecognition response of ascidians. Tunic granular cells (granulocytes) are cells in which the cytoplasm is filled with a granular material; their function is mostly unknown. Tunic pigment cells, phycocytes that contain symbiotic algae and luminocytes responsible for bioluminescence, are also present in some species (Hirose, 2009).

**LIFE HISTORY**

All colonial ascidians are hermaphroditic and can reproduce either sexually or by asexual budding. Although selfing does occur, cross-fertilization is the norm. Depending on the species, gamete release can be continuous or occurs during breeding periods that often are dictated by photoperiod (Burighel and Cloney, 1997). Sperm fertilize eggs that have been ovulated into specialized brood pouches. Embryos are brooded sometimes for weeks or months and are released as tadpole larvae. Metamorphosis occurs within hours of larval release. During metamorphosis, the anterior end of the larva bearing adhesive papillae, attaches to the substrate. The tail and notochord are reabsorbed, the branchial siphon is positioned opposite the attached larval end, and the intestine bends into its characteristic U-shape. Colony growth is asexual, resulting in all zooids within one colony being genetically identical (Manni et al., 2007).

Human-mediated transport of marine species is responsible for the successful invasion of many harbors, bays, and coastlines worldwide, and the Gulf of Maine has not been exempt (Berman et al., 1992; Bullard et al., 2007). Among the latest arrivals are
tunicates that increasingly dominate fouling communities (Dijkstra et al., 2007a, b). Currently, four colonial, non-native ascidians (*Botryllus schlosseri, Botrylloides violaceus, Diplosoma listerianum, Didemnum vexillum*) have been reported in the Gulf of Maine (Dijkstra et al., 2007a). Most colonial tunicate are considered inedible by potential predator and reproduce more quickly than other fouling organisms giving them the ability to rapidly alter benthic communities (Vance et al., 2008).
REFERENCES


CHAPTER II

A QUANTITATIVE EVALUATION OF CELLULAR CHANGES DURING FUSION EVENTS IN THE COLONIAL ASCIDIANS

**DIDEMNUM VEXillum AND POLYCLINUM CONSTELLATUM** (Tunicata)

INTRODUCTION

Although space is a limited resource in fouling communities, colonial ascidians often represent a disproportionately large component (Dijkstra et al. 2007). Their success is based on high reproductive rates, indeterminate growth, and an ability to either overgrow or inhibit recruitment of competitors. As ascidians grow, they may encounter either conspecifics or colonies of a different species. Upon physical contact, adjacent colonies of the same species may fuse into one larger colony or they may remain separate entities (Westerman et al. 2009). Fusion (or rejection) is determined by allorecognition, i.e., the ability of an organism to distinguish its own tissues from those of another individual. According to Hirose et al. (1994), allorecognition in tunicates is mediated by hemocytes circulating in the vascular system and tunic cells freely moving throughout the tunic.

Most studies of tunicate allorecognition have focused on stolidobranch species, especially *Botryllus schlosseri* (Pallas, 1766). Stolidobranchia are characterized by a common vascular system, connecting all zooids and containing several types of hemocytes (Ruppert et al. 2004). In some species (e.g., *Botryllus scalaris* Saito and Mukai, 1981, *B. sexiensis* Saito and Watanabe, 1981), allorecognition may require complete tunic fusion and the formation of a common vascular system between the two
colonies, followed by the actual exchange of blood (Cohen et al. 1998; Hirose 2003). Interactions between hemocytes and humoral factors in the blood plasma then determine if fusion is successful. Alternatively, allorecognition can occur with even minimal colony contact (e.g., *Botryloides violaceus* OKA, 1927 or *B. simodensis* SAI TO AND WATANABE, 1981) in which two colonies push against one another with some blood leakage occurring (Hirose et al. 1988). Among botryllid ascidians, these two scenarios represent the extremes of a continuum.

Aplousobranch ascidians on the other hand, lack a common blood vascular system (Ruppert et al. 2004) and therefore, depend on allorecognition mechanisms that are based on various types of tunic cells. Knowledge regarding the importance of tunic cells in the allorecognition response of aplousobranchs is limited to *Diplosoma listerianum* (MILNE-EDWARDS, 1841), *Aplidium yamazii* (TOKIOKA, 1949), and *Didemnum perlucidum* (F. MONNIOT, 1983). Indiscriminate fusion was described first in *D. listerianum* (Bishop and Sommerfeldt 1999). Because sibling, half-sibling, unrelated, and even geographically distinct colonies readily fused, Bishop and Sommerfeldt (1999) concluded that colony specificity is much less developed or possibly even absent in this species.

*Aplidium yamazii* has become the model for studying allorecognition in Aplousobranchia (Hirose et al. 1994a, b; Ishii and Hirose 2003; Ishii et al. 2008). Hirose et al. (1994b) documented seven different types of tunic cells in *A. yamazii* and established that phagocytes were the most abundant type. In fact, Ishii et al. (2008) identified phagocytes as the effector cells of allorecognition in this species.

Recently, Dias and Yokoyama (2011) demonstrated that a second tunic cell type, namely morula cells, aggregates at the natural growing edge of *Didemnum perlucidum* during an allogeneic challenge. Morula cells produce quinones, which cause a necrotic reaction at the contact site of incompatible colonies. This toxic response is thought to
limit growth of competing colonies. From these studies, it is evident that the cellular components responsible for allorecognition in aplousobranchs are quite different from those found in botryllids.

To understand aplousobranch fusion events, I examined allogeneic (pieces from to separate colonies) and isogeneic (two pieces of the same colony) challenges in *Polyclinum constellatum* SAVIGNY, 1816 and *Didemnum vexillum* KOTT, 2002 at the light microscopic level. Furthermore, I identified and quantified different tunic cell types and determined their distribution patterns at the fusion site.

**MATERIALS AND METHODS**

**Focus Species:** *Polyclinum constellatum* is found along the Atlantic coast of North and South America as far north as Bermuda and extending to Rio de Janiero, Brazil (Locke, 2009; Rocha and Costa, 2005). In the Pacific, it is present in the Hawaiian Islands, on the eastern coast of Africa (Tanzania and Mozambique), Guam, and western India, also around the Philippines extending to Hong Kong (Rocha and Costa, 2005; Lambert, 2003; Van Name, 1945; Locke, 2009). *P. constellatum* is considered an introduced species in most areas (Rocha and Costa, 2005). Colonies vary in color from dark green to purplish brown (Rocha and Costa, 2005; Van Name, 1945). The tunic is thick and gelatinous measuring 30-40 mm in height. Systems of about 20 zooids are concentrated in the upper portion of the tunic (Rocha and Costa, 2005; Van Name, 1945). Larger colonies are often attached to the substrate by the peripheral edge only with the center portion raised off the substrate like an umbrella (Van Name, 1945). Thriving on artificial substrates, this species has the ability to become a species of concern for shellfish aquaculture, although opinions differ. Rocha and Costa (2005) predict that *P.*
*P. constellatum* was most likely introduced to southern Brazil but show that colonies are small, infrequent and do not rapidly spread. Though not currently ranging as far north as Atlantic Canada, Locke (2009) regards *P. constellatum* as a likely invader and potential threat to Canadian aquaculture.

*Didemnum vexillum* is native to Japan but has been introduced to the United States, Europe, New Zealand, and Canada and shows a wide tolerance to environmental conditions (e.g., water temperatures of 31 to -24 °C; salinity of 25 - 39 ppm) (Daniel and Therriault, 2007). This tolerance, combined with a fast growth rate and few known predators, makes *D. vexillum* a threat to local biodiversity. *D. vexillum* zooids measure 1-2 mm in length and are very conspicuous in their tunic. The tunic is cream to yellow in color and contains many spicules giving the colony a white speckled appearance. Colonies encrust flat surfaces and also form long lobes or ropes hanging off the substrate. Colony mats have been observed to cover 230 square kilometers on Georges Bank (Stefaniak et al., 2009).

**Specimen Collection and Culture:** Six-cm diameter *Polycinum constellatum* colonies were collected during the summer of 2009 at the Bocas Marina, Bocas del Toro, Panama (9°20'08" N; 82°14'50" W) and maintained in flow-through aquaria of unfiltered seawater at the Smithsonian Tropical Research Institute (STRI).

*Didemnum vexillum* averaging 12 cm², were collected during the summer and fall of 2010 and 2011 at Wentworth by the Sea Marina (WM) in Portsmouth Harbor, New Hampshire (43° 04’18” N; 70° 42’37” W) and Hawthorne Cove Marina (HCM), Salem Harbor, Massachusetts (42° 31’17” N; 70° 52’55”). Colonies were maintained in flow-through aquaria of unfiltered seawater at the Coastal Marine Laboratory (CML) in New Castle, New Hampshire. Colonies collected at HCM were allowed 24-48 hours to acclimate at CML.
**Polyclinum constellatum Colony Fusion Assay:** Allogeneic (colony allogeneic assay, CAA) and isogeneic (colony isogeneic assay, CIA) combinations were used to study tunic cell distribution and abundance patterns. Ten colonies of each color morph, red and blue, were collected at Bocas del Toro Marina, Panama. Fusion assays involved cut-edge combinations of both same and different color morph combinations. Allogeneic and isogeneic assays were established by cutting colonies with a clean razor blade and abutting the pieces at the cut edge (Fig. 4).

![Figure 4](image)

**Figure 4.** Diagrammatic view of (a) isogeneic and (b) allogeneic challenges.

Colony pieces were tied to microscope slides with cotton string. Assays were repeated if one or both of the colonies died. Slides were placed vertically into flow-through aquaria of unfiltered water at ambient temperature (86 °C). Photographic records were taken of each assay at 12-h intervals under a dissecting microscope equipped with a CoolPix 990 digital camera. Samples were removed from the slides after 48 h, relaxed with menthol crystals in seawater for 2 h, the fusion junction was excised, and cut into approximately 2 mm segments. Segments were then placed in labeled vials containing 10% buffered formalin.
**Didemnum vexillum Colony Fusion Assay:** For *isogeneic* challenges (CIA), two 1.5 cm x 1 cm wide marginal pieces were cut from the edge of a single colony and abutted on a glass slide at the cut edge (A + A). The two pieces were then tied in place with cotton string and kept in separate, flow-through aquaria of unfiltered water for 48 hours at ambient ocean temperature (20 °C). No food was provided other than what was available in the seawater. Replicates were established by repeating these CIAs three times with additional pieces from the same colony (three times A + A). Independence among assays was established by repeating this experimental design two more times with pieces from different colonies (B + B and C + C, three times each). In *allogeneic* challenges (CAA), two 1.5 cm x 1 cm wide marginal pieces were cut from the edge of two different colonies and abutted on glass slides at the cut edge (D + E; F + G; H + I, three times, each). The culture conditions were the same as for isogeneic challenges.

Two types of controls were developed. First, a baseline of cell aggregation at the site of trauma (wounding) was established to quantify differences between aggregations in response to wounding vs. aggregations in response to a CAA. A marginal portion of a colony was excised and attached to a slide with cotton string. After 48 h, the colony pieces were fixed in 10% buffered formalin. This type of control was repeated three times with three different colonies. A second type of control involved the quantification of tunic cells naturally present at the colony margin. A marginal piece of a colony was excised with a clean razor blade and immediately fixed in 10% buffered formalin.

**Light Microscopy:** Fixed samples were rinsed in distilled water and dehydrated through a graded ethanol series. Samples were infiltrated first with a 50:50 polyacrylate resin (Acrylate Embedding Resin, Ted Pella) - ethanol mixture for 3-4 h, followed by infiltration with pure resin for 6-8 h, and a second fresh resin infiltration overnight. Each tissue segment was placed into a “00” gelatin capsule with the top of the colony facing down.
The capsules were filled with resin to just cover the tissue and cured overnight in a VWR 1310E oven at 60-65 °C. Capsules were allowed to cool and then filled to the top with resin and cured again overnight. Resin blocks were rough-trimmed with a Dremel 7.2V rotary tool, followed by fine-trimming with a razor blade. Thick sections (4-5 µm) were cut with a Sorvall JB-4 Microtome, using glass knives. Section were transferred to glass slides and allowed to dry after which they were stained with Epoxy Tissue Stain (Electron Microscopical Science Hatfield, PA), mounted in Cytoseal 60 (Richard-Allen Scientific), and covered with glass cover slips.

Stereology: Cell abundances and distribution patterns were documented with an Olympus C-35AD-2 microscope camera system. Using sectioned material mounted on microscope slides, three transects were established for each assay and for the controls. A grid of 0.8 cm² squares with random numbers (0-99) assigned to each square was projected over the fusion junction at a focus of 1 µm = 1 cm. Three different random number grids were used. Two single digit random numbers were obtained from a random numbers table and boxes containing these numbers were shaded. Cells within each shaded box were categorized by cell type at distances of 0 µm, 120 µm, and 240 µm from the fusion junction. Cell types were categorized into five categories, according to the terminology proposed by Hirose (2009): (i) filopodial (or net) cells, (ii) morula (or morula-like) cells, (iii) phagocytes, (iv) granular cells (or granulocytes), and (v) bladder cells.

Analysis: An ANOVA with a Tukey’s test was used to quantify differences between CAAs and CIAs, as well as wound and natural edge control groups. Cell type abundances and differences in distance from the fusion junction or from the colony periphery were also compared using an ANOVA with Tukey’s test.
RESULTS

1. *Polyclinum constellatum*

Of the 16 challenges conducted with *Polyclinum constellatum* colony fragments, 7 resulted in fusion and were used in further histological analysis. Assays in which colonies did not fuse or in which one partner died were eliminated from further analysis.

1. 1. Gross Morphology and Histology of the Fusion Junction

A series of images taken at 0, 24, and 48 h, respectively after initial contact revealed that the junction site of a CAA transformed from a highly distinct border to an area recognizable only by a difference in color (Fig. 5a-e). Tunic material completely filled the junction and the two pieces appeared as a continuous colony. Allogeneic combinations fused more readily and completely (i.e., all tunic layers fused), and survived more frequently than CIAs. Isogeneic combinations also resulted in fused colonies (Fig. 5g-h), however, only the cuticle fused while the underlying layers of the tunic did not fuse into a continuous covering.
Figure 5. Gross morphology of allogeneic and isogeneic challenges in *Polyclinum constellatum*. 
(a, b) Allogeneic contact zone at 0 h, scale bars = 5 mm and 3 mm, respectively; (c, d) at 24 h, scale bars = 1 mm; (e, f) at 48 h, scale bars = 5 mm and 1 mm, respectively. (g, h) Isogeneic contact zone at 48 h, scale bars = 5 mm and 1 mm, respectively.
At the histological level, numerous tunic cells aggregated at the junction of CAAs (Fig. 6a-c), whereas in isogeneic challenges, colonies remained distinctly separate with few tunic cells migrating to the junction (Fig. 6d-f).

![Figure 6](image)

**Figure 6.** Light micrographs of the fusion junction of *Polyclinum constellatum*. (a-c) Allogeneic contact zone showing tunic cell pattern at the fusion junction. (d-f) Isogeneic contact zone, showing cell aggregation to fusion junction. Scale bars (a, d) = 100 μm, scale bars (b, e) = 50 μm, scale bars (c, f) = 10 μm.

Isogeneic challenges (Fig. 6d-f) resulted in two abutted colony portions, sharing a thickened squamous cuticle. Necrotic tissue, tunic cells and fragments of zooids were often found between the two fragments. A continuous tunic as seen in CAAs was never observed in CIAs. Colonies appeared to be fused only superficially. When sectioned, they appeared to have grown close together and to have formed a thick edge at the junction site with minimal numbers of tunic cells present.

1.2. Tunic Cell Identification
Four different types of tunic cells were identified at the fusion junction (Fig. 7). Round morula (or morula-like) cells were larger (15 μm) than any other cell type and were completely filled with large vesicles that often stained more readily than other cellular components (Fig. 7a). Phagocytes were identified by their irregular shape and presence of small, darkened vacuoles, which are assumed to be phagosomes. These cells also had a grainy cytoplasm (Fig. 7b).

Filopodial cells (Fig. 7c) were approximately 5μm in length and characterized by long filopodia (10-15 μm). Granular cells (Fig. 7d) were identified by the presence of numerous small round vacuoles. They were differentiated from phagocytes by the number of vacuoles present; phagocytes had less than four vacuoles and granular cells...
had four or more vacuoles. Furthermore, granular cells also did not have the grainy cytoplasmic appearance characteristic of phagocytes (Fig. 7b, d).

1.3. Tunic Cell Abundance and Distribution Patterns

Overall, the most abundant cell types were phagocytes (Fig. 8), and they were more abundant in CAAs than in CIAs (p= 0.002). Morula cells represented the second most abundant cell type (Fig. 8) and again, were more numerous in CAAs than in CAIs (p=0.000). No differences were found in the abundances of granular cells (p=0.895) or filopodial cells (p=0.273) for either type of challenge (Fig. 8).

![Bar graph showing average abundances of different cell types in allogeneic and isogeneic assays. Asterisks indicate significance between allogeneic and isogeneic combinations, n. s. indicates no significance; cell types were not compared to one another for significance, error bars are two standard error (n=30; p=0.000).]

Comparing average tunic cell abundances for the two types of challenges at the fusion junction and at two distances from the junction, revealed greater numbers in CAAs than in CIAs immediately at the junction itself (0 μm) (p<0.05). No differences were found at 120 μm and 240 μm from the junction for either assay type (p>0.05) (Fig. 9).
Figure 9. Difference in abundances of all cell types at three distances from the fusion junction for allogeneic and isogeneic challenges. Asterisks indicate significance; n. s. indicates no significance, error bars are two standard error (n = 30; p<0.05).

This difference was mostly due to the presence of phagocytes, which were more abundant at the junction in either type of assay (p=0.00) (Fig. 10a). The same comparison for morula cells also revealed significance at the immediate junction but only in allogeneic challenges (p=0.000) (Fig. 10b).
2. Didemnum vexillum

Of the 36 challenges conducted, 24 resulted in fusion and were used in histological analysis. Five wound controls were established and 10 different wound edge regions of 3 different colonies were analyzed. Twelve different natural edge regions were excised from 4 independent colonies and of these 9 replicates were analyzed. Trials in which colonies did not fuse or in which one partner died were eliminated from further analysis.

2.1. Gross Morphology and Histology of the Fusion Junction and the Colony Edge

Light microscopic records at 0 and 48 h after initial contact revealed that the junction of both CAAs and CIAs transformed from a highly distinct border to a continuous colony in 70 percent of assays (Fig. 11a-d). In the remaining challenges either one or both colonies died or failed to adhere to the slide and in a very few combinations both colonies survived but fail to fuse. Fusion was considered successful if any portion of the
contact zone from one colony was fused to the other colony. After 48 h, the cut edge of
wound controls had healed and was indistinguishable from non-injured tissue (Fig. 11e).
Natural edge controls often started to grow over the lower edge of the slide (Fig. 11f). In
a majority of combinations, colonies grew over the string that tied them to the slide (Fig.
11b-f).

Figure 11. Gross morphology of assay and control groups of *Didemnum vexillum*. (a,b)
Allogeneic contact zone at 0 and 48 h; (c,d) Isogeneic contact zone at 0 and 48 h; (e) Wound
control; (f) Natural edge control. All scale bars = 10 mm.
Light micrographs revealed that tunic cells aggregated at the fusion junction (Fig. 12a-b) and that they were also present in the wound and natural edge control (Fig. 12c-d).

Figure 12. Light micrographs of challenged and control groups after 48 h of contact. (a) Allogeneic colony contact. Scale bar = 20 μm. (b) Isogeneic colony contact. Scale bar = 20 μm. (c) Wound control. Scale bar = 20 μm. (d) Natural edge control, note layer of tunic bladder cells below the cuticle. Scale bar = 50 μm. Z, zooid; M, morula cell; P, phagocyte; Mp, macrophage; Sp, spicule; B, bladder cell.

2. 2. Tunic Cell Identification

Five different types of tunic cells were identified. Filopodial cells were the smallest (8 μm in diameter) and possessed long filopodial tails (approx. 15-20 μm in length) (Fig. 13a), whereas bladder cells (Figs. 12d, 13b) were the largest of all tunic cell types (35 μm diameter). Generally, bladder cells were round and their interior was almost completely filled by a large vacuole. Their nuclei resided in a small amount of cytoplasm at the periphery of the cell (Fig. 13b). Mostly bladder cells were found in a
layer just below the cuticle (Fig. 12d). Phagocytes were characterized as irregularly shaped cells measuring 8 -16 μm in diameter (Figs. 12a-c, 13a-c) and contained easily distinguishable nuclei. The cytoplasm of phagocytes was grainy and often contained many vacuoles assumed to be phagosomes (Fig. 13c). Phagocytic macrophages were large (28 μm average diameter), round cells containing a single large vacuole (Figs. 12a-b, 13a-b). Numerous round vesicles of fairly uniform size and no noticeable nuclei characterized morula cells (Figs. 12a-c, 13a-c). These vesicles give the cells their "mulberry-like" appearance (hence the name, morula). Typically, morula cells measured 20 - 25 μm.

Figure 13. Light micrographs of Didemnum vexillum tunic cells. (a) filopodial cell; (b) bladder cell; (c) phagocyte; (d) macrophage; (e) morula cell. All scale bars = 15 μm.
2. 3. Tunic Cell Abundance and Distribution Patterns

Allogeneic and isogeneic challenges and natural growing edge controls resulted in greater average tunic cell abundances than wound controls (p=0.028) (Fig 14). Among those three assays, the greatest abundances were found in CAAs (p=0.330), while CIAs and natural edge controls had similar averages (p=0.954).

Figure 14. Average abundances of all cell types of different challenges and control groups. CAAs, n = 45; CIAs, n = 39; Wound control, n = 30; Natural edge control, n = 27; (p=0.001), error bars are two standard error.

More cells were present at the fusion site and at the natural colony edge for all combinations and control groups than at any other distance (p=0.000). There was no difference in average cell abundance at 120 μm and 240 μm from the junction or from the colony edge (p=0.266) (Fig. 15).
Within allogeneic combinations, average tunic cell abundance at the fusion junction (0 µm) was greater than at 120 µm and 240 µm from the fusion junction (p < 0.05). Isogeneic challenges reveal no differences between average cell abundance and distance from the fusion junction (p=1.00). Average tunic cell abundance in the wound control was also not significant over the three distances from the natural edge of the colony (p=1.00). Within the natural edge control, average abundance of tunic cells was greatest at the fusion junction, however a similar number of cells was also found at 120 µm from the fusion junction (p=0.27). Significance was found between cell abundances at the fusion junction and at 240 µm from the junction (p=0.05). No difference was found between 120 µm and 240 µm from the colony edge (p=1.00) (Fig. 16).
For the purpose of cell abundance calculations of individual cell types, phagocytes and macrophages were combined into one category (phagocytic cells) because of their similar function. Average cell abundances of phagocytic cells were the same among CAAs, CIAs, and the natural edge control group ($p=0.816$). However, their abundances in wound control groups were different from all other groups ($p=0.001$) (Fig. 17).

Filopodial cells were the least numerous and were found in similar numbers in CAAs, CIAs, and the natural edge control group ($p=1.000$). However, their abundances in CAAs and CIAs were different from abundances found in the wound control group ($p=0.029$ and $0.034$, respectively). No difference was found in filopodial cell abundances between wound control and natural edge control ($p=0.946$) (Fig. 17).
Average cell abundances for bladder cells were the same in CAAs, CIAs, and the wound control group (p=0.996). However, in the natural edge control group, bladder cells were found in larger proportion (p=0.000). Finally, there was no difference in average cell abundances of morula cells over all assay and control groups (p=0.998) (Fig. 17).

![Average abundances of different cell types for different combinations and control groups, all distances combined (n=141, p=0.000).](image)

**Figure 17.** Average abundances of different cell types for different combinations and control groups, all distances combined (n=141, p=0.000).

The abundances of the two most common cell types (phagocytic cells, morula cells) were also compared at different distances from the fusion junction or from the natural growing edge of the colony (Figs. 18, 19). Phagocytic cell abundances varied significantly among assay types when distance from the fusion junction or from the natural edge of the colony was considered (p=0.000) (Fig. 18). Only the wound control was different from the challenge groups and natural edge control at 0 µm (p=0.000). At 120 µm and 240 µm from the junction or colony edge, all groups showed similar
phagocytic cell abundances (p= 1.000) (Fig. 18). Within the allogeneic combination, phagocytic cell abundance at 0 μm was greater than abundances at either 120 μm or 240 μm from the fusion junction/colony edge (p = 0.002 and p = 0.000, respectively). Phagocytic cell abundances at 120 μm and 240 μm from the fusion junction/colony edge were identical (p = 0.842). CIs and control groups did not differ in their phagocytic cell abundances over the three distances (p = 1.00) (Fig. 18).

![Figure 18](image)

**Figure 18.** Comparison of phagocytic cell abundance (includes phagocytes and macrophages) at three distances from the fusion junction or the natural colony edge. Asterisks indicate significance, n.s. indicates no significance, error bars are two standard error (n= 141, p= 0.000).

Morula cells represented the second most numerous cell type (Fig. 17). Only CAAs were different from other combinations and control groups at 0 μm from the fusion junction/colony edge (p = 0.047). No differences were found among challenge and control groups at either 120 μm or 240 μm (p = 1.000 and p =1.000, respectively) (Fig. 19).
DISCUSSION

Most studies of ascidian allorecognition involving immunogenic challenges and cell type identifications have focused on botryllid species. In this taxon, allorecognition is mediated by hemocytes circulating through a common vascular system to the site of an immunogenic challenge (Mukai and Watanabe 1974; Hirose et al. 1990; Shirae et al. 1999; Hirose 2003; Rinkevich 2005). Aplousobranch ascidians on the other hand, lack a common vascular system and allorecognition is mediated via different types of tunic cells that move through the tunic. To date, cellular events in aplousobranch allorecognition have been described for three species (Bishop and Sommerfeldt 1999; Ishii and Hirose 2003; Ishii et al. 2008; Dias and Yokoyama 2011). Here I contributed information on different cell types, and their abundance and distribution patterns during CAAs and CIAs for two additional aplousobranch species.

Ascidian tunics consist of a fibrous, gelatinous matrix containing free tunic cells. A thin layer of dense fibers, forming a continuous cuticle covers the tunic matrix (Hirose et al. 1995; Hirose et al. 1997). The cuticle provides a first line of defense against

**Figure 19.** Comparison of morula cell abundance at three distances from the fusion junction or from the natural colony edge; n. s. = not significant, letters a and b indicate Tukey’s Test of Significance, error bars are two standard error (n= 141, p=0.009).
invading microbes, abrasions, and wounding. Hence, its structural integrity is critical for the protection of the zooids and as a result, cuticle regeneration is rapid (Hirose et al. 1995; Hirose et al. 1997). My study supported this notion. Within 48 h of abutting cut pieces of the same or different colonies, the cuticles in both species had fused completely. Additionally, in *Didemnum vexillum* the underlying tunic matrix also had fused, regardless of assay or control type. However, in *Polyclinum constellatum* complete fusion of all tunic layers only occurred in CAAs. In CIAs only the cuticle fused, while underlying layers remained distinctly separated by actual spaces containing necrotic tissue and fragments of zooids. Furthermore, histological examinations revealed the appearance of very few phagocytes and morula cells at the junction. It is possible that in this species, CIAs are not perceived as strong immunogenic challenges, and that as long as a regenerated cuticle protects the colony, the damage to the underlying tunic matrix itself is repaired slowly by re-growth and the activities of phagocytes.

In both species, total tunic cell abundances were greater in CAAs than in CIAs, providing evidence that tunic cells are involved in allore cognition between two different colonies. However, because no wound control trials were performed in *P. constellatum*, I could not distinguish between tunic cell mobilization due to allore cognition per se or due to a response to wounding. In *D. vexillum*, the response was clearly due to the allogeneic challenge.

Relying mostly on the classification system developed by Hirose (2009), I identified four and five different tunic cell types in *Polyclinum constellatum* and *Didemnum vexillum*, respectively. In both species, phagocytic cells (phagocytes + macrophages) were the most abundant type, and were most numerous at the immediate fusion junction. However, in *P. constellatum*, phagocytes were less numerous in CIAs, whereas there was no difference in *D. vexillum* based on assay type. This was consistent with my observation that in *P. constellatum*, only the cuticles fused during
CIAs while the underlying tunic matrices remained separated by necrotic tissue. However, because aplousobranchs lack a common vascular system, physical contact between colony pieces is required for a proper allore cognition response (Ishii et al., 2008 and Dias and Yokoyama, 2011). It is likely that without contact, fewer tunic cells are recruited to the immunogenic challenge site.

Only phagocytes were identified in \textit{P. constellatum}, whereas a second phagocytic cell, namely macrophages could be differentiated in \textit{D. vexillum}. The distinction was based on cell size, shape and the number of presumptive phagosomes. Phagocytes have been described as a common cell type in other aplousobranch ascidians, too (Hirose et al. 1994a, b; Hirose 1999, 2009; Ishii and Hirose 2003; Ishii et al. 2008; Turon et al. 2005). Functionally, phagocytes are scavenging cells, engulfing cellular debris, foreign or dead matter, and zooid fragments. They have been identified as effector cells in allorejection and may carry the recognition sites of allore cognition (Ishii et al. 2008). Such cells would then be expected in greater abundances in areas of an immunogenic challenge. My data strongly supported this hypothesis. Phagocytic cells were also numerous in the natural colony periphery of \textit{D. vexillum}. It is possible that this represents a baseline number of phagocytes at the periphery of a colony. However, the colonies were collected from heavily fouled floating docks where the natural edge of a colony is a site of constant immunogenic challenge.

In both species, the second-most abundant tunic cell type was the morula (or morula-like) cell, and similar to the phagocytic cells, morula cells also were significantly more abundant at the fusion junction than at distance in both species. Morula cells are ubiquitous among colonial ascidians (Smith 1970; Ballarin et al. 1995; Hirose et al. 1994b). They take their name from their mulberry-like appearance, which is the result of numerous small vesicles. Negative staining with periodic acid-Schiff (PAS) in \textit{Halocynthia aurantium} (PALLAS, 1787) and \textit{Cystodytes dellechiajei} (DELLA VALLE, 1877)
unequivocally revealed a lack of acid mucopolysaccharides in morula cells (Smith 1970; Rottmayr et al 2001). Hence it is unlikely that morula cells contribute structural components (e.g., fibers) to the tunic matrix as had been proposed (Endean, 1955, 1960). Instead, a defensive function is more likely. Morula cells stain positive for phenoloxidase, the enzyme that catalyzes the production of quinones (Ballarin 2008, Dias and Yokoyama 2011). The reaction results in cytotoxicity due to oxidative stress (Ballarin et al. 1998). Although I did not specifically stain for phenoloxidase, the Epoxy Tissue Stain employed contains toluidine blue, which stains proteins. The morula cells identified in this study were filled with vesicles whose contents stained with toluidine blue.

Shirae et al. (1999) distinguish a morula-cell mediated (M-type) from a phagocyte-mediated (P-type) allorejection response in different botryllid species. In an attempt to assign allrecognition function in D. vexillum to either morula or phagocytic cells (phagocytes + macrophages), I determined abundances of these cell types for different immunogenic challenges and for a wound control group. Because phagocytic cells were by far the most numerous cell type in all assay and control groups, it is tempting to assign a P-type allorecognition process to D. vexillum. However, considering phagocyte abundances by assay type and distance from the immunogenic site, I found no difference in proportions of phagocytes with the exception of the wound control at the immediate fusion junction. Hence, I concluded that the observed phagocyte abundances in D. vexillum represent baseline numbers. However, an analysis of morula cell abundances with respect to assay type and distance from the fusion junction, revealed significantly more cells at the fusion junction for CAAs. As a result, I identify morula cells as the most likely effector cells in allrecognition reactions in D. vexillum.

Ishii et al. (2008) clearly showed that phagocytes are involved in the allrecognition response of the polyclinid Aplidium yamazii. In comparison, I found that
both phagocytes and morula cells in *P. constellatum* were very abundant at the fusion junction in CAAs but only phagocytes were abundant in CIAs. These results suggest that both cell types are involved in allore cognition in this species. However, because morula cells were the prevalent cell type at the fusion junction in allogeneic challenges, this indicates morula cells are the primary effector cells in CAAs. Phagocytes then play a secondary role and are mostly present in response to wounding. Because the assay types in *P. constellatum* were of a small, limited number, it is impossible to ascertain if the rejection reactions in this species are of the M- or P-type.

Mackie and Singla (1987) describe two separate networks of elongate cells in didemnid ascidians. According to these authors, flattened filopodial cells are spaced throughout the surface layers of the tunic and their processes make contact with each other forming a regular, two-dimensional network. A second network, consisting of presumptive myocytes is located deep within the tunic and also forms sphincters around cloacal apertures (Mackie and Singla 1987). According to Hirose (2001), tunic net cells characterized by long filopodia are found in all polyclinid and some didemnid species. I observed elongate filopodial cells in both species. Although long filopodial tails clearly distinguished such cells, I never detected connections between the cells themselves or their organization into a network. However, this could be due to the fact that they were present only in very low numbers in my samples. In a later review of ascidian tunic cells, Hirose (2009) no longer distinguished between tunic net cells and the myocytes described by Mackie and Singla (1987). Tunic net cells *sensu* Hirose (2009) have been implicated in tunic contraction, especially after wounding (Hirose and Ishii 1995). Upon injury, the tunic shrinks around the wound site, promoting rapid cuticle regeneration. If shrinking and rounding of injured tissue is experimentally prevented, the cuticle will not regenerate (Hirose and Ishii 1995). Although I did not specifically measure tunic shrinkage after cutting, I did observe rounding and shrinking of the tunic in our assays.
Granular cells represented a cell type unique to *P. constellatum*, and was found in all assays. The function of granular cells is unknown (Hirose 2009). However, due to their scarcity in my assays, I conclude that there is very little evidence to infer an allorecognition function for granular cells.

Finally, bladder cells were unique to *D. vexillum* and were present in all assays and controls. Bladder cell were most numerous at the colony periphery as evidenced by the abundances of different cell types at the natural edge controls. Light micrographs of natural edge controls revealed a layer of bladder cells just below the tunic cuticle. This is consistent with findings in other didemnid species (Dionisio-Sese et al. 1997; Maruyama et al. 2003). Bladder cells are almost entirely filled by a single large vacuole, confining the cytoplasm and organelles to a small peripheral area (Hirose 1999, 2009; Rottmayr et al. 2001; Turon et al. 2005). According to Hirose (1999, 2001) and Rottmayr et al. (2001), the vacuoles of bladder cells are filled with either sulfuric or hydrochloric acid. The release of acid in conjunction with spicules embedded in the tunic is thought to deter potential predators (Stoecker 1980a, b). An acidic tunic has also been implicated in preventing epibiont recruitment, which could potentially smother the colony (Stoecker 1978).

In colonial ascidians, bladder cells are taxonomically restricted to species that harbor symbiotic prokaryotes (Hirose et al. 2004). I also occasionally observed prokaryotic cells containing chloroplasts in my histological sections. According to Maruyama et al. (2003), symbiotic *Prochloron* synthesize mycosporine-like amino acids (MAAs), which act as a sunscreen and are also passed on to the ascidian host. However, MAAs are unstable at low pH, and therefore, it is highly unlikely that they are stored in the vacuoles of the bladder cells. Instead, Maruyama et al. (2003) have localized MAAs to the small cytoplasmic region of the bladder cells. Indirect evidence for MAA storage in the cytoplasmic portion of bladder cells comes from an ultrastructural
study, which describes small vesicles with heterogeneous content confined to the cytoplasm of bladder cells (Turon et al. 2005).

Tunic cell abundances at the fusion junction were greater in both *P. constellatum* and *D. vexillum* allogeneic challenges than in isogeneic challenges inferring that tunic cells are responsible for mediating allorecognition in these species at the artificially cut edge. In this study I identify five tunic cell types and show that morula cells and phagocytes mediate allorecognition in both *P. constellatum* and *D. vexillum*.

Colony contact at the cut edge triggers an immunological response beyond what is required for wound repair. This strongly suggests tunic cells, namely morula cells are the effector cells in allorecognition reactions. However, further studies are needed to determine how morula cells regulate histocompatibility between opposing colonies and what biological cues call them into action.
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