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The marine sponge, *Hymeniacidon sinapium*, displays allorecognition of siblings during post-larval settling and metamorphosis to juveniles

L. Courtney Smith [a,b,*]

[a] The Department of Microbiology and Immunology, University of California at Los Angeles, 609 Charles E Young Drive E, Los Angeles, CA, 90024, USA
[b] The Department of Biological Sciences, George Washington University, Suite 6000, 800 22nd St NW, Washington, DC, 20052, USA

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ABSTRACT

Marine sponges, including the crumb of bread sponge, *Hymeniacidon sinapium*, display allorejection responses to contact with conspecifics in both experimental and natural settings. These responses have been used to infer immunocompetence in a variety of marine invertebrates. However, larvae and juveniles from several marine sponge species fuse and form chimeras. Some of these chimeras persist, whereas others eventually break down, revealing a period of allogeneic non-responsiveness that varies depending on the species. Alternatively, for *H. sinapium*, most pairs of sibling post-larvae and juveniles that settle in contact initiate immediate allorecognition and show the same morphological response progression as the adults. This indicates that allorecognition and response occurs during early metamorphosis. Results from *H. sinapium* and other sponge species, in addition to annotations of sponge genomes, suggest that allorecognition and immunocompetence in sponges are mediated by distinct systems and may become functional at different times during or after metamorphosis for different species. Consequently, allorecognition may not be a good proxy for the onset of immunocompetence.

1. Introduction

Substrate space in marine ecosystems is commonly limited, which brings sessile organisms such as sponges, ascidians, and bryozoans into conflict (Russ 1982; Wulff 2006). When growth and increased size correlates with the onset of reproductive capabilities and reproductive success, it requires more substrate area and often leads to competition with neighbors (Buss 1990). Contact with conspecifics (with assumed genetic dissimilarities) results in histocompatibility responsiveness or allorecognition and leads to allograft rejections. Histocompatibility reactions include molecular cues that display self, a molecular receptor system to recognize sameness or difference in the allogeneic cues, and an effector mechanism that results in either fusion upon detection of self or rejection of non-self (Grosberg 1988). These characteristics have been used to demonstrate immunocompetence in species from multiple phyla including cnidarians, echinoderms, and protochordates (Theodore 1970; Barki et al., 2002; Rosengarten and Nicotra 2011; Müller and Rinkevich 2020; Taguchi et al., 2022). In the Phylum Porifera, adult sponges consistently reject allogeneic tissues either by cytotoxicity, the formation of a physical barrier, or simple non-fusion (Smith 1988) when they come into contact naturally or under experimental conditions (Curtis 1979; Hildemann et al., 1979a Evans 1980; Hildemann et al., 1980; Hildemann and Linthicum 1981; Kaye and Ortiz 1981; Bigger et al., 1983; Buscema and Van de Vyver 1983; Neigel and Avise 1983; Van de Vyver and Barbieux 1983; Buscema and Van de Vyver, 1984a; Neigel and Schmahl 1984; Smith and Hildemann 1984; Kaye and Reiswig 1985; Mukai and Shimoda 1986; Smith and Hildemann 1986; Amano 1990; Ilan and Loya 1990; Van de Vyver et al., 1990; Mukai 1992; Humphreys 1994; Yin and Humphreys 1996; Saito 2013). Allograft fusion among conspecifics generally correlates with physical proximity and suggests that individual sponges may be fragmented clones of an original single sponge in marine systems (Curtis 1979; Evans 1980; Kaye and Ortiz 1981; Jokiel et al., 1982; Neigel and Avise 1983; Smith and Hildemann 1984; Wulff 1986; McGhee 2006) or a single genotype or 'strain' of fresh water sponges (Van de Vyver 1975; Mukai and Shimoda 1986). Alternatively, allogeneic post-larvae or newly metamorphosed juveniles of sponge species that have been investigated fuse and form chimeras suggesting that they are not capable of recognizing allogeneic contact (Warburton 1958; Ilan and Loya 1990; Maldonado 1998; Gauthier and Degnan 2008). A postulated selective benefit of chimeras, whether it is based on kin recognition of a sibling or parent or even fusion with non-kin, is increased size leading to improved

* Corresponding author. The Department of Biological Sciences, George Washington University, Suite 6000, Science and Engineering Hall, 800 22nd St NW, Washington, DC, 20052, USA.
E-mail address: csmith@gwu.edu.

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survival and an earlier onset of reproduction (Grosberg and Quinn 1986). A postulated disadvantage of chimeras is the concept of somatic cell parasitism in which cells of one genotype take over production of gametes while the other cells function as the somatic support system, which is a genetic dead end (Buss 1982).

The demosponge, *Hymeniacidon sinapium* (de Laubenfels 1930), called the crumb of bread sponge, is native to the western Pacific Ocean of Korea and Japan. It is an invasive species that was introduced to the eastern Pacific coastline of California likely through shipping and is found in intertidal rocky regions, estuaries, backwater habitats (de Laubenfels 1933; Wasson et al., 2001), and marinas. *H. sinapium* is orange, yellow, or light green and displays an encrusting to partially upright growth form on a range of substrates such as marina pier pilings (Fig. 1A) (Smith and Hildemann 1984) and has also been documented growing in the mud of Elkhorn Slough (Fig. 1B) (Fuller and Hughey 2013). It is plentiful, easily collected from marinas located along the coastline of Los Angeles California, and is tolerant of a wide range of water temperature. Autografts of the adults fuse forming a continuum of the two sponges, whereas allografts consistently undergo rejection with kinetics that are directly related to temperature (Smith and Hildemann 1984). Rejection in *H. sinapium* is first evident as an accumulation of cells at the allogeneic interface. This is similar to rejection in the Hawaiian sponge, *Callyspongia diffusa*, that shows tracks of migrating mesohyl cells, composed mostly of amoeboid archeocytes, that are oriented toward the non-self contact surface (Smith and Hildemann 1986). As the cells accumulate, they displace the aquiferous canals and the flagellated choanocyte chambers that function as the water pumps plus food capture, and the incurrent pores (ostia) close (Johnston and Hildemann 1983). Similar cell accumulations are also noted at allograft interfaces for other marine sponge species (*e.g.* (Hildemann and Linthicum 1981; Bigger et al., 1983),) and fresh water sponges (*e.g.,* (Buscema and Van de Vyver, 1984b; Mukai 1992). Cellular accumulation at allograft interfaces in *H. sinapium* is followed by the appearance of a barrier between the two sponges in addition to a disintegration of the apposed tissues that separate the individual sponges (Smith and Hildemann 1984).

Although adult *H. sinapium* show consistent rejection of allogeneic tissues, the responses in post-larval and juvenile stages of *H. sinapium* to contact with conspecifics have not been addressed. Furthermore, the onset of allorecognition during sponge development from pelagic larvae to benthic juveniles is unknown. Consequently, observations of morphological changes in adult parabioses are compared to post-larval and juvenile responses. Results described herein show that when pairs of sibling post-larvae settle together, most show allorecognition as a response to sibling contact that progresses as metamorphosis to juveniles proceeds. The morphological changes in juveniles are consistent with those observed for allorecognition in adult *H. sinapium*. The timing of allorecognition in post-larval and juvenile *H. sinapium* differs from reports for other sponge species that show fusion of larvae and juveniles that form chimeras, which last for weeks to months (Warburton 1958; Ilan and Loya 1990; Maldonado 1998; Gauthier and Degnan 2008). The variability in the onset of allorecognition capabilities in juvenile sponges suggests that aggregation factors that mediate this system in demosponges (Fernàndez-Busquets and Burger 1997; Fernàndez-Busquets et al., 1998; Grice et al., 2017) become functional at different times post metamorphosis for different sponge species.

2. Methods

Adult *Hymeniacidon sinapium* were collected intact from the Los Angeles Harbor (33.719769°N; 118.280958°W) and from the A basin of Marina del Rey (33.97089°N; 118.456218°W), California. The A basin is located on the beach side of the marina and is the closest basin to the inlet that connects to the Pacific Ocean. Autograft and allograft parabioses of adult sponges were carried out *in situ* as reported (Smith and Hildemann 1984). Other sponges of similar sizes (2.5–7.5 cm) were transferred in seawater to the UCLA campus and placed in a recirculating seawater aquarium (Instant Ocean® sea salts) that was maintained at room temperature (20–22 °C). When nine sponges (eight from Marina del Rey and one from the Los Angeles Harbor) began to release larvae, each sponge was moved to an individual floating plastic beaker with open slots on the sides that were covered with mesh to allow water flow but restricted larval movement to enable their collection. Pairs ($n = 14$) of swimming, sibling larvae released from four individual adult sponges were transferred to conical wells of a Terasaki plate (Sigma) filled with 10 µl artificial seawater (unfiltered Instant Ocean). The small size and conical shape of the well was used to induce the larvae to settle together at the bottom tip of the well. Plates were held at room temperature and larvae were observed for settling. The seawater in the wells was not changed during the observation period and food was not added. Pairs of larvae that settled in contact ($n = 7$) within 8 h of collection and transfer to the wells were imaged with the aid of a dissecting stereomicroscope (Olympus) at magnifications of 25X and 50X. The remaining pairs of larvae ($n = 7$) settled too far apart to establish contact and were

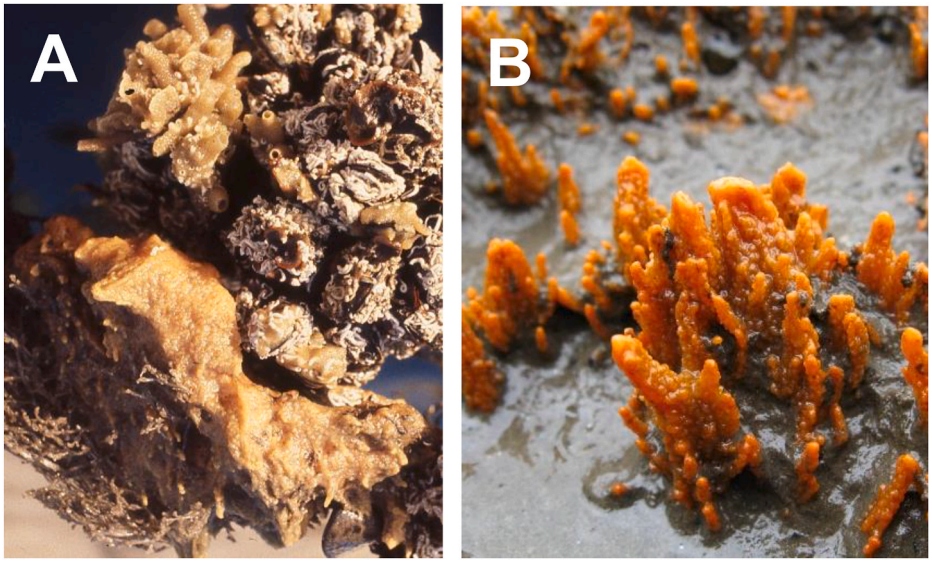

Fig. 1. *Hymeniacidon sinapium*, the crumb of bread sponge. A. An adult specimen of *H. sinapium* collected from a marina in the Los Angeles area. B. An adult *H.* sinapium growing in mud. Image credit is the World Porifera Database (https://www.marinespecies.org/porifera/index.php).

excluded from further observations. All imaging was carried out on living sponges.

3. Results

3.1. The morphology of allorecognition for adult Hymeniacidon sinapium

Autograft fusion and the kinetics of allograft rejection has been reported previously (Smith and Hildemann 1984), however images of the morphological changes were not published. To provide a framework to understand the morphology of post-larval and juvenile fusion or allorecognition, images that illustrate the changes that occur at contact interfaces between adult sponges are presented (Fig. 2). Autograft parabioses of adult sponges consistently resulted in fusions by 1–2 days (Fig. 2A and B). However, upon allogeneic contact, allorecognition in adult *H. sinapium* became discernible with a migration and accumulation of cells at points of contact between the allogeneic tissues (Fig. 2C and D), the timing of which was dependent on temperature. The appearance of tissue at the allogeneic contact points appeared more dense. This change was consistent with infiltrating mesohyl cells that replaced the aquiferous canals and choanocyte chambers. This was similar to the initial stages of allograft rejection noted for other species (*e.g.*, see the images in Johnston and Hildemann 1983)) and was a key morphological change in the tissues that preceded rejection.

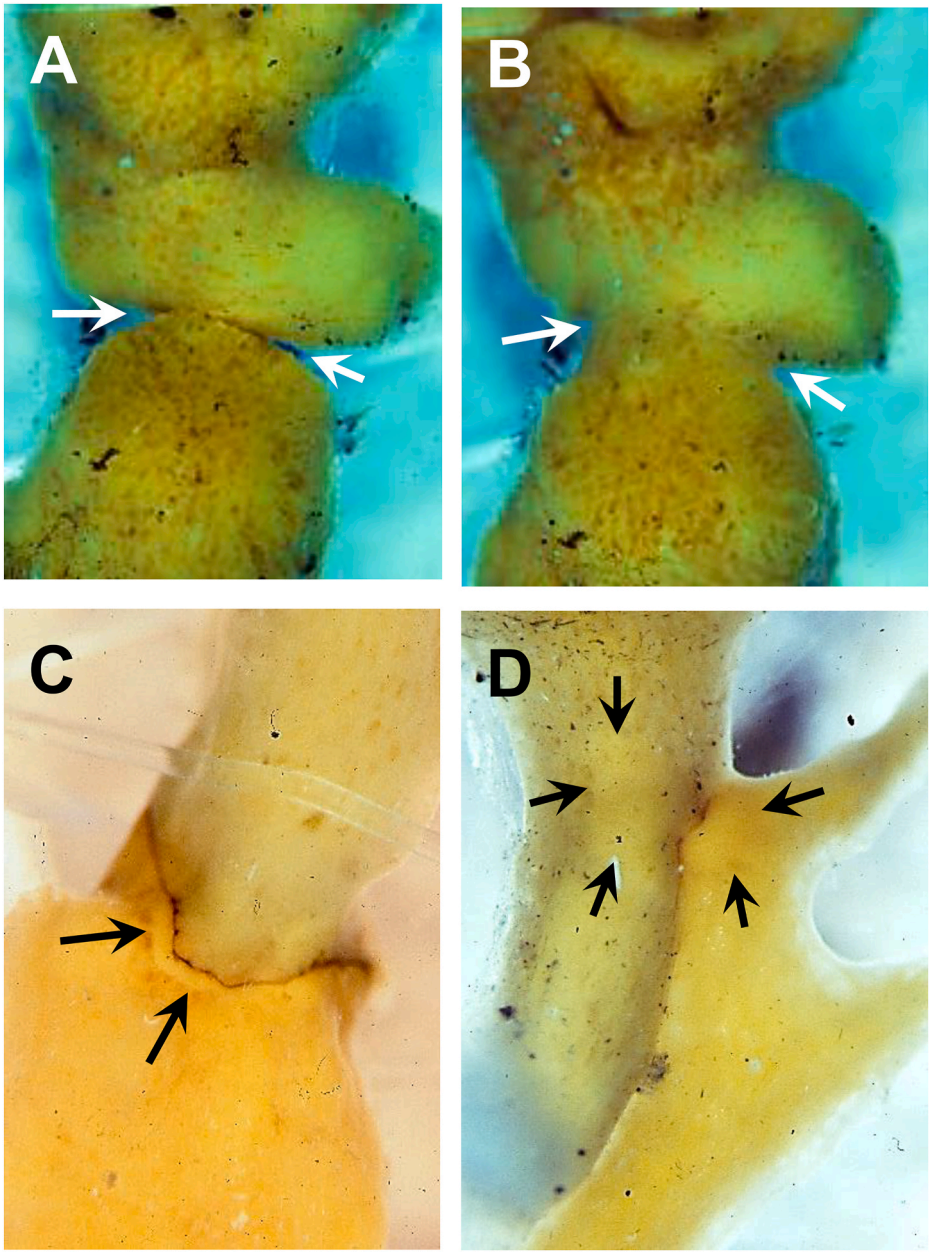

Fig. 2. Parabioses of adult *Hymeniacidon sinapium* demonstrates autograft fusion and allograft recognition. A. Autograft parabiosis at 0 h shows that the sponges are in contact (arrows). B. Autograft parabiosis at 5 days shows that the tissues have fused (arrows) into a single functioning sponge. C. Allograft recognition results in an infiltration of cells from both sponges that accumulate at the interface. The cellular infiltrate is most easily discerned in the lower sponge (arrows). D. Allograft recognition by both sponges shows cellular infiltrates at the interface (arrows). The infiltrate is more prominent in the sponge on the right. These images were, in part, the basis for the description of the allorejection kinetics in (Smith and Hildemann 1984), which did not include images of adult sponge parabioses.

3.2. Allogeneic contact recognition in post-larvae and juveniles matches that in the adults

Larval sponges develop in brood chambers within adults for many species and are released as free swimming parenchymella larvae that are assumed to be the outcome of sexual reproduction (Simpson 1984) and serve as the dispersal life stage for these sessile animals (Ilan and Loya 1990; Leys and Degnan 2002; Whalan et al., 2005; Abdul Wahab et al., 2011). Sperm released into the surrounding water are captured by choanocytes in the sponge aquiferous system and transferred to eggs in brood chambers for fertilization (Bergquist 1978; Simpson 1984; Degnan et al., 2015). Hence, swimming larvae released from individual parent sponges were assumed to be genetically diverse based on the haplotype of the egg. Furthermore, they were likely to be half siblings based on fertilizing sperm released from multiple sponges that would impart additional genetic diversity. However, full sibling larvae may also have been released based on the possibility of egg fertilization by sperm from the same nearby male sponge (Degnan et al., 2015). Pairs of siblings that settled in contact were observed for morphological changes consistent with allorecognition. The developmental stages that were observed included post-larvae that had settled and had begun to spread but had not developed an aquiferous system, and juvenile sponges that had metamorphosed, had developed visible water canals of the aquiferous system, and were assumed to be filter feeding (Leys and Degnan 2002). Juveniles also showed evidence of the mesohyle that are spaces between the external pinacoderm and the internal water canals and contain a number of different cell types including archeocytes (Smith and Hildemann 1984); reviewed in (Smith 1988)). Results for post-larval

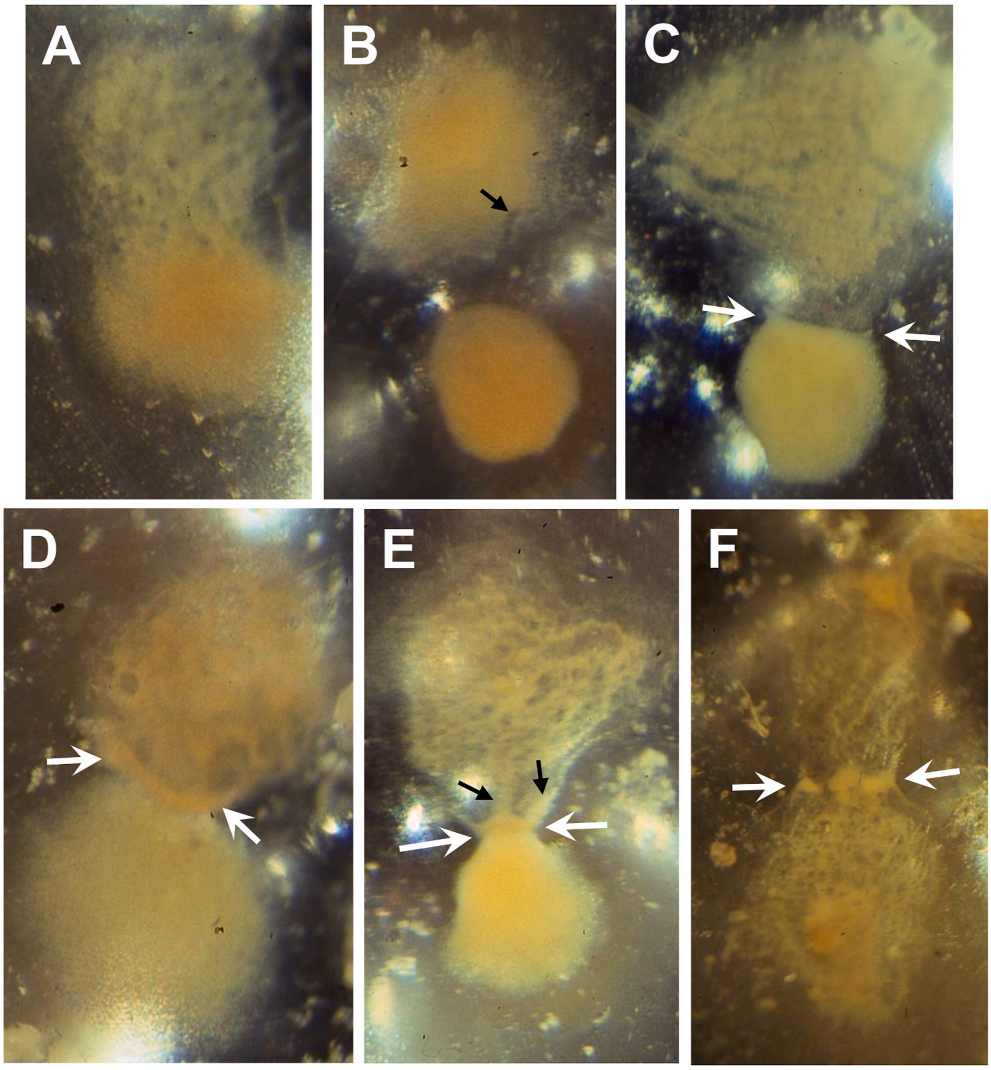

Fig. 3. Sibling larvae released from individual adult *H. sinapium* either fuse or respond to allogeneic contact upon settling and metamorphosing to juvenile sponges. A. Fusion of siblings. The upper metamorphosed juvenile fused with a spreading post-larva shown below. B. Two post-larvae settled near each other and have not initiated an allogeneic response. The upper post-larva is spread and in the process of metamorphosis showing the beginnings of an aquiferous system located at the edges (arrow). The lower post-larva has settled and is only beginning to spread. Neither shows evidence of responses to contact with the other. C. The initial phase of allorecognition. The upper juvenile has completed metamorphosis and shows the aquiferous system throughout the body. The lower post-larva has settled and is early in the process of spreading. The pair shows a linear interface between them (arrows) with no evidence of cellular accumulations at the interface. D. The second phase of allorecognition. The upper juvenile has completed metamorphosis. The lower post-larva has settled and spread but has not completed metamorphosis. The pair are in contact and the upper juvenile shows an accumulation of cells at the interface (arrows). E. Another example of the second phase of allorecognition shows the upper juvenile that has completed metamorphosis and the lower post-larva that has settled and has started to spread. The juvenile shows cellular migration tracks (black arrows) and cell accumulation at the interface (white arrows). The cells of the spreading post-larva appear more dense at the interface with the juvenile compared to the distal edges away from the contact interface. F. The third phase of allorecognition. Both juveniles have completed metamorphosis and show allorecognition responses with cell accumulations at the interface (arrows).

and juvenile pairs (n = 7) that settled in contact illustrated fusion or allorecognition that appeared as three stages as the response progressed (Fig. 3). For two pairs of siblings, a yellow juvenile and an orange post-larva in the process of spreading resulted in fusion (one fused pair is shown in Fig. 3A) suggesting that these pairs may have shared surface self-recognition cues. The remaining five pairs demonstrated allorecognition in response to allogeneic contact that correlated with the process of post-larval settlement, spreading, and metamorphosis to juveniles (Fig. 3B–F). A pair of post-larvae that settled near each other were in two different stages of metamorphosis and showed no evidence that allorecognition had occurred (Fig. 3B). The upper post-larva had spread and had begun to develop water canals along the edges, whereas the lower post-larva had settled, had started to spread but had no detectible aquiferous system or other attributes of a juvenile. The initial phase of allorecognition was evident when a juvenile was in contact with a settled post-larva, which resulted in a linear interface between the two (Fig. 3C). This suggested that allorecognition had occurred and that the juvenile and post-larva were unlikely to fuse, but that neither had initiated an observable response to contact. The second phase of the response was apparent for a juvenile that was in contact with a spread post-larva (Fig. 3D). The juvenile showed an accumulation of cells along the contact zone, whereas the post-larva did not show a similar cellular reaction at the interface. A different pair in the second phase of allorecognition showed a similar response in which a juvenile was in contact with a partially spread post-larva (Fig. 3E). Both showed an accumulation of cells at the interface, however, in this case, the juvenile showed cell tracks oriented toward the interface with the post-larva. Finally, the third phase of allorecognition between two juveniles resulted in the accumulation of cells at the contact zone from both sponges (Fig. 3F). Overall, this series suggested that responses to allogeneic contact by post-larvae and juveniles proceeded from an initial interaction at the contact interface, to an accumulation of cells in juveniles from tracks of migrating cells toward the interface. This allogeneic response in juveniles (Fig. 3C–F) matched allorecognition responses in adults (Fig. 2C and D).

4. Discussion

Settling post-larvae and newly metamorphosed juveniles of *H. sinapium* demonstrate allorecognition upon contact with the appearance of cell tracks and cellular accumulation at allogeneic interfaces, which is similar to allorecognition in the adults. This suggests that these early life stages for this species have a functional allorecognition system capable of detecting cues of proximal conspecifics and that the juveniles have a cellular effector system that is activated by allogeneic contact. This result is unlike allogeneic fusion for post-larvae and juveniles for all other sponge species in which it has been investigated (Warburton 1958; Ilan and Loya 1990; Mukai 1992; Maldonado 1998; McGhee 2006; Gauthier and Degnan 2008), suggesting that allorecognition does not function at early life stages for these species. Furthermore, lack of allorecognition leading to larval or juvenile fusion has been documented for other sessile invertebrates including scleractinian corals, gorgonians, colonial hydrozoans, and colonial ascidians (*e.g.,* (Hidaka 1985; Shenk and Buss 1991; Frank et al., 1997; Barki et al., 2002; Fuchs et al., 2002; Chadwick-Furman and Weissman 2003; Wilson and Grosberg 2004; Casso et al., 2019). Consequently, at what point during the life stages does allorecognition become functional for species that show allorejection as adults? The onset of allorecognition and duration of sponge chimeras that result from fusions of post-larvae or newly metamorphosed juveniles has been investigated for a few species. In some sponges, chimera status is maintained beyond the observation periods of one to several months in which the allogeneic cells in the chimera appear to function as a unit (Maldonado 1998; McGhee 2006). In other species, a subset of larvae or juveniles fuse, whereas others reject, as noted for *H. sinapium*, and differences have been attributed to the level of genetic similarity or the level of allorecognition function (McGhee 2006). The combination of fusions and rejections for pairs of post-larvae and juveniles of *H. sinapium* may correspond to whether they are full or half siblings. Transient fusion of chimeras has also been reported for sponges in which chimeras cease to function as a unit suggesting the onset of allorecognition (Mukai 1992; Gauthier and Degnan 2008). For example, chimera breakdown of the Australian sponge, *Amphimedon queenslandica*, appears after a week or two with the onset of an internal competition among the allogeneic cells that is followed by their separation into different areas of the chimera, or a separation into different sponges, which may lead to the death for one or both sponges (Gauthier and Degnan 2008). Transient chimeras of cnidarian species also show variations in the onset of allorecognition (Shenk and Buss 1991; Frank et al., 1997; Fuchs et al., 2002). Transient fusion identifies a time period in which chimeric sponges do not demonstrate capabilities for detecting and/or responding to conspecifics. During these periods, chimeras survive, grow, and spread over a substrate before the onset of allorecognition. For *H. sinapium*, fusion is observed but whether these chimeras will eventually undergo allorecognition followed by separation of the allogeneic cells or death of the chimera was not investigated. In other pairs of siblings, *H. sinapium* shows that allorecognition is active upon post-larval settling and metamorphosis to juveniles and consequently, for these combinations of siblings there is no period in which the species lacks allorecognition capabilities. Whether chimera formation or immediate allorecognition and response correlates with effective competition for space, growth, and reproduction is not known. Immediate allorejection of conspecifics may avoid later conflicts between the genetically dissimilar cells that may ensue with the onset of allorecognition. Perhaps *H. sinapium* has optimized the benefits of either sibling fusion or allorejection leading to success in survival in the shallow waters of the eastern Pacific Ocean.

Allorecognition of sponge conspecifics is regulated by an expanded multi-gene family that encodes highly polymorphic aggregation factors (AFs), which have scavenger receptor cysteine-rich domains, and interact with polymorphic cell surface aggregation receptors (ARs) (Müller and Zahn 1973; Fernàndez-Busquets et al., 1996; Fernàndez--Busquets and Burger 1997; Pancer et al., 1997; Blumbach et al., 1998; Conaco et al., 2012; Grice et al., 2017). The significant variability among and mismatches between AFs correlates directly with cell-cell adhesion (or aggregation) that underpins allorejection or non-fusion (Curtis and Van de Vyver 1971; Van de Vyver 1975; Fernàndez-Busquets and Burger 1997; Blumbach et al., 1998; Müller et al., 1999). As a non-self detection system, allorejection has been accepted as a measure of immunocompetence for a wide range of animals including sponges because it displays visual evidence of non-self recognition that is easily documented (*e.g.* (Hildemann et al., 1979b; Grosberg 1988)). However, if allorecognition equates to immunocompetence, chimera formation described above for some sponge species predicts the absence of immunocompetence, which leads to the corollary that juveniles are not immunocompetent. Furthermore, it follows that non-immunocompetent juveniles will succumb to infection by a wide range of microbes in the environment; pathogens or opportunists. However, rampant infection has not been reported for post-larvae and juveniles. This includes the case of *A. queenslandica* that does not succumb to lethal infection during the transition from post-larvae to juveniles when they show an increased diversity of the microbiome compared to all other life stages (Fieth et al., 2016). This transition period also corresponds to when chimera formation occurs (Gauthier and Degnan 2008) based on the failure of allorecognition.

Evidence for, and the composition of sponge immune systems have resulted from annotations of sequenced sponge genomes that have identified homologues of expanded immune gene families encoding a variety of pathogen recognition receptors (PRRs) and members of immune signaling pathways (Müller and Müller 2003; Wiens et al., 2007; Gauthier et al., 2010; Hentschel et al., 2012; Yuen et al., 2014; Degnan 2015; Pita et al., 2018). The life stage transition from post-larva to a juvenile corresponds with a significant upregulation of genes that

encode innate immunity proteins in *Amphimedon queenslandica* and correlates with the invasion microbes from the local microbial community that initially results in a complex microbiome in juveniles (Conaco et al., 2012; Fieth et al., 2016). The correlation between immune activation and modifications to the juvenile sponge microbiome infers the involvement of the immune functions of the sponge to protect the host and to control, modify, and eventually to maintain a stable microbiome. It is noteworthy that this occurs at the same time as when juveniles form chimeras (Gauthier and Degnan 2008). Although the microbiome of *H. sinapium* is not known, the fusion of some juveniles to form healthy chimeras suggests immune function in the absence of allorecognition.

5. Conclusion

Sponges have two distinct non-self recognition systems that function through different sets of proteins (AFs and ARs vs. PRRs and other immune proteins) that detect different categories of non-self (conspecifics vs. pathogens) and activate different types of responses (rejection vs. immunity). Allorecognition and immunocompetence appear to become active at different times during post-larval settling and metamorphosis to juveniles for many sponge species that have been investigated. The delay in allorecognition that enables chimera formation may not correspond with the onset of immunocompetence. Consequently, allorecognition may not be an appropriate gauge of immunocompetence. Although this concept appears consistent for some demosponge species, it may be applicable to a broader set of sessile marine invertebrates such as colonial tunicates and hydroids.

CRediT authorship contribution statement

L. Courtney Smith: Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Data availability

Data will be made available on request.

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