Revealing the Systematic Nature of Coral Attachment to Reef Substrates

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Research Article

Keywords: Reef-building coral colonies , periodic sexual reproduction , coral attachment model

Posted Date: October 18th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-957311/v1>

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Abstract

Reef-building coral colonies propagate by periodic sexual reproduction and continuous asexual fragmentation. The latter depends on successful attachment to the reef substrate through modification of soft tissues and skeletal growth. Despite decades of research examining coral sexual and asexual propagation, the contact response, tissue motion, and cellular reorganisation responsible for attaching to the substrate via a newly formed skeleton have not been documented. Here, we correlated fluorescence and electron microscopy image data with 'live' microscopic time-lapse of the coral tissue biomechanics and developed a multiscale imaging approach to establish the first "coral attachment model" (CAM) - identifying three distinct phases that determine the timing and success of attachment during asexual propagation: (i) an initial immune response, followed by (ii) fragment stabilisation through anchoring by the soft tissue and (iii) formation of a "lappet appendage" structure leading to substrate bonding of the tissue for encrustation through the onset of skeletal calcification. In developing CAM, we provide new frameworks and metrics that enable reef researchers, managers and coral restoration practitioners to evaluate attachment effectiveness needed to optimise species-substrate compatibility.

Introduction

Coral reef development begins with the establishment of new reef-building corals onto reef substrates via the settlement of coral planulae ¹⁻³ or (re-) attachment of fragments broken from adult colonies ⁴⁻⁶. The initial attachment of fragments can determine how coral reefs recover postdisturbance ^{7,8} and is therefore a key factor in regulating reef resilience to stressors. However, the biological processes required for effective coral fragment attachment to substrates remain largely unknown despite an ongoing need to establish the fundamental biology of attachment to progress decades of research on coral growth and reef development ^{4,6,9}.

Colony fragmentation, commonly caused by physical disturbances (e.g., storms; ^{10,11} and aided by biological factors (e.g., bioeroders; ¹²), can result in new independent fragments reattaching to form new adult colonies through asexual reproduction ^{4,5,10}. Fragments that successfully form these adult colonies can begin reproducing and contributing to reef populations from 1-2 years ^{4,13}. Successful attachment is inhibited by biological and environmental factors such as substrate type ^{14,15}, substrate mobility and water movement ^{8,16,17}. To date, fragment attachment has broadly been defined as "the first basal tissue" growing onto a substrate ¹⁸, with success quantified as the extent of basal tissue visible at the coral-substrate interface ^{18,19}. While these broad considerations for what defines attachment have been useful to compare survivorship, this description does not consider the different phases of the coral attachment process or the degree to which the fragment is 'attached', namely, whether the attachment consists of only soft tissues or whether a new skeleton is present on the contact surface.

Several factors have been suggested to play a role in the effective attachment of a coral fragment. These include (i) an immune response to promote growth and protect the colony against pre-existing debris,

organisms, and/or pathogens on the substrate^{20–24}; (ii) passive stabilisation of the fragment via tissue anchoring, interlocking with the substrate or low water currents to reduce soft tissue damage due to constant movement^{8,16,25,26}; (iii) maintenance of extracellular matrices (ECM), which facilitates cell–cell and cell–substrate adhesion and the controlled deposition of a calcium carbonate skeleton^{27–30}; and/or (iv) the transition from the outer surface body wall SBW into a basal body wall (BBW) possessing the specialised cells required for ECM production and skeletal precipitation^{31,32}. However, whether or when these factors play a role in the attachment and if the process or part of a sequence has not been evaluated due in part to the difficulty of a dynamic process observation at multiple scales.

Most microscopy-based studies of corals either rely on protocols where the soft tissues are removed from the calcareous skeleton or where fixed coral tissues are decalcified^{31,33–35}. Consequently, studies of the complex three-dimensional cellular and tissue interactions between the coral skeleton and adjacent tissue are rare^{36,37}. Knowledge of such interactions can greatly add to our understanding of fundamental coral biology and innate adaptability needed to improve strategies for the conservation of coral reefs³⁶. Live and static imaging is an effective way to detail the structures of reef-building corals at the macro, tissue and cellular levels^{30,36,38–40}. Recent advancements in imaging have provided vital insights into coral calcification, biomechanics, and cellular plasticity^{30,36,38–40}. These works highlight the ongoing importance of developing noninvasive techniques for dynamic imaging at high temporal resolutions, such as in vivo video and time-lapse microscopy and novel static methods^{36,39,40}. The utility of the in vivo observation is maximised when used in combination with novel sample preparations for cellular and mineralized composites (skeleton) to maximise the observable capacity for static microscopy, including images produced by electron microscopy. Such knowledge opens the possibility for the amplification of natural processes for conservation-based biotechnology approaches. The utility of the in vivo observation is maximised when used in combination with novel sample preparations for coral that allow cellular and mineralized composites to improve the observable capacity for static microscopy (e.g., electron microscopy).

Existing approaches to coral outplanting in small-scale targeted sites (meter scale) have suggested that coral fragment success is dictated by the durability of the fragment's attachment^{15,18,41} and that success can be improved with a stable point of contact between the coral fragment and the substrate^{14,19}. To date, one study¹⁸ has examined fragment attachment processes within the first month of deployment. The paucity of observations of coral attachment through the initial stages of contact obscures the systematic nature by which the coral fragment changes from the time of substrate contact; therefore, the tissue and skeletal processes and factors that promote or inhibit rapid and robust attachment are absent from the literature. If aquaculture and outplanting are to improve return on effort for targeted sites^{42,43} and be viable for large-scale interventions for coral reef restoration, it is vital to assess coral responses to external environmental factors with appropriate spatial and temporal resolution.

Outplanting coral is accelerating globally as a means to actively rehabilitate degraded reef sites⁴⁴. The efficacy of such planting practices relies on robust and rapid attachment of “outplants” – either coral

fragments or nursery reared colonies – to existing substrates^{14,18,19,45}. The cost-effectiveness and scalability of these practices in turn rely on the “attachment success” of new coral material. What is also significant is that few restoration studies have attempted to quantify the rate and extent of attachment^{14,18,42}, which in part reflects a lack of knowledge of how attachment is conserved as a biological process across diverse coral taxa-reef and substrate interfaces. The development of a ‘coral attachment model’ (CAM) provides the fundamental baseline to resolve the systematic stages that cumulatively give rise to attachment success and, in doing so, a means to identify more rigorous metrics of attachment success to optimise planting success. Past research to evaluate attachment biology has been fundamentally constrained by histological methods that only allow for mutually exclusive imaging of either coral skeleton or soft tissues separate from one another. Therefore, we used novel integrated sampling methods and high-resolution time-resolved images spanning light, fluorescence and electron microscopy to present the first CAM, using *Acropora millipora*, a common Indo-Pacific reef forming coral and the frequent focus for reef research^{46–48} and restoration initiatives^{9,42}.

Results

Three distinct phases of morphological changes and cellular and tissue remodelling were observed during fragment attachment (Fig. 1): phase one: immune response leading to fragment attachment – 0-5 days; phase two: soft tissue anchoring and fragment stabilisation – 3 to 8 days; and phase three: lappet appendage development and calcification leading to substrate bonding and encrustation – 5 to 12 days. Each phase initiates sequentially at all contact points in all samples. The variation in the timing of the different phases is detailed below. The complex nature of the coral shape resulted in spatial variation at each contact point between the fragment and substrate. Definitions of the various terms above will be provided below.

Phase one: immune response on contact.

Gross anatomy

The attachment process begins when the fragment is first placed in contact with the substrate. The fragment soft tissue then undergoes an innate immune response (i.e., cell proliferation, mucus release) to respond to the foreign body and wound formation (Fig. 2). Insertion of the substrate produces wounds in the soft tissues via abrasion (Fig. 2), which then heal over the subsequent 2-3 days. The abraded surface body wall (SBW) tissue, in contact with the substrate, becomes thicker (approx. 100-150 µm) compared to the standard SBW (approx. 40 µm) (Fig. 2).

Multiple localised mucus excretions occur at the contact points, most of which eventually dissolve into the water column. Some mucus is visually distinct mucus that remains as a layer surrounding the tissues or as fibrous strands (Fig. 2). This mucus layer is more durable until it is physically or hydrodynamically dislodged and, over time, becomes more visible, turning from transparent to white and eventually brown.

There is a significant increase in mesenterial filament activity within the tissue wounds, and external to the body cavity may help clean the substrate⁴⁹ and wounds⁵⁰. The mesenterial activity also coincides with the timing and location of the durable mucus secretions (Fig. 2D) (Supplementary Movie S1). Mesenterial filaments build-up at the underside or distal edge of the SBW and gain access to the environment surrounding the coral fragment. Here, a temporary cinclide-like⁵¹ opening develops in the SBW through which multiple mesenterial filaments extend using a spiralling motion (Fig. 3) (Supplementary Movie S2). Time-lapse imagery documents the macrobiological processes that underlie tissue recovery, substrate sterilisation, foreign material removal – all processes that are driven by micro-anatomical changes.

Micro anatomy

The proximal and temporal relationship between the durable mucus and mesenterial filaments observed during time-lapse (gross anatomy) was further supported by autofluorescence imaging, which documented the mesenterial filaments external to the body cavity present with-in with the mucus biofilms (Fig. 3A and B). Histological analysis of the filaments also confirmed that the cnidoglandular band possesses numerous secretory gland cells (Fig. 4), which are capable of producing variations in mucus development²³ and digesting matter⁵² (Fig. 4E). Tissue sections of the enlarged tissues observed via time-lapse were viewed with scanning electron microscopy (SEM) (Fig. 4) and confirmed that the enlarged tissues observed via time-lapse (Fig. 4C) were from a rapid proliferation of supporting cells (also known as epitheliomuscular cells or stem cells), algal symbionts in the gastrodermis, and three distinct types of secretory gland cells (or perhaps supporting cells that have differentiated similar vesicles) that can be used in mucus development⁵³, adhesion and digestion and/or protection (Fig. 4)^{52,54,55}.

Phase two: soft tissue anchoring and fragment stabilisation.

Soft tissues have undergone a localised immune response (phase one) to promote growth of the SBW at the contact point. Here, the tissue at the leading edge of growth expands with pulses of extension and increases the contact surface area of the tissue (Supplementary Movie S3) (Fig. 5), actively stabilising the fragment. Proliferation of the supporting cells enables the biomechanics of the expansion and growth of the fragment's cytoskeleton, which leads to the development of a complex tissue morphology. The anchored tissues form an enclosed, sealed area so that the SBW can transition into a BBW for extracellular matrices to develop and skeletal calcium carbonate (CaCO₃) precipitation.

Gross anatomy

Towards the end of phase one, the tissues were observed to undergo inflation and contraction-like pulsing until the tissues became anchored to the substrate (Fig. 5) (Supplementary Movie S3), thereby stabilising the coral fragment to the substrate²⁵ and initiating phase two. Stabilisation by anchoring produces a relatively weak attachment, as the tissues were observed retracting or detaching during deployment of large numbers of mesenterial filaments at the anchoring tissues or during slight movements of the

substrate or fragment. The coral fragments possessed multiple contact points where anchoring initiated; however, the rate and degree of soft tissue anchoring varied among contact points, with some points delaying development until other contact points reached the bonding stage (phase 3). The anchored tissues inherently created an enclosed space at the interface between the tissue and the reef substrate. We documented coral skeleton (stage 3) formation in this space, which suggests that it is sealed from the surrounding environment enough that the ECM can develop free of seawater or other external influences. Systematic deployment of mesenterial filaments begins once the sealed space is created, which removes the enclosed SBW via autolytic processes (Fig. 6) (Supplementary Movie S4). Only after this systematic deployment of filaments did skeletal precipitation begin at these sites. No evidence of skeleton production before autolysis was observed in any samples.

Micro anatomy

Anchoring, active stabilisation and the localised pulsed contractions observed in the time lapse were enabled by the continued proliferation of supporting cells (hereinafter referred to as epitheliomuscular cells) (Fig. 5). The epitheliomuscular cells from phase one continue to develop through phase two and underpin the development of the enlarged, undulated soft tissue anchor (Fig. 5). Continued development of secretory gland cells (including mucocytes) appears to aid this adhesion through surface sterilisation and mucus production^{52–56} (Fig. 5). Autolysis removed the enclosed tissues/epidermis of SBW, leaving space for the development of the calicodermis on the remaining gastrodermal gland and completing the transition from an SBW to a BBW required for generation of the ECM and subsequent CaCO_3 precipitation (Fig. 6). SEM and fluorescence microscopy observations showed that vestigial epidermal cells (discussed in phase 3) – left over from the original epidermis - were present in the newly developed calicodermis, and symbiont cells were removed from the enclosed space by the mesenterial filaments (Fig. 6C). Mesenterial filaments possess several secretory cells that are capable of cell digestion and mucus production (Fig. 3, 6)^{52–56}.

Phase three: calcification and lappet appendage development leading to substrate bonding and encrustation.

Anchored tissues initiate precipitation of the incipient basal skeleton, and the distal edge of these soft tissues develops into a complex lappet appendage that enables secondary skeletal growth thickening and encrustation (Fig. 7). In *A. millipora*, the lappet appendage is defined by a thickened folded SBW lip approximately 1.25 mm across, resembling a lappet structure³⁴. Barnes (1972) defined a lappet as a highly mobile distended fold over the rim of the vertical epitheca that forms at the junction of the SBW and BBW and is responsible for the vertical precipitation of the epitheca from the initial basal plate. The epithecate lappet possesses four cell types: columnar-supporting cells, mucus cells, nematocysts, and muscular-epithelial cells (Fig. 8), which steadily change from the types found in the SBW to the types found in the BBW (Fig. 4). In *A. millipora*, the lappet appendage provides three primary functions: (i) it

allows the anchored tissues to begin movement along the surface (pulsed contractions); (ii) it maintains this movement while also preserving the enclosed/sealed areas required for ECM development and colony health; and (iii) it is responsible for the expansion of the initial basal skeleton and costae wall development, which in turn leads to colony basal expansion or substrate 'bonding' (Supplementary Movie S5).

Gross anatomy

A lappet appendage developed at the edge of the encrusting rim, appears to be discoloured or lighter, is polyp free and, like the anchoring tissues, appears enlarged compared to the standard SBW (Fig. 7). The lappet appendage was capable of systematic pulsed contractions or undulations that rolled around the appendage at the encrusting rim (Supplementary Movie S5), presumably helping it move forward.

Micro anatomy

At the microscale, the lappet appendage is the junction between the SBW (protection) fragment and the BBW (for skeletal precipitation) (Fig. 7). The lappet appendage itself is characterised by a larger than usual surface body wall, an undulated tissue morphology at its base (Fig. 7) and a non-intrusive ($<5\text{ }\mu\text{m}$) poorly defined calicodermal epithelium that extends from the transition zone of the lappet appendage and sits between the lappet undulations and initial skeleton that formed on the substrate (Fig. 7). A complex epitheliomuscular cell network is responsible for the lappet appendage's thicker SBW and intricate undulated morphology (Figs. 7, 8). The proliferation of epitheliomuscular cells is also likely to facilitate the ability of the lappet appendages to pulse and therefore move along the surface. The poorly defined calicodermal epithelium followed a non-traditional epithelial arrangement by not possessing an adjacent gastrodermis but appeared to be a continuation of the calicoderm. This layer was closely aligned with the initial skeleton in all samples despite some retraction of the tissues. The unique calicodermal epithelium's location under the lappet's undulated tissue, which does not possess calicodermal cells for skeletal production, suggests that calicodermal continuation was responsible for the precipitation of the initial skeleton on the reef substrate (Fig. 7). The thin and nonintrusive structure of the lappet appendage continuation of calicodermis does not appear to impede the mechanical grip of the lappet appendage.

The coral skeleton, including the initial skeleton and secondary growth, conforms closely to the surface of the substrate (Fig. 9). The microstructure of the sectioned skeleton is similar to clypeotheca^{25,26}, with skeleton development indicating growth that broadly progressed away from damaged zones of coralla. The organic rich basal skeletal layer nearest to the substrate with a thickened skeletal layer and growth bands in the composite needle shaped biocrystals indicate the direction (RAD) (Fig. 7B, 9. B-D). The skeleton also thins to a single basal layer on the distal parts of the contact points of the coral fragment and substrate. Incipient costal structures form out of the thickest areas of the skeletal encrustation perpendicular to the substrate (Fig. 7E).

The costae wall of the coral fragments begins to develop in the lappet appendage as rapid accretion deposits (RADs) (Fig. 9). The costae wall nucleates from pocket calicoblastic cells (calicoblasts) that

develop on the gastrodermis of the lappet appendage's SBW, in between it and the epidermis (Fig. 7F), similar to the calicoblastic cell proliferation after autolysis observed in phase 2. The gastrodermis separates from the SBW of the lappet appendage as the lappet appendage develops and moves over the substrate with newly formed calicoblasts proliferating in place of the epidermis (Fig. 7F). Because costae walls are vertical and do not form as a horizontal layer across the encrusting rim, the lappet appendage can appear 'deflated' without the costae developing.

Discussion

The standard body wall of Scleractinian corals develops as two histologically separate regions^{31,32,51}: the surface body wall (SBW), which is in contact with ambient seawater, and the basal body wall (BBW), which rests next to the skeleton (Fig. 4A). Both the SBW and BBW are comprised of two epithelial layers - separated by a mesoglea - an epidermis (environmental barrier) and a calicodermis (skeleton production), respectively, and a shared gastrodermis (nutrient acquisition and storage). The results discussed here for *A. millipora* show an example of a sequence cellular reorganisation in reaction to an immovable foreign object in Scleractinia and the process responsible for skeletal anchoring to the substrate.

The cellular process to facilitate the attachment of coral fragments has not been examined with detailed spatial and temporal resolution. Despite being recognised as a prominent mechanism by which reefs accumulate new coral cover. Elucidating such a process has the potential to impact efforts to rapidly scale interventions for reef recovery through effective (re)attachment of propagated or "opportunity" fragments to reef substrates^{9,19}. For example, there is a need to identify the phases at which robust and effective attachment occurs. The combination of time-lapse, light and electron imaging has merged the behaviour of coral fragments with the ontogeny of their cells and skeleton. From these observations, we propose a novel coral attachment model (CAM) comprised of three sequential phases. The CAM provides an additional framework to help determine opportunities for biological manipulation and optimisation of protocols during the initial phase of outplanting⁵⁷ and establish integrated metrics of "success" that account for attachment strength over time to determine outplanting return-on-effort (RRE) scores^{42,43}. These advancements have the potential to streamline protocols, reducing the time and cost of outplanting and consequently presenting opportunities to improve outplanting scalability. However, this model is derived from a single species and needs to be tested across a multitude of coral taxa to assess broader applications.

Phase one: immune response on contact.

Initial contact with the substrate is abrasive^{4,8}, causing wounds to form in the coral tissues (Fig. 2) as a result of direct tissue contact with foreign materials^{4,25,58}. Wound formation and foreign material contact in *A. millipora* triggered a localised immune response characterised by mesenterial filament deployment (Fig. 3), cellular proliferation and mass movement (Fig. 4), and mucus development (Fig. 2 and 3), which

are all phenomena observed in studies involving tissue damage in other reef building corals, such as *Porites lutea*, *P. lobata* *Acropora aspera*, *A. pulchra* and *A. polystoma*. *Montipora patula* *Acropora millipora*^{22,23,59–62} displayed a high degree of tissue and cellular plasticity, which suggests the presence of a highly coordinated signalling response and cellular communication during tissue contact with foreign materials, early wound healing, and overall tissue recovery^{24,50,63}. This in turn indicates that Scleractinia may possess regeneration potential similar to that documented in other cnidaria, such as Actinaria and Hydrozoa.

Histological analysis identified two main types of cells at the centre of epidermal proliferation: supporting cells and suites of secretory gland cells (Fig. 4). Prior to our study, only the proliferation of fibroblasts and amoebocytes had been previously linked to the immune response in coral²¹, whereas we identified the specific proliferation of supporting cells and what – according to previous work - appear to be secretory gland cells type 1, 2 and 4^{52,53,64} (Fig. 4). Of the two cell types, the supporting cells, which also function as epitheliomuscular cells^{65–67}, comprised the bulk of the rapidly proliferating cells during phase one. Supporting cells are the fundamental cells forming the structural foundation for tissue development and, as stem cells, form the platform for the differentiation of new cell types⁶⁵. Supporting cells can also be sites of nutrient acquisition^{51,68} via phagocytosis while also comprising the outer surface of the coral, thereby forming direct contact with the substrate³¹. The proximity of the supporting cells to the substrate and their role in nutrient acquisition⁵¹ may indicate that they remove foreign material from the substrate surface, helping maintain homeostasis. This was supported by the presence of type 4 gland vesicles present at the apical surface (Fig. 4), which appeared to be developing in the supporting cells. Nevertheless, the ongoing and localised proliferation of the supporting cells expands the fragment cytoskeleton⁶⁵, resulting in improved mechanical strength (biomechanics) and morphological changes that facilitate soft tissue anchoring (phase two) and skeletal bonding to the substrate (phase three). This process fundamentally confirms previous suggestions for an intrinsic relationship between the immune response and locally accelerated growth in reef-building corals²⁰.

The second cell type documented – secretory gland or goblet cells - includes type 1, type 2 and 4 gland cells,^{52,53,64} where the latter appeared to be vesicles containing supporting cells that terminated at the coral apical surface (Fig. 4). When viewed using electron microscopy, the type 4 gland cell vesicles next to the reef substrate consisted of foreign matter in varying stages of degradation. The tissue cell population is a reflection of the function of that tissue⁶⁹. Hence, we can infer that these cells remove foreign debris and function to protect the epidermis from foreign materials, including benthic organisms, bacteria, or pathogens. Perhaps either by producing zymogen vesicles capable of exocytosis⁵², which are also known to migrate to the apical surface of the cell⁵⁴, or lipopolysaccharide endotoxins that protect the epithelium from bacteria^{55,70}. However, further analysis will be required to verify their exact function.

Typically, type 1 and 2 gland cells (Fig. 4) have been associated with key developmental processes, including digestive enzyme development (type 1) and mucus development and adhesion (type 2)^{52–54, 64},

which should aid the immune response and fragment survival^{23,62}. The presence of type 1 and 2 gland cells in the developing epidermis may therefore also partially explain the increased mucus development observed from the coral fragment and the nonsoluble mucosal gel that formed as a thin layer on the substrate. Time-lapse and confocal fluorescence microscopy illustrate an increase in both soluble (SML) and nonsoluble mucus gels. The latter forms a fine, durable layer on the substrate during the first 24 to 48 hours and lasts until it is physically dislodged. The location of the mucus gel on the substrate and timing of its development may indicate a role similar to that of the surface mucus layer (SML) and macrosized mucus sheets (also exposed to physical disturbance) in providing a physiochemical barrier to promote beneficial microbes and antimicrobial activity resulting in near sterile environments (Johnston and Rohwer, 2007).

Deployment of mesenterial filaments was observed with cell proliferation and external mucus production (Supplementary Movie S1). These structures are well-established sites for digestion and colony defence^{51,71–73}. During this study, the mesenterial filaments appeared to clean the fragment wound sites and adjacent substrate, presumably to aid fragment homeostasis during the first 48 hours of substrate contact. Such cleaning and mucus production roles for the mesenterial filaments were supported by histological analysis, whereby the *A. millipora* mesenterial filaments possessed supporting cells, nematocysts and numerous secretory gland cell types (type 1 and type 2; mucocytes) (Fig. 3) used in mucus production and digestion. Our observations also confirm previous reports^{49,74} of filament cleaning substrates prior to colony growth and expansion and that mesenterial structures are involved in wound cleaning⁵⁰. The cleaning and mucus production roles of the mesenterial filaments (and the supporting cells and gland cells of the epidermis) during this early phase thus appear vital for fragment survival and the eventual transition into phase two (anchoring) and hence a critical component of the immune response for our model coral (*A. millepora*).

Phase two: soft tissue anchoring and fragment stabilisation.

The onset of Phase 2 was characterised by soft tissue anchoring to the substrate, thereby initiating stabilisation of the coral fragment²⁵. SEM images indicated that anchoring was enabled through continued localised proliferation of supporting cells and secretory gland cells (Fig. 5) that may aid with adhesion or further the removal of benthic material (phase one). Anchoring was underpinned by continued development of epitheliomuscular cells advancing the fragment cytoskeleton morphology (undulated and thicker), mechanical strength and localised inflation and contraction (pulsing), which together promote soft tissue development required for anchoring. While inflation and contraction biomechanics are commonplace in Scleractinian corals^{75–78}, perhaps more obvious in corals with larger tissue volumes, it is rare to observe such complex movements from taxa with low tissue volumes, such as those in the Acroporiid family. However, such pulsing combined with the unique undulated tissue morphology of the SBW may help channel surrounding seawater away from the substrate surface as the tissues grip and contract^{79,80} – although this is highly speculative. Helping to ultimately create an

enclosed space at the interface between the tissue and the substrate that is weakly sealed off from the surrounding seawater (Fig. 5). An enclosed space could serve multiple functional roles, such as allowing contact between the calicoblasts and the precipitation site, producing an enclosed environment required for extracellular matrix to develop (ECM) or the development of adhesive mucus or glycoprotein gels isolated from ambient seawater, and/or supplying an environment for autolysis and the transition from the surface body wall (SBW) into a skeleton producing the basal body wall (BBW). Verification of the various roles of the enclosed space will require further investigation.

The deployment of mesenterial filaments to the enclosed tissue interface of the anchored tissues led to the digestion and autolysis of the SBW tissues captured by documented time-lapse microscopy. Any remaining gastrodermis formed specialised calicoblasts to complete the transition from the SBW to a BBW necessary to initiate precipitation of the skeleton. Creating a stronger bond to the substrate and robust attachment^{81–84}. Patches of vestigial cells endemic to the epidermis were present in the newly developed calicodermis, suggesting that in some cases, only the epidermis of the SBW and not the gastrodermis was removed during autolysis. By not removing the gastrodermis, the coral should in principle limit energy expenditure and the time required for skeletal encrustation and maintain a level of tissue coverage should it become dislodged before the transition is complete. In some cases, there was a secondary localised deployment of mesenterial filaments for autolysis to areas where the initial skeleton did not develop, which would then trigger skeletal precipitation beginning at these sites.

Phase three: calcification and lappet appendage development leading to substrate bonding and encrustation.

Phase three of CAM begins with the development of a complex lappet appendage at the rim of the anchored tissues, leading to substrate encrustation by reorganised tissue and secondary skeletal growth. The lappet appendage (Fig. 7) develops at the edge of the anchored tissues and consists of thicker, highly muscular epithelial layers (Fig. 8) that form as undulations at its base. Similar undulations appear in the lappet appendage of epithecate coral, although they appear to be less complex³⁴. We observed similar undulations developing in the anchoring tissues in Phase 2, making it therefore possible that such undulations in Phase 3 may operate similarly to those in Phase 2⁸⁰. A semipermanent gripping mechanism relying on undulations rather than perpetual cellular mechanisms would presumably allow for more rapid lappet appendage expansion and contraction (pulsed contractions) while moving over the surface without the need to consistently repair or replace attaching cells³⁵. Directly behind the undulated tissues, the lappet appendage begins transitioning into a traditional BBW consisting of a gastrodermis and calicodermis in a similar fashion to the lappet appendage in epithecate corals³⁴. At this transition point, the lappet appendage extends a thin (<5 µm) poorly defined continuation of the calicoderm that sits under the undulations at the base of the lappet appendage and has not been described in epithecate coral^{34,85}. The calicoblasts in the membrane aided the development of the initial skeleton (Fig. 7)

beneath the lappet appendage, while the undescribed anchoring cells found in the membrane anchored to the initial skeleton – helping maintain the sealed environment as the lappet appendage pulses and moves over the surface.

Our SEM and fluorescence images document the first lappet appendage in nonepithecate reef-building coral, and for the first time, a lappet has been described in basal and costal development. A lappet has been previously documented in the epithecate corals *Montastrea annularis*, *Porites astreoides*, *Gardineria spp.* and *Isophyllia spp.* and was always responsible for the *vertical* advance of the coral epitheca (outer wall) directly from the initial basal plate^{34,85}. Horizontal skeletal advance during the development of clypeotheca - an epithecate-like defensive skeletal wall found in a select few colonial corals - was hypothesised to be the result of a lappet-like structure²⁶. The lappet appendage for *A. millipora* is likely somewhere in-between its role in epithecate development and hypothesised role in clypeotheca development - providing forward advance of the coral tissue similar to the epithecate lappet but doing so in a horizontal orientation akin to clypeotheca development. The basal deposits in *A. millipora* share similarities with clypeothecal growth (Fig. 9), which would protect the colony during tissue damage or from invasion by parasites and disease while the fragment attaches^{25,26}. Epitheca and the lappet are documented in the juvenile stage of most modern Scleractinia but absent in most mature colonies because larger species have evolved into more integrated colonies that require rapid regeneration⁸⁶. We have shown for the first time that a lappet appendage exists in adult reef-building corals, suggesting functional evolution in the lappet appendage from epithecate development to colony protection and a substrate attachment role that improves attachment surface area and colony stability, which in turn allows more integrated colonial development and expansion of colonial colony sizes.

Methodology

Coral collection and aquaria experiments

Five independent colonies of the reef-building coral *Acropora millipora* (Ehrenberg, 1834) sourced from coastal waters (16.9186° S, 145.7781° E) off the northern Great Barrier Reef, Australia (*Cairns Marine*, Cairns, Australia) were acclimated together for one month in a 500 L closed system aquaria at Queensland's University of Technology (QUT). Tropic Marin Pro © salt-based seawater was maintained at 25±1°C and 1.023 – 1.025 sg (specific gravity). Water flow was maintained at 200 L hour using a wave-maker (Tunze, Germany). Light was delivered via Radion LED units (Ecotech, Pennsylvania, USA) on a 12 h:12 h light:dark cycle peaking in intensity around midday (4 hours) at 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$, measured at the coral surface using an underwater quantum flux reader (Apogee, Utah, USA). A biological sump of Marine Pure™ (CerMedia, New York, USA), *Caulerpa* sp. and coral rubble that together maintained inorganic nutrient concentrations (NO_3^+ , PO_4^{3-} , NH_4^+) at very low to undetectable levels (< 0.1 ppm), monitored by Red Sea © multitest titrations every three days. CaCO_3 concentration was maintained between ca. 400-420 ppm through the addition of calcium chloride (CaCl_2) (Seachem Laboratories,

Georgia, USA). The carbonate alkalinity and magnesium concentration were determined by Red Sea © multitest titration and maintained between 125 and 150 ppm and 1280 +/- 50 ppm, respectively.

Following the acclimation period, each colony was divided into fragments ($n = 25$), each retaining three to 10 branches with a maximum fragment length and width of 7 cm, using Dremel Cordless Rotary Tool (Bosch, USA). All fragments were acclimated for an additional two weeks in a separate, closed 50 L experimental system (Fig. 10a) maintained in an identical manner as for the original 500 L holding tank (above) to allow recovery from the fragmentation process. All fragments were maintained on the glass bottom of the tank and gently repositioned every two days to ensure that no fragments began attachment prior to the start of experiments. Chemically inert and pH neutral kiln fired (2000°C) ceramic plugs (Ecotech, Pennsylvania, USA) of a consistent size (12 mm x 12 mm x 12 mm), shape (hexagon) and surface texture were used as substrates to avoid the physical and potential environmental variables (e.g., existing algae colonisation, fouled surfaces) that may affect the attachment process when using rubble or live rock. Opaque black ceramic was used to improve time-lapse imaging by limiting areas of high exposure caused by the standard white surface.

To view the internal physiology of the fragments that were otherwise obstructed by the opaque ceramic plug, an additional five trials were conducted using clear glass slides as a substrate. Time zero was considered the first point of contact of coral with the ceramic substrate. In nature, the orientation of fragments that fall from parent colonies onto the surrounding substrate is random. Therefore, the substrate was positioned either between the branches (Fig. 10b and 10d) or at the surface/base of the fragment (Fig. 10c), ensuring contact with both the higher growth apical tips and slower growth base tissues and axial polyps, and in a position that would not move.

Time-lapse microscopy

Time-lapse microscopy was used to observe processes on the outside of the coral, including soft tissue behaviour and gross anatomy, during the experiment using a Dino-Lite AM7915 portable light microscope (Dino-Lite, New Taipei City, Taiwan). Specimens observed via time lapse were positioned close to the glass walls of the aquaria and within the focal distance of the time lapse microscopes situated on the outside of the aquaria. The microscopes examined the interface between the ceramic plug or glass and coral surface. Images were captured every 2 min for 28 days, with Playback set at 15 frames per second. To determine mesenterial filament density changes over 7 days, an image was collected at sunrise, midday and sunset and then analysed using ImageJ to determine the surface area change of the mesenterial filaments over the total image surface. Changes in camera focus, location or sample movement due to water currents and equipment error rendered most time-lapse invalid for this analysis. Linear and equal adjustments in contrast and brightness have been applied to some images in this manuscript.

Autofluorescence and electron microscopy

Scanning electron microscopy (SEM) and confocal autofluorescence methods were used for histology and skeletal imaging. Two specimens from the pool of fragments were removed from the aquaria every three days over the 28-day experimental period to be dehydrated and embedded in resin. Sample preparation was adapted from Clode et al. (2006), Marshall and Wright (1993), and McCutcheon et al. (2018) to observe coral tissue and skeleton as well as the ceramic plug in one section. Samples were fixed in a solution comprising 3% paraformaldehyde, 3% glutaraldehyde and 94% saltwater. Each fixed sample was then placed into 0.1 M sodium cacodylate ($C_2H_7AsO_2$) solution before staining with both 1% osmium tetroxide (OsO_4) to create contrast for imaging and ruthenium red ($Cl_6H_{42}N_{14}O_2Ru_3$) to fix mucopolysaccharides. Samples were then dehydrated and embedded in Spurr's resin according to the Pelco Biowave osmium staining and embedding protocol and protocols modified from McCutcheon et al. (2018) specific to this study (Appendix 1). Samples were then placed in an oven and left at 60°C to polymerize for 3 to 7 days (where the rate of polymerization for any given sample was dependent on the volume of resin). Initial attempts to dehydrate the samples using standard biowave practices did not remove all moisture from the samples, which in turn caused moisture pockets to form in the resin during polymerization. We therefore tripled the dehydration steps and added an additional acetone dehydration phase (Table S1).

Once set, the resin blocks were cut to expose a cross section of the intrusion/coral interface using a diamond saw. Cross-sectioned surfaces of fixed and embedded tissue, coral skeleton and ceramic plug were polished using 3 M wet/dry sandpaper (CAMI, from 400 to 2000 grit) followed by a lapidary polishing wheel and 1 μm aluminium oxide compound on a cloth pad. Samples were then rinsed using deionized water (DI) and placed into an ultrasonic cleaner to remove fine particles from the surface. In the case of SEM, samples were etched by being placed into 1% formic acid solution for ~10 seconds before rinsing in DI to expose the skeletal microstructure⁸⁷.

Microanatomy and autofluorescence of the coral fragments embedded in resin were observed using an *A1R HD25* confocal autofluorescence microscope (Nikon, Tokyo, Japan). Excitation wavelengths of 405 nm, 488 nm, 561 nm and 640 nm were delivered using a N4S 4-laser unit. Emission bandwidths were set at 425 – 475 nm (cyan autofluorescence protein), 500 – 550 nm (green autofluorescence protein), 500 – 620 nm (red autofluorescence protein) and 663 – 738 nm (near infrared). Laser power was set at 1 to 1.5 for 10x to 20x magnification and 2 - 4 for 40x to 60x magnification. Gain was held between 90 - 115 depending on the magnification for cyan, green and red emissions and between 115 - 140 to image the weaker near infrared emission. Tissue morphology cells and skeletal microstructure were imaged using a Sigma Field Emission SEM (Zeiss, Oberkochen, Germany). Samples were imaged using Zeiss's backscatter detector (BSE) under variable pressure vacuum at 20 kV. Microanatomical analysis identified the fragments typical and atypical cell populations and soft tissue morphology, the presence and absence of tissues, and skeletal microstructure and development.

Declarations

Acknowledgements

We would like to acknowledge Karyn Gonano's sizable contribution in reviewing and guiding the academic writing of this article. The authors acknowledge the facilities and scientific and technical assistance from the Microscopy Australia (MA)-linked lab and the Institute of Health and Biomedical Innovation (IHBI) at the Central Analytical Research Facility (CARF) and Queensland University of Technology, Brisbane, Australia. We would also like to thank QUT's Environmental, Geological and Biological Sciences technical services team for use of their laboratory space and equipment.

Author contributions

B.M.L. and L.D.N. designed the project. B.M.L. carried out all data collection, sample preparation, microscopy, and analysis. B.M.L. curated all figures and videos and wrote the paper, which was reviewed and edited by D.J.S., P.J.P., and L.D.N. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Data and materials availability

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

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Figures

Figure 1

Schematic graphic of the three phases of attachment over 21 days from both the macro view and the obscured underside and with the timing of each stage and their characteristics. (a to c) Macro view of coral attachment through three stages: immune response leading to fragment attachment, soft tissue anchoring and fragment stabilisation, calcification and lappet appendage development leading to bonding and encrustation. (e to f) Micro view of the underside in three stages: calcification and lappet appendage development leading to substrate bonding and encrustation. (h) A heat map of observations of timing (days) and the processes within each stage.

Figure 2

Stills from time-lapse light microscope movies displaying the processes that characterise the immune response phase, i.e., tissue enlargement, mucus release, wound cleaning (0-5 days). (a) Wounds (Wd) formed by abrasion are cleaned by mesenterial filaments (MF) as the surrounding immunocompromised tissues become enlarged (Enl). (b and c) The mesenterial filaments are present external to the body cavity and maintain contact with the immediate substrate, helping clean the surface and the tissues of dangerous debris and pathogens as the compromised tissues become further enlarged (2-3 days). (d) Mucus (Mu) secretions formed on the substrate surface surrounding the compromised tissues appeared to change colour over time – from transparent to white or brown. (e) The mesenterial filament activity outside the body cavity was consistently higher during the first five days, a process that remained consistent for all samples observed attaching to the glass slides.

Figure 3

Confocal fluorescence microscopy images, backscatter electron microscopy images and stills from time-lapse microscopy movies highlighting the distribution, cell composition and mesenterial behaviour during the immune response phase. (a and b) Mesenterial filaments (MFs) were present in the newly formed wounds caused by the insertion of the substrate and adjacent to the mucus barrier as it formed. (c, d and e) A diverse number of secretory cells (Sec) were present in the mesenterial filaments of *A. millipora*, and their proximity to the wounds and enduring mucus indicates a role in cleaning and mucus formation 49,53. (g and h) For mesenterial filaments to move through the SBW and extend beyond the body cavity, they employ a twisting or corkscrewing motion to slide out of the cinclide-like (Cin) openings (Supplementary Movie S2). Skeleton; Sk, SBW; surface body wall, Nm; nematocyst.

Figure 4

Backscatter electron microscopy images and stills from time-lapse movies comparing *A. millipora*'s regular SBW with the enlarged tissues during the immune response phase. (a) A standard surface body wall (SBW) of *A. millipora* showing the two epithelial layers (epidermis; Ep, gastrodermis; Ga) separated by the mesoglea (Mes) and the cells and processes that characterise these layers; supporting cells (Sup), intercellular vacuole (Va) or spaces in between each cell, nematocysts (Nm), electron-dense (white) type 2 gland cell (Mu), Symbiodinium spp (Sym) and the soluble surface mucus layer (SML). (b) Higher magnification image of electron-dense vesicles (white) in standard type 2 gland cells (mucocytes). (c and d) The soft tissue became enlarged at the points of contact between the coral and the substrate. (d) Electron images of the enlarged tissues showed a large proliferation of thin supporting cells and Symbiodinium sp, a lack of intercellular vacuoles due to densely packed supporting cells and the

proliferation/differentiation of type 4 (yellow) and type 1 and 2 (blue) gland cells/vesicles (Sec). (e and f) Electron image showing the vesicles (Sec4) of type 4 gland cells with matter trapped inside and mucocytes (Sec2). (g) Closer look at the densely packed epitheliomuscular supporting cells and their nuclei.

Figure 5

Backscatter electron microscopy images and stills from time-lapse microscopy movies comparing A. millipora's regular tissues with the enlarged anchored tissues. (a) The enlarged tissues (Enl) (Phase one) develop into a soft tissue attachment (Anc) that anchors and helps stabilise the fragment. (b and c) The anchoring process creates a sealed or enclosed (Enc) environment where the SBW or epidermis can be safely removed and a BBW can form. (d and e) The anchoring tissue took on a complex undulated morphology that likely assists tissue anchoring. The shifting morphology of these tissues is down to an ongoing proliferation of supporting epitheliomuscular cells (Emc) and (f and g) to a lesser extent gland cells, which may also aid with adhesion (Sec). Ep; epidermis, Ga; Gastrodermis, Mu; mucus.

Figure 6

Confocal fluorescence microscopy images and stills from time-lapse microscopy movies highlighting the distribution, cell composition and mesenterial behaviour during the immune response phase. (a) The fragment deploys mesenterial filaments en-masse (MF) or as smaller concentrated balls to begin removing the epidermis of the sealed SBW in contact with the substrate. (b) Autolysis leaves behind a transparent gastrodermis where skeletal producing cells (calicoblasts) can develop. (c) The mesenterial filaments in the anchored or compromised tissues can be seen with increased numbers of symbionts in the mesentery filaments. Sk; skeleton.

Figure 7

Backscatter electron microscopy images and stills from time-lapse microscopy movies highlighting the location, morphology, and function of the lappet appendage vital for encrustation of the substrate and for forming an enduring bond. (a and b) The lappet appendage (Lap) is located on the distal edge of the encrusting rim and is responsible for both initial basal precipitation and costae development (Cos). (c) The lappet appendage is a complex structure possessing an SBW that is thicker than the coral regular SBW. (d) Higher magnification view of the lappet appendage shows a complex morphology consisting of 4 key characteristics: (i) tissue undulations (Und) with densely packed cells on its underside, (ii) a transition zone (Trn) where the SBW transitions into the BBW, (iii) a 'pocket' of calicoblastic cells (Cos) for costae development (Cos) – this is not always present as some areas are not actively precipitating costae, and (iv) a thin, poorly defined continuation of the calicoderm (Ext) that sits between the lappet

appendage and the substrate surface and is responsible for the first layers of skeleton (InSk). (e) Higher magnification view of the transition zone (Trn) and the poorly defined calicoderm extension (Ext). Calicoderm extension is nontraditional in that it does not possess a gastrodermis directly adjacent to the corals, which is the standard tissue arrangement. Both epithelial layers of the SBW primarily consist of supporting epitheliomuscular cells, while the BBW consists of a standard calicodermis and gastrodermis. As the lappet appendage slowly migrates, it leaves behind a trail of new BBW to continue colony growth and skeletal thickening. (f) The 'pocket' of cells responsible for the costae wall (Cos) forms between the lappet appendage SBW epidermis (Ep) and gastrodermis (Ga) (Blue) at the mesoglea.

Figure 8

Backscatter electron microscopy images highlighting the primary cell composition of a newly developed lappet appendage. (a) The base of the developing lappet appendage (right) and the trailing basal body wall (BBW, left). (b) The standard BBW consists of two epithelial layers separated by the mesoglea (M); the calicodermis (Ca) primarily consists of cuboidal calicoblasts and a gastrodermis (Ga). (c) A pocket of vestigial epidermal cells that has not yet been removed by mesenterial filaments is still present in the BBW trailing the new lappet appendage. (d) The lappet primarily consists of epitheliomuscular cells (Emc) that attach to the mesoglea (M) by their filamentous myofibrils (Myo). The heightened musculature gives the lappet its complex undulated morphology and the mechanical ability to pulse and perhaps grip the surface.

Figure 9

Confocal fluorescence microscopy images and backscatter electron microscopy images highlighting the costae wall and initial skeletal layer development in the lappet appendage. (a) A confocal fluorescence image of the lappet appendage overlaid with the corresponding SEM image showing the morphology of the lappet appendage and its relationship to the costae (Cos), initial basal deposits (InSk) and the basal skeleton (Sk). (b and c) First, the lappet appendage deposits an initial skeleton (InSk) via the lappet

appendage poorly defined calicoblastic extension; then, as the lappet appendage moves forward, the trailing calicodermis produces a layer of skeleton (Sk) (arrows show the direction of the thickening), building a stronger basal attachment. The lappet appendage can generate rapid accretion deposits (RADs) (b) that form the costae walls (Cos) and further the robustness of the skeleton/attachment. (C) The lappet appendage's poorly defined calicoblastic extending layer (a) (Ext) appears to produce a protuberance (Prt) in the initial skeleton (InSk). (d) The smooth initial skeleton layer possesses characteristics similar to those of *Clypeotheca* (Clp) and can form a keystone structure (Key) in a similar fashion to dissepiments, which can indicate irregular development 26. CCA; crustose coralline algae, Sub; substrate.

Figure 10

Digital graphic of the aquaria setup for the time-lapse and macro images of the ceramic clast and the glass substrate in the fragments. (a) The interface between the coral fragment tissue and the substrate was recorded (time-lapse) in a research aquaria setup with LED light for 30 days using light microscopes. (b and c) The ceramic substrate was placed either between the branches or at the surface/base of the fragment to maximise contact with both the higher growth areas and slower growth base tissues. (d) The

glass substrate was lighter and less hydrodynamic than the ceramic, which made it easier to dislodge via water flow and therefore had to be placed between the branches for added stability.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [MovieS1MesenteriafilamentsInterval60seconds30fps.mp4](#)
- [MovieS2MftwistingInterval60seconds30fps.mp4](#)
- [MovieS3AnchoringInterval60seconds30fpsx500.mp4](#)
- [MovieS4AutolysisInterval60seconds30fps3.mp4](#)
- [MovieS5Pulsedencrustationandcostaedevelopment2.mp4](#)
- [SUPPLIMENTARYMATERIAL.docx](#)