

## Review

# Towards an integrated network of coral immune mechanisms

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Reef-building corals form bio-diverse marine ecosystems of high societal and economic value, but are in significant decline globally due, in part, to rapid climatic changes. As immunity is a predictor of coral disease and thermal stress susceptibility, a comprehensive understanding of this new field will likely provide a mechanistic explanation for ecological-scale trends in reef declines. Recently, several strides within coral immunology document defence mechanisms that are consistent with those of both invertebrates and vertebrates, and which span the recognition, signalling and effector response phases of innate immunity. However, many of these studies remain discrete and unincorporated into the wider fields of invertebrate immunology or coral biology. To encourage the rapid development of coral immunology, we comprehensively synthesize the current understanding of the field in the context of general invertebrate immunology, and highlight fundamental gaps in our knowledge. We propose a framework for future research that we hope will stimulate directional studies in this emerging field and lead to the elucidation of an integrated network of coral immune mechanisms. Once established, we are optimistic that coral immunology can be effectively applied to pertinent ecological questions, improve current prediction tools and aid conservation efforts.

**Keywords:** invertebrate immunity; coral immunology; melanin; coagulation; TOLL-like receptors; immune cells

## 1. INTRODUCTION

Human-induced local impacts and climatic changes are threatening the persistence of coral reefs [1]. This is largely due to a coincident increase in occurrences of compromised coral health, such as disease and the breakdown of symbiosis with *Symbiodinium* algae, known as bleaching [2]. As immunity determines, the ability of an organism to resist and eliminate infection and to recover from injury, it can be used as a predictor of compromised health susceptibility [3,4]. Therefore, understanding immune mechanisms will likely enable better insight into coral declines at individual, population and ecological scales. However, coral immunology is an emergent field, with many disjointed studies and lacks a comprehensive synthesis of the current knowledge [5].

Reef-building corals (Order Scleractinia, Class Anthozoa) fall within the phylum Cnidaria, and therefore not only occupy a vital ecological niche, but also a basal position in metazoan phylogeny. Concomitantly, innate immunity is an evolutionarily ancient system, suggesting that the origins of well-documented immune mechanisms of bilaterian organisms may reside within the basal phyla [6]. However, cnidarians' anatomical simplicity and phylogenetic distance from mammals, led to assumed genomic simplicity [7], and thus they have been overlooked as immunological models. However, it has recently become apparent that anthozoans possess a genomic complexity more similar to vertebrates than model invertebrates (e.g. *Drosophila melanogaster*; [8]), suggesting that the field

of immunology may benefit from an understanding of cnidarian immunity [9].

This review defines the current field of coral immunology by placing it within the context of the better-established field of invertebrate immunology. In doing so, we highlight key knowledge gaps and provide order to the recent surge of studies, many of which remain overlooked by the fields of coral biology and/or invertebrate immunology. In the light of the urgent need for mechanistic explanations of compromised coral health, we also develop a comprehensive framework for future coral immunological studies, emphasizing the necessity of establishing a solid foundation of fundamental knowledge, to encourage the rapid and effective expansion of this vital field.

## 2. INVERTEBRATE INNATE IMMUNITY

Invertebrates have evolved innate immune mechanisms to resist infection and maintain tissue integrity, but lack the adaptive immune systems of vertebrates [9]. An innate immune response delivers an immediate reaction to the presence of non-self and/or compromised tissue integrity in three broad phases: *recognition* of a threat (detection of non-self; allorecognition), *signalling pathways* to activate appropriate defence mechanisms and *effector responses*, which are responsible for eliminating the threat ([10]; figure 1). Key effector responses that will be addressed in this review include antimicrobial peptides (AMPs), melanin synthesis, coagulation and immune cell activation. Although the innate immune phases can be employed in response to numerous immune activators, such as

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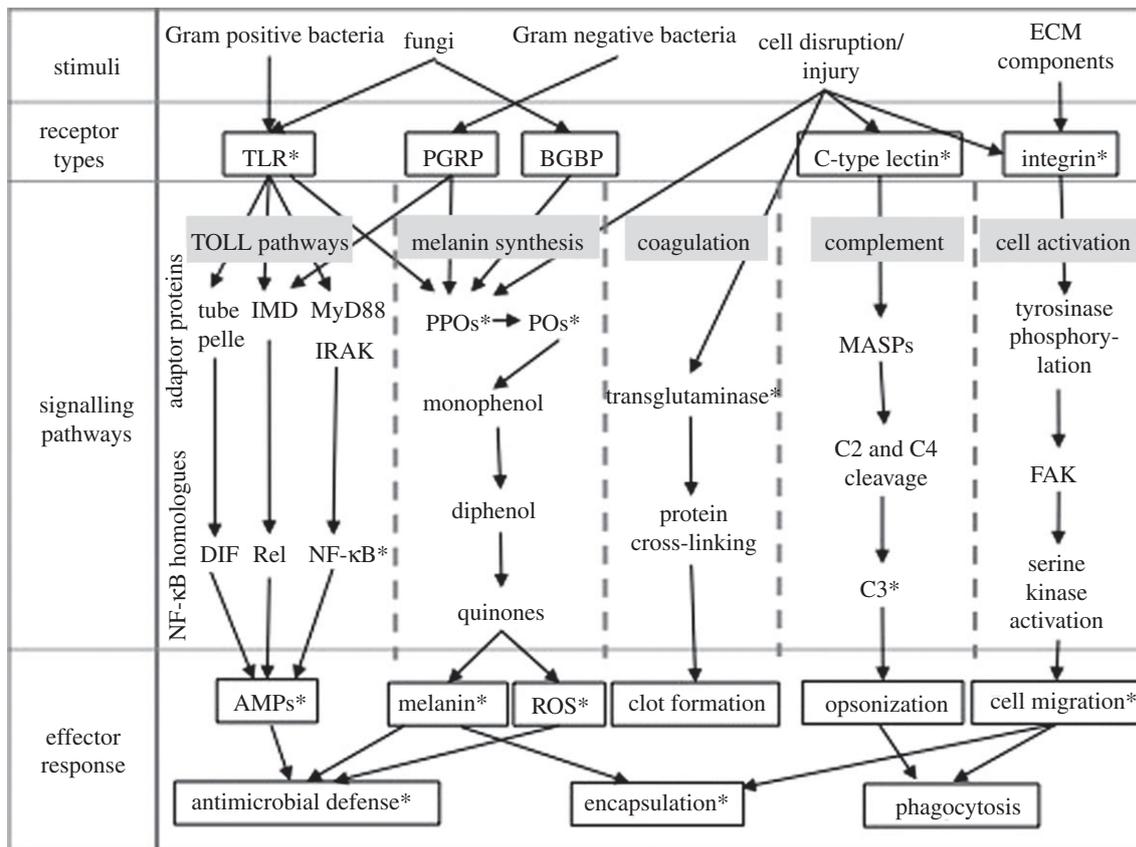


Figure 1. A simplified overview of the main invertebrate immunity components (synthesized from [11] and [12]). Starred (\*) components have been identified within anthozoans. TLR, TOLL-like receptor; PGRP, peptidoglycan recognition protein; BGBP, beta-glucan binding protein; IMD, immunodeficiency; MyD88, myeloid differentiation primary-response protein 88, PPOs, prophenoloxidas; POs, phenoloxidas; MASPs, mannose binding lectin-associated serine proteases; FAK, focal adhesion kinases; AMPs, antimicrobial peptides; ROS, reactive oxygen species.

parasitism [13], allorecognition (see reviews by [14] and [5]) and potentially by symbiosis establishment [2], innate immunity is perhaps most commonly referred to in response to infectious disease and injury. In this review, we therefore focus on the network of immune mechanisms invertebrates employ in response to these latter two immune activators.

### (a) Recognition

Invertebrates employ a suite of pattern recognition receptors (PRRs) and soluble proteins to recognize a broad array of pathogenic microbes and cellular disruption ([15]; figure 1). PRRs bind to conserved pathogen-associated molecular patterns (PAMPs) [16], such as the lipopolysaccharides (LPS; [7]). Each receptor binds to one or more PAMP and is responsible for inducing appropriate signalling pathways (figure 1). Numerous PRRs have been identified within invertebrates, and several recognition-related genes have been identified within corals during microarray and transcriptional studies in response to environmental stress, e.g. tachylectin [17] and a spondin [18]. However, to date, only three have been specifically investigated as immune-related genes: TOLL-like receptors (TLRs), lectins and integrin ([19,20]; figure 1).

#### (i) TOLL-like receptors

TLRs are highly conserved trans-membrane proteins that recognize PAMPs and activate signalling for AMP synthesis ([21]; figure 1). TLRs consist of a leucine-rich repeat (LRR) outer domain, which determines the PAMPs

they bind to [22], and cytoplasmic components with a TOLL/interleukin-1receptor homology (TIR) domain (e.g. adaptor protein MyD88) that activate downstream signalling pathways [15,21]).

Consistent with their high evolutionary conservation, TLRs have been identified across the invertebrates, for example, within Porifera [23], Nematoda [21], Echinodermata and Arthropoda (see review [24]). Within Cnidaria, complete TLRs (with both TIR domains and LRRs) have only been characterized within two species, one within the anemone *Nematostella vectensis* [20] and five TLRs in the coral *Acropora digitifera* [25]. However, *N. vectensis* has five additional TIR domains [20,25,26], while *Hydra* sp. possesses two [27,28] and *Acropora millepora* has one within an investigated transcriptome [20]. Although only identified within two cnidarians, the presence of TLRs needs to be confirmed within a representative suite of species and their role within coral immunity needs to be corroborated with functional studies, which are conspicuously absent [20]. While searches of available transcriptome and genome databases would provide a starting point, elucidation of coral TLR stimuli and the resultant responses would represent a great leap towards a full understanding of the coral immune network.

#### (ii) Lectins

C-type lectins belong to a superfamily of carbohydrate-binding proteins that are dissolved within the extracellular matrix (ECM) or are cell surface receptors [29] and are

important to a diversity of innate immune functions [11]. Lectins activate signalling pathways that lead to cell adhesion and opsonization [29], induce the melanin-synthesis (phenoloxidase, PO) cascade [30] and AMP production [31].

Many C-type lectins, such as the mannose-binding lectin (MBL), have been identified within numerous invertebrates, including the Nematode *Caenorhabditis elegans* and Arthropods, e.g. *D. melanogaster* (reviewed by [11]). C-type lectins have also been identified within the cnidarians *N. vectensis* [32], *Hydra* sp. [33] and corals, including *Montastraea faveolata*, *A. millepora* [34], *Oculina varicose* [35] and *Porites astreoides* [18]. In addition to its identification within *A. millepora*, the mannose-binding-like lectin gene, Millectin, showed increased expression when exposed to a bacterial challenge and *Symbiodinium* endosymbionts [19]. This represents one of the few studies confirming cnidarian immune gene homologue function, and suggests an involvement in symbiosis. In order to provide further insight into the role of this lectin within the coral immunity network, downstream pathway activation and the resultant effector responses need to be investigated.

### (iii) Integrins

Integrins are trans-membrane alpha beta heterodimers that mediate interactions between cells and the ECM [36]. In addition to fundamental roles in development, integrins are involved in multiple immunological cellular processes, such as cell migration and differentiation, as well as fibrillar matrix formation and signal transduction [37].

Integrins are found throughout the invertebrates, including within Arthropoda; *D. melanogaster* [38] and Porifera; *Suberites domuncula* [39]. Within Cnidaria, three alpha and four beta integrin subunits have been identified in *N. vectensis*, indicating at least three complete integrins [8,32]. *Hydra magnipapillata* has at least one alpha and beta integrin present [28] and two beta subunits have been identified in the hard coral *A. millepora* [20,40,41]. Although the diversity of cnidarian integrins is more complex than first thought [41], functional studies need to be conducted. The presence and diversity of integrins also needs to be confirmed within a broader suite of coral species.

## (b) Signalling pathways

### (i) Antimicrobial peptide synthesis pathways

AMPs rapidly kill a broad spectrum of microbes and are produced by all multi-cellular organisms [42]. Most organisms have multiple AMPs that are produced by different pathways, e.g. TOLL or IMD pathway ([12]; figure 1). Insect AMP synthesis signalling pathways are activated via the TIR adaptor proteins and comprise a suite of components, including a protein complex family of transcription factors known as nuclear factor kappa B (NF- $\kappa$ B)/Rel and their corresponding inhibitors ([21]; figure 1). In addition to insects and other higher invertebrates (e.g. Mollusca; [43]), TOLL pathway components have been found within ancestral metazoans, including Porifera [44]. Within the cnidarian *N. vectensis*, multiple TOLL pathway components have been identified, including an NF- $\kappa$ B inhibitor (I $\kappa$ B), NF- $\kappa$ B, a mitogen-activated protein kinase (MAPK) and a nucleotide-binding domain (reviewed by [21]). Consistent with the lack of a complete TLR within *H. magnipapillata*, only one pathway component was identified, potentially suggesting gene loss

[20]. Three TOLL pathway components were located with an *A. millepora* transcriptome [20]. These studies demonstrate the presence of this pathway within non-bilaterian animals for the first time, confounding hypotheses that immune-related TOLL evolved after the divergence of Nematoda [21]. However, the role of cnidarian TOLL pathway components in immunity cannot yet be confirmed as functional studies are lacking.

### (ii) Proteolytic cascades

*Melanin-synthesis pathways.* The melanin-synthesis pathways, synonymous with the prophenoloxidase (PPO) system, are responsible for cytotoxic defence and the melanization (encapsulation) of foreign bodies (reviewed by [45]; figure 1). Melanin synthesis is triggered by pathogens, their derived products and upon injury, leading to cleavage of PPO into PO. Melanin synthesis is then the rapid outcome of a cascade involving the catalysis of monophenol hydroxylation and diphenol oxidation by various POs, as well as autocatalytic reactions ([45]; figure 1). PPOs, and POs, exist in various isoforms that represent different components of, potentially several, melanin-synthesis pathways, which likely have differing functions [46,47]. While the tyrosinase-type melanin synthesis pathway has been the most intensively investigated with regard to invertebrate immunity [48,49], an invertebrate laccase-type pathway has also been documented [50]. However, aside from a likely role in cuticle formation, roles of the laccase-type pathway remain to be elucidated [46,50].

Although traditionally studied within the Arthropoda [46,51], melanin-synthesis pathways are present across the invertebrates, including Mollusca [50] and Annelida [52]. A melanin-synthesis pathway was the first classic invertebrate immune response to be investigated within corals, with PO and PPO activities documented in two hard coral species, *A. millepora* and *Porites* sp. [13], and the gorgonian sea fan *Gorgonia ventalina* [53,54]. Increased melanin-synthesis pathway activities in compromised [13], infected [53] and thermally stressed tissue [55] were also demonstrated. Subsequently, PO activity was documented within 22 healthy Indo-Pacific hard and soft corals and, in conjunction with other immunity parameters, can be used to predict coral families' susceptibility to both disease and bleaching [4]. Significantly, functional studies [55] and the potential use of melanin-synthesis activity as a predictive tool demonstrate the role of this pathway not only in coral resistance to infection but also to bleaching.

Further demonstrating the importance of melanin-synthesis within corals, POs and PPOs indicating the presence of both laccase and tyrosinase-type melanin pathways have been identified within suites of Indo-Pacific and Caribbean corals [56,57]. However, field sampling of diseased *A. hyacinthus* colonies has raised currently unresolvable questions of the role of baseline immunity in resisting disease versus immunocompetence in pathogen elimination [58]. Similarly, the variable responses of PPOs to LPS and thermal treatments among three Caribbean coral species indicates that they likely use this innate immunity component distinctly [59], reinforcing the need for multi-species approaches to coral immunology studies.

*Coagulation cascades.* Coagulation ensures the rapid re-establishment of tissue integrity after injury [60] to

prevent fluid loss and infection [45]. Transglutaminases are essential coagulation enzymes and function by forming a gel upon interaction with plasma proteins (reviewed by [45]). Transglutaminases have been identified within molluscs [61] and a suite of arthropods (reviewed by [62]). Coagulation remains poorly investigated within anthozoans, with transglutaminase activity only documented within one reef-building coral *Porites cylindrica* [57]. Therefore, coral transglutaminases need to be conclusively identified within a suite of corals and their role within coagulation functionally tested.

### (iii) Immune cell activation

Immune cells communicate via chemical signalling within both their internal and external environments [36] and are primarily responsible for phagocytosis and encapsulation (figure 1). Immune cell activity cross-links with multiple effector responses, and thus immune cells have numerous activators, including the lectin complement network and integrin signalling [12].

The complement system forms a bridge between innate and adaptive immunity and results in opsonization (figure 1), whereby phagocytosis is enhanced via host tagging of pathogens [63]. In invertebrates, the complement system can be activated by the lectin and alternative pathways, which both culminate in the activation of  $\alpha$ 2-macroglobin protein C3 [11]. The lectin pathway (figure 1) is triggered by C-type lectin receptors, e.g. MBL, which leads to the cleavage of pathway components C4 and C2 to form C3 [11,63]. The alternative pathway does not involve receptors, but is activated by C3 binding to micro-organisms [11,63]. Components of the complement system, such as C3 and homologues that contain a thioester protein (TEP), have been located within a diverse range of invertebrates, e.g. echinoderms and arthropods (reviewed by [64]). C3 has been identified within several anthozoans; *Swiftia exserta* [65], *N. vectensis* [32] and the hard corals *A. millepora* [20,66] and *Porites lobata* [18], and is thus indicative of lectin-mediated cellular immune functions within corals. However, although C3 genes have been included in expression studies of thermal stress [18,67], functional studies targeting the role of C3 in coral immunity are lacking.

Integrins signal both mechanically via cell anchorage and chemically by outside-in signalling into the cell [36]. Integrin signalling induces the activation of focal adhesion kinases (FAKs; figure 1), a central component of cell migration and cell-cycling [68]. FAK associates with other signalling molecules [68,69], causing phosphorylation and signalling, which leads to a diverse array of immune cell-related effector responses ([68]; figure 1). Though integrin signalling pathway components have been identified within many invertebrates, including arthropods [70] and echinoderms [71], they remain unidentified within cnidarians. The lack of information on cnidarian integrin pathway components, and thus cell signalling, represents a significant gap in our current knowledge of coral immunity.

## (c) Effector responses

### (i) Antimicrobial peptide activity

AMPs are located within granular immune cells and on epithelial layers [42] and act by disrupting microbial cell membranes [72]. A suite of AMPs is usually associated

with a single organism, such as the seven types described for *Drosophila* [73]. AMPs have been identified in a wide variety of invertebrate phyla, including within the Porifera (*Discodermia* sp.), Arthropoda, e.g. *Panopeus vannamei* and Echinodermata, e.g. *Strongylocentrotus droebachiensis* and the Cnidarian jellyfish *Aurelia aurita* (reviewed by [74]). Within *H. magnipapillata*, three AMPs have been identified, despite the proposed loss of representative TOLL pathway components [20], and are produced in response to a variety of pathogens, including proteobacteria and *E. coli* [27,75]. Conversely, no AMPs have been documented within the anemone *N. vectensis*, despite the suite of TOLL pathway components being located within the genome [20], though this is likely an artefact of incomplete investigation, rather than an actual absence of AMPs. One AMP (Damicornin) was recently identified in the hard coral *Pocillopora damicornis*, and demonstrated activity in response to both Gram-positive bacteria and fungi [76].

Although AMPs have only been confirmed in one anthozoan (*P. damicornis*), bactericidal activity has been documented for numerous anthozoans [55,59,77]. In addition to AMPs, these bactericidal activities may be due to mucus-associated antibiotic-producing microbes, small peptides and secondary metabolites (reviewed by [78]). The origin of coral bactericidal activity needs to be determined in order to accurately interpret results of inoculation studies where, for example, bactericidal activity of coral tissue decreased upon exposure to a pathogen elicitor [59]. Similarly, further studies are needed to elucidate the suite of AMPs that are potentially synthesized by hard corals, and the pathogens they eliminate.

### (ii) Melanin synthesis

The products of melanin-synthesis pathways (melanin and cytotoxic intermediates; figure 1) are central to invertebrate immunity [46]. Melanin creates a physical barrier, encapsulating invading organisms, protecting healthy tissue and providing structural support during tissue repair [46]. Cytotoxic by-products, including reactive oxygen species (ROS), assist in antimicrobial defence by immobilizing and killing pathogens [46,79], but are also capable of causing self-harm [45,80].

Melanin-associated encapsulation and structural support usually involves the degranulation of immune cells within which the melanin-synthesis pathway is active [81], such as crystal cells of insects [48] and haemocytes of crustaceans [82]. For anthozoans, melanization was first documented within the sea fan *G. ventalina* as a barrier against a fungal infection [54], and Mullen *et al.* [83] described the amoebocytes involved. In the same species, aggregations of amoebocytes were documented around fungal infections, and their granular content was confirmed to be melanin [53]. Melanin-containing granular cells were subsequently identified in hard corals (*Porites* spp.), and their higher densities in compromised and infected tissue supports a role in immunity [13,84]. Subsequently, melanin-containing cells have been found within a suite of healthy Indo-Pacific corals (Scleractinia and Alcyonacea), suggesting their likely ubiquity among anthozoans [4]. A recent study confirmed the role of these cells in an injury response, via degranulation to form a rudimentary clot and subsequent aggregation and/or proliferation to provide structural reinforcement [85].

Cytotoxic aspects of melanin-synthesis are less explored within corals and would provide a complementary avenue for future studies. Similarly, the possible ecological implications of the variation in abundance of melanin-containing granular cells [4,13,59,85] and their likely involvement in bleaching resistance [4] warrants further exploration.

### (iii) Clot formation

The effector response associated with the activation of the coagulation pathway is, primarily, the formation of an insoluble clot [62]. Clot formation is an excellent example of cross-linking among immune pathways, with clots formed by transglutaminase activity that become melanized for stability [62] and involves the aggregation of immune cells [81].

Clot formation has been investigated within many invertebrates, including Arthropods such as *Drosophila* [81]. Within cnidarians, while observations of tissue regeneration [86,87] suggest that clot formation must initially occur, clotting has only been confirmed within one hard coral, *P. cylindrica* [85]. While this clot formation coincided with melanin and immune cell activity, its initiation by transglutaminase was not documented [85] and thus needs to be confirmed.

### (iv) Immune cells

Invertebrate immune cells become activated in response to foreign organisms and injury [88] to phagocytose and/or encapsulate pathogens and re-establish tissue integrity. Immune cells with phagocytic potential have been identified across the invertebrates, e.g. within Arthropoda [81] and Echinodermata [89]. During wound repair, after clot formation, specialized immune cells infiltrate the wound site and phagocytose cell debris and foreign organisms [90]. Infiltrated cells then proliferate, forming granulation tissue, which consists of multiple cell types, collagen and a basic ECM, and provides a platform for re-epithelialization [81,89]. Although there are differences among species, these cellular phases of wound healing have been documented within numerous invertebrates [81,91].

Within the Cnidaria, cells with phagocytic potential were identified in the sea anemone *Actinia equina* and the soft coral *S. exserta* over 10 years ago [92,93]. An investigation of the gorgonian *Plexaurella fusifera* provided preliminary insights into the processes and cells involved in anthozoan wound healing [94]. However, within hard corals, investigations of immune cells have been hindered by the assumption that they are small and confined to the acellular mesoglea. Despite this hindrance, as described earlier, melanin-containing granular cells (granular amoebocytes) have been characterized within a host of anthozoans [4,13,55,85]. Additionally, the immune cells and phases involved in wound healing were recently documented for the hard coral *P. cylindrica* [85]. However, the full suite of coral immune cells and their functional repertoire, particularly in response to pathogenic invasion, has yet to be understood.

## 3. CORAL-SPECIFIC IMMUNE COMPLEXITIES

Coral obligate endosymbionts, *Symbiodinium*, provide valuable energetic resources. However, how this fundamental relationship is established, maintained and

broken down, remains to be conclusively resolved, but likely involves immune mechanisms of both partners [2]. While some recent studies hint at a relationship between host immunity and symbiosis [4,19,55,66], this has yet to be directly investigated. Significantly, immune involvement in symbiosis suggests that understanding immune mechanisms will likely provide insight into questions pertaining to coral bleaching.

An additional aspect of coral biology that remains unresolved is the function of fluorescent proteins (FPs). Nonetheless, FPs are often used as a measure of thermal stress due to their rapid responses to environmental changes [95,96]. Many hypotheses have been proposed for the role of these eye-catching proteins, for example, in *Symbiodinium* photoprotection [97], and more recently as components of an immune response, potentially with antioxidant function. This latter hypothesis stemmed from the higher abundance of FPs in injured [13] and infected coral tissue [84] and from evidence that they scavenge oxygen radicals more efficiently than other proteins [98]. While these recent findings do not conclusively elucidate FP function, they support the hypothesis that FPs are part of an immune response, which frequently induces oxidative stress [80]. The potential role of FPs in immunity should therefore be considered as the field of coral immunology expands, and will hopefully lead to the elucidation of their biological function.

## 4. A FRAMEWORK FOR DEVELOPING THE FIELD OF CORAL IMMUNOLOGY

The progression of the field of coral immunology has recently been criticized for its paucity and lack of functional studies [5]. While the gaps in our current knowledge support these statements, coral immunology is an emerging field and thus such deficiencies are currently to be expected. As a detailed knowledge of fundamental coral biology is essential for accurately answering pressing climate-related questions, elucidating coral immune mechanisms and their functions, should be a research priority. We therefore propose a three-stage framework for comprehensively developing the field of coral immunology (figure 2) by progressively building on layers of knowledge so that pertinent questions on coral declines can be accurately addressed.

The first stage of the framework aims to define coral immunity by elucidating the suite of immune mechanisms anthozoans use. We propose that this aim should be addressed by establishing the presence or the absence of common invertebrate immune mechanisms within corals, using a multi-species and multidisciplinary approach to target key gaps in our current knowledge, such as immune cell characterization. The expected imminent proliferation of new, and exhaustive searches of current coral transcriptome and genome databases will undoubtedly expedite this stage. Additionally, potential coral-specific immune mechanisms should also be considered, e.g. FPs, perhaps in conjunction with stage 2 of the framework.

The aim of stage 2 is to confirm the role of the identified mechanisms (stage 1) within coral immunity by conducting controlled experiments (figure 2). As controlled experiments will determine immune component function and biological relevance, stage 2 is crucial to the development of coral immunology and should be

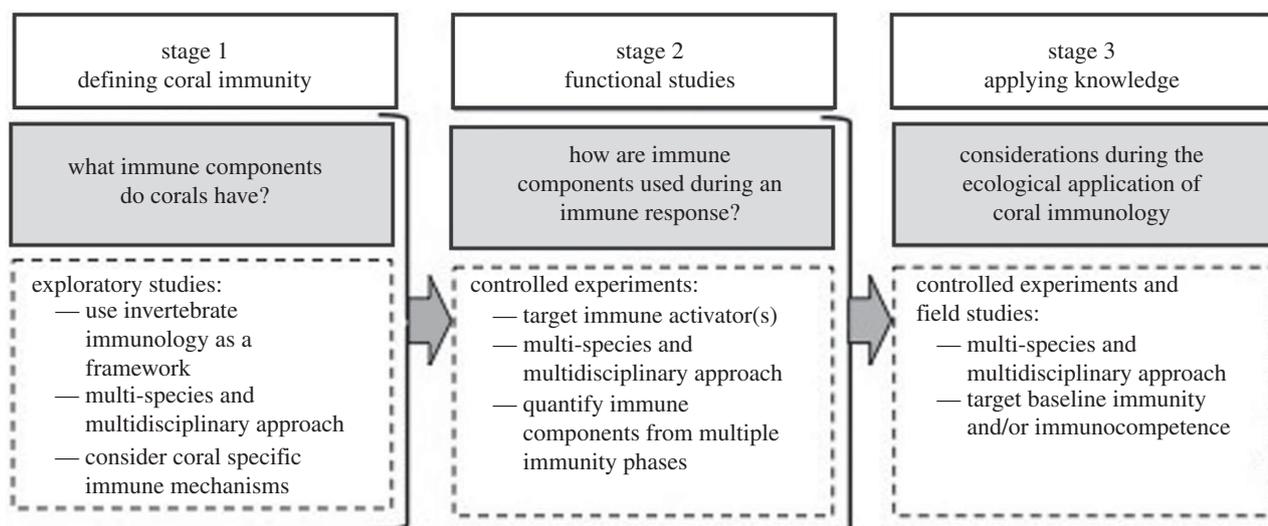


Figure 2. A proposed framework for the development of coral immunology.

executed and published with stage 1 [13,53]. During the design of the controlled experiments, the type of immune activator (e.g. injury or parasitic infection) to be used needs to be considered, as the biological relevance of different immune mechanisms will likely vary among them. Ideally, a multi-species and multidisciplinary approach should also be taken to maximize the information output and to account for species-specific differences. Additionally, quantifying immune mechanisms from more than one immune phase will increase the likelihood of elucidating interrelationships among components, and investigating multiple immune pathways will help to unravel cross-linkages within the immune network.

Once stages 1 and 2 have been addressed, the resultant information can be used to effectively tackle ecological scale and climate-related questions (stage 3) by providing clearer interpretations of findings. Ecological aspects of immunology can begin to be addressed, such as variations within and among populations, species and over time, and the role of baseline immunity in resisting disease versus the immunocompetence that fights it. Current coral immunology is only just at the cusp of these ecological applications with the melanin-synthesis pathway [4,59].

## 5. CONCLUSIONS

Corals possess components of the established innate immune phases and key invertebrate immune pathways. However, the gaps in the current knowledge are numerous, with the most striking being the lack of functional studies of proposed coral immune components. The proposed framework of future research offers a logical and systematic approach that will enable the rapid and effective development of the field, and additionally enhance coral biological and general immunological knowledge. Moreover, as immunology likely underpins health-related coral declines, its inclusion into coral biology and ecology will enhance our ecological understanding and predictions of how climatic changes will likely shape our future reefs.

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