

# Innate Immunity, Environmental Drivers, and Disease Ecology of Marine and Freshwater Invertebrates

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prophenoloxidase pathway

## Abstract

Despite progress in the past decade, researchers struggle to evaluate the hypothesis that environmental conditions compromise immunity and facilitate new disease outbreaks. In this chapter, we review known immunological mechanisms for selected phyla and find that there are critical response pathways common to all invertebrates. These include the prophenoloxidase pathway, wandering phagocytic cells, cytotoxic effector responses, and antimicrobial compounds. To demonstrate the links between immunity and the environment, we summarize mechanisms by which immunity is compromised by environmental conditions. New environmental challenges may promote emergent disease both through compromised host immunity and introduction of new pathogens. Such challenges include changing climate, polluted environment, anthropogenically facilitated pathogen invasion, and an increase in aquaculture. The consequences of these environmental issues already manifest themselves as increased mortality on coral reefs, pathogen range expansion, and transmission of disease from aquaculture to natural populations, as we summarize in a final section on recent marine epizootics.

## INTRODUCTION

Host-pathogen interactions are strongly governed by the environment (Harvell et al. 2004). Recent increases in disease outbreaks among marine organisms (Ward & Lafferty 2004) may be caused either by introduction of new pathogens or a change in the environment (Harvell et al. 2004, Lafferty et al. 2004, McCallum et al. 2004). Host resistance is a first line of defense and its success is a major determinant of whether a new pathogen or a changed environment will result in a disease outbreak. Thus the hypothesis that changing environmental conditions compromise host immunity and lead to disease outbreaks should be carefully considered. This review critically evaluates this hypothesis by examining recent advances in immune mechanisms of marine invertebrates, the known interactions of immunity and environment, and the evidence for compromised immunity in recent outbreaks of disease in the ocean.

The invertebrate immune system is based on self/nonself recognition and cellular and humoral processes. To date, no true adaptive components have been identified in these innate systems (Soderhall & Cerenius 1998), though elements suggesting memory and specificity are seen in invertebrates as basal as sponges (Bigger et al. 1982, Hildemann et al. 1979). Exploration of immunity in invertebrates is dominated by mechanistic studies of model organisms, with little attention to natural populations (Little et al. 2005). Thus, identifying what is known about key components of the innate immune system in different invertebrate phyla, and how these are affected by changing environmental conditions, remains a critical priority. Invertebrates are especially attractive study systems owing to the nonadaptive nature of their innate immune systems, the propensity they have for straightforward experimental manipulation, and the diversity of environments they inhabit.

From invertebrate model systems we have learned that the primary components of innate immunity fall into three categories that define the effectiveness of an immune response. First, the organism distinguishes between self and nonself; second, the organism mounts a defensive response that can kill or disable the invader; and finally, the organism recognizes and can eliminate its own damaged or diseased cells. These requirements lead to the three essential components of innate immunity: phagocytosis (cell-mediated); activation of humoral responses leading to opsonization, melanization, and coagulation (cell-free); and the production of humoral antimicrobial compounds (cell-free).

Below we review immunity in phylogenetically widespread invertebrates, including basal phyla such as Porifera and Cnidaria and representative phyla from Lophotrochozoa, Ecdysozoa, and Deuterostomia. We focus on marine phyla that are ecologically, environmentally, and commercially relevant, that are not model organisms, and that have suffered substantial losses because of disease outbreaks. Most of the groups we select are affected by changing climate, water quality, and pollutants. We then examine how environmental factors, namely climate change and environmental pollution, modulate these immune responses. Finally, we explore the ecological effects of diseases in invertebrates by documenting disease outbreaks in natural populations.

## PRIMARY ELEMENTS OF THE INNATE IMMUNE SYSTEM IN MARINE AND FRESHWATER INVERTEBRATES

In the following and in **Table 1**, we describe by phylum the known elements of the innate immune system in marine invertebrates, focusing on the following where known: (a) recognition responses such as lectins, pattern recognition proteins, cell adhesion factors; (b) cell-free or humoral responses characterized by antibacterial peptides, small molecule antifungal and antibacterial compounds; (c) cellular responses, such as physio-chemical barriers (melanin), phagocytosis, granulated vesicles, antioxidant enzymes, and adhesive proteins; (d) communication and integration of immune function pathways including prophenyloxidase, complement system, cytokines, eicosanoids, and Toll-like receptors (TLRs), for example; and (e) evidence for primitive immune memory and specificity.

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**TLR:** Toll-like receptor

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### Phylum Porifera

Sponges are the most basal of the Metazoa. Not only are true tissues absent in Porifera, but most body cells are totipotent—capable of changing function and form as needed. Thus despite the fact that they are large, multicellular animals, in many ways they function like organisms that are unicellular in complexity.

**Recognition.** As early as the latter part of the nineteenth century, Wilson (1891) demonstrated the remarkable capacity of sponge cells to reaggregate after being dissociated. This surprisingly complex behavior was later found to involve a number of molecules and signaling pathways. Adhesion of sponge cells involves the molecules galactin, integrin, fibronectin or fibronectin-like proteins, and collagen (Brower et al. 1997, Labat-Robert et al. 1981, Muller et al. 1999). In the sponge *Microciona prolifera*, calcium ions serve as an intracellular messenger in stimulus-response coupling during cell-cell aggregation (Dunham et al. 1983, Weissman et al. 1986), and carbohydrate self-recognition mediates cellular adhesion (Haseley et al. 2001). Allogenic rejection has a cellular component: Interactions between incompatible sponges activate exopinacocytes (cells forming the external surface of the sponge) and various mesohyl cells, some of which contain the secondary metabolites so common in sponges (Gaino et al. 1999).

**Responses: cell-free.** A large number of bioactive compounds, many of which have potential pharmacological applications (e.g., antimicrobial, antifungal, anti-inflammatory, antitumor, and cytotoxic bioactivities) have been discovered in sponges. Some of these compounds are products of the sponge cells themselves, and some are secreted by symbionts (e.g., Becero & Paul 2004, Rifai et al. 2004).

**Responses: cell-based.** Archeocytes are wandering, totipotent, amoeboid cells capable of differentiating into virtually every other cell type present in a sponge. They are large, phagocytic cells that play a major role in digestion, defense, and food transport. As such, they possess digestive enzymes, including protease, lipase, and amylase, and can accept phagocytized material from other cells, for example, the choanocytes

**Table 1 Invertebrate immune parameters**

Organism/Phyla	Pathogen/elicitor	Immune parameter characterized	Reference <sup>2</sup>
Porifera			
Sponge <i>Ephydatia mulleri</i>	N/S <sup>1</sup>	Self-recognition, cell-cell adhesion, fibronectin-like protein	1
Sponge <i>Microciona prolifera</i>	N/S	Carbohydrate self-recognition, cellular adhesion and aggregation, activation of protein kinase C, calcium signaling	2
Demosponge <i>Geodia cydonium</i>	N/S	Adhesion molecules: galactin, integrin, fibronectin, collagen; allogenic rejection involves upregulation of phenylalanine hydroxylase, an enzyme initiating the pathway to melanin synthesis	3
Sponges (four spp.)	N/S	Alloimmune memory revealed by consistently accelerating second-set graft rejections	4
Sponge	N/S	Enhanced self/nonself recognition upon second exposure to foreign sponge tissue	5
Sponge	N/S	Activation of exopinacocytes and mesohyl (granule-bearing) cell types	6
Sponge <i>Suberites domuncula</i>	Gram-positive bacteria	Activation of endocytosis, lysozyme release	7
Cnidaria			
Sea anemone	N/S	Self/nonself recognition	8
Sea anemone <i>Anemonia viridis</i> , Coral <i>Goniopora stokesi</i>	N/S	Antioxidant enzymes, superoxide dismutase, catalase, glutathione peroxidase associated with granulated vesicles	9
Sea anemone <i>Aiptasia pallida</i>	N/S	Nitric oxide synthase activity	10
Gorgonian coral <i>Swiftia exserta</i>	Foreign particles (inert)	Activation of phagocytic cells	11
Gorgonian coral <i>Gorgonia ventalina</i>	<i>Aspergillus sydowii</i> (fungal pathogen)	Melanin, lipid antifungal and antibacterials	12
Scleractinian coral	N/S	Immune specificity, primitive memory	13
Scleractinian coral	Cyanobacteria, other microbes	Mucus accumulation and shedding, secretion of antimicrobial compounds, nitric oxide synthase activity	14
Gorgonian and scleractinian corals	Bacterial	Wound healing by motile phagocytic cells, tissue reorganization	15
Gorgonian coral <i>Plexaurella fusifera</i>	N/S	Wound healing by amoebocytes	16
Hydroid <i>Hydractinia</i>	Fungal, nematode pathogens	Chitinase expression	17
Sea anemone	Gram-negative bacteria	Amoebocytes engage in phagocytosis, reactive oxygen species production, soluble microbials	18
Mollusca			
Marine bivalves	Bacterial pathogens	Phagocytosis, humoral components (lectins, lysozymes), reactive oxygen species, small antimicrobial peptides	19

(Continued)

Table 1 (Continued)

Organism/Phyla	Pathogen/elicitor	Immune parameter characterized	Reference <sup>2</sup>
Marine bivalves	Protozoan <i>Perkinsus</i>	Phagocytosis and encapsulation by hemocytes, secretion of toxic polypeptides, opsonization	20
Clam <i>Ruditapes philippinarum</i>	Self-nonsel recognition, protozoan <i>Perkinsus</i> sp.	Self/nonsel recognition by lectins, lectin binds specifically to the surface of purified hyphospores from <i>Perkinsus</i> spp.	21
Marine bivalves	Bacterial	Hemocytes activity; mobile, highly clumping, phagocytic, chemotactic, and bactericidal	22
Oyster <i>Crassostrea virginica</i>	Bacteria: <i>Vibrio</i> spp., (normal gut flora)	Cell-free hemolymph agglutinizes <i>Vibrio cholerae</i> , but not 79 other bacterial strains	23
Oyster <i>Crassostrea gigas</i>	Viruses	Oyster hemolymph has detectable antiviral activity against HSV, IPNV	24
Oyster <i>Saccostrea glomerata</i>	Protozoan <i>Marteilia sydneyi</i>	Phenoloxidase activity induced and suppressed in infected areas	25
Bivalves, <i>Mytilus edulis</i> , <i>Chlamys islandica</i>	Bacterial	Lysozymes and small antibacterial proteins	26
Crustacea			
Crustaceans, arthropods	Self-nonsel	Induction of prophenoloxidase triggered by lipopolysaccharides, peptidoglycans	27
Crayfish	N/S	Cell adhesion, encapsulation, nodule formation, degranulation, opsonization, and phagocytosis	28
Horseshoe crab <i>Tachypleus tridentatus</i>	Wound-healing	Induced coagulation cascade, prophenoloxidase, Toll-like receptor	29
Horseshoe crab <i>Tachypleus tridentatus</i>	Bacterial	Pathogen recognition: lectins	30
Crab <i>Carcinus maenas</i>	Bacterial/microbial	Hemocytes produce antibacterial protein	31
Spiny lobster	Gram-negative bacteria	Induced bactericidal molecules. Memory: secondary bactericidal responses enhanced over primary responses	32
Cladoceran <i>Daphnia magna</i>	Bacterial	Specificity: strain-specific immunity conferred to offspring	33
Copepod <i>Macrocylops albidus</i>	Parasitic tapeworm	Memory and specificity	34
Shrimp, blue crab, crayfish	Variety of viruses	Antiviral substances bind specifically to a variety of RNA and DNA viruses	35
Shrimp <i>Penaeus monodon</i>	Bacterial, variety of viruses	Lysozyme activity and antimicrobial peptides. Protein with C-lectin-like domain with strong antiviral activity	36
Echinodermata			
Sea star	Bacterial	Lysozyme activity, reactive oxygen species production	37
Sea star <i>Asterias rubens</i>	Nonspecific	Primitive cytokines and cytokine receptors	38
Sea urchin <i>Lytechinus pictus</i>	Nonspecific; evidence of memory	Immune response to grafted tissue similar to vertebrates, accelerated rejection rates with second-set allografts	39

(Continued)

**Table 1** (Continued)

Organism/Phyla	Pathogen/elicitor	Immune parameter characterized	Reference <sup>2</sup>
Sea urchin	Microbial	Humoral agents: lectins, agglutinins, and lysins. Complement proteins analogous to vertebrate complement C3	40
Sea urchin <i>Strongylocentrotus purpuratus</i>	Bacterial	Complement homologue SpC3 functions as Opsonin	41
Urochordata			
Colonial ascidians	Self/nonself	Allorecognition, same colonies fuse while nonrelated colonies form lesions. C-type lectins	42
Tunicates <i>Halocynthia aurantium</i> , <i>Styela plicata</i>	Bacterial	Antibacterial peptides	43
Tunicate	Lippopolysaccharides	Cell migration, chemotaxis	44
Tunicates <i>Ciona intestinalis</i> , <i>Botlenia villosa</i>	N/S	Toll-like receptors	45

<sup>1</sup>N/S, not specific.

<sup>2</sup>1) Labat-Robert et al. 1981; 2) Dunham et al. 1983, Weissman et al. 1986, Haseley et al. 2001; 3) Muller et al. 1999; 4) Bigger et al. 1982; 5) Hildemann et al. 1979; 6) Gaino et al. 1999; 7) Thakur et al. 2005; 8) Lubbock 1980; 9) Hawkrigde et al. 2000; 10) Morall et al. 2000; 11) Olano & Bigger 2000; 12) Petes et al. 2003, Kim et al. 2000a,b; 13) Hildemann et al. 1977; 14) Koh 1997; 15) Bigger & Hildemann 1982, Mullen et al. 2004; 16) Meszaros & Bigger 1999; 17) Mali et al. 2004; 18) Hutton & Smith 1996; 19) Canesi et al. 2002; 20) Villalba et al. 2004; 21) Bulgakov et al. 2004; 22) Pruzzo et al. 2005; 23) Tamplin & Fisher 1989; 24) Olicard et al. 2005; 25) Peters & Raftos 2003; 26) Olsen et al. 2003, Cerenius & Soderhall 2004; 27) Nilsen et al. 1991; 28) Johanssen 1999, Holmblad & Soderhall 1999; 29) Nagai & Kawabata 2000, Nagai et al. 2001, Inamori et al. 2004; 30) Saito et al. 1997, Inamori et al. 1999; 31) Relf et al. 1999; 32) Weinheimer et al. 1969, Evans et al. 1968, Evans et al. 1969; 33) Little et al. 2003; 34) Kurz & Franz 2003; 35) Pan et al. 2000; 36) Sotelo-Mundo et al. 2003, Bachere et al. 2000; 37) Bachali et al. 2004, Coteur et al. 1999; 38) Legac et al. 1996; 39) Coffaro & Hinegardner 1977; 40) Gross et al. 1999, Al-Sharif et al. 1998; 41) Clow et al. 2004; 42) Khalturin et al. 2004; 43) Jang et al. 2002, Tincu et al. 2003; 44) Raftos et al. 1991; 45) Azumi et al. 2003.

**PPG:** peptidoglycan

(specialized collar cells). As the primary macrophage of the sponge, however, they are nonselective in their phagocytosis, thus not specifically immune cells. Nonetheless, intracellular digestion functions as a powerful component of immunity in sponges. Sponge lysozyme is released by mesohyl-type cells after exposure to peptidoglycan (PPG) from the cell walls of Gram-positive bacteria (Thakur et al. 2005).

**Primitive immune memory.** Consistent with the early discovery of self-recognition in sponges, isografts of sponges fuse compatibly, but allografts are invariably rejected. In addition, reaction times of sponges to allografts on second and third exposure are reduced, suggesting short-term immune memory. Indeed, although sponges lack an “organized” circulatory system, the immunologic memory spreads rapidly through the body of the sponge and lingers for several weeks (Bigger et al. 1982, Hildemann et al. 1979). Finally, in the desmosponge *Geodia cydonium*, allogenic rejection involves the upregulation of phenylalanine hydroxylase, which is an enzyme initiating the pathway to melanin formation (Muller et al. 1999).

### Phylum Cnidaria

The Cnidaria comprise a diverse phylum that includes jellyfish, soft and stony corals, sea anemones, and the laboratory model system *Hydra*.

**Recognition.** Sessile colonial invertebrates, like the Cnidaria, have the ability and basic need to distinguish between their own tissues and those of unrelated conspecifics (Hidaka 1985). Typically, genetically identical or closely related colonies will fuse, whereas unrelated conspecifics reject. Cnidarians employ a diverse set of responses against allogenic tissues; these include extrusion of mesenterial filaments, and growth of sweeper tentacles and hyperplastic stolons. Allorecognition responses within the hydractinians are by far the best understood, and thus may serve as an example (Grosberg et al. 1997). There are three possible outcomes after contact between two *Hydractinia* colonies: fusion, transitory fusion, and rejection (Cadavid 2005). In fusion, compatible colonies adhere to one another and can eventually share a gastrovascular system. In rejection, fusion does not occur and interacting tissues swell owing to migration of nematocysts to the contact area (Cadavid 2005, Cadavid et al. 2004).

**Responses: cell-free.** The antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) are associated with granulated vesicles, the endosymbiotic algae, and with cnidae (nematocysts). SOD and CAT are localized in all forms of cnidae. The presence of both SOD and CAT in the accumulation bodies of endosymbiotic algae is consistent with a hypothesized role of these bodies in digestion and cell-aging (Hawkrigde et al. 2000). Nitrate oxide synthase activity has been observed in the sea anemone *Aiptasia pallida* (Morall et al. 2000). In the hydroid *Hydractinia* sp., chitinase is expressed in epidermal tissues following metamorphosis in response to fungal pathogens and nematode parasites; it is not detectable in larval stages, however (Mali et al. 2004). Gorgonian corals produce antifungal and antibacterial compounds (Jensen et al. 1996; Kim et al. 2000a,b). Crude extracts from the sea fan *Gorgonia ventalina* inhibit germination of fungal spores and have antibacterial activity against the bacterium *Listonella anguillarum* (Kim et al. 2000a). Antifungal and antibacterial activity is highest at the colony edges (youngest tissue) and lowest in the medial and central portions of the fan (Kim et al. 2000b). Lipid extracts from scleractinian corals are active against six species of marine bacteria (Koh 1997).

**Responses: cellular.** Like the sponges, cnidarians also possess motile phagocytic cells. In gorgonian and scleractinian corals, amoebocytes aid in wound healing and tissue reorganization (Bigger & Hildemann 1982). In the Scleractinia, these amoebocytes are few, scattered within mesoglea. In gorgonians they are abundantly scattered throughout the thicker mesoglea (Mullen et al. 2004). In gorgonians, trauma induces activation of phagocytic cells, including granular amoebocytes; it furthermore induces phagocytosis in cells not usually phagocytic. Phagocytic cells in this case include granular amoebocytes, epidermal cells, sclerocytes, mesogleal cells, and gastrodermal cells (Olano & Bigger 2000), all of which respond to the presence of bacteria. In sea anemones challenged with Gram-negative bacteria, amoebocytes engage in phagocytosis, reactive oxygen species (ROS) production, and production of soluble microbials (Hutton & Smith 1996). In *Plekaurella fusifera*, motile amoebocytes aid in wound healing (Meszaros & Bigger 1999). In gorgonian corals, infection by *Aspergillus sydowii* induces a band of melanization (a physical barrier formed from

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**SOD:** superoxide dismutase

**CAT:** catalase

**GPX:** glutathione peroxidase

**ROS:** reactive oxygen species

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polymerized quinones) locally around the fungal lesion as well as nodule formation (Mullen et al. 2004, Petes et al. 2003).

**Primitive immune memory.** Tissue transplantation immunity in scleractinian and gorgonian corals has a specific (short-term) memory component, as shown by significantly accelerated rejection rates for second-set grafts (Hildemann et al. 1977, Salter-Cid & Bigger 1991).

## Phylum Mollusca (Bivalvia)

The molluscs are a diverse phylum of coelomates that includes the familiar snails, slugs, clams, mussels, and octopi. We limit discussion here to the Bivalvia (clams, oysters, mussels, etc.). Owing to their filter-feeding habits, bivalves accumulate large numbers of bacteria, which are both a source of nourishment and an immune challenge.

**Recognition.** As in other invertebrates, bivalves possess a suite of adhesion molecules that aid in self/nonself recognition and bind reversibly to carbohydrate-containing molecules of foreign cells. The properties of lectins and their recognition roles in bivalve host defense are well documented in several commercial species, including mussels and oysters (Bulgakov et al. 2004, Fisher 1992). Among these lectins is the recently isolated manila clam lectin, which binds to the surface of hyphospores from *Perkinsus* sp., a protozoan parasite of manila clams and other bivalves (Bulgakov et al. 2004).

**Responses: cell-free.** Among the many weapons in the chemical arsenal of bivalves are humoral defense factors such as agglutinins (lectins), ROS, antimicrobial peptides, and lysozymal enzymes (Canesi et al. 2002). Bivalve lysozymes have both immune and digestive function (abundant in both the stylus and digestive gland); immune-defense and digestive functions may thus act contemporaneously, as bacteria filtered from the water represent both nourishment and a threat. In bivalves, multiple lysosymes are involved in self-defense against pathogenic bacteria (Olsen et al. 2003). For example, in the bivalve *Chlamys islandica*, an antibacterial lysosyme-like protein (chlamysn) isolated from the viscera inhibits all growth of Gram-positive and -negative bacteria. Interestingly, this protein is active at cold temperatures but remains stable and active when heated (Nilsen et al. 1999). In addition, bivalve hemocytes secrete antimicrobial polypeptides; hemocytes from the clam *Tapes decussatus* secrete a polypeptide that is toxic to *Perkinsus* sp. (Villalba et al. 2004). There is relatively little literature on the antiviral defenses of bivalves; protease-inhibiting peptide isolated from the Pacific oyster *Crassostrea gigas* was effective against HIV-1 (Lee & Maruyama 1998), and oyster hemolymph was found to have broad antiviral activity against HSV, IPNV, and other viruses (Olicard et al. 2005).

**Responses: cellular.** Circulating cells or hemocytes are primarily responsible for immune defense. Hemocytes are mobile, clumping, phagocytic, and chemotactic



(Pruzzo et al. 2005). Bacterial susceptibility to hemocyte attack in different bivalve species may be due to variation in phagocytic hemocyte affinity to different bacterial products, the presence (or absence) of appropriate opsonizing molecules, and the abilities of hemocytes to bind to the surface of the bacteria (Canesi et al. 2002). Surface interactions between bacteria and hemocytes lead to rapid bacterial clearance. Bacterial cells with fimbria (fringes) are cleared more rapidly owing to enhanced cell-cell adhesion with hemocytes (Canesi et al. 2001). Hemocytes in the eastern oyster, *Crassostrea virginica*, recognize and phagocytose *Perkinsus marinus* cells. However, in contrast to filtered bacteria, hemocytes have only limited ability to kill *P. marinus* merozoites, the reproductive life-stage of the parasite (La Peyre 1993).

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**LPS:** lipopolysaccharides  
**proPO:** prophenoloxidase  
**PO:** phenoloxidase

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## Phylum Arthropoda (Class Crustacea)

The Arthropoda comprises coelomate organisms with a diversity of form, size, and lifestyle. Crustaceans (including crabs, lobster, and shrimp) are the most abundant and ecologically relevant in the context of innate immunity of the marine arthropods.

**Recognition.** Fibrogen-related molecules in hemolymph function as self/nonself recognition lectins in the horseshoe crab (Gokudan et al. 1999, Kairies et al. 2001). Horseshoe crab lectins recognize specific antigens of bacterial lipopolysaccharides (LPS) (Inamori et al. 1999, Saito et al. 1997). Conversion of prophenoloxidase (proPO) to active phenoloxidase (PO), and thus initiation of the melanization pathway, is thought to be part of the self/nonself recognition system in arthropods, and is triggered by very small amounts of molecules such as LPS, peptidoglycan, and  $\beta$ -1,3 glucans (Cerenius & Soderhall 2004).

**Responses: cell-free.** Crustacean hemocytes produce an array of antimicrobial compounds as an efficient means of protection against systemic coelomic infection. Granular hemocytes in the shore crab (*Carcinus maenas*) produce an antibacterial protein active against Gram-positive marine bacteria (Relf et al. 1999). In the spiny lobster and rock lobster, induced bactericidal molecules have broad activity, covering a wide range of pathogens (Tzvetnenko et al. 2001, Weinheimer et al. 1969). In shrimp, blue crab, and crayfish, antiviral substances bind specifically to a variety of RNA and DNA viruses (Pan et al. 2000). In the white shrimp *Penaeus monodon* lysozyme activity is reported, and penaeidins, antimicrobial peptides, isolated in the white shrimp are active against viruses, including Parvo-like viridae, Picornaviridae, and Baculoviridae (Bachere 2000, Bachere et al. 2000, Sotelo-Mundo et al. 2003).

**Responses: cellular.** Cell adhesion is necessary for immune responses, including encapsulation, nodule formation, opsonization, and phagocytosis [observed in crayfish (Johanssen 1999) and cladoceran (Little et al. 2004)]. Hemolymph proteins in crustaceans may facilitate the cell adhesion, which leads to initiation of phagocytosis and encapsulation. For example, peroxinectin, a hemolymph protein in crayfish (stored in granular hemocytes) is released concomitant with proPO activation, triggering cell adhesion, encapsulation, enhanced phagocytosis, and opsonization of foreign cells.

Peroxinectin and extracellular SOD may cooperate during respiratory burst to destroy ingested parasites (Holmblad & Soderhall 1999). Upon activation of the proPO pathway, enhanced cell adhesion activity is observed in black tiger shrimp hemolymph (Sritunyalucksana et al. 2001).

**Communication and integration of immune responses.** A primary means of defense in arthropods, including crustaceans, is the melanization of pathogen and damaged tissue, which is orchestrated by PO. The activation of the proPO pathway is triggered by the presence of potential pathogens, which in turn signals the induction of many other immune responses, including generation of cytotoxic and antimicrobial factors, and compounds that facilitate opsonization and encapsulation (Cerenius & Soderhall 2004). The TLR  $\epsilon$ Toll plays a role in cell-signaling after wounding in horseshoe crab (Inamori et al. 1999). Horseshoe crab coagulation cascade promotes proPO activation, leading to conversion of hemocyanin to PO, and PO participates in wound healing, that is, repair of damaged exoskeleton (Nagai & Kawabata 2000, Nagai et al. 2001).

**Primitive immune memory.** There is evidence from a number of Crustacea that is suggestive of immune memory. Secondary bactericidal responses to Gram-negative bacteria are enhanced over primary responses in the spiny lobster (Evans et al. 1968, 1969). In addition, there are signs of immune specificity associated with adaptive immunity in higher metazoa: strain-specific immunity is conferred to offspring in the cladoceran *Daphnia magna* (Little et al. 2003) by mothers infected by a bacterial pathogen. Evidence of specificity and memory is found in copepods infected with a tapeworm parasite, with reduced success of infection on consecutive exposures to antigenically similar parasites (Kurz & Franz 2003).

## Phylum Echinodermata

This phylum comprises some 7000 identified living species, among these the familiar sea star and sea urchin. They are coelomate Deuterosomes, are radially symmetric (fivefold symmetry) as adults, possess a calcareous endoskeleton arising from mesodermal tissue, and a distinguishing coelomic water-vascular system composed of fluid-filled canals.

**Recognition.** Empirical evidence implying the existence of mechanisms for determining self/nonself is seen in the rejection of unrelated conspecific allografts in sea urchins. In the sea urchin *Lytechinus pictus*, allografts of unrelated conspecifics were rejected within a month; second and third set grafts were rejected within 12 days, even after two-months delay following the first graft (Coffaro & Hinegardner 1977).

**Responses: cell-free.** Echinoderm coelomocytes produce a battery of humoral factors, including lectins, agglutinins, lysins, and soluble microbials (Gross et al. 1999). A sea star (bacterial) lysozyme has been identified, with the inferred function of the

digestion of bacteria (Bachali et al. 2004). In the sea star, ROS produced by sea star immunocytes is stimulated by direct contact with bacteria or bacterial wall proteins (Coteur et al. 1999).

**Responses: cellular.** Echinoderms, like all other metazoans, possess motile coelomocytes, some of them phagocytic. The coelomocytes are produced in the the axial gland (axial organ), which is thought to be an ancestral lymphoid organ (Legac et al. 1996). Coelomocytes can be divided into five categories by morphology and physiology: spherulocytes, crystal cells, progenitor cells, vibratile cells, and amoebocytes (phagocytes) (Nair et al. 2005). As they are differentiated cells, coelomocytes are not specifically immune cells; in asteroids (as amoebocytes) and in urchins they phagocytose nonselectively, scavenging waste products from the body fluids. Yet in purple urchins, coelomocytes mount the cellular response to immune challenge via opsonization and phagocytosis, encapsulation, and production of antimicrobial agents (Gross et al. 1999). The cellular immune response of the sea star *Asterias rubens* consists of two differentiated amoebocyte lines: phagocytic macrophage-like cells and lymphocyte-like cells. Experiments show that the lymphocyte-like cells, upon stimulation, further subdivide into analogues of the vertebrate B and T cell lines (Legac et al. 1996). B-like and T-like cells have specific, nonoverlapping roles in cellular immunity (Leclerc & Brillouet 1988). Further empirical evidence for specialized function in sea star immune cell types is found in more recent experiments employing bacterial challenge. Sea star coelomocytes (amoebocytes) were observed to differentiate into two types, one of which responds very rapidly to bacteria; by contrast, the second is immunomodulated, responding in a density-dependent fashion to the pathogen (Coteur et al. 2002).

**Communication and integration of immune response.** There is evidence both of primitive complement and cytokine-like systems in the echinoderms. A simple complement system has been identified in purple sea urchin that is homologous to the vertebrate alternative pathway, is inducible by a challenge, and further activates coelomocytes (Gross et al. 1999). Sea urchin coelomocytes secrete complement proteins analogous to vertebrate complement component C3 (Al-Sharif et al. 1998). Coelomocyte gene expression in response to LPS results in a diverse complement system (Nair et al. 2005). Sea urchin complement homologue SpC3 functions as an opsonin (Clow et al. 2004). In the sea star *Asterias rubens*, molecules with interleukin-like function (IL-1 and IL-2-like) and receptors for these molecules have been identified (Legac et al. 1996).

**Primitive immune memory.** Evidence exists of immune memory in echinoderms. The sea urchin rejects allografts of unrelated conspecifics within 30 days. Acceptance of allografts is only observed in inbred animals, with the likelihood of acceptance increasing with the degree of inbreeding. In addition, there is accelerated rejection of second-set allografts, suggesting both primitive memory and specificity (Coffaro & Hinegardner 1977).

## Phylum Chordata, Subphylum Urochordata (Tunicata)

As members of the phylum Chordata, Tunicates or Urochordates are bilaterally symmetric, possessing a dorsal notochord and pharyngeal slits at some point in their development. As chordates, the Tunicata have been unusually well studied because they are the closest of the invertebrate phyla in body plan and development to vertebrates, and thus deserve mention here.

**Recognition.** The tunicates employ a cell-based allogenic response, and natural killer (NK)-type cells expressing a C-type lectin binding domain are deployed in the task of recognizing and eliminating allogenic tissue (Khalturin et al. 2004). Pairs of *Botryllus schlosseri* colonies placed together either fuse, or develop lesions and reject within 24 hours. Participating in the rejection of incompatible colonies are the morula cells (see below), which gather at the site of tissue interaction and cause the formation of points of rejection (Khalturin et al. 2004). Elements of the lectin-mediated activation pathway such as galectins, C3-like component and *Masp*-like protease have been isolated from the tunicate *Clavelina picta* (Vasta et al. 1999).

**Responses: cell-free.** Many antimicrobial peptides have been isolated from hemocytes of the Urochordata; the following are select examples. Halocidin was isolated from the solitary tunicate *Halocynthia aurantium*. Halocidin and its synthetic congeners demonstrated antibacterial activity against the antibiotic-resistant bacteria *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Jang et al. 2002). Plicatamide is a small octapeptide found in the hemocytes of *Styela plicata*, which exhibited fast-acting, broad antimicrobial activity (Tincu et al. 2003). Genes for the lytic pathway have been identified in the *Ciona intestinalis* genome. This pathway consists of soluble hemolymph proteins with similar domains and organization to terminal vertebrate complement components C6–C9 (Azumi et al. 2003).

**Responses: cellular.** The tunicates are endowed with a suite of differentiated hemocytes, all with specialized function. In the solitary tunicate *Styela clava* there are two types of granulocytes, phagocytic hyaline cells, and lymphocyte-like hemoblasts (Sawada et al. 1993). Although hyaline cells are most aggressively phagocytic, granulocytes also actively phagocytize, and are more mobile and abundant. Hemoblasts are lymphocyte-like cells, and are also thought to function as proliferative stem cells. These observations are recently confirmed and expanded in the colonial ascidian *Botrylloides leachi*, which was found to have hemoblasts as a circulating stem cell pool and at least five specialized hemocyte differentiation pathways. These pathways lead variously to a phagocytic cell line (hyaline cells and macrophage-like cells), a cytotoxic line (granulocyte-like cells and morula cells), a vacuolated cell line involved in storage of catabolites, compartment amoebocytes, and a second granulated cell line including highly migratory trophic cells (Cima et al. 2001).

**Communication and integration of immune response.** An interleukin 1-like molecule (cytokine-like) stimulates proliferation of tunicate immune cells, both

granulocyte and lymphocyte-like (Raftos et al. 1991). In addition, bacterial LPS significantly enhances the cell migration in tunicate hemocytes, and directional chemotaxis is stimulated by two tunicate hemolymph proteins. Hemocyte migration under chemotactic stimulation is both directional and two to three times faster than untreated controls (Raftos et al. 1998). The tunicates *Ciona intestinalis* and *Boltenia villosa* utilize TLRs and the corresponding signaling pathways (Azumi et al. 2003, Davidson & Swalla 2002).

## Synopsis

Ability to distinguish self is a basic property of all metazoans. It is well developed in sponges, the most basal of the Metazoa, and is a cell-based system involving a number of molecules and signaling pathways, including a calcium-dependent signaling system and the molecules galactin, integrin, fibronectin-like proteins, and collagen (Brower et al. 1997, Labat-Robert et al. 1981, Muller et al. 1999). Sessile colonial invertebrates, including most Cnidaria, distinguish between their own tissues and those of unrelated conspecifics. Closely related (sharing fusibility alleles) or genotypically exact colonies fuse, whereas less-related conspecifics reject (Grosberg et al. 1997). In the Crustacea, self/nonself recognition arises both through fibrogen-related molecules in hemolymph that function as self/nonself recognition lectins (Gokudan et al. 1999, Kairies et al. 2001) and TLR-mediated activation of the proPO pathway leading to melanization. Note that the cnidarians possess TLRs, but lack key transcriptional factors belonging to the NF- $\kappa$ B family, which are required in Toll-signaling pathways (Zheng et al. 2005). These animals also activate the melanization pathway, but it is likely mediated in a slightly different fashion given the incomplete nature of the Toll-signaling pathway in the Cnidaria.

Phagocytosis is perhaps the most primitive mode of defense, and wandering phagocytic cells are found in all metazoans. As observed in the Porifera, phagocytosis is primarily a means of acquiring nutrients with a secondary defense function. It thus seems likely that in more complex animals, nutritional phagocytosis evolved to be (or was co-opted as) a means of immune defense. Note that in the cnidarians, trauma induces phagocytosis in cells that normally have other functions. Cnidarian amoebocytes respond to presence of local bacterial invaders by becoming phagocytic and engaging in production of soluble microbials (Hutton & Smith 1996). Coelomate invertebrates have specialized cell lines. The most complex cellular differentiation described here is observed in the deuterostome tunicates and echinoderms, as both possess proliferating lymphocyte-like immune cells as well as nonproliferating phagocytic cell lines. In the crustaceans and other coelomates, enhanced phagocytosis appears to be tied to the PO pathway; upon activation of this pathway, heightened phagocytosis along with cell adhesion and encapsulation are triggered (Zheng et al. 2005).

Chemical warfare is another important defense tool for invertebrates. Sponge and cnidarian cells, or the symbionts they host, secrete a large number of antioxidants and bioactive compounds, many of which have potential pharmacological applications (Becero & Paul 2004, Rifai et al. 2004). Animals possessing a true body cavity (here,

the molluscs, the crustaceans, the echinoderms, and the tunicates) likely developed the ability to secrete antimicrobial peptides, mediated by a signaling cascade employing TLRs, as an efficient means of protection against systemic coelomic infection (Zheng et al. 2005). Note that in coelomate invertebrates, for example the sea urchin, coelomic cavity fluid is rapidly cleared of microbes by phagocytic activity, suggesting an efficient signaling mechanism.

## ENVIRONMENTAL FACTORS AND IMMUNOCOMPETENCE

Environmental stressors have long been thought to negatively impact invertebrate immune systems, although information linking environmental pressures, reduced immunity, and disease outbreaks are few (LeMoullac & Haffner 2000, Malham et al. 2003). Altered environmental conditions can affect immunity directly, by changing components of the immune responses, or indirectly, by inducing general stress responses. In invertebrate studies, deciphering the effects of environmental stressors as direct or indirect responses on immunity is complex and remains relatively mechanistic. These types of studies, however, represent the first step toward integrating specific physiological information with immune function, and thus permit prediction and modeling of the potential effects of environmental factors on immune response. In **Table 2** and this section, we review documented links between environmental stress and impaired immunity in experimental situations and in natural populations of marine and aquatic invertebrates.

### Chemical Pollutants

The field of ecotoxicology aids in the understanding of how chemical pollutants affect immunocompetence of invertebrates (see review by Galloway & Depledge 2001). Many studies link exposure to heavy metals, polycyclic aromatic hydrocarbons, and pesticides to altered immune function in laboratory studies, but establishing a direct link between changes in immune function and increased susceptibility to disease has remained elusive. In addition, the studies of the effects of anthropogenic stress and pollutant stress in natural populations in situ remain scarce (Galloway & Depledge 2001).

Studies using bivalves such as the mussel *Mytilus edulis* (Coles et al. 1995, Pipe et al. 1999), the oyster *Crassostrea virginica* (Anderson 1994, Baier-Anderson & Anderson 2000), crustaceans including the prawn *Macrobrachium rosenbergii* (Cheng & Wang 2001) and the lobster *Nephrops norvegicus* (Hernroth et al. 2004), and echinoderms such as sea star *Asterias rubens* (Coteur et al. 2005), have demonstrated that cellular responses are affected by the metals copper, cadmium, and manganese. These studies are laboratory-based and employ controlled dosages of the pollutant, yielding straightforward results. Hemocyte counts and phagocytic activity are observed to decrease and the production of ROS to increase with heavy metal stress. When natural populations of invertebrates are exposed to environmental pollution, the data are more ambiguous. In three French rivers, Vasseur & Leguille (2004) transported

**Table 2 Environmental factors and immunocompetence**

Stressor	Organism	Immune function measured	Reference*
Pollutants			
Biocides	Oyster <i>Crassostrea virginica</i>	Hemocyte activation, phagocytosis, reduced pyridine nucleotides and ROS production	1
Copper	Mussel <i>Mytilus edulis</i>	Hemocyte counts, pathogenic activity	2
Copper	Oyster <i>Crassostrea virginica</i>	Hemocyte function, ROS production	3
Copper sulfate	Prawn <i>Macrobrachium rosenbergii</i>	Hemocyte density, PO activity, respiratory burst	4
Cadmium	Mussel <i>Mytilus edulis</i>	Immune response alteration	5
Manganese	Lobster <i>Nephrops norvegicus</i>	Hemocyte counts and degranulation, PO activity, expression of the Runt-domain protein	6
Cadmium	Sea star <i>Asterias rubens</i>	Phagocytosis, production of ROS	7
Natural pollution salinity gradient	Sea star <i>Asterias rubens</i>	Coelomic amoebocyte concentration (CAC) and production of ROS by amoebocytes	8
Harbor dredging	Shrimp <i>Crangon crangon</i>	Total hemocyte count, PO activity	9
Sites contaminated with heavy metals, sewage and industrial outfalls	Mussel <i>Mytilus edulis</i>	Molecular characteristics of the hemocytes, phagocytosis, superoxide generation	10
Natural river pollution	Bivalve <i>Unio</i> sp.	Glutathione, glutathione reductase, selenium dependent glutathione peroxidase, nonselenium-dependent glutathione peroxidase, lipid peroxidation	11
Immunostimulants	Lobster <i>Homarus gammarus</i>	Granulocyte activity in vitro	12
Physical and Chemical Changes			
Temperature	Bivalve <i>Mya arenaria</i>	Mitochondrial respiration, energetic coupling to phosphorylation, production of ROS in mitochondria	13
Temperature	Bivalve <i>Laternula elliptica</i>	ROS production by mitochondria	14
Temperature	Sponge <i>Axinella polypoides</i>	Electrophysiological methods, ADP-ribosyl cyclase, abscisic acid production	15
Temperature	Coral <i>Acropora grandis</i>	Intracellular calcium and a series of stress-proteins, including heme oxygenase	16
Seasonal Temperature	Crab <i>Carcinus maenus</i>	Hemocyte antibacterial activity	17
Temperature	Shrimp <i>Penaeus californiensis</i>	Hemolymph proPO and plasma protein	18
Temperature and copper	Mussel <i>Mytilus edulis</i>	Total and differential hemocyte counts, production of intracellular superoxide, phagocytosis	19

(Continued)



Table 2 (Continued)

Stressor	Organism	Immune function measured	Reference*
Temperature and copper	Coral <i>Porites cylindrica</i>	Stress measured as respiration and primary production	20
Temperature, pollutants, injury	Sea Urchin	Coelomocytes expression of heat shock protein (hsp70)	21
Elevated pO <sub>2</sub> and solar irradiance	Sponge <i>Petrosia ficiformis</i>	Oxygen radical scavengers; superoxide dismutase, catalase, glutathione peroxidases and total oxyradical scavenging capacity (small molecules)	22
Hypoxia	Oyster <i>Crassostrea virginica</i>	Production of ROS in hemocytes to produce	23
Hypoxia	Shrimp <i>Penaeus stylirostris</i>	Hemocyte counts, hemocyte differentiation, respiratory burst, PO activity, mortality	24
Hypercapnic hypoxia	Crab <i>Callinectes sapidus</i>	Hemolymph clearance of bacteria	25
Hypercapnic hypoxia	Shrimp <i>Litopenaeus vannamei</i> , <i>Palaemonetes pugio</i>	Total hemocyte count, mortality	26
Hypoxia and TBT	Oyster <i>Crassostrea virginica</i>	Total hemocyte counts, mortality, ROS production, lysozyme activity	27
Other Disturbances			
Mechanical disturbance	Abalone <i>Haliotis tuberculata</i>	Number of circulating hemocytes and migratory activity, phagocytosis, respiratory burst, noradrenaline and dopamine levels	28
Wounding	Amphipod <i>Gammarus pulex</i>	PO activity	29
Wounding and feeding conditions	Crustacean <i>Daphnia magna</i>	PO activity	30

\* 1) Baier-Anderson & Anderson 2000; 2) Pipe et al. 1999; 3) Anderson 1994; 4) Cheng & Wang 2001; 5) Coles et al. 1995; 6) Hernroth et al. 2004; 7) Coteur et al. 2005; 8) Coteur et al. 2003; 9) Smith et al. 1995; 10) Dyrinda et al. 1998; 11) Vasseur & Leguille 2004; 12) Hauton & Smith 2004; 13) Abele et al. 2002; 14) Heise et al. 2003; 15) Zocchi et al. 2001; 16) Fang et al. 1997; 17) Chisholm & Smith 1994; 18) Vargas-Albores et al. 1998; 19) Parry & Pipe 2004; 20) Nyström et al. 2001; 21) Matranga et al. 2000; 22) Regoli et al. 2000; 23) Boyd & Burnett 1999; 24) Le Moullac et al. 1998; 25) Holman et al. 2004; 26) Mikulski et al. 2000; 27) Anderson et al. 1998; 28) Malham et al. 2003; 29) Plaistow et al. 2003; 30) Mucklow & Ebert 2003.

the bivalve *Unio* sp. to control sites and to sites up- and downstream from sources of pollution. They measured antioxidant and lipid peroxide levels. Only in one of three rivers did bivalves downstream from a pollutant source exhibit decreased antioxidant activity and higher lipid peroxidation indicative of impaired immune response. The other two rivers were equally if not more contaminated, but the bivalves did not show any significant changes in antioxidant levels, illustrating the difficulty in examining one environmental parameter, such as pollution, in a continually changing environment.

In an English study, Dyrinda et al. (1998) examined mussels from known contaminated sites and comparable uncontaminated sites, and measured immune components



including total hemocyte count, hemocyte phagocytic activity, generation of superoxide anion, N-acetyl-glucosamidase activity, chymotrypsin activity, lysosomal volume, peroxidase, and PO activity. Of the immune parameters, only hemocyte production of superoxide anion and glucosaminidase were different in mussels from all three contaminated sites over uncontaminated sites, while other immune parameters were different at only one or two sites. Again, this study of natural populations in contaminated sites exemplifies the variability of immune defenses between different environmental locations and different levels of contamination. Although results from these studies are ambiguous relative to controlled experiments, they are nonetheless extremely important in characterizing impaired immune response in invertebrates associated with contamination, and in establishing appropriate invertebrate indicator species and relevant immune measurements.

## Temperature

Temperature stress, both warming and cooling, can compromise physiological and immune function in freshwater and marine organisms. In the bivalves *Mya arenaria* (Abele et al. 2002) and *Laternula elliptica* (Heise et al. 2003), temperature stress caused mitochondrial decoupling owing to ROS membrane damage. This type of physiological stress can impair defense responses against pathogens or lead to direct oxidative damage to tissues. In the sponge *Axinella polypoides*, temperature stress induced a signaling cascade that involved heat-gated cation channels and the stimulation of abscisic acid, a scavenger of reactive oxygen (Zocchi et al. 2001). In the coral *Acropora grandis*, heat stress induced the production of heat shock proteins and heme-oxygenases (Fang et al. 1997). A more direct influence of temperature on disease was characterized in the mussel *Mytilus edulis* (Parry & Pipe 2004), the freshwater prawn *Macrobrachium* (Cheng & Chen 1998), and the brown shrimp *Penaeus californiensis* (Vargas-Albores et al. 1998). In the mussel, temperature and copper stress caused significant decrease in hemocyte function to clear the bacterial pathogen *Vibrio tubiashii*. Increasing temperature and pH between 8.8 and 9.5 caused increased mortality in the freshwater prawn infected by bacteria relative to controls. Increasing water temperature by approximately 12°C above ambient for the brown shrimp caused decreased activity of the prophenyloxidase system (Vargas-Albores et al. 1998). Also important when considering temperature stress is that decreases as well as increases in temperature may compromise immunity, and seasonal temperature change leaves certain invertebrates vulnerable to disease at specific times of the year. For example, the antimicrobial properties of hemocytes from the crab *Carcinus maenus* were the least active when water temperatures dropped in February and reached their highest activity in August (Chisholm & Smith 1994).

For organisms with obligate algal symbionts, temperature stress uniquely affects immune function and general physiology. Invertebrates including anemones, sponges, and corals harbor photosynthetic dinoflagellates that can cause daily changes in the oxidative state of the host tissues (Regoli et al. 2000; Richier et al. 2001, 2003). This has led to symbiotic organisms having an enhanced or more efficient antioxidant system and increased tolerance to oxidative stress. Although these studies have

not examined the relationship of oxidative stress tolerance to disease, the role of ROS and the ability to regulate the oxidative state of tissues during pathogen invasion and utilize reactive oxygen as a respiratory burst is an important aspect of defense. For example, in amoebocytes isolated from sea anemones, phagocytosis and production of reactive oxygen conferred antibacterial activity and reduced growth of the Gram-negative bacterium *Psychrobacter immobilis* (Hutton & Smith 1996).

Although symbiosis may provide enhanced tolerance to changes in the oxidative state of tissues, which in turn can provide some immune protection, symbiosis gone awry can cause a state of hyperoxia and harm the tissue of the host. The disruption of the fine balance between symbiont and host can have dire physiological consequences. The coral bleaching phenomenon is an example of the complete breakdown between symbiosis, causing mass mortality and potentially leaving corals vulnerable to disease. Cellular events concomitant with bleaching include the release of intact endoderm host cells and the symbionts they contain, suggesting that thermal stress causes dysfunction in cnidarian host cell adhesion (Gates et al. 1992). The direct physiological and mechanistic link between water temperature warming, bleaching, and impaired immunity in corals has not yet been examined and is further discussed in this article.

## Environmental Hypoxia

High summer temperatures and continued climate warming can cause more frequent seasonal hypoxia, which can be accompanied by elevated levels of CO<sub>2</sub> and lowered pH. Hypercapnia, hypoxia, and low pH can affect benthic, sessile organisms, especially those living in estuarine habitats, and these conditions have been associated with mortality and disease outbreaks (Boyd & Burnett 1999). Several groups have conducted studies to understand the association of disease-related mortality and hypoxic conditions. In the oyster *Crassostrea virginica* (Boyd & Burnett 1999), hypoxia caused a reduction in the ability of oyster hemocytes to produce ROS, thereby reducing their ability to kill pathogen invaders. In another study, *Crassostrea virginica* (Anderson et al. 1998), naturally infected with the parasite *Perkinsus marinus*, were subjected to the pollutant tributyltin and hypoxia. Although tributyltin alone did not cause mortality above control levels, hypoxia exacerbated this effect and increased oyster mortality synergistically with tributyltin. The effects of hypercapnia and hypoxia on bacterial clearance was tested in the crab *Callinectes sapidus* (Holman et al. 2004) and the shrimps *Litopenaeus vannamei* and *Palaemonetes pugio* (Mikulski et al. 2000). In these experiments organisms kept in hypercapnic hypoxia were exposed to live pathogenic bacteria. In the shrimp *Litopenaeus vannamei*, and in the crab *Callinectes sapidus*, total hemocyte counts following bacterial infection were reduced in the hypoxia treatments. In both shrimp species, mortality was increased relative to controls in the hypercapnic hypoxia treatments.

The relationship between hypoxia and ability to fight pathogens is very important when considering the long-term effects of increasing ocean acidity and water

temperature. Benthic sediment habitats play crucial roles in ecosystem processes (by providing the water column with key nutrients, for example) and the bacterial and large animal communities they support are important to maintaining this function. Bioturbation of the sediments by shrimp, crabs and urchins is crucial to nutrient exchange to support phytoplankton growth and subsequently other members of the food chain. If disease occurrences aggravated by more frequent hypoxia cause a shift in the sediment-dwelling invertebrate communities, the impact on the benthic ecosystem water column and even characteristics of the topography and sediment of a tidal flat may be large (Mouritson et al. 1998).

## Physical Injury

Fluctuations in environmental conditions are not the only extrinsic factors that can affect immunity. Physical injury, wounds, and food supply can alter immune parameters. Henry & Hart (2005, and references therein) discuss the effects on immunocompetence of wound regeneration in corals, sponges, and other invertebrates. They suggest that the resources allocated for allorecognition and cell-mediated immune responses may be depleted during regeneration of wounds. In addition, cells involved in both wound healing and immunity, such as amoebocytes (Olano & Bigger 2000) and archeocytes, may be limited in their ability to fight disease during active regeneration of wounds. This constitutes double jeopardy, as wound sites provide entry for pathogenic microorganisms.

Malham et al. (2003) studied the mollusc *Haliotis turberculata* using a mechanical disturbance similar to handling in aquaculture to evaluate the effects of physical pressure on the immune system. The effects of this stress were transient, but marked decrease in hemocyte numbers, migratory ability, and phagocytic ability of *Haliotis* hemocytes were observed. Their experiments demonstrate a direct link between physical stress and impaired immunity. Whether the physical stress is natural, such as in the case of predation or hurricanes, or anthropogenic injury, such as bottom trawling or handling in aquacultural settings, these factors cannot be ruled out when considering susceptibility to disease and may contribute not only to impaired immunity but may provide opportunistic pathogens entryway into the organism.

Very few studies provide a direct link between any meaningful component of immune function and susceptibility to disease. In work with the crustacean *Gammarus pulex* (Plaistow et al. 2003), animals with more naturally occurring wounds (as measured by counting melanized spots) exhibited less proPO activity, indicating a decreased ability to produce melanin as a cellular response to infection. In the copepod *Daphnia magna* (Mucklow & Ebert 2003, Mucklow et al. 2004), experimentally wounding individuals induced higher levels of proPO 24 hours postwounding. Higher levels of proPO were also observed in well-fed *Daphnia*. Furthermore, those animals with higher proPO activity were more resistant to infection by the bacterial pathogen *Pasteuria ramosa*. Mucklow & Ebert's experiment exemplifies the link between health of the organism and resistance to infection as measured by an important immune parameter, melanin production.

## Synopsis

The use of field populations to study the links between anthropogenic effects, impaired immunity, and disease is complicated by natural variability in phenotype and genotype, seasonal variation in level of defense, and local environmental variation in study sites. For the field of ecotoxicology this is an ongoing issue; laboratory experiments clearly demonstrate causal links between exposure to pollutants and impaired immune function, but verification of these relationships in the field remains problematic (Galloway & Depledge 2001). As demonstrated in this review, field surveys of natural populations exposed to pollutants can be ambiguous and often raise questions about the nutritional and reproductive status of the organism studied. In addition there are the confounding effects of local environment and season. Future challenges are to resolve these issues so relationships between anthropogenic effects and disease are more conclusive and thus may inform conservation and environmental management efforts.

Also important to conservation and environmental management are the effects of a changing climate on immune parameters. The main outcomes of a warming ocean are decreased oxygen levels in coastal waters and increased prolonged temperature anomalies. Although several studies show that hypoxia can directly affect immunocompetence of marine invertebrates, the same cannot be said for temperature effects. Based on terrestrial models, temperature can affect pathogen growth and range, which in turn can increase the severity of disease (Harvell et al. 2002), but the mechanistic links between temperature and immune function are not yet resolved.

Many studies that do incorporate temperature, pH, and other environmental conditions into the study of invertebrate disease are driven by the aquaculture industry. In shrimp and oyster aquaculture disease limits production and therefore economic returns (Bachere et al. 2004). Problems that have risen from the aquaculture industry have exposed the lack of existing knowledge on basic immune defenses of invertebrates. In culture, invertebrates are densely populated, subject to changing environmental conditions, such as in oxygen and pH, and increased concentration of potential pathogens (Bachere et al. 2004). To counteract these problems and prevent disease, some industries have begun adding immunostimulants to the diets of the farm-raised invertebrates. In one study of this practice (Hauton & Smith 2004), the immunostimulants (glucans, bacterial cell walls) intended to boost immune response in the lobster *Homarus gammarus* turned out to be mildly cytotoxic to lobster hemocytes.

## DISEASE OUTBREAKS INVOLVING MARINE INVERTEBRATES

The major disease outbreaks recorded in marine and freshwater invertebrates over the past 30 years are summarized in **Table 3**. Diseases of marine invertebrates are the focus of this review, as there are fewer recorded events in freshwater invertebrates, and include outbreaks in invertebrates from most phyla, from sponges to echinoderms. The groups best represented are those of commercial or ecological

importance, such as mollusks, crustaceans, corals, and echinoderms. This unequal distribution of disease outbreaks among phylogenetic groups and emphasis on commercially important groups suggests that diseases of marine invertebrates are seriously understudied, especially if there are no cataloged cases of disease outbreaks in major groups like polychaetes, urochordates, or nematodes. Similar imbalance occurs with freshwater invertebrates. Although records of disease or parasite outbreaks exist for snails (Lively & Jokela 1996) and the crustacean *Daphnia* (Ebert 2005, Mitchell et al. 2004, Mucklow & Ebert 2003), there are scant records of diseases for the majority of freshwater invertebrates. The work of Ebert and Little's groups on epizootics of *Daphnia* represents some of the most comprehensive work in a natural, aquatic invertebrate population. *Daphnia magna* is infected with three microsporidian parasites, and a bacterial pathogen (*Pasteuria ramose*) (Ebert 2005). Several of these infections reach epizootic proportions under different environmental conditions. Overall, the greatest epizootics correlated with warm temperatures and fishless conditions, both situations that trigger high host densities (Ebert 2005). Capaul & Ebert (2003) detected microevolutionary change in laboratory microcosms of *D. magna* populations exposed to microsporidians. Although the frequencies of the different clones shifted, it is not known if these populations became more resistant after selection. Mucklow & Ebert (2003) showed that wounded *D. magna* have upregulated PO. This group has done pioneering work on evolutionary ecology of disease in natural populations of freshwater *Daphnia*, and yet important questions remain about the links between immunity and disease susceptibility.

Observations of diverse invertebrates in the ocean suffering epizootics prompt the question, do other freshwater molluscs, such as unionid clams, or even zebra mussels, escape disease outbreak? Or is it merely that such events remain undetected and underreported in freshwater? Understanding the true distribution of disease among invertebrate groups is important because it yields clues to the interplay between immunity and disease risk. For example, documenting what we suspect is a disproportionate level of disease outbreaks in corals might suggest either a particular vulnerability to disease in cnidarians, or an unusual change in environmental conditions, such as temperature stress, which allows opportunistic pathogens to invade.

Another pattern observable in **Table 3** is the paucity of data on viral diseases. Typically pathogens reported to cause outbreaks in marine invertebrates are bacterial or protozoan, with fungi also accounting for significant mortality, even though viruses are present in seawater at concentrations between  $10^6$ – $10^8$ /ml seawater (Wilson et al. 2005). Most of the data available on viral diseases and immunity comes from commercially important invertebrates. Viral diseases are known in farmed shrimp and in some bivalves (Lightner & Redman 1998, Renault & Novoa 2004, Shields & Behringer 2004, Stentiford et al. 2004), and viral-like particles have been reported in the coral *Pavona danai* (Wilson et al. 2005) and the sea anemones *Metridium senile* and *Anemonia viridis* (Wilson & Chapman 2001, Wilson et al. 2005). To date no viral diseases are reported to cause cnidarian die offs and no antiviral defenses in this group have been identified. Antiviral compounds have been detected in oysters (Olicard et al. 2005), and in the shrimp *Fenneropenaeus indicus*, where oral administration of inactivated white spot syndrome virus protects the animal from subsequent infection

**Table 3** Disease outbreaks among natural populations of selected marine invertebrates adapted and updated from Kim et al. 2005. For each reported outbreak, location, pathogen identity, if known, and environmental correlates are shown: T = temperature, ND = no data, sal = salinity, turb = turbidity, hur = hurricane, ? = unknown.

Start date	Host species	Outbreak location	Pathogen identity	Estimated mortality (%)	Env. correlates	Reference*
1938	Sponges	N Caribbean	fungus?	70-95	ND	1
1946	<i>Crasostrea</i> (oyster)	Gulf Coast, USA	<i>Perkinsus marinus</i>	extensive	high T, sal	2
1967	<i>Crasostrea angulata</i> (oyster)	France	iridovirus	extensive	ND	3
1975	<i>Helaster</i> (starfish)	W USA	?	>90	high T	4
1980	<i>Strongylocentrotus</i> (urchin)	NW Atlantic	amoeba?	>50	ND	5
1980	<i>Ostrea</i> (oyster)	Netherlands	<i>Bonamia ostreae</i>	extensive	ND	6
1981	<i>Acropora</i> (coral)	Caribbean-wide	bacterium?	>90	ND	7
1982	<i>Gorgonia</i> (coral)	Central America	?	extensive	high T	8
1982	<i>Halotis</i> (abalone)	Australia	<i>Perkinsus</i> sp.	extensive	high T	9
1983	Scleractinian Corals	Caribbean-wide	microbial consortium	seasonal	?	10
1983	<i>Patinopecten</i> (scallop)	W Canada	<i>Perkinsus qugwadi</i>	extensive	ND	11
1983	<i>Diadema</i> (urchin)	Caribbean-wide	bacterium?	>95	high T	12
1985	<i>Paralithodes platypus</i> (crab)	?	herpes virus	?	?	13
1985	<i>Halotis</i> (abalone)	NE Pacific	?	>95	high T	14
1986	<i>Ostrea chilensis</i> (oyster)	New Zealand	<i>Bonamia exitiosa</i>	>90	dredging	15
1987	<i>Ruditapes philippinarum</i> (clam)	France	<i>Vibrio tapetis</i>	?	T	16
1988	<i>Argopecten</i> (scallop)	N Caribbean	protozoan	extensive	ND	1

1989	<i>Argopecten</i> (scallop)	E Canada	<i>Perkinsus karlsoni</i>	extensive	ND	17
1991	<i>Macrobrychium rosenbergii</i>	Taiwan	<i>Metschnikowia bicuspidata</i>	>50	ND	18
1995	<i>Strongylocentrotus</i> (urchin)	Norway	nematode?	~90	ND	19
1995	<i>Gorgonia</i> (coral)	Caribbean-wide	fungus	extensive	ND	20
1995	<i>Diabocenia</i> and others (coral)	Florida, USA	bacterium	<38	seasonal	21
1996	<i>Diploria</i> and others (coral)	Puerto Rico	bacterium	extensive	seasonal, hur	22
1996	<i>Tapes decussatus</i> (clam)	NW Spain	<i>Perkinsus olseni</i>	?	high T	23
1997	<i>Litopenaeus setiferus</i> (shrimp)	SE USA	WSSV Virus	<10	ND	24
1999	Gorgonian corals	NW Mediterranean	protozoan/fungi?	>20	high T	25
2000	<i>Crangon crangon</i> (shrimp)	Scotland	bacilliform virus (CeBV)	100	?	26
2002	<i>Montipora</i> (coral)	Australia	bacterial consortium	extensive	high T	27
2002	<i>Panulirus argus</i> (lobster)	SE USA	virus	≤37	?	28
2002	<i>Homarus americanus</i> (lobster)	NE USA	not isolated	extensive	high T	29
2002	<i>Echinopora/Montipora</i> (coral)	E Africa	fungi?	extensive	ND	30
2003	krill	NW USA	<i>Collinia</i> sp (ciliate)	ND	ND	31
2004	<i>Mercenaria</i> (clams)	New York, USA	QPX	<10	ND	32

\* 1) Galtsoff et al. 1939, Smith 1939; 2) Sornat 1996; 3) Renault & Novoa 2004; 4) Dungan et al. 1982; 5) Miller & Colodey 1983, Scheibling & Stephenson 1984, Jones & Scheibling 1985; 6) van Banning 1991; 7) Antonius 1981, Gladfelter 1982; 8) Guzmán & Cortéz 1984; 9) Lester et al. 1990; 10) Rützler et al. 1983, Carlton & Richardson 1995; 11) Bower et al. 1998; 12) Lessios et al. 1984; 13) Sparks & Morado 1986; 14) Lafferty & Kuris 1993; 15) Cranfield et al. 2005; 16) Paillard 2004; 17) McGladdery et al. 1991; 18) Su et al. 2005; 19) Skadsheim et al. 1995; 20) Nagelkerken et al. 1997; 21) Richardson et al. 1998; 22) Bruckner & Bruckner 1997; 23) Villalba et al. 2005; 24) Chapman et al. 2004; 25) Cerrano et al. 2000; 26) Stentiford et al. 2004; 27) Jones et al. 2004; 28) Shields & Behringer 2004; 29) Dove et al. 2004a; 30) Dove et al. 2004b; 31) Gómez-Gutiérrez et al. 2003; 32) Dove et al. 2004b.

by white spot virus (Bright et al. 2005). In addition, the physiological effects of viruses on infected organisms of commercial value are being documented; for example oxidative stress and depressed levels of antioxidant defenses were detected in shrimp infected with white spot virus (Mohankumar & Ramasamy 2006). From the work on commercial invertebrate species, inferences may be drawn about the viral immunity of related, ecologically important species.

Also evident (**Tables 2 and 3**) is the observation that increasing temperature may be an important environmental factor and a possible facilitator of disease outbreaks in the ocean, even though understanding of the mechanistic effects of temperature on immunity is in its infancy. Below we further examine hypotheses behind this linkage, using two disease systems as examples, molluscan infection by *Perkinsus* and coral bleaching and disease.

- *Perkinsus*. Well-documented outbreaks involving temperature as a facilitator involve the protozoan *Perkinsus* spp. in various commercially important bivalve species. *Perkinsus* suppresses host immune responses, such as lysozyme activity, as well as causes hemocyte mortality (Romestand et al. 2002). *Perkinsus* outbreaks represent a clear example of disease range expansion due to climate warming. In both the Southeast and in Northern Europe the geographic range of the *Perkinsus* spp. that infects oysters expands in warmer months and is geographically limited by cold winters (Cook et al. 1998). *Perkinsus* spread in eastern oyster (*Crassostrea virginica*) populations from New York to Maine during warming winters in the 1990s (Cook et al. 1998). These range expansions combined with the effects of warming waters and increased hypoxia on bivalve immunity (Anderson et al. 1998, Parry & Pipe 2004) make the *Perkinsus* epizootic a compelling example of the complex interactions between host immunocompetence, parasite resilience, and environment. Gaffney & Bushak (1996) showed variation in resistance among strains of oyster exposed to *Perkinsus*, although the mechanism of immunity is unknown.
- Coral bleaching. The best example of climate warming specifically facilitating disease outbreaks in compromised hosts is likely to be corals. Corals have undergone massive bleaching events that are directly driven by warm sea surface temperatures (Hoegh-Guldberg & Salvat 1995). Coral bleaching occurs when the symbiotic algae are expelled from coral tissue during thermal stress, causing the coral to appear white or bleached. This is a nonlethal event unless the temperature stress is of protracted duration. However, bleaching is widely hypothesized to trigger susceptibility to opportunistic microorganisms, because stressed hosts are known to be more susceptible to disease. In situ observations (Bruckner & Bruckner 1997, Harvell et al. 2001, Jones et al. 2004; see also Selig et al. 2006) show disease outbreaks occurring concurrently with bleaching events when warmer than usual water temperatures are present. For example, in the 1998 El Niño warming event in the Caribbean, Harvell et al. (2001) recorded a mass mortality of the gorgonian, *Briareum asbestinum*, caused by a cyanobacterial pathogen attacking bleached colonies. Jones et al. (2004) recorded a similar event in scleractinian corals on the Great Barrier Reef in



2002. Selig et al. (2006) documented a link between warm temperature anomalies and disease outbreaks of white syndrome in Australia in 2002.

Missing from these studies, however, is an understanding of why heat-stressed corals are more susceptible to disease. Whether warm water temperatures directly and detrimentally interfere with immune components or indirectly affect the organism through the bleaching phenomenon remains unclear. What is apparent, however, is that the sensitivity of symbiotic organisms to temperature variation may leave corals unusually susceptible to changes in physiological state and immune function. Efforts to unveil this link between temperature stress and coral disease require careful experimentation with host immune responses and with pathogen virulence and infectivity. Pathogen response to increased temperatures may be a key element in the dynamics of coral diseases. For example, *Aspergillus sydowii*, the fungal pathogen of the sea fan disease aspergillosis, grows at a faster rate at higher temperatures (Alker et al. 2001), and the bacterial pathogen of hard corals, *Vibrio coralliilyticus* (Ben-Haim et al. 2003), produces more lytic proteins when grown at the elevated temperatures that increase its virulence. Adhesion ability, a critical virulence factor in the causative agent in coral bleaching (*Vibrio shiloi*) is also temperature sensitive (Toren et al. 1998). In addition to adhesion, production of anti-algal toxins and SOD (which detoxifies superoxide anion) are also temperature-dependent virulence factors, which seem to be induced in *V. shiloi* by elevated seawater temperatures (Banin et al. 2003).

### Linking Immunity and Disease Outbreaks in Nature

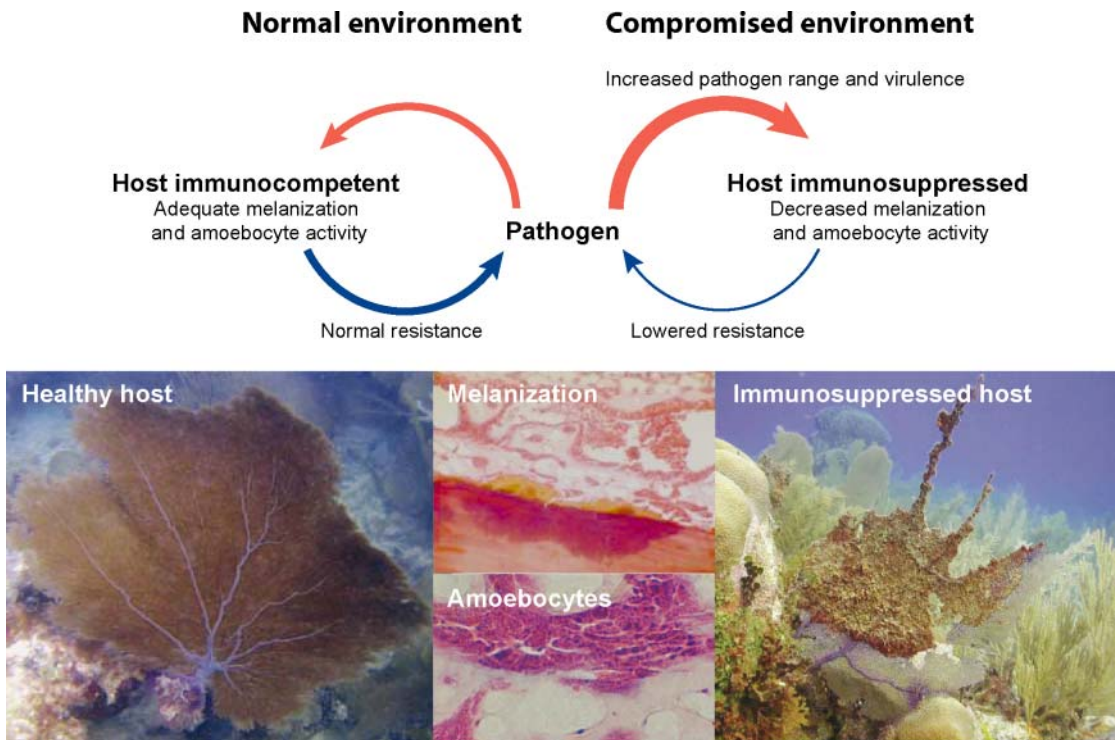
Although there are considerable data on invertebrate immunity (Table 1) and many documented disease outbreaks in nature (Table 3), these two areas have not been appropriately linked to address two important questions:

- What are the major ecologically important immunological measures?
- How is the probability of infection related to immunity of an organism in a changing environment?

Ecologists studying disease in natural populations continue to seek biologically relevant, quantifiable measures of immunocompetence that are appropriate for field deployment. However, the search for a common measure of immunity in invertebrates is complicated by the sheer diversity of the invertebrate fauna in body plan, function, life history, and the option of measuring either cellular or humoral components of immunity. In the case of oysters, hemocyte function measured as hemocyte counts, phagocytic activity, and production of ROS are proven measures of immunity (Anderson 1994, Bachere et al. 2004, Baier-Anderson & Anderson 2000, Boyd & Burnett 1999). In crabs or lobsters, hemocytes phagocytize bacteria and secrete antibacterial compounds (Bachere et al. 2004, Holman et al. 2004). In corals, it is still unclear which measures of immunity are most predictive of resistance to a given pathogen, as both cellular and humoral components have an influence on bacterial and fungal pathogens, and critical immune components may vary with pathogen. Thus in the study of invertebrate immunity, the tools used by ecologists will

undoubtedly differ depending on the organism, pathogen, and ecological setting. Some good examples are outlined in **Table 2**, where immunity in natural populations of crustaceans (Le Moullac & Haffner 2000; Smith et al. 1995) and bivalves (Dyrynda et al. 1998; Vasseur & Leguille 2004) was measured using a suite of cellular immune responses. In *Drosophila melanogaster*, multiple components of antibacterial immunity vary in natural populations and affect successful infection by the bacterial pathogen, *Serratia marcescens* (Lazzaro et al. 2004).

Another ongoing problem with the study of immunity in natural populations is the lack of diagnostics for many microbially based diseases. Improvements in these diagnostic methods will put tools in the hands of ecologists to evaluate and study disease outbreaks as they are occurring. This in turn can lead scientists to discover the environmental cofactors implicated in an outbreak, as well as the levels of immunocompetence in the host invertebrates. Hence, using field-deployable immunity assays, we can measure immune parameters in situ in natural populations. We can then begin to estimate the effects of natural selection on invertebrate immunity, estimations that will lead to studies of the evolution of immunity in natural populations (Altizer et al. 2003).



**Figure 1**

A hypothetical model of the effects of changing environmental conditions on the immune responses and resistance of the sea fan *Gorgonia ventalina* to pathogens. Outcome of this interaction is either a healthy immunocompetent host or an immunosuppressed host with disease. Photographs by L. Mydlarz and J. Bruno.

## CONCLUSIONS

Invertebrates are phylogenetically diverse and have evolved a multiplicity of efficient defense strategies to defend against microbial attack. Marine invertebrates are especially challenged by their environment with high bacterial and viral loads, pollutants that can spread from their point source, and through changing states of oxygenation and temperature. Data on the effects of environmental factors on immunity are scarce and mechanistic, and are typically focused on economically valuable species. However, the study of marine invertebrate ecological immunity is advancing rapidly, and critical signaling pathways and cytotoxic responses are being elucidated. From the data presented in this review it is clear that several defense and signaling mechanisms are important in the innate immune responses to changing environment and pathogens. Examples are the proPO pathway and the ability of immune and phagocytic cells to clear or encapsulate pathogen invaders (**Figure 1**). However, there are almost no field studies estimating how these components vary in nature, or how variation in immunity affects susceptibility to infection in natural populations.

Thus, via collaborative efforts between ecologists, immunologists, cell biologists, and physiologists, we may expand the current understanding of innate immunity in naturally occurring, ecologically important species. With greater understanding of the connections between environment and organismal immunity, we can make predictions about the effects of changing climate and environment on immunocompetence and disease outbreaks.

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# Contents

Birth-Death Models in Macroevolution <i>Sean Nee</i> .....	1
The Posterior and the Prior in Bayesian Phylogenetics <i>Michael E. Alfaro and Mark T. Holder</i> .....	19
Unifying and Testing Models of Sexual Selection <i>Hanna Kokko, Michael D. Jennions, and Robert Brooks</i> .....	43
Genetic Polymorphism in Heterogeneous Environments: The Age of Genomics <i>Philip W. Hedrick</i> .....	67
Ecological Effects of Invasive Arthropod Generalist Predators <i>William E. Snyder and Edward W. Evans</i> .....	95
The Evolution of Genetic Architecture <i>Thomas F. Hansen</i> .....	123
The Major Histocompatibility Complex, Sexual Selection, and Mate Choice <i>Manfred Milinski</i> .....	159
Some Evolutionary Consequences of Being a Tree <i>Rémy J. Petit and Arndt Hampe</i> .....	187
Late Quaternary Extinctions: State of the Debate <i>Paul L. Koch and Anthony D. Barnosky</i> .....	215
Innate Immunity, Environmental Drivers, and Disease Ecology of Marine and Freshwater Invertebrates <i>Laura D. Mydlarz, Laura E. Jones, and C. Drew Harvell</i> .....	251
Experimental Methods for Measuring Gene Interactions <i>Jeffery P. Demuth and Michael J. Wade</i> .....	289
Corridors for Conservation: Integrating Pattern and Process <i>Cheryl-Lesley B. Chetkiewicz, Colleen Cassady St. Clair, and Mark S. Boyce</i> .....	317

The Population Biology of Large Brown Seaweeds: Ecological Consequences of Multiphase Life Histories in Dynamic Coastal Environments <i>David R. Schiel and Michael S. Foster</i> .....	343
Living on the Edge of Two Changing Worlds: Forecasting the Responses of Rocky Intertidal Ecosystems to Climate Change <i>Brian Helmuth, Nova Mieszkowska, Pippa Moore, and Stephen J. Hawkins</i> .....	373
Has Vicariance or Dispersal Been the Predominant Biogeographic Force in Madagascar? Only Time Will Tell <i>Anne D. Yoder and Michael D. Nowak</i> .....	405
Limits to the Adaptive Potential of Small Populations <i>Yvonne Willi, Josh Van Buskirk, and Ary A. Hoffmann</i> .....	433
Resource Exchange in the Rhizosphere: Molecular Tools and the Microbial Perspective <i>Zoe G. Cardon and Daniel J. Gage</i> .....	459
The Role of Hybridization in the Evolution of Reef Corals <i>Bette L. Willis, Madeleine J.H. van Oppen, David J. Miller, Steve V. Vollmer, and David J. Ayre</i> .....	489
The New Bioinformatics: Integrating Ecological Data from the Gene to the Biosphere <i>Matthew B. Jones, Mark P. Schildbauer, O.J. Reichman, and Shawn Bowers</i> .....	519
Incorporating Molecular Evolution into Phylogenetic Analysis, and a New Compilation of Conserved Polymerase Chain Reaction Primers for Animal Mitochondrial DNA <i>Chris Simon, Thomas R. Buckley, Francesco Frati, James B. Stewart, and Andrew T. Beckenbach</i> .....	545
The Developmental, Physiological, Neural, and Genetical Causes and Consequences of Frequency-Dependent Selection in the Wild <i>Barry Sinervo and Ryan Calsbeek</i> .....	581
Carbon-Nitrogen Interactions in Terrestrial Ecosystems in Response to Rising Atmospheric Carbon Dioxide <i>Peter B. Reich, Bruce A. Hungate, and Yiqi Luo</i> .....	611
Ecological and Evolutionary Responses to Recent Climate Change <i>Camille Parmesan</i> .....	637

## Indexes

Cumulative Index of Contributing Authors, Volumes 33–37 .....	671
Cumulative Index of Chapter Titles, Volumes 33–37 .....	674