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REVIEW

The role of histones in the immune responses of aquatic invertebrates

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Abstract

Histones are primary components of eukaryotic chromatin and highly abundant in all animal cells. In addition to their important role in chromatin structure and transcriptional regulation, histones contribute to innate immune responses. In several aquatic invertebrate species, as well as in many other invertebrate and vertebrate species, the transcripts for core histones are upregulated in response to immune challenge and exposure to environmental stressors. Histones show antimicrobial activity against bacteria and parasites *in vitro* and *in vivo* and have the ability to bind bacterial lipopolysaccharide and other pathogen-associated molecules. Several mechanisms regulating and facilitating the antimicrobial action of histones against pathogens have been described in vertebrate and some invertebrate species, including the production of Extracellular Traps (ETs) and the accumulation of histones in lipid droplets that can be selectively released in response to immune stimuli. Further studies are needed to determine the mechanisms of action of histones in immune responses in aquatic invertebrates and investigate the potential use of histones in the treatment of infectious diseases in aquaculture.

Key Words: Antimicrobial responses; bivalves; histones; immunity; invertebrates; mollusks; pathogenic challenge

Introduction

Histones are major components of chromatin in eukaryotic cells, playing key roles in DNA replication, repair, and recombination, transcriptional regulation, and cell growth control (Parseghian and Luhrs, 2006). The core histones (H2A, H2B, H3, and H4) constitute the basic structure of the nucleosome by forming an octameric complex, whereas the linker histones (H1) are involved in the generation of the higher-order chromatin structure which seal loops of DNA and maintain the nucleosome structures condensed in a compact conformation. Due to their important structural role, histones are very abundant in animal cells and most are highly conserved evolutionarily throughout eukaryotes. Interestingly, several other roles for histones have recently been described, including roles in cellular signaling and innate immunity (Parseghian and Luhrs, 2006; Brinkmann and Zychlinsky, 2012). Histones have long been known to have antimicrobial activity (Miller *et al.*, 1942; Hirsch, 1958), probably due to their cationic charge, which they share with many antimicrobial peptides. We review here evidence supporting a role for histones in antimicrobial defenses, with a special focus on aquatic invertebrate species, many of them of commercial interest for the aquaculture industry. We also speculate on the potential mechanisms of action of histones in the immune responses of invertebrate species based on evidence from studies in vertebrates and a few model and non-model invertebrate species. The evidence provided in this review and others (*e.g.* Smith *et al.*, 2010; Noga *et al.,* 2011) suggests that histones may be a target for use in the treatment and prevention of infectious diseases in aquaculture.

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Immune challenge leads to up-regulation of histones

Recent advances in gene library construction, sequencing, and cDNA microarray technology have enabled the characterization of gene expression patterns in non-model species like aquatic invertebrates in response to a variety of environmental stressors, including immune challenges. These studies have revealed a large number of immune-related sequences, including the homologues for many genes involved in pathogen recognition, signaling, antioxidant and antimicrobial responses, and apoptosis, showing that aquatic invertebrates express many of the same genes that have previously been described in other invertebrates and vertebrates in response to viral, bacterial, or parasitic challenge (Messier-Solek *et al.,* 2010; Gosh *et al.,* 2011; Li *et al*., 2011; Romero *et al*., 2012). Among the many immune-related genes that have been described in these studies, several histone genes have been shown to be highly upregulated in aquatic invertebrates in response to pathogenic challenge and/or to possess antimicrobial activity (Table 1). An up-regulation of histone gene expression in response to immune challenge is not restricted to aquatic invertebrates, but is a feature shared with several other invertebrate and vertebrate species. To provide some examples, rhesus macaque monkey kidney epithelial cells exposed to monkey pox virus, showed a steep up-regulation of histone genes (Alkhalil *et al.*, 2010), while zebrafish skin exposed to bacterium *Citrobacter freundii* showed significant up-regulation of linker histone-like protein H1M (Lu *et al*., 2012).

These patterns of differential expression of histones in response to immune challenge suggest a potential role for histones in innate immunity. Immune challenges, however, are not the only conditions in which histone up-regulation has been observed in aquatic organisms. For example, histones are up-regulated in rapidly growing oyster larvae (Meyer and Manahan, 2010), in oyster adults and larvae in response to many different environmental stressors (Chapman *et al.,* 2011; Zhang *et al.,* 2012), and in mussels maintained along a copper pollution gradient in the field (Dondero *et al.,* 2006), suggesting that histone gene up-regulation may be a general response to environmental stress (Robinette and Noga, 1998). There is additional evidence, however, supporting a role for histones in innate immunity. We review this evidence in the sections below.

A role for histones in immune responses: Histones have antimicrobial and LPS-binding activity.

The first report of the effects of histones on bacteria was by Miller *et al.,* (1942), who showed that calf thymus histones and histone-like cationic proteins (protamines) inhibited respiration in Gram-positive and Gram-negative bacteria. Hirsch (1958) demonstrated the antimicrobial activity of arginine-rich histones (H3 and H4) against a variety of bacteria under different pH and salt concentrations. Decades later, Hiemstra *et al.,* (1993) identified three molecules with antimicrobial activity isolated from the lysosomal fraction of murine macrophages as lysine–rich histones H1 and H2B, suggesting that histones may have a role in immune defenses.

Subsequently, molecules with antimicrobial activity isolated from cells and tissues from a variety of organisms were identified as histones or histone-derived fragments. Antimicrobial molecules identified as histones (H1, H2A, H2B) by peptide sequencing have been described, among others, from a diverse set of tissues from humans and other terrestrial vertebrates (reviewed in Parseghian and Luhrs, 2006; Kawasaki and Iwamuro, 2008) and the tissues of many aquatic vertebrates (finfish, reviewed in Smith *et al.,* 2010). Many peptides with antimicrobial activity are derived from the proteolytic digestion of intact histones. These include buforins I and II, originally isolated from the Korean frog *Bufo bufo gargarizans* (Cho *et al.*, 2002; Park *et al*., 1996; Cho et al., 2009), oncorhyncin II, an antimicrobial peptide derived from histone H1 isolated from rainbow trout (Fernandes *et al.*, 2004), parasin I (residues 1-19 of histone H2A), isolated from the skin of catfish *Parasilurus asotus* (Park *et al.*, 1998), and hipposin (residues 1-51 of histone H2A), isolated from the Atlantic halibut *Hippoglossus hippoglossus* (Birkemo *et al.*, 2003).

Histones, either purified from biological samples or produced using recombinant technology, show antimicrobial activity *in vitro* against a variety of pathogens, including Gram-negative bacteria like *Aeromonas* and *Vibrios,* Gram-positive bacteria, fungi, viruses, and protozoa (reviewed in Kawasaki and Iwamuro, 2008). For example, histone (H2B) purified from skin secretions of *O. mykiss* exhibits powerful anti-bacterial activity against Gram-positive bacteria with a minimal inhibitory concentration (MIC) in the submicromolar range (Fernandes *et al*., 2002). Purified olive flounder histone H1-like protein extract from testis shows antimicrobial activity against Gram-negative bacteria with minimal effective concentrations (MECs) of 1.4-12.0 μg/ml, Gram-positive bacteria with MECs of 2.8-30.0 μg/ml, and yeast *Candida albicans* with a MEC of 2.0 μg/ml (Nam *et al.*, 2012). Furthermore, recombinant human histones H2A and H2B efficiently kill promastigotes of the parasites *Leishmania amazonensis*, *L. major*, *L. braziliensis*, and *L. mexicana* (Wang *et al.*, 2011). Histones also have antimicrobial activity against several protozoan fish pathogens, including water molds (*Saprolegnia* sp.) and the dinoflagellate *Amyloodinium ocellatum* (Noga *et al.,* 2011). There is relatively less evidence of antimicrobial activity for histones H3 and H4. Purified histones H2A and H4 from human meconium have antimicrobial activity (Kai-Larsen *et al.*, 2007). In addition, histone H4 contributes to the antimicrobial activity of extracts of SEB-1 sebocytes against *Staphylococcus aureus,* and recombinant histone H4 showed antimicrobial activity against *S. aureus* and *Propionibacterium acnes* (Lee *et al.,* 2009).

Invertebrate histones and histone-derived peptides also show antimicrobial activity against a wide range of microorganisms. A mix of core histone proteins H2A, H2B, H3, and H4, isolated from the

Table 1 Evidence supporting a role for histones in immune responses in aquatic invertebrate species

Species	Histone Type	Accession number	Upregulation to challenge with	Technology used	Evidence for antimicrobial activity	Reference
Biomphalaria glabrata	H4	DQ117979	*Echinostoma caproni*	Mass spectrometry and cDNA	Not done	Bouchut *et al*., 2007
Chlamys farreri	H2A (39 N-terminal aa)	DQ418455	Not induced by bacteria	cDNA	Yes (*in vitro*)	Li *et al*., 2007b
C. farreri	H1oo	-	Acute viral necrobiotic virus	SSH	Not done	Chen *et al*., 2013
Crassostrea virginica	H4	HM130521	*Perkinsus marinus*	SSH	Yes (*in vitro* and *in vivo)*	Dorrington *et al.*, 2011
C. virginica	H2B	BG624428	*P. marinus*	EST (Microarray)	Not done	Wang *et al*., 2010
C. virginica	H3.3	BG624455	*P. marinus*	EST (Microarray)	Not done	Wang *et al*., 2010
C. virginica	H2B-1	-	Not done	HPLC	Yes (*in vitro*)	Seo *et al*., 2010
C. virginica	H2B-2, 3, 4	-	Not done	HPLC	Not done	Seo *et al*., 2011
Haliotis discus discus	H2A-derived Abhisin	EF103384	Bacteria G+/-	cDNA	Yes (*in vitro*)	De Zoysa *et al*., 2009
Macrobrachium rosenbergii	H2A	HG001454	Viral and Bacterial	HTG	Yes (*in vitro*)	Arockiaraj *et al.,* 2013
Meretrix casta and 4 other spp.	H2A Molluskin	HQ720143, HQ720145-HQ720148	Not done	PCR	No	Sathyan *et al.*, 2012
Ruditapes philippinarum	H2A	06-R-H12 (Clone number)	*Perkinsus olseni*	EST	Not done	Kang *et al*., 2006

Abbrevations: aa: amino acids; cDNA: complementary DNA; EST: expressed sequence tags; HPLC: high pressure liquid chromatography; HTG: high throughput genomics,; SSH: suppression subtractive hybridization.

hemocytes of the Pacific white shrimp, have antimicrobial activity against *Micrococcus luteus*. Complete inhibition of the growth of a Gram-positive bacterium was observed at 4.5 μM of purified histone H2A. On the other hand, a mixture of histones H2B and H4 was active at 3 μM (Patat *et al.*, 2004). Recently, a recombinant form of H2A from the freshwater prawn *Macrobrachium rosenbergii* was shown to possess antimicrobial activity against several Gram-negative and Gram-positive bacteria (Arockiaraj *et al.,* 2013). In mollusks, histone H2B from the American oyster *C. virginica* has strong activity against Gram-negative *Vibrio parahaemolyticus* and *V. vulnificus,* two

human pathogens present in seafood (Seo *et al.,* 2010). An ortholog for buforin-I (1-39 residues of H2A), characterized in the scallop *Chlamys farreri*, has broad-spectrum growth inhibitory activity against both Gram-negative and Gram-positive bacteria (Li *et al.*, 2007). Further, abhisin derived from N-terminus of the abalone histone H2A (80% amino acid identity with buforin I), inhibits the growth of Gram-positive and Gram-negative bacteria and yeast (De Zoysa *et al.*, 2009). We have shown that recombinant histones H4 and H2B from the African clawed frog, *Xenopus laevis,* which in the case of histone H4 is 100% identical at the amino acid level with those of other species such as the American oyster *Crassostrea virginica,* have antimicrobial activity against the Gram-negative bacteria *Escherichia coli* and *Vibrio anguillarum,* and the Gram-positive *Micrococcus luteus* at micromolar concentrations (Dorrington *et al.,* 2011). Histone H4 protein levels of oyster in hemocyte lysate and cell free hemolymph are significantly increased in oysters experimentally challenged with the protozoan parasite *Perkinsus marinus*, but neither histone H2B nor histone H4 inhibit the growth of this pathogen of oysters at the highest concentration tested (20 μM). These results suggest that *P. marinus* may have evolved resistance to the antimicrobial effects of histones (Dorrington *et al.,* 2011). Alternatively, histone H4 up-regulation may be a general response to the stress caused by the pathogenic challenge (Chapman *et al.,* 2011; Zhang *et al.,* 2012) or histone H4 may have an indirect role in immunity against this protozoan parasite. Further research needs to be done to determine the role of histones on immune defenses in oysters. Interestingly, delivery of yeast cells expressing the recombinant American oyster histone H4 into the gut of brine shrimp *Artemia salina* artificially challenged with *V. anguillarum* showed a significant and dose-dependent decrease of the bacterial load in brine shrimp (Dorrington *et al.*, 2011). These results support the idea that histones are potentially useful molecules in the development of novel drugs for therapeutic use against pathogenic infections in aquaculture (Smith *et al.,* 2010; Noga *et al*., 2011). Further, these experiments validate the use of oyster histone H4 in a yeast feed-based delivery system for the treatment of bacterial infection in aquaculture applications.

Another fascinating property of histones is their lipopolysaccharide (LPS) binding ability. In humans, histones H2A and H2B show dose-dependent inhibition of the endotoxin activity of LPS through binding to and the blocking of both the core and lipid A moieties of LPS (Augusto *et al.,* 2003). Therefore, binding of histones to LPS released from Gram-negative bacteria may also block the production of cytokines such as tumor necrosis factor alpha (TNF-α) that can often leads to fatalities from toxic shock in humans (Kim *et al.*, 2002). The LPS binding site is located in the C-terminal region of histones and all histones (but H2A1 and H4 in particular) from calf thymus are able to bind LPS, with a greater affinity than the LPS-binding antibiotic polymixin B (Augusto *et al.*, 2003). Histones also have the ability to bind viral proteins like the Human Immunodeficiency Virus (HIV) envelope glycoprotein gp120 and its receptor CD4 (Mamikonyan *et al.,* 2008). Evidence of the potential role of histones as pattern recognition receptors (PRRs) is the identification and characterization in catfish of a histone-like protein (similar to linker histone H1) in the membranes of non-specific cytotoxic cells (NCAMP-1) that recognizes bacterial DNA, oligodeoxynucleotides, and polyguanosine motifs, and has antimicrobial activity (Connor *et al.,* 2009). Further research is needed to determine if histones have similar properties in invertebrate species.

Mechanisms of action of histone and histone derived compounds in immunity

Organisms have evolved specific mechanisms tightly regulating the motility and presence of free histones in the cytoplasm, membranes, and extracellular fluids (Parseghian and Luhrs, 2006), as well as facilitating the antimicrobial activity of histones against pathogens. One of such mechanisms is the formation of Extracellular Traps (ETs), which was first described in vertebrate neutrophils. ETs are part of the innate immune response and function through the release by dying neutrophils of granular proteins (such as elastase and myeloperoxidase) and chromatin (DNA and histones) that form extracellular fibers to trap and kill both Gram-negative and Gram-positive bacteria, yeast, and parasites (Brinkmann *et al.*, 2004; Urban *et al.,* 2006; Brinkmann and Zychlinsky, 2007; Guimaraes-Costa *et al.,* 2009; Urban *et al.,* 2009; Brinkmann and Zychlinsky, 2012; Saffarzadeh *et al.,* 2012). These fibers are covered in histones, which bring them into contact with bacteria more effectively, facilitating their antimicrobial and LPS-binding activities. Neutrophils releasing ETs undergo a particular cell death process named NETosis that appears distinct from apoptosis and necrosis, is induced by inflammatory stimuli and pathogen-associated molecular patterns (PAMPs) such as LPS and phorbolmyristate acetate (PMA), and depends on the formation of radical oxygen species. During neutrophil cell death byNETosis, intracellular organelle membranes disintegrate leading to the formation of a complex composed of nuclear and cytoplasmic components (Fuchs *et al.,* 2007; Metzler *et al.,* 2011; Mesa and Vasquez, 2013). This phenomenon of NETs production has also been described in fish such as the fathead minnow (Palic *et al.*, 2007a), zebrafish (Palic *et al.*, 2007b), goldfish (Katzenback and Belosevic, 2009), and carp (Pijanowski *et al.*, 2013). Production of ETs has also been observed in immune cells other than neutrophils (Goldman and Medina, 2012). The formation of ETs has been also reported in a few invertebrate species, including the greater wax moth *Galleria mellonella* (Altincicek *et al.,* 2008), marine crabs (unpublished results reported in Smith *et al.,* 2010), and the Pacific white shrimp (Ng *et al.,* 2013). In *G. mellonella,* a small percentage of hemocytes appear to be the source of nets containing extracellular nucleic acids in the hemolymph during coagulation, which then induce degranulation of granulocytes and bacterial entrapment (Altincicek *et al.,* 2008). These studies suggest that a system of defense homologous to the NETs is present in

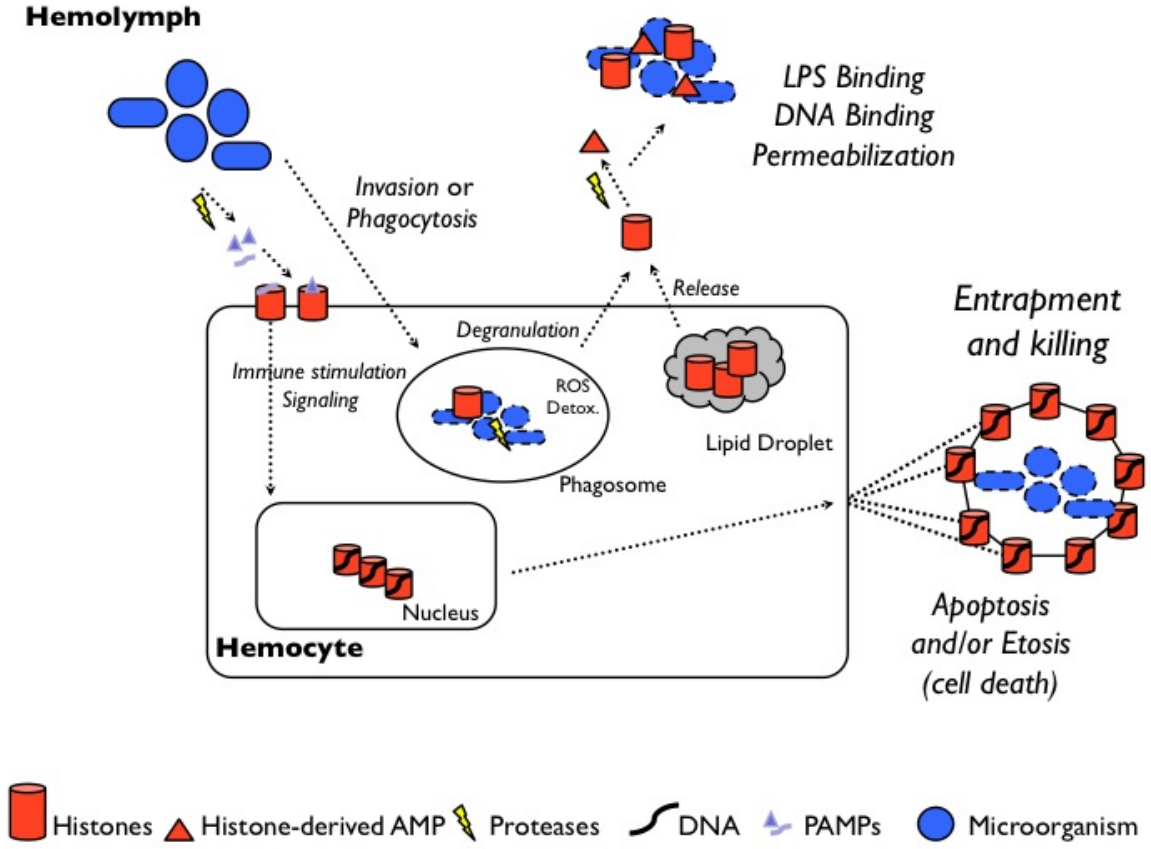

Fig. 1 Potential roles and mechanisms of action of histones on immune responses in invertebrates. See text for a detailed explanation. AMP: Antimicrobial peptide; ET: extracellular trap; LPS: bacterial lipopolysaccharide; PAMP: pathogen-associated molecular pattern; ROS: Radical Oxygen Species.

several invertebrates and may be an ancient defense mechanism (Smith *et al.,* 2010).

An alternative mechanism of action for histones in immunity has been recently described in the fruit fly *Drosophila melanogaster.* Histones bound to lipid droplets (fat storage organelles) in the cytosol of cells are released upon immunological stimuli, killing bacteria. Furthermore, genetically modified flies lacking histones in lipid droplets are more susceptible to bacterial infection, and droplet-bound histones enhance fly survival to bacterial challenge (Anand *et al.*, 2012). These authors also describe that exposure of mice to bacterial stimuli leads to accumulation of histone in lipid droplets in the liver, suggesting that this may be an ancient immune defense strategy conserved through evolution (Anand *et al.*, 2012).

Another potential role for a histone on immunity has been described in an invertebrate model species, the nematode *Caenorhabditis elegans* (Studencka *et al.,* 2011). A variant of linker histone H1 (HIS-24) interacts with heterochromatin protein 1 to regulate the transcription of many immune-related genes. Interestingly, bacterial challenge leads to localization of a proportion of HIS-24 to the cytoplasm of intestinal cells in infected worms, which would facilitate the interaction of the histone with pathogens. These changes in localization of HIS-24 were dependent on the methylation status of the protein (Studencka *et al.,* 2011).

Based on the existing evidence in vertebrate and invertebrate organisms presented in this review, we hypothesize that histones could have multiple mechanisms of action in invertebrates (Fig. 1). Exposure of hemocytes to immune stimuli leads to up-regulation of the expression of histone genes. Histones may be released after hemocyte cell death caused by apoptosis or a process similar to ETosis in invertebrate hemocytes. Additionally, in hemocytes and possibly in other cells, histones stored in lipid droplets may also be released to extracellular spaces upon immune stimuli. Histones and fragments of histones derived from proteolytic activity, in combination with other molecules released into the hemolymph, would then be able to exert their antimicrobial and LPS-binding activities against extracellular pathogens. Based on their ability to bind DNA and LPS, histones may also be present on the surface of hemocytes, serving as receptors for Pathogen Associated Molecular Patterns (PAMPs). Finally, histones may also be localized to granules and other structures in the

cytoplasm upon pathogenic challenge to become involved in pathogen killing intracellularly within phagosomes, in combination with Radical Oxygen Species (ROS) and other antimicrobial molecules.

Concluding remarks

Evidence supporting a role for histones in immune responses in invertebrate and vertebrate species include: 1) up-regulation in response to immune challenge, 2) biochemical identification of molecules with antimicrobial activity as histones or histone-derived peptides and 3) the ability of recombinant histones to kill a variety of pathogens and bind bacterial LPS and other PAMPs. The mode of action of histones in immunity appears to involve mechanisms that facilitate direct contact of concentrated histones with pathogens, such as the release of extracellular traps (ETs) as shown in many vertebrates and a few invertebrates (Altincicek *et al.,* 2008; Brinkman *et al*., 2004; Brinkman and Zychlinsky 2007; Ng *et al.,* 2013) or the formation of lipid droplets in *Drosophila* (Anand *et al.,* 2012).

It has been postulated that antimicrobial histones have evolved as an ancient innate defense system component against pathogenic microorganisms that may have been coopted from the structural role of these abundant proteins as components of the chromatin structure of eukaryotic organisms (Kawasaki and Iwamuro, 2008; Smith *et al.,* 2010). It will be important to study the mechanisms that specifically regulate the transcription of selected histone genes in response to pathogenic challenge. Histones are encoded by multigene families that occur in clusters (Marzluff *et al*., 2002; Albig *et al*., 2003), allowing for the differential expression of selected genes for histones subtypes (Alami *et al.*, 2003). It will also be important to study the potential role of post-transcriptional modifications on histone localization to immune-relevant organelles (Ouvry-Patat and Schey, 2007; Studencka *et al.,* 2011).

Furthermore, due to the high levels of amino acid conservation of several histones, and in particular histone H4, between many diverse species, these proteins and peptides can be considered universal antimicrobial agents. Histones and histone-derived peptides could be potentially useful targets in the development of novel drugs for the prevention and treatment of infectious diseases in the aquaculture industry. Further studies should be done to determine mechanisms of action of histones in the immune responses of aquatic invertebrates, which would aid in the improved design of treatments (Smith *et al.,* 2010; Noga *et al.,* 2011).

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