

Review Article

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Effects of Stress among Shrimp Post-Larvae stocked at High Stocking Density in Nursery Culture System: A Review

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ABSTRACT

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Since the recent past, intensive shrimp culture has become widely spread and applied because of the diminishing farming land and to regulate proper discharge/processing of wastewater for monitoring environmental conditions. These systems tend to culture shrimps at high stocking density which is one of the most important factor in shrimp culture, and bear the potential to influence growth and survival of shrimp due to the stress response induced by crowding. Aquatic animals are likely to suffer from oxidative stress when cultured under high stocking densities as well as during pH fluctuations, decrease in temperature, salinity fluctuations, environmental hypoxia and re-oxygenation, bacterial invasion. Moreover, high stocking density increases the chance of disease outbreaks in shrimp ponds. Inactivation of antioxidant enzymes in infected shrimps can lead to oxidative stress and tissue damage leading to system failure and sudden death. However, an elevated expression of antioxidant enzymes including Superoxide dismutase, Catalase, Glutathione peroxidases and Heat shock protein (HSP) are observed in shrimps under high stocking densities to mitigate the negative effects of oxidative stress. In this review we have discussed about the effects of stocking density in nursery system on stress and change in the expression of antioxidant enzymes and non-enzyme molecules.

Introduction

Commercial shrimp farming has gained momentum especially in the Asian countries where there are vast brackish and marine water resources enabling easy cultivation of shrimps for domestic consumption and export. Moreover, in the recent years, nursery phase has been incorporated in culture systems for obtaining size uniformity which is desirable for marketing shrimps, early disease

diagnosis, better scope for monitoring water quality and health status of Post Larvae(PL), managing adequate feed ration to reduce wastage, reducing the grow-out period (Garzade *et al.*, 2004; Mishra *et al.*, 2008). In commercial shrimp farming, high stocking densities are favourable for enhancing production and sustaining economic feasibility in aquaculture. However, rapid intensification of culture systems have led to disease outbreaks due to degraded water

quality and accumulation of organic matter in the pond bottom (Mishra *et al.*, 2008). Moreover, Sun *et al.*, (2016) reported that high stocking densities can result in stress among animals due to overcrowding, adverse social interactions like competition for food and grazing area, resulting in lowering their metabolic rate, weakening their immune responses, and inhibiting their individual growth rate. Aquatic animals especially the crustaceans are more prone to suffer from oxidative stress when cultured under high stocking densities. And the endogenous antioxidant defence grid plays a vital role in protecting against the lethal free radicals. This system is composed of enzymes and other (non-enzyme) molecules that scavenge Reactive oxygen species(ROS), including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and the early stress biomarker HSP70 (Gao *et al.*, 2017). Increasing stocking density causes undesirable changes on aquatic animals like oxidative stress which disrupts their cell structure by lipid peroxidation of the phospholipid bilayer as well as causes protein oxidation, modifications in nitrogenous bases of DNA. Antioxidant enzymes like Catalase, Superoxide Dismutase and Glutathione peroxidase as well as HSP70 can serve as biomarkers of crowding stress response in shrimps. The objective of this review article is to focus on the differential expression patterns of these genes due to crowding stress in shrimp nursery systems.

Oxidative stress and free radicals production

Oxidative stress is a cellular condition which occurs due to physiological imbalance between the levels of antioxidants and oxidants (free radicals or reactive species) in favour of oxidants (Fig. 1). When the production of free radicals exceeds the level which the body's natural antioxidant defence

mechanisms can deal with; a cellular oxidative environment is spontaneously generated which elicits the rapid oxidation of essential biomolecules like DNA, protein and lipids, leading to tissue damage followed by system failure and death (Ighodaro *et al.*, 2018). The major oxidants that can cause oxidative stress to biomolecules are hydroxyl radical ($\cdot\text{OH}$); superoxide anion (O_2^-), singlet oxygen radical ($^1\text{O}_2$), peroxy radical ($\cdot\text{ROO}$), nitric oxide radical (NO) and lipid peroxy radical ($\cdot\text{LOO}$) as well as peroxy nitrate (ONOO^-), trichloromethane ($\cdot\text{CHCl}_3$) and hypochlorous acid ($\cdot\text{HOCl}$) leading to radical induced toxicity.

Oxygen is prone to free radical formation because of its electronic structure which bears two unpaired electrons in separate electronic orbitals. Oxygen radicals such as superoxide anion (O_2^-) and singlet oxygen, ($^1\text{O}_2$) are easily generated from the consecutive reduction of molecular oxygen via step-wise addition of electrons. Some normal physiological processes that lead to formation of oxygen radicals are mitochondrial energy production pathway (MEPP). These radicals are formed as oxygen is reduced down the electron transport chain which is located in the inner mitochondrial membrane. Oxygen radicals are also produced as fundamental metabolites in cascades of enzyme catalysed reactions. Hypoxic or/and hyperoxic condition in cells can also produce numerous oxygen-derived radicals. Besides, Sung *et al.*, (2014) reported that a couple of therapeutic drugs that enter water bodies through sewage discharge such as acetaminophen and ibuprofen can cause oxidizing effects on cells, consequently leading to formation of oxygen radicals through the activity of drug metabolizing enzymes known as cytochrome P-450 system. Free radicals are mostly produced for advantageous reasons, like they are used for destruction of microbes and pathogens by white blood cells. ROS like O_2^- ,

H₂O₂, and ·OH are produced during phagocytosis (also known as the respiratory burst), which plays an important role in microbicidal activity. It has been reported by Ighodaro *et al.*, (2018) that oxygen radicals are involved in various intercellular and intracellular signaling pathways. In particular, signal transduction pathways, such as AP-1 and NF-κB, are known to be activated by ROS, which leads to the transcription of genes involved in cell growth regulatory pathways which include antioxidant enzyme genes such as CAT, GPx and SOD (Meyer *et al.*, 1993). It has also been studied that superoxide anion and hydrogen peroxide can act as mitogens, thereby enhancing the rate of DNA replication and cell proliferation in a variety of cultured cells. However, in excess amounts, oxygen-derived radicals are very detrimental to living beings. Besides being injurious themselves, they are also capable of generating other free radicals like Reactive oxygen species (ROS) and Reactive Nitrogen species (RNS) that are even more fatal.

Role of antioxidant defence grid in Hepatopancreas of shrimps

The shrimp body encloses a complex antioxidant defence system that relies on endogenous enzymatic and non-enzymatic antioxidants. These molecules collectively act against free radicals to resist their damaging effects to vital biomolecules and ultimately body tissues. The role and effectiveness of the first line defence antioxidants which primarily include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) is crucial especially in reference to super oxide anion radical (*O₂) which is generated under stressful events like high stocking densities. This oxidative stress leads to lipid peroxidation, protein oxidation, modifications in nitrogenous bases of DNA (Ighodaro *et al.*, 2018). Peroxidation of membrane lipids lead to loss of membrane fluidity and elasticity,

impaired cellular functioning, and even cell rupture. Oxidative damage to DNA causes alterations in DNA bases. Guanine has a high oxidation potential than the other three nitrogenous bases, making it susceptible to oxidation by superoxide anion (O₂²⁻) and hydroxyl radical (·OH). Oxidation of Guanine produces 8-hydroxy-deoxyguanosine, which blocks DNA replication due to base pairing defects. If left unrepaired, the modifications of DNA bases can lead to genetic defects. Protein oxidation, on the other hand, can cause fragmentation at amino acid residues, formation of protein-protein cross-linkages, and oxidation of the protein backbone which ultimately leads to loss of function (Fig. 2). To counteract ROS-induced damage of biomolecules, cells have developed various levels of defence mechanisms that act to prevent or repair such damage.

Levels of antioxidant defence systems

Antioxidant molecules may be of radical preventive, radical scavenging and radical induced damage repairing types.

They can be categorized as first line defence antioxidants, second line defence antioxidants, third line defence antioxidants and fourth line defence antioxidants.

First line defence antioxidants

These are a collection of antioxidants that act to prevent or suppress the formation of free radicals or reactive species in cells. They have the potential to rapidly neutralize any molecule with chances of developing into a free radical or any free radical with the ability to induce the production of other radicals. Three key enzymes: superoxide dismutase, catalase and glutathione peroxidase are top on the list. These enzymes respectively dismutate superoxide radical, and breaks down hydrogen peroxides to water and O₂.

Second line defence antioxidants

This group of antioxidants is generally known as scavenging antioxidants as they can scavenge active radicals to inhibit chain initiation and break chain propagation reactions. They neutralize or scavenge free radicals by donating electrons to them, and in the process, themselves become free radicals but of lesser damaging effects. These 'new radicals' are easily neutralized and made completely harmless by other antioxidants in this group. Antioxidants of this group include glutathione, uric acid, ascorbic acid which are hydrophilic and alpha tocopherol (vitamin E) and ubiquinol which are lipophilic in nature.

Third line defence antioxidants

This category of antioxidants only comes into effect after free radical damage has occurred. They are de novo enzymes which repair the damage caused by free radicals to biomolecules and reconstitute the damaged cell membrane. They perform 'clean up duty', that is they identify, breakdown and remove oxidized or damaged proteins, DNA and lipids, to prevent their accumulation which can be toxic to body tissues. Popularly known members of this group include the DNA repair enzyme systems (polymerases, glycosylases and nucleases), proteolytic enzymes (proteinases, proteases and peptidases).

Fourth line defence antioxidants

These 'antioxidants' involves an adaptation mechanism in which they utilize the signals required for free radicals production and react to prevent the formation of such free radicals.

The signal generated from the free radicals induces the formation and transport of an appropriate antioxidant to the right site.

Roles of antioxidant enzymes and molecules in preventing oxidative stress within cells

Superoxide dismutase (SOD)

Superoxide dismutase (SOD) is the first detoxification enzyme and most powerful antioxidant in a cell. It is an important endogenous antioxidant enzyme that acts as a component of first line defence system against reactive oxygen species (ROS). It catalyses the dismutation of two molecules of superoxide anion (*O_2) to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2), thus converting the harmful superoxide anion into a less hazardous form. It is present in mitochondria, cytosol and peroxisomes of hepatopancreas of shrimps. The enzyme protects body cells and tissues from excessive oxygen & nitrogen radicals and other harmful agents that promote cell death (Ighodaro *et al.*, 2018).

Catalase

It catalyses the degradation or reduction of hydrogen peroxide (H_2O_2) to water and molecular oxygen, consequently completing the detoxification process initiated by SOD. This enzyme is located mainly in the peroxisomes of hepatopancreas of crustaceans. Hydrogen peroxide though at low amounts tends to regulate some physiological processes such as signaling in cell proliferation, cell death, carbohydrate metabolism, mitochondrial function, however, at high concentrations it has been reported to be very deleterious to cells. Hence, the ability of CAT to effectively limit H_2O_2 concentration in cells underlines its importance in the aforementioned physiological processes as well as being a first line defence antioxidant enzyme (Ighodaro *et al.*, 2018).

Glutathione peroxidase

Glutathione Peroxidase (GPx) is an important intracellular enzyme that breakdown hydrogen peroxides (H_2O_2) to water; and lipid peroxides to their corresponding alcohols. Therefore they play a crucial role in inhibiting lipid peroxidation process, thereby protecting cell structure and integrity. It resides in mitochondria and cytosol of hepatopancreatic cells. It is a component of first line defence system.(Ighodaro *et al.*, 2018).

Heat Shock Proteins (HSP70)

Heat shock proteins (HSP) are highly conserved group of proteins that are synthesized in response to different forms of stress. (Robert *et al.*, 2003).The HSP can non-covalently bind exposed hydrophobic surfaces of non-native proteins and perform essential biological functions under both physiological and stressful conditions. General functions attributed to HSPs include: preventing protein aggregation under physical stress; serving as molecular chaperones in protein transport between cell organelles; and contributing to the folding of nascent and altered proteins. Although most HSP70 are constitutively expressed, their expression is however upregulated by various physiological perturbations or stressors (e.g. elevated temperature, hypoxia, heavy metals, radiation, calcium increase and microbial infection). Because of the high sensitivity to changes in the environment, HSP is suggested as an early biomarker of exposure in ecotoxicological studies. They act as molecular chaperones, maintaining homeostasis and acting against the proteotoxic effects. HSP70 play important roles in resisting environmental stresses and stimulating innate immune system; the only system on which the invertebrates rely on. HSP is found to be both constitutive and inducible(highly stress-inducible), and is mostly expressed in haemocytes and almost

all tissues including muscle, stomach, heart, hepatopancreas and gills. These proteins have also been associated with inhibition of viral replication. Increased expression of HSPs, particularly HSP70, is considered as a good biomarker for detecting changes in metabolic activity.

Effect of stocking density on the regulation of antioxidant enzymes and molecules

Increasing stocking density causes undesirable influences on aquatic animals like oxidative stress which disrupts their cell structure by lipid peroxidation, as well as oxidation of proteins and DNA. Antioxidant enzymes like Catalase, Superoxide Dismutase and Glutathione peroxidase as well as HSP70 can serve as biomarkers of crowding stress response in shrimps. Oxidative stress and tissue damage due to inactivation of antioxidant enzymes in infected shrimps can result in system failure and sudden death. Slow growth and low survival rate of shrimps under high stocking density is generally observed. SOD and CAT activities in hepatopancreas in small individuals increases remarkably with the increase in stocking density, indicating a chronic response to crowding stress. It has been proposed that hepatopancreatic lipid peroxidation and CAT activity can also serve as stress biomarkers of black tiger shrimp cultivated in intensive and extensive systems. It has been documented by Li *et al.*, (2006) that Phenoloxidase (PO) activity in shrimp serum increased as stocking density was increased. Similarly, Hepatopancreatic HSP70 mRNA expression also increases significantly with increase in density of shrimps (Gao *et al.*, 2017). It has been reported that HSP 70 mRNA expression of the large individuals are usually lower than that of the small individuals in higher stocking density treatments indicating that crowding stress in the small individuals was stimulated by the dominant large individuals.

It has also been demonstrated that the immune system responds vigorously to infection by pathogens as well as to different forms of stress with an increased expression of HSP70 (Valentim *et al.*, 2014). Aksakal *et al.*, (2011) reported that increasing stocking density caused inhibition of antioxidant enzymes and elevation of HSP70 mRNA levels in rainbow trout. Therefore, the detection of SOD, CAT, HSP 70 levels reflects the antioxidant status in shrimps. Physiological and cellular stress responses depend on changes in the concentration of proteins and genes that play an important role in the stress response process, such as the molecular chaperones heat shock proteins (HSPs). Besides high stocking density, oxidative stress in shrimps can also be caused by pH & temperature fluctuations, low salinity, environmental hypoxia and re-oxygenation, bacterial challenge. Thus under such conditions, an up-regulation in the SOD, CAT, GPx, HSP70 mRNA expression in the hepatopancreas, gills and muscle of shrimps have been reported by Wang *et al.*(2010) and Taylor *et al.*, (2011).

Physiological impacts of stress on Shrimp PLand mitigation technologies

High stocking density can increase competition for food and viable space between individuals, leading to the establishment of size variation among shrimps which increases cannibalism during moulting especially among juveniles at night, thus reducing survival rate (Wu *et al.*, 2001). High density also compromises shrimp health, thereby increasing the risk of disease outbreak and difficulty of management .Moreover, size variation is not desirable for marketing shrimps which directly puts a negative impact on profitability.

Two technologies are generally employed to reduce these negative impacts on shrimps in high density nursery systems, thus enhancing

production and sustaining economic feasibility in aquaculture.

The first technology is Biofloc system where highly oxygenated nursery ponds are fertilized with carbon-rich sources like molasses to maintain a C: N ratio of 6:1. This triggers the predominant appearance of heterotrophic bacterial biota. The bacteria that inhabit bioflocs assimilate the dissolved toxic nitrogen compounds in the water, which are generated by shrimp excretion and the decomposition of organic matter into bacterial biomass. Furthermore, biofloc serves as an important feed supplement in the shrimp diet, which is highly proteinaceous and also serves as an alternative energy source. Bacteria and their products exert an immunomodulatory effect on shrimps, which in turn increases the survival and resistance in farmed animals, even during stressful conditions like high stocking densities (Silva *et al.*, 2015). This technology helps in providing high productivity through the use of high stocking densities, and little or no water exchange, which in turn reduces the emission of effluents to the environment and increases the biosafety level during the culture period.

During a study by Wasielesky *et al.*, (2013), shrimps were stocked at four different stocking densities 1,500, 3,000, 4,500 and 6,000 shrimps/ m² in a biofloc-based nursery system for 35days. Biofloc system with the highest stocking density (6,000 shrimps/ m²) experienced least mean final weight, least specific growth rate, least percentage survival, highest FCR and lowest productivity thus making it unsuitable for commercial production. The reduced growth and survival of cultured Penaeid shrimp at high densities is related to a combination of factors such as increase in competition for the same space and natural food sources, higher events of cannibalism, degradation of water quality and accumulation of anaerobic sediment. The

highest FCR revealed that this parameter is influenced by the high stocking densities, and it also reflects the ability of shrimps to graze on the microbial community may decrease or be less efficient at such high densities (4,500 and 6,000 shrimps/ m²). During the last week of nursery rearing the survival of shrimps was reduced with the highest densities due to lack of viable space, even in biofloc system. Percentage survival was higher with stocking densities of 1,500, 3,000 animals/ m². The average daily specific growth rate, mean final weight, percentage survival, final biomass values were highest for the lowest stocking densities (T1500 and T3000). Similar studies were carried out by Silva *et al.*, (2015), to find out the best stocking density for nursery rearing of *L. vannamei* in a BFT system using similar stocking densities. Since in these

experiments the water quality and the availability of natural food were similar for all treatments because the biofloc recirculation system used was same for all the culture systems. Thus, the specific growth rate was influenced by the space limitation to which the shrimps were subjected, showing an inverse relationship with the increase in stocking density. According to the results obtained, it is possible to nurse *Litopenaeus vannamei* in a BFT system at stocking densities of up to 4,500 shrimp/m² with minimal reduction in the percentage survival of the cultured shrimps. Thus, the findings revealed that the best stocking density in terms of optimum growth rate, survival, FCR, productivity and final biomass was 3000 shrimps/m².

Figure.1 Imbalance between free radicals and antioxidants in biological system (High level of free radicals and low level of antioxidant)

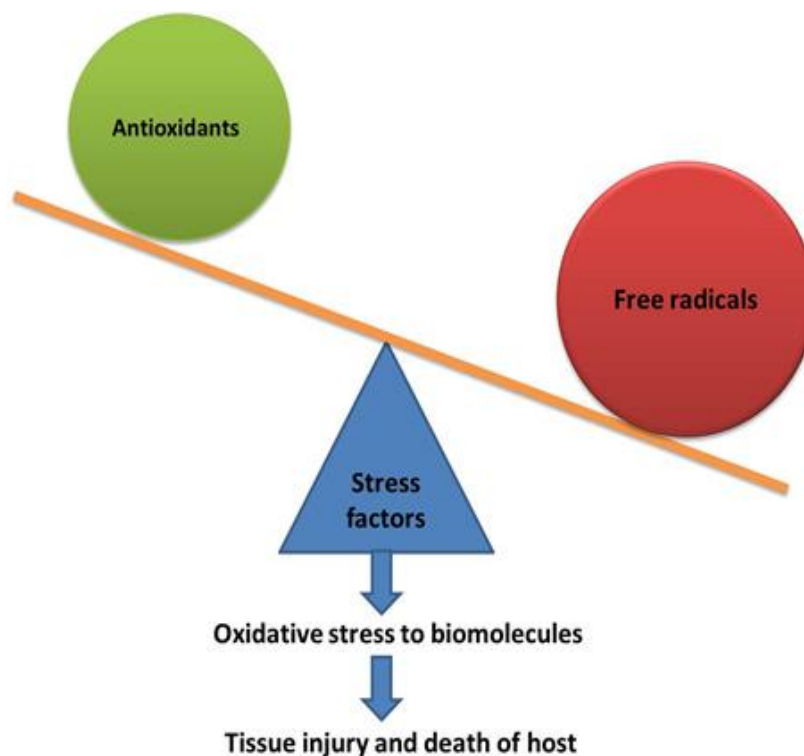
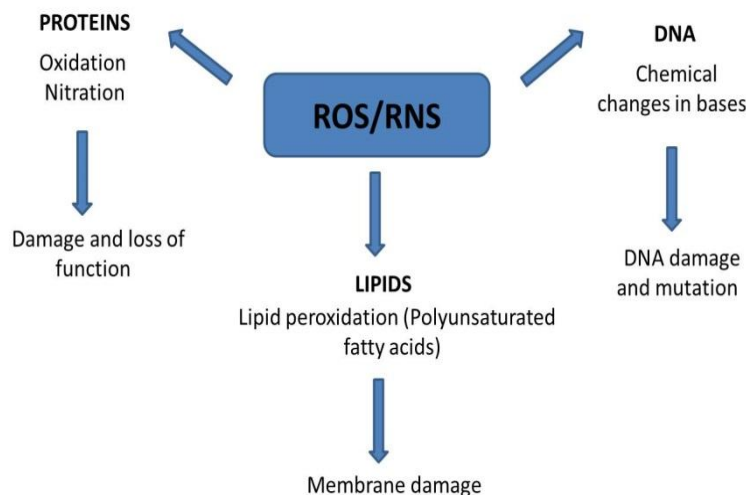


Figure.2 Effects of reactive oxygen species on major biomolecules



The second technology in demand is the application of Artificial Substrate. Nursery production may be enhanced by the addition of artificial substrate to increase the surface area upon which shrimps can graze, to serve as refuge for moulting shrimps as well as serves as an additional substrate for nitrifying bacteria. Increased grazing area helps in better growth (Moss *et al.*, 2004). High stocking densities have potential negative effects on growth of *L.vannamei* as shrimp growth is density dependent during the nursery phase, i.e. lowest stocking density will have better growth rate than the highest stocking density. The major cause of this reciprocal relationship between stocking density and shrimp growth are decreased grazing area, decreased availability of natural food and increased cannibalism, poor water quality. However, this problem can be mitigated to certain extent by using Aqua MatsTM. It is covered with particulate organic matter (POM) to which bacteria, microalgae, protozoans remain attached. These organisms along with POM serve as an important food source for *L. vannamei*. Moreover, these substrate contain nitrifying bacteria that can

oxidise toxic ammonia and nitrite produced in shrimp culture systems due to feed degradation and excretion by shrimps into nitrate, which is the utilizable form of nitrogen (Antony *et al.*, 2006). Thus, in the presence of this artificial substrate increased growth and weight gain, lower FCR can be seen.

In an experiment conducted by Moss *et al.* (2004), three stocking densities were employed to stock tanks with and without artificial substrate. In the absence of artificial substrate, mean final weight of shrimp stocked at lowest density was 46% greater than the mean final weight of shrimp stocked at highest density without substrate. Shrimps stocked at lowest density with substrate were larger than shrimps stocked at highest density with substrate. However, in presence of an artificial substrate, the mean final weight of shrimps stocked at highest density was only 8% lower than the mean final weight of shrimps stocked at lowest density without substrate. Final weight gain was greater in treatments with substrate than without substrate. Aqua MatsTM can therefore be used

to mitigate the potential negative effects of high stocking density on growth of *L. vannamei* in nursery systems.

In conclusion, high stocking density is extremely desirable for commercial shrimp farming. The major drawback of this intensive aquaculture is that it causes stress among shrimps leading to low survival and growth rate, increased risk of disease outbreak and difficulty in management. The crowding stress often results in oxidative damage to biomolecules which are the integral components of a cell. In order to mitigate this lethal damage as much as possible, an elevated expression of antioxidant enzymes including Superoxide dismutase, Catalase, Glutathione peroxidases and Heat shock protein (HSP) are observed in shrimps under high stocking densities. However, under extreme stressful conditions this antioxidant defence system crashes leading to death of the host. Biofloc technology and addition of artificial substrate can somewhat help in the mitigation of these adverse effects.

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