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Biomolecules as energy source during salinity acclimation and pathogenic infected the PLs of *Penaeus monodon*

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Abstract
The tiger shrimp *P. monodon*, is the major species cultured in worldwide. It is the largest, fastest growing species compare to other penaeid shrimp s, it has high protein content and taste, due to this reason it has high market demand. The culture of *P. monodon* in sea water (30 ppt) may cause disease problems, particularly white spot or vibriosis, because of this problem shrimp farmers keep moving towards sea water to freshwater environment. During salinity adaptation, energy-demanding mechanisms for hemolymph osmotic and ionic regulation are activated. Thus, the main goal of present research work is to identify the possible involvement of biomolecules as an energy source in the osmotic adaptation process and the pathogenic infected shrimps. Results shows that Gill and muscle biomolecules were significantly higher in 30 ppt and reduced in fresh water (0 ppt) salinity levels. Similarly, we observed that PLs were acclimated to low saline water, diseases cannot spread in freshwater conditions. Our studies represents biomolecule levels gradually decreased from high salinity to low salinity stress levels and also pathogenic infected shrimps.

Keywords: *P. monodon*, WSSV, *Vibrio harveyi*, Lipids, Proteins

1. Introduction
Farming shrimps in freshwater can help eliminate disease-causing organisms from marine environment and also enables farming in inland areas that are difficult for seawater. In general, white spot syndrome virus and vibriosis (*V. harveyi*) caused total mortality in cultured systems. Previous studies have shown that pathogens cannot spread or cause disease outbreak in freshwater conditions. Therefore, cultivating PLs in freshwater can help to overcome the challenge of disease outbreaks, thereby improving production of PLs of P. *monodon.* Therefore, the cultivation of Penaeus *monodon PL was improved.* Pathogenic and salinity stress induced by changes in environmental conditions requires homeostatic regulations that induce biochemical alterations in tissue of PLs. Furthermore, both stress conditions require the PLs ability to provide enough fuel to tissues in order to meet with a higher energy demand. This study shows that what energy source is used and which biomolecules are mobilizing and providing this energy source is an important issue to understand metabolic aspects of homeostatic regulations during stressful situations. As an increase in salinity from 0.5% to 28%, the total proteins in the rotifer *Brachionus plicatilis* decreased with simultaneous increase in the free amino acid content [1]. Even though glycogen metabolism is linked with osmotic adaptation, it does not seem to be the only source of energy in *C. granulata* during osmoregulation [2]. The present article, describes the role of biomolecules and involvement during both stress conditions.

2. Material and Methods
21. Experimental Protocol
Group I: Control Animals: PLs of *P.monodon* uninfected animals were gradually acclimated to low salinity levels.

Group II: *Vibrio harveyi* challenged Animals: PLs were challenged with *Vibrio harveyi*,

these animals were gradually acclimated to low salinity levels

Group III: White Spot Syndrome Virus challenged Animals: PLs were challenged with WSSV, these animals were gradually acclimated to low salinity levels.

2.2. Pathogen challenging Test

V. harveyi strain brought from Chandigarh (MTCC No-3438). Bacterial strain was inoculated in sterile TCBS broth, incubate 24 h for bacterial growth. After incubation 10^{-4} (cfu/ml) broth were added to the PLs rearing tub for challenging experiments. Likewise, WSSV infected shrimp haemolymph were collected and injected into PLs for challenge.

2.3. Experimental Animals and Design: PLs were collected from shrimp farm in Muthukam bakam village, Nellore District, Andhra Pradesh and sent to the laboratory in aerated plastic containers and PLs were maintained in laboratory tanks for a week in filtered sea water (30 ppt). During experimental period the PLs were fed with commercial pelleted feed for a day. After the PLs acquired 10 mg, they were separated and gradually acclimated from 30 ppt to six salinity levels, i.e. 25, 20, 15, 10, 5 and 0 ppt. The PLs were randomly stocked into 5 tubs with each density of 20 PLs per tub with four replicates. Every day we remove at least 25% of sea water then equal percentage of tap water was aerated before added to the tub to adjust different salinity levels.

2.4 Sample Collection: shells were removed, gills were separated and muscle tissues were collected quickly. Tissues were washed in ice-cold normal saline. 10% homogenate of tissues were prepared at 4 °C in the homogenizing buffer (0.5 mM Sucrose, pH 7.8). Tissues were homogenized in pre-cooled mortar and pestle. The homogenate was centrifuged at 1,000 rpm for 10min at 4 °C in a cooling centrifuge to sediment tissue debris. Again the supernatant was centrifuged at 10,000 rpm for 10 min at 4 °C to obtain the clear supernatant which was used as sample.

2.5. Biochemical Estimations

2.5.1 Total lipids: lipids were estimated by the method of [3]. Gill and muscle were collected from PLs, weighed to the nearest mg, placed at 60 ^{0}C in an hot air oven for one day and dry weights were noted. A known amount of dry, powdered tissue was homogenized in chloroform: methanol (2:1) mixture (16 ml). The contents were placed in 25 ml of test tubes and kept in a water bath at 60^{0}C. The contents were allowed to boil for 5 min and cooled to room temperature. The volume was made up to 25 ml with chloroform and 10 ml normal saline (0.9%) was added to the contents then the tubes were shaken vigorously. The lower aliquot was collected into a pre-weighed beaker and weighed again. The weight of the residue in the beaker is equal to the amount of lipid present in the sample. Total lipid content was expressed as mg lipid/gm wet weight of tissue.

2.5.2 Total protein: It is estimated by the method of [4]. 2% (w/v) homogenate of gill and muscle tissue was prepared separately in 10% TCA and centrifuged at 1000 rpm for 15 mins. The supernatant was discarded and the residue was collected and dissolved in 5 ml of sucrose solution. 4 ml copper reagent was added to 0.5 ml of this solution then the sample was allowed to stand for 10 mins and finally, 0.4 ml Folin phenol reagent was added. The optical density of the colour developed was measured using a spectrophotometer at 600 nm against the blank. Values were expressed as mg protein/ gm wet weight of tissue.

2.5.3 Total Carbohydrate (TCHO): Carbohydrate levels are estimated by the method of [5]. 5% homogenate of the muscle and 2% homogenate of the gill were prepared separately in 10% Trichloroacetic acid (TCA). The homogenates was centrifuged at 1000 rpm for 15 min at 4^{0}C. 4 ml of anthrone reagent was added to 0.2 ml of supernatant and boiled for 15 mins. The colour was read at 620 nm in a spectrophotometer using the blank. Values were expressed as mg carbohydrate /gm wet weight of tissue.

2.5.4 Glycogen: Glycogen levels are estimated by the method of [5]. 10% homogenates of the gill and muscle were prepared separately in 5% TCA and centrifuged at 1,000 rpm for 15 mins at 4 ^{0}C. 1:5 volume of supernatant and 95% ethanol was added and allowed to stand overnight in refrigerator (4 ^{0}C). After complete precipitation, the contents were centrifuged again for 15 mins at 1,000 rpm. The supernatant was discarded and the residue was dried, then it was dissolved in 1 ml of distilled water. 5 ml of anthrone reagent was added to each test tube. The test tubes were kept in boiling water bath at 40 ^{0}C for 15 mins and allowed to cool to room temperature. The colour was read at 620 nm in a spectrophotometer against the blank. Values were expressed as mg glycogen/ gm wet weight of tissue.

2.6 Statistical Analysis: Results are presented as mean± standard error of mean (SEM). Difference between the control and treatment means were compared by one way analysis of variance (ANOVA), followed by Duncan's new multiple range test. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1 Lipids: Shrimps subjected to 0 ppt stress showed a significantly decreased in total lipid level were observed in muscle (43.5±0.02 to 10.2±0.02 mg/g) and gill (28.6±0.12 to 5.6±0.05 mg/g) compared to controls. Similar decreasing levels were observed in *V. harveyi* challenged shrimps subjected to hypo osmotic stress respectively. Muscle lipid levels were decreased from 30.6±0.21 to 8.5±0.05 mg/g and 36.6±0.04 to 9.5±0.12 mg/g in gill were recorded. Viral infected shrimps shows gradual decrease of lipids in muscle (36.6±0.04 to 9.5±0.12 mg/g) and gill (22.4±0.01 to 5.4±0.11 mg/g) were registered at 0 ppt salinity compared to high salinity (Figure-1 & 2).

3.2 Proteins: Figure (3 & 4) present results on the concentration of proteins decreased from 187.9±0.02 to 87.6±0.11 mg/g in muscle and 32.3±0.12 to 7.6±0.25 mg/g in gill were recorded respectively under salinity stress PLs. Following *V.harveyi* challenged shrimps shows significantly decreased protein concentration in muscle (150.8±0.21 to 78.6±0.05 mg/g) and 26.4±0.01 to 7.1±0.13 mg/g in gill at low salinity adaptation were observed. Similarity same decreasing trend was followed by WSSV infected shrimps at 0 ppt salinity. The mean±SD values from 167.9±0.23 to 82.6±0.23 in muscle and 26.4±0.05 to 7.1±0.13 mg/g in gill of viral infected shrimps were recorded. Higher protein concentration was recorded in infected shrimps held at 30 ppt

compared to under salinity stress respectively.

3.3 Carbohydrates: Figure (5 & 6) The results represented a gradual decrease register from 20.5±0.11 to 2.9±0.11 in gill and 56.4±0.10 to 25.6±0.01 mg/g in muscle carbohydrate concentration was noted in shrimps at hypo osmotic stress ($p<0.05$) compared to control group. When challenged with Pathogen decreasing was observed in the carbohydrate concentration in shrimps at all reducing salinities compared to under salinity stress shrimps. *V.harveyi* challenged shrimps maintained at 0 ppt registered comparatively lower carbohydrate levels in muscle 22.7±0.24 to 23.4±0.02 and gill 10.5±0.15 to 2.4±0.32 mg/g were recorded respectively. Similarly viral infected shrimps shows same decreasing trend was followed. The carbohydrate levels were progressively reduced from 48.6±0.14 to 4.6±0.13 in muscle and 15.6±0.02 to 2.6±0.03 mg/g in gill respectively in viral infected shrimps maintained at 0 ppt.

3.4 Glycogen: The results evidently show that the concentration of glycogen decreased significantly in shrimps subjected to hypo osmotic stress ($P< 0.05$) and increased at control group in muscle and gills. The values 68.4±0.12 to 34.6±0.11 mg/g in muscle and 27.6±0.11 to 5.2±0.02 mg/g in gill were recorded at low salinity levels. *V.harveyi* challenged groups shows decreased glycogen concentration in muscle from 50.2±0.32 to 29.1±0.02 and 16.5±0.22 to 4.2±0.01 mg/g in gills at 0 ppt salinity adaptation. Glycogen levels were significantly lower at reducing salinities in under salinity stress compared pathogenic challenge shrimps. Viral infected PLs showed a reducing from 57.9±0.15 to 32.2±0.04 mg/g glycogen levels in muscle and 20.5±0.02 to 4.7±0.21 mg/g in gill were recorded respectively (Fig- 7 & 8).

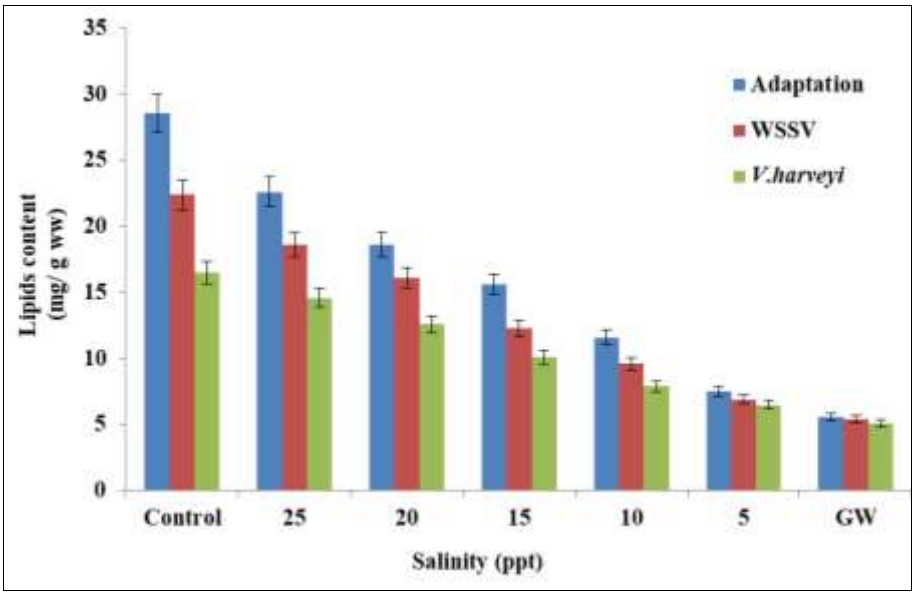

Fig 1: Lipid content in the gill of PLs at different salinity levels and diseased with *V. harveyi* & White Spot Syndrome Virus. (Value are mean± SD of 6 observations; $p< 0.05$).

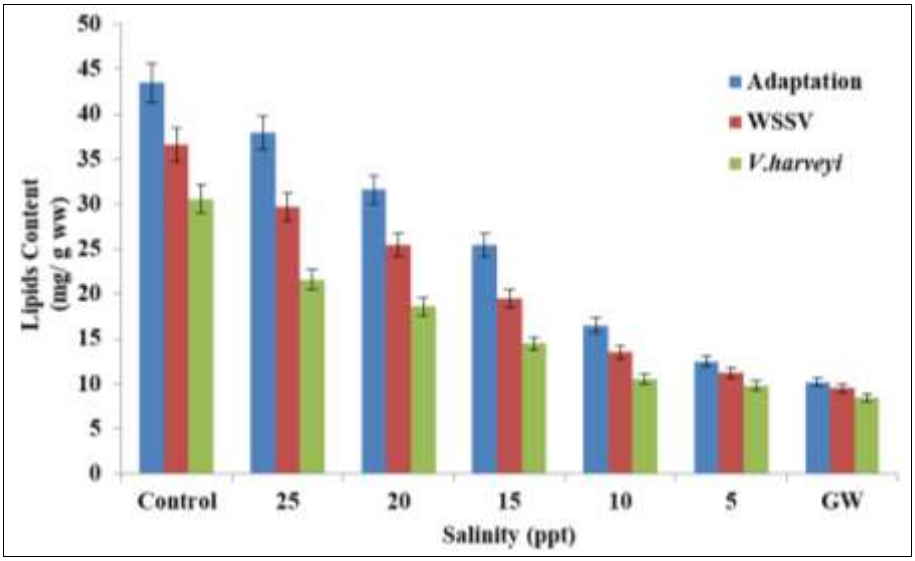

Fig 2: Lipid content in the muscle of PLs at different salinity levels and diseased with *V. harveyi* & White Spot Syndrome Virus. (Value are mean± SD of 6 observations; $p< 0.05$).

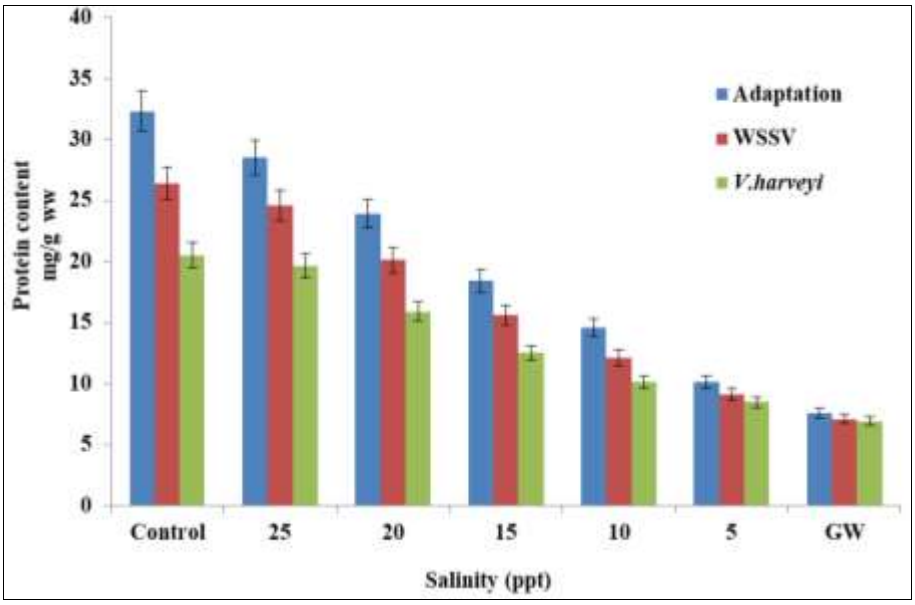

Fig 3: Protein content in gill of PLs at to different salinity levels and diseased with *V.harveyi* & White Spot Syndrome Virus. (Value are mean± SD of 6 observations; $p< 0.05$).

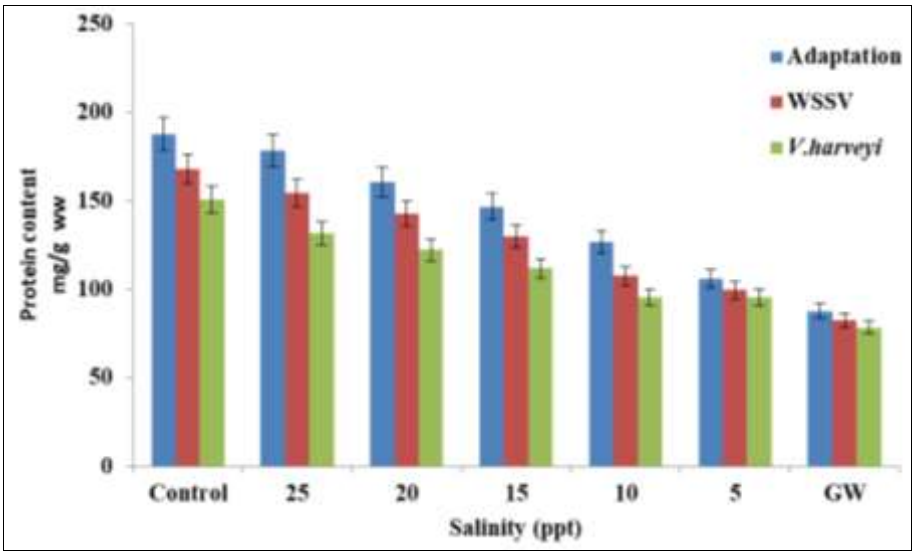

Fig 4: Protein content in muscle of PLs at different salinity levels and diseased with *V. harveyi* & White Spot Syndrome Virus. (Value are mean± SD of 6 observations; $p< 0.05$).

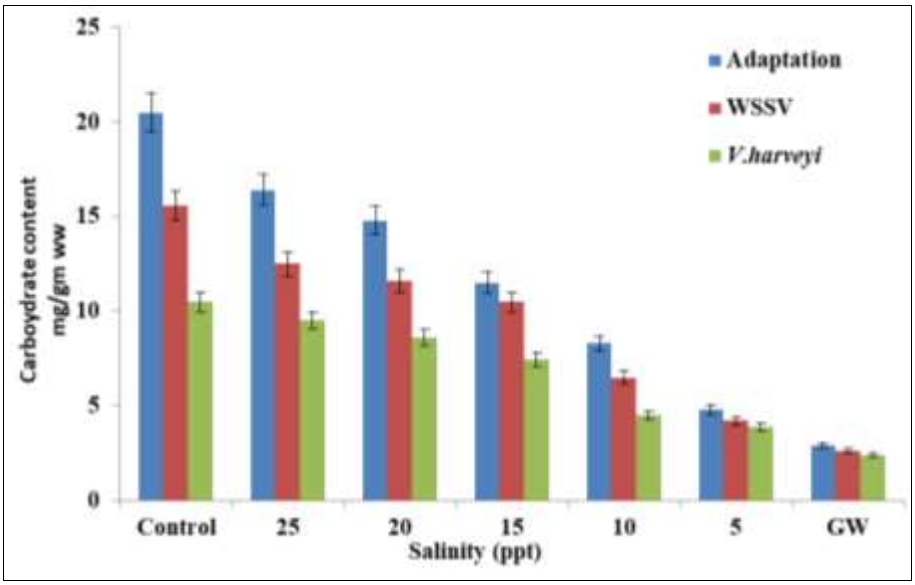

Fig 5: Carbohydrate content in gill of PLs at different salinity levels and diseased with *V. harveyi* & White Spot Syndrome Virus. (Value are mean ±SD of 6 observations; $p< 0.05$).

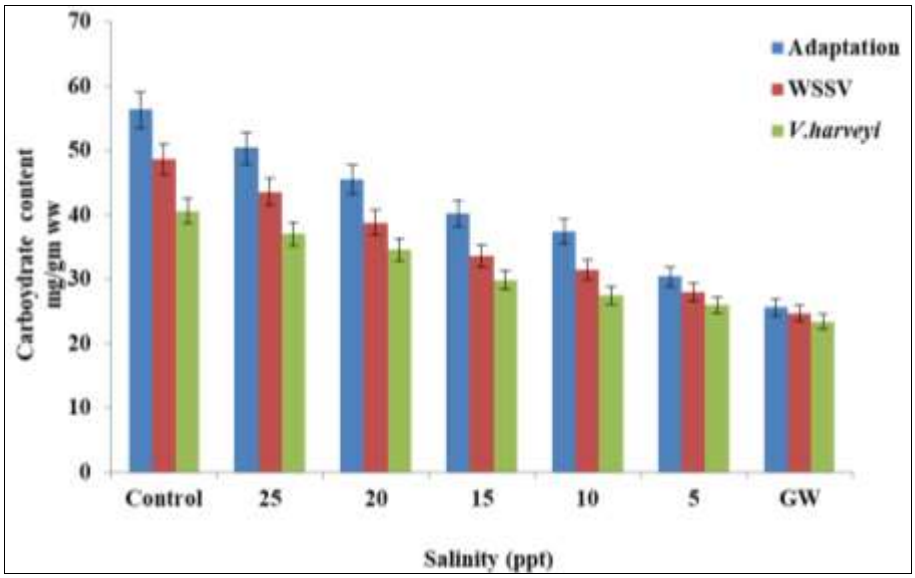

Fig 6: Carbohydrate content in muscle of PLs at different salinity levels and diseased with *V.harveyi* & White Spot Syndrome Virus. (Value are mean±SD of 6 observations; $p< 0.05$).

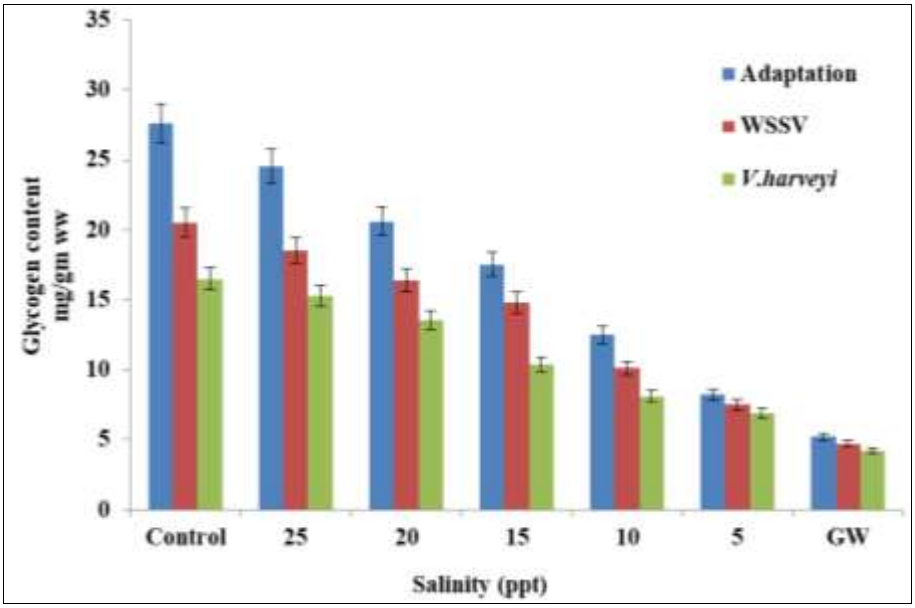

Fig 7: Glycogen content in gill of PLs at different salinity levels and diseased with *V.harveyi* & White Spot Syndrome Virus. (Value are mean±SD of 6 observations; $p< 0.05$).

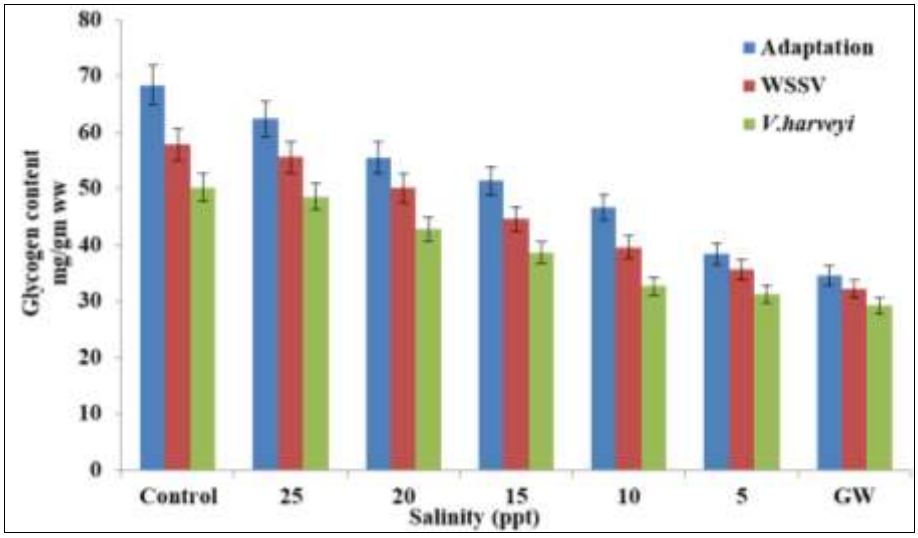

Fig 8: Glycogen content in muscle of PLs at different salinity levels and diseased with *V.harveyi* & White Spot Syndrome Virus. (Value are mean±SD of 6 observations; $p< 0.05$).

4. Discussion

The levels of different bio molecules such as proteins, carbohydrates, lipids and glycogen in muscle and gill tissues of PLs gradually acclimated to different salinity levels were observed. Salinity strongly affected the biomolecules levels in crustaceans [6-7]. When crustaceans placed in an medium of low osmotic pressure, energy-consuming processes are required to maintain the internal osmotic pressure constant. These molecules are playing major role in the cellular metabolism by serving as fuel and providing energy to the cells. Fluctuations in salinity reflect fluctuations in the energy demands of the animal, changes in the bio molecules metabolism that would meet the changing energy demands may be expected to stress [8-9]. Present study indicate that depletion in the levels of total proteins, carbohydrates, lipids and glycogen by 50% in low saline water acclimatization.

During salinity acclimation, lipids play a metabolic role in providing energy for osmoregulation processes and they are also important in maintaining the structural and physiological integrity of cellular and sub cellular membranes. Similarly depletion of proteins represents, the mobilization of protein as a source of energy in the tissues. At low salinity, shrimp must use protein as source of amino acids to maintain the osmotic pressure and growth [10, 11] reported that decrease in the protein content of zoea 1 decapods crustacean larvae at the lowest salinity. The potential use of protein as a metabolic reserve has also been suggested by [12] for marine zooplankton. The decrease in protein reserves after 4-day exposure to low salinity supports the hypothesis that *N. integer* can actively use protein as an energy source under stress conditions.

Carbohydrates play an important role in the cellular metabolism by serving as fuel and providing energy to the cells. The Oxidation of carbohydrates to meet the energy demands. This is important to maintain internal osmotic pressure of the organism to enable efficient osmoregulation in order to prevent water accumulation and salt loss from their body. Glucose delivers fast energy in the form of ATP via the process of glycolysis and oxidative phosphorylation and is the major circulating carbohydrate in crustaceans [13]. Although lipid and protein utilisation is variable in crustaceans, generally, carbohydrate is used before lipid and protein as the preferred fuel for metabolic processes [14]. Glycogen is the important energy source in both vertebrates and invertebrate, especially during environmental fluctuations [15, 16, 17]. The possibility of high levels of glucose and carbohydrate in haemolymph might be due to the transport of glycogen and carbohydrate from gill and muscle to haemolymph. During stress, shrimp use glycogen as a source of energy [18-19]. The depletion of carbohydrate occurred earlier in gill is more than in the muscle of *Oreochromis mossambicus* [20].

Pathogenic challenged PLs have additional burden on the biomolecules requirements of the animals contributing to a significant reduction in the levels of these molecules in the tissue of gill and muscle. Following Pathogenic infected control groups (30 ppt), there was an overall reduction in the levels of tissue biomolecules in PLs maintained at 30 ppt and gradually increased with low salinities. Most probably, biomolecules were transported to the haemolymph from gill and muscle to meet the energy demands towards infection [21]. Observed a reduction in the total carbohydrate and glucose levels in muscle and hepatopancreas of WSSV-infected *F. indicus* and a corresponding increase in the haemolymph. Compared to those at 30 ppt, a general reduction in the biomolecules levels could be observed in shrimps under salinity stress and following pathogenic challenged shrimps.

Our results represents significant depletion in the levels of total carbohydrates, glycogen, total proteins and as well as lipids during low salinity acclimatization, clearly indicates that the utilization of these bio molecules provide energy for new environmental adaption via influence of osmoregulation. Significant reduction of tissue metabolites in infected shrimp under salinity stress may be attributable to deviation of energy flow toward supporting osmotic adjustment because they are under both stress conditions.

5. Conclusion

The present study revealed that both stress conditions (salinity adaptation and pathogen challenge) can change some biomolecule levels in muscle and gills of PLs gradually acclimated to fresh water environment. This study supports that a gradual acclimation of PLs can grow without pathogenic infections. In the future, production has to be assessed up to the harvesting stage to validate the farming potential of shrimp in fresh water environments. Fresh water practices must be helpful to minimize outbreaks of infections in shrimp farms.

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