

Neuroendocrine and immunotoxicity of polyaromatic hydrocarbon, chrysene in crustacean post larvae

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Abstract

Polyaromatic hydrocarbons are a group of chemical pollutants which cause a significant threat to the living organisms in estuaries and marine ecosystems. We report the effect of chrysene, a major PAH pollutant found in Cochin Estuary along the southwest coast of India, on the neuroendocrine and immune gene expression of the post larvae (PL-25) of *Penaeus monodon*. The PL- 25 of *P. monodon* were administered with feed coated with increasing concentrations of chrysene (1, 2 and 3 µg/ g) for 10 days and the gene expression was studied on 7th, 11th and 15th day. The PL exposed to chrysene showed moulting stress and changes in the levels of moult inhibiting hormone 1 (MIH1) indicated by irregular moulting in the experimental tanks. At the molecular level, the higher concentration of chrysene induced two-fold upregulation of neuroendocrine (MIH I) and downregulation of immune (ProPO and crustin) gene on the 7th day of exposure. The expression of MIH I gene returned to normal level on withdrawing the experimental feed (on 11th and 15th day), while continued downregulation of ProPO and crustin were observed on the 11th day. The results of the present study indicate that the micro-gram levels of PAH can impinge the neuroendocrine and immune system of the *P. monodon*, which may induce morbidity and mortality to the larvae in polluted coastal ecosystems. Therefore, more attention may be given to avoid PAH pollution in the estuaries to maintain a healthy ecosystem and to protect the animals from extinction.

Keywords: Polyaromatic hydrocarbon; Chrysene; *Penaeus monodon*; immune system; proPO; neuroendocrine; MIH, pollution; Cochin Estuary.

Introduction

The coastal regions across the globe are susceptible to increasing levels of pollution due to large scale development in industrialization and urbanization. Estuaries, along the coastal regions, need special attention as they function as the connecting point between the terrestrial and open ocean. Any pollutants reaching the estuaries will ultimately reach marine ecosystems and can cause equal harm to marine organisms as well. The diversity of the pollutants reported from the estuaries and marine ecosystems include heavy metals, polyaromatic hydrocarbons, pathogens and recently microplastics. Polyaromatic hydrocarbons (PAHs), which include chrysene, pyrene, phenanthrene, naphthalene and benzo (a) anthracene, are a group of pollutants originated from petroleum-related industries and reach the estuary through various routes including oil tankers, shipping vessels, the release of industrial effluents etc. The intensity of PAH pollution might aggravate in future because of the increasing number of ports, shipping vessels and industrial development. Several studies have reported a higher level of PAH in the sediments and water column of estuarine and riverine environments worldwide, for example Exe and Humber in UK, Seine in France, Urdaibai and Plentzia estuary in Spain, York river-VA estuary in USA, Gulf of Thailand, Yangtze estuary in China, Mundaú-Manguaba in Brazil, Estero de Urias in Mexico, and Cochin estuary in India (Herrmann and Hubner 1982; Fernandes et al. 1997; Zhou et al. 1998; Orbea et al. 2002; Countway et al. 2003; Boonyatumanond et al. 2006; Liu et al. 2008; Maioli et al. 2010; Jaward et al. 2012; Ramzi et al. 2017).

The response of organisms to PAHs varies depending on the type and concentration of the chemical, duration of exposure and the age of the animals. The benthic organisms like polychaetes have the chances for prolonged exposure to PAHs as they are sedentary while the shrimps and fishes can move away from the polluted regions. In any case, the toxicity of these endocrine disrupting molecules is initiated by their irreversible binding to lipophilic sites in the cell and slow penetration through cell walls, disrupting cellular processes (Neff 1979). Impairment of osmoregulation and inhibition of moulting leading to death has been reported among the juveniles of crabs and shrimps (Mothershead and Hale 1992; Oberdörster et al. 2000; Oberdörster et al. 1999) while toxic effects of PAHs also include respiratory stress and inhibition of feeding among copepods and mussels on exposure to sub lethal concentrations (Lotufo 1997; Wirth et al. 1998). The bioassay based studies reported the lower growth rate in *Littopenaeus vannamei* on exposure to PAH polluted sediment collected from Mexican estuary for 21 days (Jaward et al. 2012). The PAHs impacted swimming ability, feeding activity and phototactic response (Laramore et al. 2016) among Pink shrimp larvae *Farfantepenaeus duorarum* to Macondo canyon 252 crude oil and Corexit dispersants. Coastal

fishery populations in the Gulf of Mexico, USA were vulnerable to oil spills and PAH pollution both in their spawning grounds and in estuarine nursery areas which had large impacts on recruitment and eventually on adult population (Whitehead et al. 2011). Similarly, 1989 *Exxon Valdez* oil spill in Prince William Sound, Alaska, coincident with the spawning of Pacific herring population caused the herring's decline and the population has still not recovered (Thorne and Thomas 2008).

Although the above studies provide an overall picture on the impact of PAH pollution on the population stock of organisms in the natural ecosystems, cellular and molecular investigations are inevitable to discuss more on the impact of these chemicals on the life cycle of organisms, bioaccumulation and subsequent long term impact on the ecosystem. Recent studies also reported the alterations in the metabolism-related genes (CYP1A1, GST, SOD) controlling the antioxidant enzyme activities among white shrimp *L. vannamei* on exposure to benzo (a) pyrene (Ren et al. 2015b). In the present study, we investigate the impact of PAH, chrysene on the immune and neuroendocrine system of the post larval stage of *Penaeus monodon* found abundantly in Cochin Estuary (CE), situated along the southwest coast of India. The seasonal migration of gravid female *P. monodon* to the CE to lay eggs and the return of young ones to the sea after spending their larval and post larval stages in the estuary is a part of their life cycle. However, the stocking density of *P. monodon* has been showing a decreasing trend indicated by their reduced contribution (1.4 % in 2012 to 0.84 % in 2014) to the total crustacean fishery catch from CE (Asha et al. 2014). The CE is susceptible to PAH pollution from the adjacent oil refineries, ships arriving in the Cochin Port, tourists and fishing boats plying the estuary. An increase in the level of PAHs, chrysene, from nanogram (193.29 ng/ g) to microgram (2.816 µg/g) levels has been reported from the sediments near the rearing grounds of shrimp larvae in 2006 (Latha 2006). An overall increase in the level of total PAHs in the sediment from 93.82 ng/ g in 2002 (Munga 2002) to 9.626 µg/ g in 2017 (Ramzi et al. 2017) has been reported. It is hypothesized in the present study that such an increase in the level of PAH in the ecosystem may impinge the growth and immune system of the larval stages of *P. monodon* which may result in a reduction of their distribution in the estuary.

Methods

Experimental animals

Healthy post larvae (PL 25) of *P.monodon* were obtained from Azhikode shrimp farm hatchery, Kerala, India. These PL were reared from a single brooder collected from the pristine waters of Andaman Sea. The standard procedures of larval rearing were followed with regular monitoring of hydrographical parameters (pH, temperature, salinity, ammonium and nitrite), microbial pathogens

and ~ 25 % of water was exchanged every day. The larvae collected were acclimatized in tanks containing aerated seawater having a salinity of 15psu, and the water quality was maintained within the preferred conditions for the PL (pH 6.8 - 7.8; total ammonia < 0.1 mg/ L; nitrite < 1.0 mg/ L; total alkalinity (CaCO₃) 75 - 125 mg/ L; and total hardness >5000 - 6000 mg/ L) by exchanging one third of the water in each tanks with fresh sea water. The PL was fed daily, twice with commercially available larval feed (Higashimaru, India).

Experimental setup

The experimental feed was prepared by coating commercially available shrimp larval feed (PL-25 pellet, Higashimaru, India) with chrysene (Sigma, USA). Here the chrysene dissolved in acetone (1000 µg/ ml) was mixed with feed to achieve the final concentrations of 1, 2 and 3 µg/ g and shade dried. A feed coated with acetone alone (0.001%) was also prepared in the same way. The experimental animals were distributed into five groups, each group containing triplicate tanks and each tank contained 30 animals. Three groups were assigned for feeding with experimental diets and animals in each group were fed separately with experimental diets containing 1, 2 and 3 µg/ g chrysene. The experimental diet was administered ad libitum, twice in a day for ten days. The animals in the fourth group were maintained with normal feed (control 1) and the fifth group was maintained with feed coated with acetone alone (control 2). The administration of experimental feed was stopped on the 10th day and the animals were maintained for an additional five days on the normal diet. The shrimps were observed for moulting irregularities, feeding response and survival. Three animals from each tank were sacrificed on 7th, 11th and 15th day and were stored in *RNAlater* solution (Sigma, USA) at -20 °C for RNA extraction.

RNA extraction and cDNA synthesis

The total RNA was extracted from the tissue samples of the animal using commercially available Trizol (Ambion, USA). The genomic DNA was removed by treating total RNA with RNase free DNase (0.2 U) for 10 min at 37 °C followed by enzyme inactivation at 75 °C for 10 min. The purity and concentration of thus extracted RNA were confirmed by measuring the absorbance (Abs 260/280 nm) in a nanodrop (ND1000 spectrophotometer V3.8). An aliquot of 1 µg total RNA was reverse transcribed in 20 µl reaction volume using Monster kit first strand cDNA synthesis kit (Epicentre, USA) following the instruction protocol.

Neuroendocrine and immune-related gene expression assay

Expression of three target genes moult-inhibiting hormone (MIH I), Prophenoloxidase (ProPO) and Crustin (Antimicrobial peptide gene) was measured by RT-PCR method. The target genes were

amplified using the primer sets for MIH I (Vrinda et al. 2016), ProPO and crustin (Dechamma 2015) (Table 1). The PCR was conducted in a 25 µl reaction volume using EmeraldAmp GT PCR master mix (Takara Bio USA, Inc) with 1 µl cDNA template, forward and reverse primers of selected genes (10 pmol/ µl each). The PCR was carried out in a Thermal cycler (Takara, Takara Bio USA, Inc) with an initial denaturation at 94 °C for 2 min followed by 25 cycles of denaturation at 94 °C for 40 s; annealing at 56 °C for 30 s; and extension at 72 °C for 30 s followed by a final extension at 72 °C for 7 min. The amplified PCR products were resolved by 1 % agarose gel electrophoresis. The PCR products were cloned in pJET1.2 (CloneJet PCR cloning kit-ThermoFisher Scientific, USA) following the manufacturer's instructions. Three clones were randomly sequenced on Applied Biosystems ABI 3730x1 DNA analyser. Sequences were confirmed by NCBI-BLAST search. The confirmed positive clones of MIH I, crustin and prophenoloxidase were used for plasmid extraction to set as standards for standard curve and melt curve analysis for real-time PCR.

Absolute quantitative real-time PCR (qRT-PCR) was carried out in a Roche Lightcycler 96- well Real-time PCR system (Roche, USA). Absolute quantitative real-time PCR was chosen over relative quantitative real-time PCR, as the β -actin (housekeeping gene) showed significantly variable results in the post larval stage used. Similar observations were made earlier by Sellars et al. (2007), when analysing the β -actin and 18S rRNA genes from the embryonic, larval, post larval and gonad samples of Kuruma shrimp. The PCR amplification was performed in a total volume of 10 µl, containing 2 x SYBR Premix Ex Taq II (Tli RNase H Plus) (Takara, USA), 1 µl of cDNA (1/25 diluted) and 10 pm/ µl of each primer. The qRT-PCR program, was 95 °C for 600 s followed by 45 cycles of 95°C for 10 s, 56°C for 10 s and 72°C for 10 s and completion with a dissociation (melt curve) analysis of amplified products performed at the end of each cycle to confirm the amplification and detection of confirmed PCR product. cDNA of shrimp larvae collected from test and control tanks on Day 0, 7, 11 and 15 were used for analysis of MIH I, ProPO and crustin. Three plasmids (2974bp backbone) containing MIH I (119 bp), crustin (119 bp) and ProPO (122 bp) were used to make 10 fold dilutions from 1.88×10^9 (MIH I), 1.31×10^{10} (crustin) and 5.21×10^8 (ProPO) copies of plasmid DNA down to one copy of starting molecule. Plasmid DNA standard curve (Supplementary figures 1, 2 and 3) equations obtained were used to calculate the absolute copy number of three genes in test and control samples. Each sample was run in triplicates for each gene and days of the experiment. The absolute quantification data obtained was analyzed with Roche Lightcycler 96 software (Roche, USA).

Enzyme-linked immunosorbent assay (ELISA) of Moulting Inhibiting Hormone I (MIH I)

The MIH I levels in the tissue of PL-25 exposed to different concentrations of chrysenes was detected by ELISA technique using the polyclonal antisera against MIH I of *P.monodon*. The details

of ELISA are explained somewhere else (Vrinda et al. 2016). Briefly, the tissue of animals on day 15 of experiment was macerated with phosphate buffered saline (500 µl) and the supernatant of extract was used as antigen for ELISA. A 100 µl of the supernatant was coated in a 96-well ELISA plate followed by 12 h incubation at 4 °C. The free sites were blocked with 100 µl BSA (5 % in PBS) for 1 h at 25 °C. The primary antisera (100 µl) of MIH I (mf-PmMIH I - 1: 12,800 dilution) was added and incubated for 1 h at 25 °C. A 100 µl of conjugated rabbit anti-mouse IgG-HRP (1: 2000 dilution) in 3 % BSA/PBS was added and incubated for 1 h. The substrate TMB (100 µl: 3, 3', 5, 5' - tetramethylbenzidine base, Sigma USA) was added and incubated for 10 min at 25 °C. Development of blue colour was observed and the reaction stopped by adding 50 µl of 1 M H₂SO₄. The yellow colour was measured at 450 nm in ELISA reader (Tecan Infinite M200). All reactions were done in triplicate and the plate was washed thrice in between each step with PBST (phosphate buffered saline pH 7.2 with 0.01% Tween 20). The level of MIH I expression in the tissue samples were delineated from the standard curve and trend line analysis of MIH I (Supplementary figure 4).

Statistical analysis

ANOVA was analyzed using the XLSTAT program. The significant variations were depicted by “*” for $p < 0.5$, “***” for $p < 0.005$ and “****” for $p < 0.001$. Moulting was visually observed as the number of animals moulted per day in all tanks. The moulting rate of moults per day was calculated (Kibria 1993). The range of moults was calculated as an average number of animals moulted in test and control tanks for a period of 1-5, 6-10 and 11-15 days.

Results

Effect of Chrysene on survival and moulting of *P. monodon* larvae

The impacts of chrysene on the general growth performance of the shrimp larvae are given in figure 1 and 2. The oral administration of chrysene didn't show any significant influence on the survival of the larvae during the first six days, while it reduced to less than 76 % in the group exposed to 3 µg/ g in 10 days. Morbid and dead larvae were found in experimental tanks exposed to 3 µg/ g from 6th day onward and continued till the 10th day that is the 3rd day after the withdrawal of experimental feeding. The moulting was found to be irregular in the experimental groups compared to the control group. Frequent moulting was indicated by many moulted animals found in the experimental group treated with 3 µg/ g of chrysene. The number of moulted animals was found to be reduced in experimental groups in three to four days after the withdrawal of experimental feeding.

Expression of Neuroendocrine and immune gene and MIH I hormones

The influence of chrysene on the expression of genes responsible for MIH I, ProPO and crustin genes were assessed during the feeding of chrysene spiked feed (7th day of experiment) and one day (11th day of experiment) and four days (15th day of experiment) after withdrawing the feed. Irregular expressions of MIH I gene was observed in the experimental animals during the feeding with chrysene spiked feed (Fig. 3). A two-fold increase in the MIH I gene expression was observed in experimental animals on the 7th day of the experiment. The copy number of genes in 1 μ l of cDNA extracted from control 1, 1, 2 and 3 μ g/ g of treated groups on 7th day was in the order of 3.82×10^3 , 8.02×10^3 , 9.10×10^3 and 8.94×10^3 , which decreased drastically on 1st day after withdrawal of the experimental feed to 5.60×10^3 , 2.51×10^3 , 2.50×10^3 and 3.24×10^3 in comparison to control 1. The copy number of MIH I became comparable with control 1 animals on the 15th day of post-experimental feeding.

The immune gene (ProPO) also showed an irregular expression during experimental feeding (Fig. 4). ProPO gene expression was significantly ($p < 0.05$) downregulated from 1.75×10^3 in control 1 to 1.16×10^3 , 9.73×10^2 and 1.12×10^3 in 1, 2 and 3 μ g/ g exposure to chrysene (on 7th day of experiment). The downregulation of ProPO continued even after one day of post-withdrawal of experimental feeding from 7.16×10^2 (control 1) to 3.52×10^2 (1 μ g/ g), 1.28×10^2 (2 μ g/ g) and 6.03×10^2 copies (3 μ g/ g), while an upregulation of the ProPO gene was observed on the fourth day after the withdrawal of experimental feeding (i.e. 15th day of the experiment).

The crustin gene also was downregulated with exposure to chrysene (Fig. 5). The expression of crustin decreased with increasing concentration of chrysene with maximum reduction in groups treated with 3 μ g/ g (1.15×10^4) on the 7th day of the experiment in comparison to 1.10×10^6 copies per microliter (control 1) cDNA. Although recovery of gene expression was observed on withdrawing the exposure to chrysene, the gene copy numbers were lower compared to the control 1 group on the 11th and 15th day of post-experimental feeding. On the 11th day of post-experimental feeding, copy numbers of the crustin gene in experimental animals fed with 2 and 3 μ g/ g were 6.85×10^5 and 3.08×10^5 copies per microliter of cDNA respectively compared to that of 1.06×10^6 copies per microliter of cDNA in the control animals. On day 15, the crustin gene expression was observed to be significantly reduced in experimental groups with more significant ($p < 0.005$) reduction evident in the groups treated with 2 and 3 μ g/ g. The copy number of genes in 1 μ l of cDNA extracted from control 1, 1, 2 and 3 μ g/ g treated groups were in the order of 3.52×10^5 , 1.95×10^5 , 6.44×10^4 and 5.13×10^4 respectively.

The expression of neuroendocrine hormone MIH I in the tissue of PL-25 on day 15 of exposure was estimated by ELISA. The MIH I hormone was detected in the animals kept in control (2.86 ng/ml) as well as those exposed to 1 (1.53 ng/ml) and 2 $\mu\text{g/g}$ (3.52 ng/ml) concentration of chrysene while it showed significant reduction ($p < 0.05$) in animals exposed to 3 $\mu\text{g/g}$ (-1.28 ng/ml) (Fig. 6).

Discussion

In the present study, we report the toxicity of chrysene at the molecular level on the growth and immune response of the larval stages of *P. monodon*. The expression of the genes responsible for the growth (MIH I) and immune system (proPO and crustin) were analysed in animals exposed to different concentrations (1, 2 and 3 $\mu\text{g/g}$) of chrysene in a bioassay. It was recorded in previous studies that the larval and juvenile stages of crustaceans are more sensitive to PAH than adults (Tatem et al. 1978). Exposure to higher concentrations (i.e. in mg/L) of PAH mixtures was reported to cause mortality in the larval stages of grass shrimp (Wirth et al. 1998), *Pandalus borealis* (Bechmann et al. 2010) and Pink shrimp *Farfantepenaeus duorarum* (Laramore et al. 2016). In the present study, we observed that the micro-gram concentration of chrysene (i.e. 1, 2 and 3 $\mu\text{g/g}$) doesn't induce severe mortality to the larvae of *P. monodon*, but they can impinge the growth and immune system of the animal.

Here we considered MIH I gene as an indicator of the growth of the larvae, as it controls the expression of the moulting hormone ecdysone (Chang 1993). It was observed that the exposure to chrysene induced alterations in the expression of MIH I gene, the gene was upregulated during exposure and became comparable to control on withdrawing the exposure. We observed a similar irregular pattern in the moulting when the larvae were exposed to the chrysene. Many moulted animals were found in the experimental groups during exposure to chrysene while the number reduced on withdrawing the exposure. This indicates that the exposure to small doses can influence the growth of the animal, the irregular moulting may induce stress to the animal leading to their improper growth and mortality. A small percentage of mortality observed in experimental tanks could also be due to the stress associated with moulting. The healthy post larval shrimps (2-5 g, PL 25) follow the moulting cycle in every 7 days and variations in this cycle have been reported from hatcheries and shrimp farms in response to various environmental stresses (Chow and Sandifer 1991). The irregularly moulted shrimps may remain soft shelled for a long time and may become more prone to the infections leading to death. Although the function of MIH I is to inhibit the moulting process, we found irregular moulting when the MIH I was upregulated with chrysene. This is intriguing and could be attributed to the ability of chrysene to bind to both the ecdysone receptor and moult-inhibiting hormone receptor simultaneously causing detrimental effect to the animal

(Oberdörster et al. 1999; Zou 2005). Alterations in moulting pattern in response to PAH exposure have also been reported earlier from crabs and shrimps (Mothershead and Hale 1992; Oberdörster et al. 2000; Oberdörster et al. 1999). Based on the studies in Tanner crabs exposed to crude oil, it has been postulated that the PAH can inhibit normal moulting due to interactions with the metabolism of ecdysone hormone (Oberdörster et al. 2000). The interference of PAH on moulting was further confirmed by inhibition of MIH I observed in animals exposed to 3 µg/g of chrysene. The inhibition/downregulation of MIH I might have resulted in the irregular moulting in the animals.

The immune genes prophenoloxidase and crustin were significantly downregulated upon exposure to chrysene. Continued downregulation of ProPO and crustin gene expression even after withdrawing the experimental feed indicates the adverse effect of chrysene on the immune system of the larval stages of *P.monodon*. Similar results were reported from the juvenile *Litopenaeus vannamei* on exposure to the PAHs such as Benzo (a) pyrene (0.03, 0.3 and 3µg/ L) and chrysene (0.3, 2.1 and 14.7 3µg/ L). The tendency to recover the proPO activity after the withdrawal of chrysene indicates that the toxic effects of lower concentrations of PAHs are temporary and the animal may recover its health with the withdrawal of pollutant. A similar trend was observed in the studies of Ren et al. (2014), where the immune activities recovered after 10 to 15 days. On the other side the higher concentrations of PAHs were found to impair the phagocytosis, phenoloxidase, α₂-macroglobulin and antimicrobial activities of haemolymph in shrimps (Ren et al. 2014).

The present study along with the previous reports indicates that the PAHs can induce a direct or indirect effect on the health of shrimps. At higher concentrations, the PAH can induce a direct impact on the health of shrimps leading to morbidity and mortality. At lower concentrations, the PAH can impinge the growth and immune system of the animal at the molecular level, causing the animal more susceptible to the infections. The results of the present study are based on a bioassay and the animals have the choice to swim away from the pollutants in natural systems. This is evident if the continuous reduction in the stocking population or growth rate of shrimps in Cochin Estuary (Aswathy 2008) is analysed in comparison with the level of PAH in the estuary. A reduction in the compound growth rate of penaeid shrimp from 2.83 % in 1985-1996 to -5.96 % in 1997-2006 period has been reported from the CE (Aswathy 2008). The available reports show the accumulation of PAH in the water column and sediment of Cochin Estuary during the same period, indicated by an increase from 193.29 ng/g in 2002 to 2.816 µg/g in 2006 (Latha 2006). Even higher concentrations of PAH have been reported recently from the CE (Ramzi et al. 2017). Looking into the life cycle of *P. monodon*, the gravid females migrate from sea to the offshore to lay eggs, followed by offshore planktonic larval phase (14 – 20 days), an estuarine, benthic post larval and juvenile phase (six

months), a coastal sub-adult phase (5 - 6 months) and return for the oceanic adult phase. The *P. monodon* are benthic in nature and feed voraciously on the organic matter in the surficial sediments. The supplementation of feed with chrysene could replicate the effect of PAH in sediment to the animal. Majority of the PAH studies in invertebrates have been undertaken exposing the animals to PAHs in the water column (Oberdörster et al. 2000; Farre´ and Barcelo´ 2003; Bellas et al. 2008; Bechmann et al. 2010; Ren et al. 2014; Ren et al. 2015a; Ren et al. 2015b; Laramore et al. 2016) whereas few studies were based on exposing the PAHs in sediments (Jaward et al. 2012; Wirth et al. 1998). The present study indicates that the microgram levels of PAH can impinge the immune and endocrine system of the post larval stages of shrimps at the molecular and hormonal level. It is assumed that the PAH in the surficial sediment of the CE might play significant role in retracting the migration of *P. monodon* and could be a reason for the reduction in their stocking density.

Conclusion

The present study shows that the microgram levels of PAH chrysene are toxic at the molecular level to the endocrine and immune systems of the larval stages of *P. monodon*. Such an effect may induce morbidity and mortality to post larvae in estuarine systems and otherwise, the gravid females will avoid laying eggs in such an environment. Either case the condition can affect the stocking density of shrimp larvae in the estuaries which may affect the ecosystem and shrimp industry. Therefore, more attention may be given to avoid PAH pollution in the estuaries to maintain a healthy ecosystem and to protect the animals from extinction.

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Conflict of Interest: The authors declare that they do not have any conflict of interest.

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Figure legends

Fig. 1 Percentage survival of *P. monodon* post larvae at concentrations 1, 2 and 3 $\mu\text{g/g}$ for a period of 15 days. Statistical significance depicted as “*” for $p < 0.05$.

Fig. 2 Moulting range of *P. monodon* post larvae at concentrations 1, 2 and 3 $\mu\text{g/g}$ for a period of 15 days in comparison to the range of moults in control 1. Statistical significance depicted as “***” for $p < 0.005$.

Fig. 3 Absolute quantitative RT-PCR of neuroendocrine hormone gene MIH I in *P.monodon* post larvae at concentrations 1, 2 and 3 $\mu\text{g/g}$ for time interval of day 0, 7, 11 and 15 in comparison to the control 1 (A- Control 1, B-1 $\mu\text{g/g}$, C- 2 $\mu\text{g/g}$, D- 3 $\mu\text{g/g}$). Statistical significance depicted as “****” for $p < 0.001$ and “***” for $p < 0.005$.

Fig. 4 Absolute quantitative RT-PCR of immune gene ProPO in *P.monodon* post larvae at concentrations 1, 2 and 3 $\mu\text{g/g}$ for time interval of day 0, 7, 11 and 15 in comparison to the control 1 (A- Control 1, B-1 $\mu\text{g/g}$, C- 2 $\mu\text{g/g}$, D- 3 $\mu\text{g/g}$). Statistical significance depicted as “***” for $p < 0.005$ and “*” for $p < 0.05$.

Fig. 5 Absolute quantitative RT-PCR of immune gene/antimicrobial peptide gene Crustin in *P.monodon* post larvae at concentrations 1, 2 and 3 $\mu\text{g/g}$ for time interval of day 0, 7, 11 and 15 in comparison to the control 1 (A- Control 1, B-1 $\mu\text{g/g}$, C- 2 $\mu\text{g/g}$, D- 3 $\mu\text{g/g}$). Statistical significance depicted as “****” for $p < 0.001$, “***” for $p < 0.005$ and “*” for $p < 0.05$.

Fig. 6 MIH I hormone level variations in tissue extract of *P.monodon* PL-25 at concentrations 1, 2 and 3 $\mu\text{g/g}$ for time interval of day 15 in comparison to the control 1. Statistical significance depicted as “*” for $p < 0.05$.

Sl no.	Gene	Primer sequence (5'-3')	Tm (°C)	Amplicon size (bp)	Reference
1.	MIH I	F- TAGTGCGTGTGTGTGAGGAT R- CCTGTTGGCAGCCTTTAGAC	56	119	Vrinda et al. 2016
2.	Crustin	F- AGTTCCTGGAGTTGGAGGTGGATT R- ACCTCGTTCTGCAGTAATTGCACTC	56	119	Dechamma, 2015
3.	ProPO	F- CGGTGACAAAGTTCCTCTTC R- GCAGGTCGCCGTAGTAAG	56	122	Dechamma 2015

Table 1 Primer details of three genes used for absolute quantitative real-time PCR analysis.

Figures

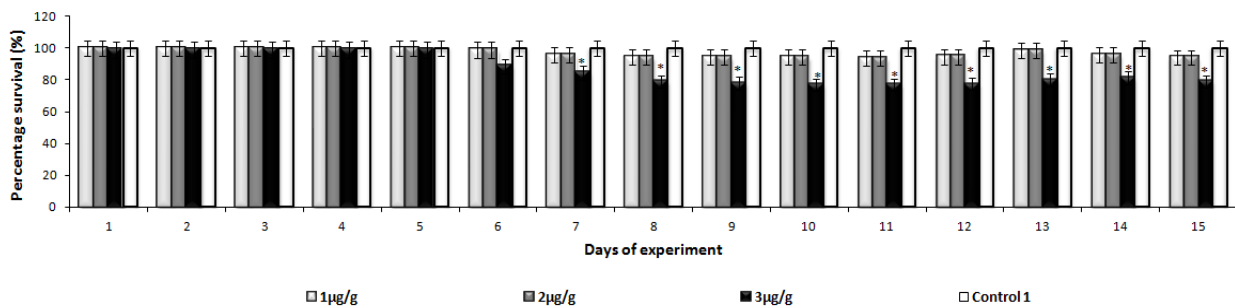


Fig. 1

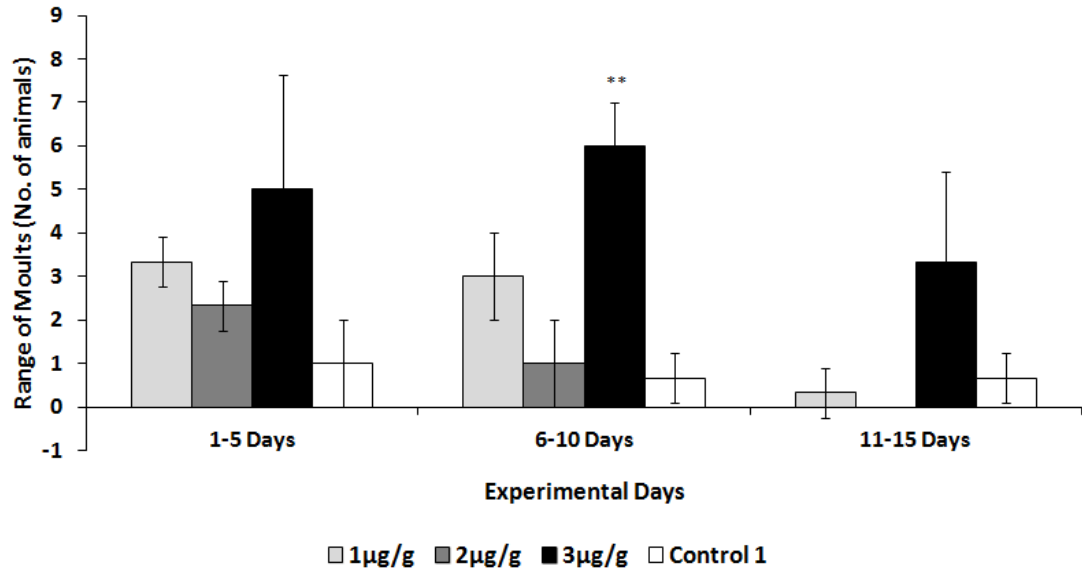


Fig. 2

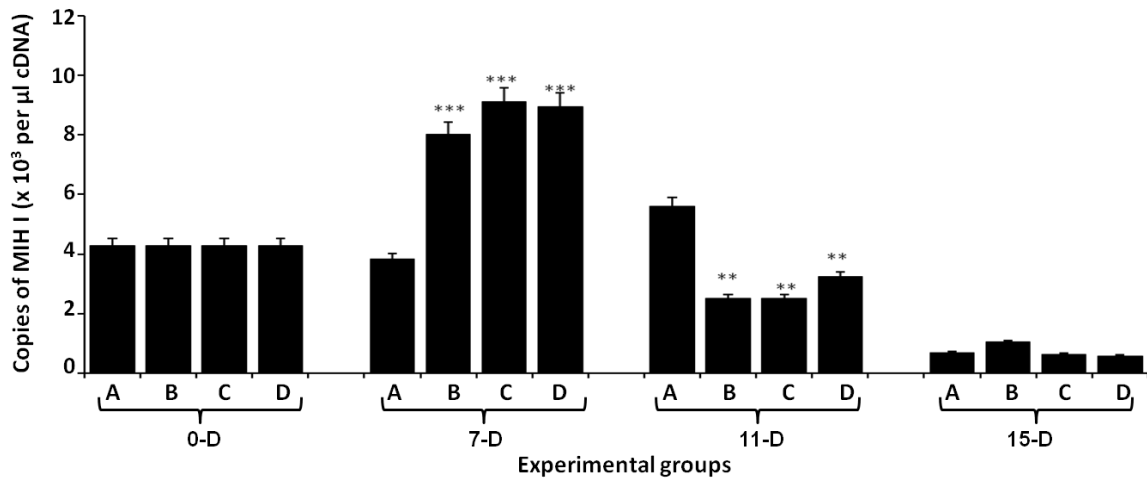


Fig. 3

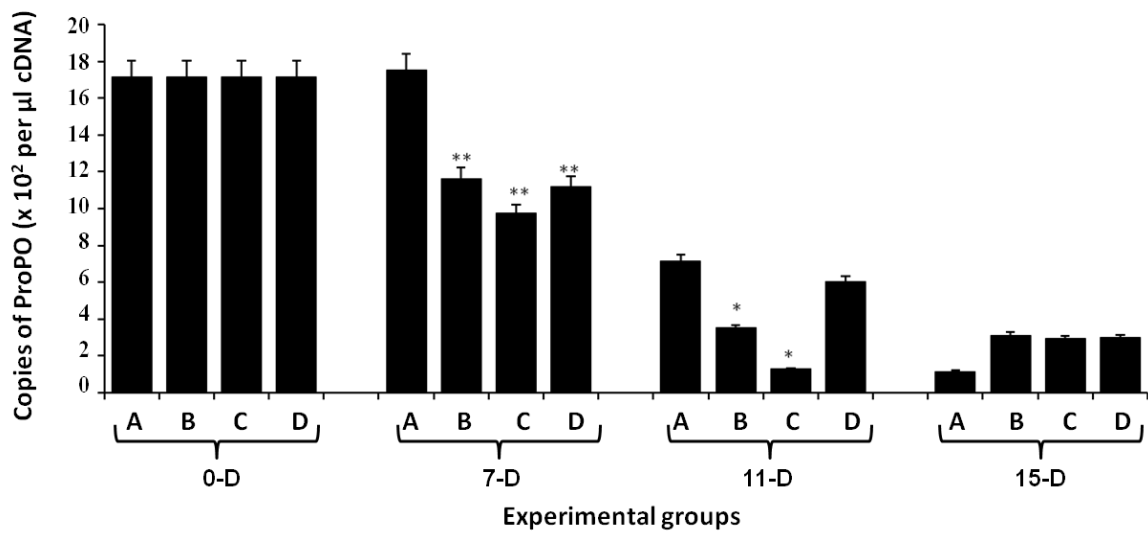


Fig. 4

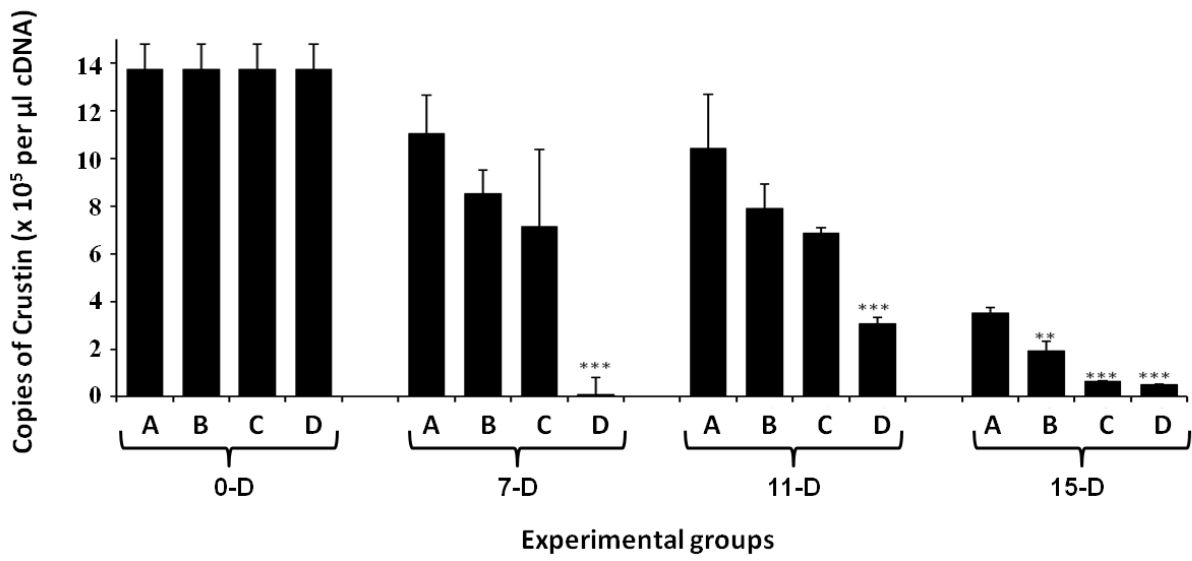


Fig. 5

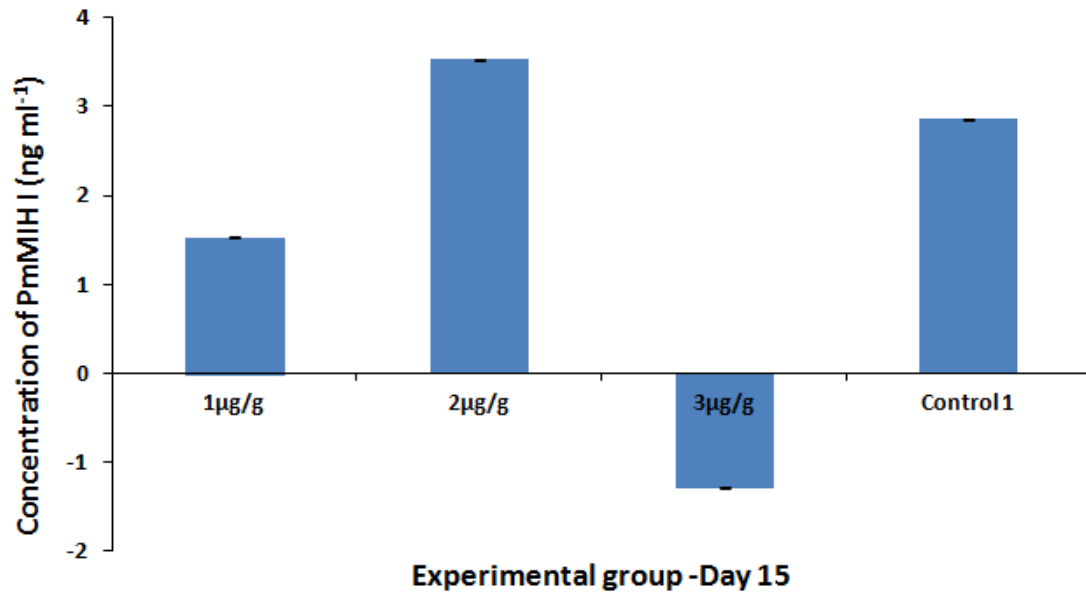


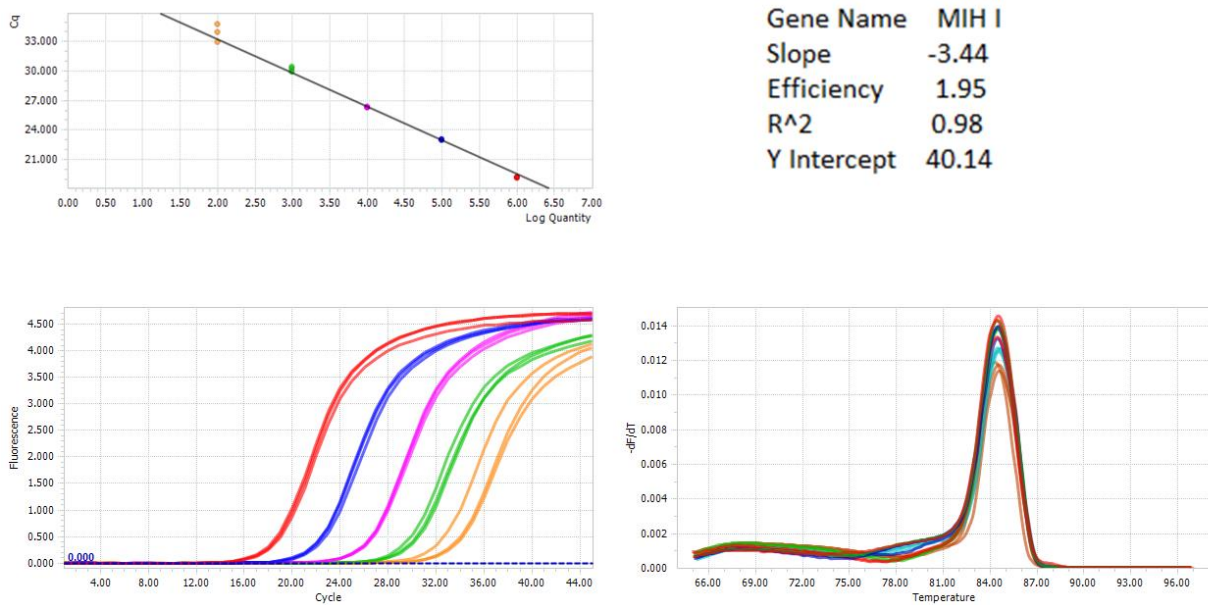
Fig. 6

Supplementary figure legends

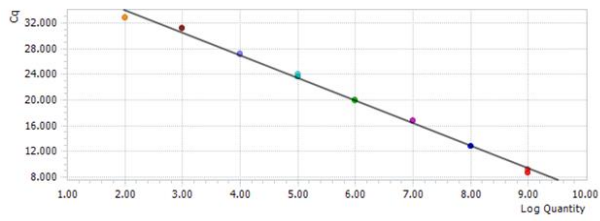
Suppl Fig. 1 Standard curve analysis, amplification curve and melt curve analysis of MIH I gene for absolute quantitative real-time PCR.

Suppl Fig. 2 Standard curve analysis, amplification curve and melt curve analysis of ProPO gene for absolute quantitative real-time PCR

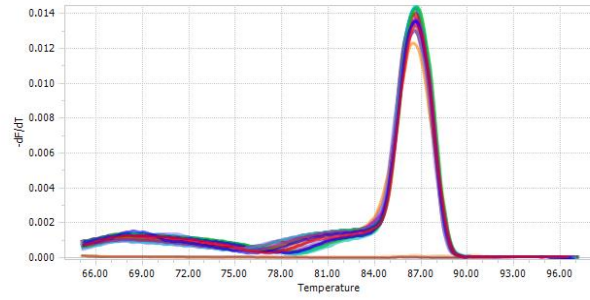
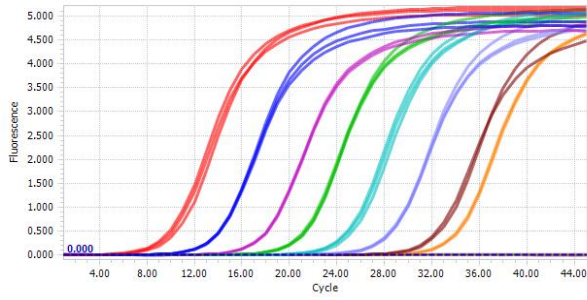
Suppl Fig. 3 Standard curve analysis, amplification curve and melt curve analysis of Crustin gene for absolute quantitative real-time PCR



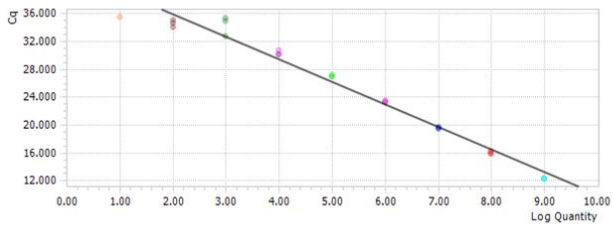
Suppl Fig. 1



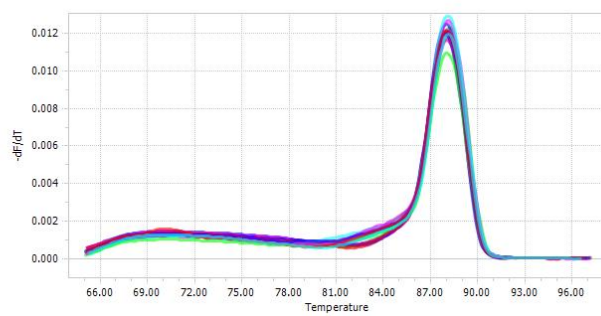
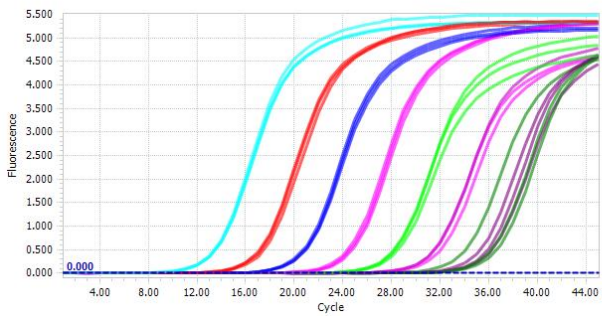
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Efficiency 1.98
R² 0.96
Y-Intercept 40.72



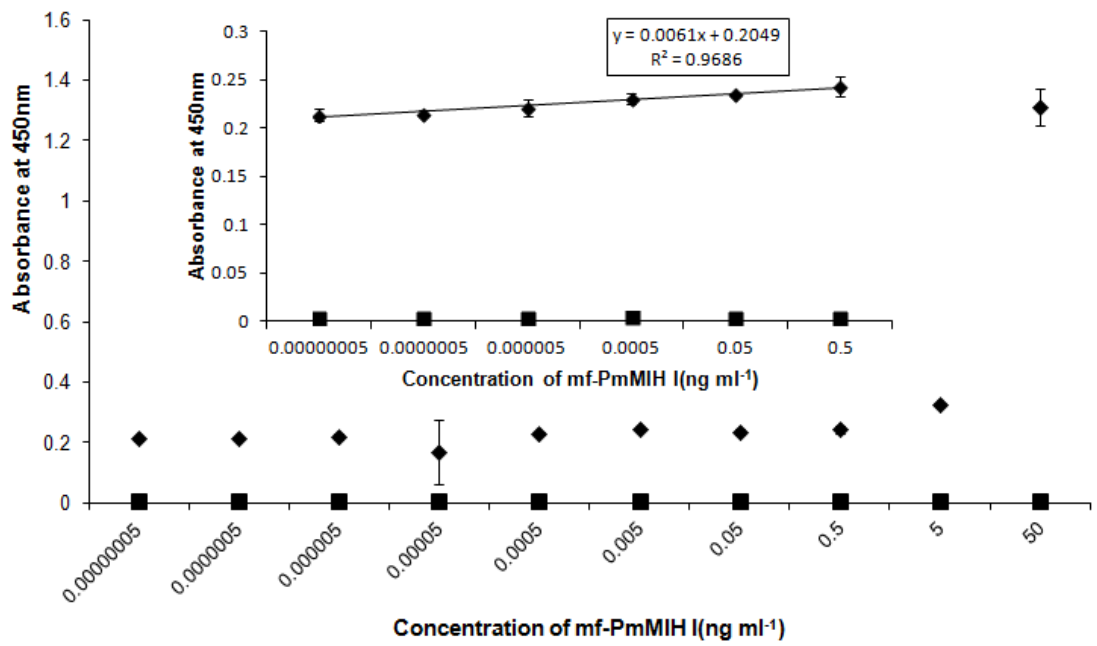
Suppl Fig. 2



Gene Name Crustin
Slope -3.2418
Efficiency 2.03
R² 0.97
Y-Intercept 42.35



Suppl Fig. 3



Suppl Fig. 4