Multifarious Biomarker Approach Elucidate Stocking Density Mediated Stress, Modulates Growth Attributes in Cage Reared Rohu, *Labeo Rohita* (Hamilton)

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Abstract

The present study was conducted for 240 days to evaluate the effects of stocking density based on growth attributes, digestive enzymes, muscular composition, biochemical and physiological responses of *Labeo rohita* fingerlings in tropical inland open water cages. *L. rohita* (30.35±1.08 g) were randomly distributed into three treatments, namely low stocking density, LSD (10 m^{-3}), medium stocking density, MSD (20 m^{-3}) and high stocking density, HSD (30 m^{-3}) in triplicates. Fish were fed twice daily with CIFRI CAGEGROW® floating feed (crude protein-28%, crude fat-4%). Fish growth and feed efficiency was higher ($P<0.05$) in LSD, however MSD registered higher yield. Amylase and protease activity reduced whereas lipase activity increased with increasing stocking density. Muscle crude protein and crude fat formed inverse correlation. The fillet quality deteriorated at higher stocking densities based on Muscle pH, drip loss and frozen leakage rate. The stress biomarkers level (glucose, cortisol, superoxide dismutase and catalase) increased in serum under crowding condition. Glutamate oxaloacetate transaminase and glutamate pyruvate transaminase in serum was significantly increased in HSD. Serum protein level decreased with increase in stocking densities. Body ionic imbalance (Na^+, Cl^- and K^+) observed under crowding stress. Based on growth attributes and multiple biomarker responses, *L. rohita* @ 10 m^{-3} found to be optimum density for inland open water cage culture.

Introduction

Stocking density optimization is a prerequisite for the development of protocol for practice of any candidate fish species and it differs based on species and its life stages, types of production system and management practice followed [1]. Over stocking produces significant stress which leads to growth retardation, poor health, lower survivability and yield loss [2,3]. The stocking density and growth are inversely correlated owing to the concurrence for food and space which in turn elucidate stress in fish [4]. However, understocking leads to poor production due to underutilization of available space and resources [2,5]. As the cage culture is an intensive farming system, the apparent efficiency of culture systems can be maximized by increasing the densities to optimal level [6,7].

Stressor in aquaculture covers a broad and diverse range of biotic and abiotic factors. Stocking density mediated stress has known physiological and behavioural consequences for the cultured fish [8]. The biochemical responses of fish to menacing stimuli are regularly controlled by physiological modification of the nervous and humoral system in order to maintain body homeostasis [9]. Fish subjected to stressors can drop the muscular flesh quality [10], decreased in digestive enzymes [11] and altered the serum biochemical parameters [12]. Stress can also increases adrenaline which rise blood pressure resulting increase the blood flow through gill lamellae and alter the exchange of ions [13].

Cage culture in inland open water is considered as relatively recent aquaculture innovation, has rapidly expanded during the past decades in fresh, estuarine, and marine open water bodies. Due to pressures for aquatic products across the globe, cage culture is presently undergoing swift changes [14,15]. In India, reservoirs and wetlands are the untapped open water resources suitable for freshwater cage culture [16]. India is bestowed with 19,370 reservoirs covering 15 states with an estimated area of 3.51 million ha surface area at its full capacity [17]. Utilizing a modest fraction of their surface area, cage culture can accord notable quantity of fish production to the total fish production basket of the country. At present, *Pangasianodon hypophthalmus* occupies the centre stage in inland cage culture in India. However, problems and prospects of this species in cage culture cannot be neglected. Farmed *P. hypophthalmus* from India fetches low prices in the domestic market and export as well [18]. Hence diversification of species in inland cages in India, which is having good market demand, is need of the hour [17].

Indian major carp is the mainstay of the Indian aquaculture contributing 80% of the total country aquaculture production [19], of which rohu (*Labeo rohita*) is in greater preference by the consumers. Despite a huge prospective for species diversification, the culture of *L. rohita* has been only restricted in pond and tanks and no scientific information available on its performances in cage culture. With this background, the present study was undertaken to bring out the optimum stocking density of *L. rohita* based on its growth attributes and by examining variety of endpoints to measure its physio-biochemical responses in inland open water cage culture. This is the first report on grow out cage culture of *L. rohita* in reservoirs. The study will be extremely beneficial for aquaculturist and researcher for standardizing the cage culture protocol of *L. rohita* in tropical reservoirs of India and Southeast Asian countries.

Result

Growth performance and feed efficiency

The growth characteristics among the groups were significantly differed ($P<0.05$) (Table 1). Higher growth performances in terms of FBW, WG and AGR were recorded in lower stocking density ($P<0.05$). The value of SGR was (1.29±0.01 g) in LSD followed by MSD (1.21±0.01 g) and HSD (1.07±0.03 g). Increase in stocking density led to decline in fish survival percentage ($P<0.05$) from LSD (88±0.11%) to HSD (66±0.18%). Fish reared at LSD reported higher ($P<0.05$) feed and protein utilization (PER and FCE) than MSD and HSD. On contrary, the lowest FCR (1.96±0.01) displayed in LSD while medium and high density showed significantly deleterious FCR value ($P<0.05$).

Serum biochemical indices

Post hoc analysis signifies that the level of serum glucose markedly increased ($P<0.05$) with increase in stocking density (Fig. 2A). The glucose concentration was ranged from 25.14±1.02 $mgdL^{-1}$ to 42.19±0.68 $mgdL^{-1}$ from LSD to HSD. The SOD level was increased significantly ($P<0.05$) in HSD (39.31±0.43 U mg $protein^{-1}$) and the extent of increase in SOD was 15% from LSD (Fig. 2A). There was no significant ($P>0.05$) changes in SOD level among lower and medium density. The CAT value was significantly ($P<0.05$) increased in MSD (29.93±0.81 U mg $protein^{-1}$) and HSD (35.69±0.06 U mg $protein^{-1}$), than LSD (26.80±0.58 U mg $protein^{-1}$) (Fig. 2A). Cortisol level demonstrated apparently higher in HSD with a factor of 29.89%, in comparison to LSD ($P<0.05$). However cortisol level did not vary among MSD and HSD (Fig. 2A).

The concentration of SGOT and SGPT in LSD and MSD varied significantly from HSD (P<0.05) as presented in table 2, however the level did not vary among low and medium density. The sum of total serum protein decreased significantly (P<0.05) with increasing stocking density from 3.29±0.01 gdL^{-1} in LSD to 2.33±0.08 gdL^{-1} in HSD (Table 2). Albumin, which is an important carrier for various hormones like thyroid, steroids and fatty acids varied from 0.96±0.01 gdL^{-1} to 0.72±0.01 gdL^{-1} from lower to higher stocking density. Serum globulins responsible for blood clotting and immunological functions proclaimed a decreasing trend from lower to higher stocking density. The ratio of albumin and globulin was found to be non-significant (P>0.05) amongst LSD and MSD but significant with HSD (Table 2).

The serum electrolytes like Na^+, K^+ and Cl^- were monitored and their values were represented in table 2. The level of Na^+, altered among lower and higher density treatments. Chloride ion decreased with the increased stocking density. Significant difference of K^+ and Cl^- values (p<0.05) have been observed in the lower and higher stocking density.

The serum thyroid level was estimated using the parameters T3 and T4 (Fig. 2B). The T3 value was not differed (P>0.05) significantly except in HSD (2.16±0.03 $ngmL^{-1}$), however T4 was decreased (P<0.05) with increase in stocking density and the lowest value recorded in HSD (3.68±0.06 $ngmL^{-1}$). The IGF1 value was highest at LSD (155.52±3.92 $ngmL^{-1}$) and it was significantly (P<0.05) declined in both MSD and HSD (Fig. 2C).

Digestive enzymes

The gut amylase, protease and lipase activities were analyzed and shown in figure 2D. Amylase was found to be inversely proportional to the stocking density and the value ranged from 19.30±0.35 U mg $protein^{-1}$ to 22.48±0.41 U mg $protein^{-1}$ from HSD to LSD. Protease, which is responsible for breakdown of proteins into smaller polypeptides or single amino acids did not differ (P>0.05) among LSD (10.25±0.12 U mg $protein^{-1}$) and MSD (9.46±0.18 U mg $protein^{-1}$) however it was markedly differed in HSD (7.88±0.05 U mg $protein^{-1}$). The lipase activity was found to be indifferent among MSD and HSD but varied in LSD (P<0.05).

Chemical composition of muscle and flesh quality

The muscular chemical compositions at different stocking densities were measured and presented in table 3. The crude protein content showed significant variation between HSD and LSD. The decreasing trend of crude lipid content among the treatments was observed though the values were insignificant between LSD and MSD. Stocking density had no impact (P>0.05) on moisture and ash content. The muscular pH declined with increase in stocking densities where the LSD showed the highest pH value (6.50±0.23) and lowest at HSD (5.8±0.17) as shown in table 3. Muscular DL and FLR decreased with increasing stocking density and the values are significantly differ between LSD and HSD (Table 3).

Nucleotide ratio

The ratio of RNA:DNA is an important sign of stress in the fishes as shown in the figure 2E. The quantity of DNA did not vary significantly (P>0.05), however the quantity of RNA varied significantly (P<0.05) among LSD (1.33±0.08 μgmg^{-1}) and HSD (1.56 ±0.12 μgmg^{-1}). The nucleotide ratio (RNA:DNA) was found to be decreased with increasing stocking density (P<0.05).

Univariate and multivariate statistical analysis

Pearson correlations among the parameters are depicted in figure 3A. The correlation coefficient suggested that correlated parameters are closely associated and their behaviours are inter-dependent to each other. This correlation was further confirmed by principal component analysis (PCA). Two PC were obtained having eigenvalues >1. PC1 and PC2 constitutes eigenvalues 32.23 and 4.76 and the variance 87.12% and 12.88% respectively. PC1 showed strong positive correlation among FBW, WG, SGR, AGR, survival, PER, FCE, amylase, protease, K^+, Cl^-, RNA, RNA:DNA, moisture, CF, pH, serum protein, albumin, globulin, T3, T4, and IGF1 (Fig. 3B). However, a negative correlation exist among FCR, lipase, CP, DL, FLR, serum glucose, cortisol, SGOT, SGPT, SOD and CAT (Fig. 3B). PC2 didn't show any significant correlation among the vital parameters. Hierarchical clustering dendrogram formed two groups among the three stocking densities. Cluster one noticed between LSD and MSD while HSD formed a separate cluster (Fig. 3C).

Discussion

Stocking density, nutrition and appropriate culture environment are considered as vital factor which affects the fish growth and production [20] however overcrowding negatively affect the growth of fish [21]. In the present study, stress led by crowding has obvious effect on growth attributes like FBW, WG, SGR, AGR and survival which are significantly different among LSD, MSD and HSD. The present findings proclaimed that the feed utilization and protein efficiency ratio was found significantly greater in lower stocking density, however, higher density had a deleterious impact on FCR, FCE and PER. Growth retardation, poor production and feed utilization in HSD could be the result of crowding which triggered a raising demand for energy to activate the physiological functions to combat the stress by decreasing appetite and food intake, and led to reduction in the available energy for growth [22–24]. The major studies on evaluation of stocking density were observed on various tropical as well as temperate fishes and also in different culture systems. The similar effect of crowding stress on growth and feed utilization was also observed in common carp, *Cyprinus carpio* [25–27]; grass carp, *Ctenopharyngodon idella* [28,29]; olive barb, *Puntius sarana* [7]; Amazon fish, *Colossoma macropomum* [30] and Nile tilapia, *Oreochromis niloticus* [31,32]. On contrary, many authors could not establish any relation between stocking density and growth attributes in silver perch, *Bidyanus bidyanus* [33], Atlantic sturgeon, *Acipenser oxyrinchus* [34]. Thus stocking density (10 m^{-3}) of *L. rohita* ensured the best growth performances in term of weight gain, survival and feed and protein utilization.

Glucose is considered as an indicator of secondary stress response in fishes [35,36]. Stressors such as transportation, stocking density, confinement and bad handling have found to be responsible for increase in blood-glucose and whole body-glucose level. The increased level of catecholamine results in activation

of glycogenolysis and gluconeogenesis, which ultimately leads to rise of glucose level in blood [37]. It is a secondary stress response which shifts the glucose to body tissue in order to cope with restoration of energy demanding activity. In the present study, increased blood glucose level (P<0.05) from LSD to HSD was observed with an increment in stocking density followed by crowding stress. Similar findings on stocking density mediated stress elevates blood glucose level observed in olive barb, *P. sarana* [7], Gilthead seabream, *Sparus auratus* [38]; Senegalese sole, *Solea senegalensis* [39]; Chinese sturgeon, *Acipenser sinensis* [40]; common carp, *C. carpio* [25,26] and Asian seabass, *Lates calcarifer* [41].

Free radicals and reactive oxygen species (ROS) are produced continuously under stressful conditions to combat the damage of antioxidant abilities and also act as a scavenger of excessive superoxide generated in the body. SOD and CAT played a key antioxidant enzyme in the animal defence system, to function against oxidative stress, and with the increase in stress; its value tends to ascend [42,43]. SOD level in serum of *L. rohita* at higher density increased significantly. Similar to the observations with an increment of SOD and CAT with increased stocking density observed in cage reared olive barb, *P. sarana* [7], common carp, *C. carpio* [25,26], Nile tilapia, *O. niloticus* [11], GIFT strain of Tilapia [44], and dourado, *Salminus brasiliensis* [9].

Cortisol released as an indicator of chronic and acute stress which elevates the expenditure of energy and hence the stored somatic energy expenses also subsequently increase, which ultimately led to growth retardation in fish [25,41]. The cortisol level of *L. rohita* elevated at high density (P<0.05). Moreover increase the level of cortisol and glucose were associated with the reduction in muscular fat. In concordance with the present study, growth retardation and cortisol level positively correlated in olive barb, *P. sarana* [7], common carp, *C. carpio* [25–27, 45]. GIFT strain of Tilapia [44],. In contrast to the present finding, cortisol level remained unaffected in African catfish, *Clarias gariepinus* [46] at higher density, but decreased in Nile tilapia, *O. niloticus* in biofloc based systems [11].

The major thyroid hormones, triiodothyronine (T3) and thyroxine (T4) play an important role in growth and development of fishes [47] which are majorly affected by crowding stress, hypoxic condition and starvation [48]. The crowding stress plays a major role in elevation of cortisol level and the increase in cortisol level has a negative feedback mechanism on hypothalamus-pituitary-interrenal axis thus decreasing the peripheral circulation of T3 and T4. Unless the thyroid hormones are not in free form, they cannot be utilized by the fish, leading to poor growth. The elevated crowding stress led the fishes towards growth retardation, also towards less availability of thyroid hormone in fish serum, which was observed in the present study. In our study, the decrease in level of T3 and T4 with increase in stocking density was supported by the earlier findings in Amur sturgeon (*Acipenser schrenckii*), mosquito fish (*Gambusia holbrooki*), channel catfish (*Ictalurus punctatus*) and olive barb (*P. sarana*) [7,49–51] respectively.

The IGF1 axis has a prominent role in regulations of various physiological responses in fishes [52,53]. Due to the secretion of IGF 1, growth hormone has a prominent impact on growth regulation in fishes [54]. Due to the stress factors like overcrowding in the present study, the somatic growth was inhibited significantly in MSD and HSD; also these groups of fishes were encountered with significantly lower IGF 1 concentration in their serum. The findings were consistent with earlier studies where stress affects the secretion of IGF in tilapia, *Oreochromis mossambicus*; Chinese sturgeon, *A. sinensis*; Amur sturgeon, *A. schrenckii*; Senegalese sole, *S. senegalensis* [40,55–57] respectively. The result divulged that high cortisol level under stressful conditions ,inhibition the secretion of growth hormone and IGF 1 [58].

SGOT and SGPT, both are ubiquitous aminotransferases in the mitochondrion of fish and used as indicator of hepatic ruination [59]. The enzymes are coerced to be released into bloodstream when the liver gets damaged. In the present finding, SGOT and SGPT activity upraised in *L. rohita* due to crowding stress in HSD; however the change in such values among LSD and MSD was not varied significantly. This result might also reflect that increase of these enzymes in the serum was occurred due to over utilization of hydrocarbons in order to meet the stress mediated energy demands by fish. Similar types of outcome also observed in olive barb, *P. sarana* [7], GIFT strain of Tilapia [21,60], Channel catfish, *Ictalurus punctatus* [51], Marbled eel, *Anguilla marmorata* [61], Amur sturgeon, *A. schrenckii* [62], Nile tilapia, *O. niloticus* [63] and rohu, *L. rohita* [64,65].. Those means any kind of unpleasant living condition or stress situations could lead to the injury of the hepatic function.

Total protein, albumin and globulin content in the serum plays a pivotal role in fish innate immune response; chiefly during the stressful conditions such as dietary irregularity, high stocking density, infections due to disease and other environmental stress factors [66].These parameters are also used as indicator of humeral defence system [67]. The drop in serum protein, albumin and globulin and their ratio in the higher stocking density (HSD) was a clear image of compromised innate immunity which may be due to inhibition of protein synthesis, liver cell lesions, kidney dysfunction or malnutrition. The crowding stress has also badly affected the serum protein and albumin concentration in Nile tilapia, *O. niloticus* [31] and common carp, *C. carpio* [25].

Neuroendocrine system controls the acute and chronic stress responses which release catecholamines and cortisol [68], both of the steroid are responsible for controlling the ionic regulation in fish body by the ion concentrations and their exchange between the body and surrounding environment called osmoregulation [69]. The responses of fish to the stress are related to the neuroendocrine system, which is a critical part of osmoregulatory adaptations [70]. This present study showed that the Na^+ level did not vary (P>0.05) among the treatments except LSD and HSD however, the levels of Cl^- and K^+ decreased with increasing stocking density and the lowest value were noticed in high density. The result may be attributed by excessive blood flow in gills and the permeability of the epithelium, resulting in ionic losses in freshwater fish [71]. A similar finding was recorded in Atlantic salmon, *Salmo salar* [72], however contrasting results have seen in case of salmon smolts, *Salmo salar* [73] and rainbow trout, *Oncorhynchus mykiss* [74].

The digestive enzymes were affected by increase in stocking density, since the crowding stress could force the fish body metabolism to channelize their energy towards coping of stress conditions. Thus, it can also imply that the digestion and utilization of feed in fishes could be affected by crowding stress caused by higher stocking densities [25]. In present study, the amylase activity was decreased significantly with increasing stocking density. Crowding stress also caused decline in amylase activity in olive barb, *P. sarana* [7]; common carp, *C. carpio*, [25]; rainbow trout, *Oncorhynchus mykiss* [75] and turbot, *Scophthalmus maximus* [76]. The decrease in lipase activity with increase in stocking density among the treatments with *L. rohita* indicates that, the utilization of body fat

during crowing stress led to increase in lipase activity. In present study, due to crowding stress, protease and lipase activity in fishes were found declining with increase in stocking density, although the change was not significant among LSD and MSD. The crowding stress has also been found affecting the digestive enzymes of fishes like common carp, *C. carpio* [25]; marbled eel, *A. marmorata* [61] and rainbow trout, *Oncorhynchus mykiss* [75].

Energy metabolism in fish body can also be indicated by flesh composition [77]. In present study, deterioration in growth parameters suggests the decline in metabolism. A number of factors can be a reflection to change in body flesh composition of the stocked fishes, including water quality, crowding stress, nutrient availability, feed intake and the follow up utilization [32]. In current study, significant decline of lipid extract was noticed in HSD which can be attributed due to chronic crowd stressing in cages followed by lesser uptake of feed resulting lower accumulation of lipid in fish fleshes. In other hand, crowding stress enhanced the process of lipid metabolism in order to meet growing demand of energy followed by decreased lipid content in some other fish species as Amur sturgeon, *A. Schrenckii*[78]; juvenile blunt snout bream. *M. amblycephala*[23] and Nile tilapia, *O. niloticus*[79]. In contrast, lipid content increased with increasing stocking density in African catfish, *Clarias gariepinus* and rainbow trout, *O. mykiss*[80,81]. The dissimilar results of these above studies could be due to the differences in fish species, age, size and the exposure time of crowding stress. Crude protein content of fish muscle was increased significantly in HSD in comparison to LSD. The present result corroborate with [61] in *A. marmorata* where crude protein content increases (P>0.05) with increment in stocking density.

The muscle pH is an important flesh quality parameter and under stressful condition, fishes produce low pH muscle [10,82,83]. In present study, lower pH was exhibited in higher densities. It is possibly induced by chronic crowding stress which stimulates lactate acid formation in muscle [51,83]. Similar findings of lower pH and water holding capacity were obtained in case of rainbow trout, *O. mykiss* [81]. The DL and FLR in HSD displayed a significant increasing trend, which indicate the deterioration of flesh quality attributed by lower muscular pH. According to [51], DL and FLR is inversely correlated to muscular pH in channel catfish, *I. punctatus*.

RNA, as a reflection of protein synthesis and expected to increase with an increase in the growth of somatic tissues [84], whereas DNA, which is a genetic carrier molecule, and the quantity remains constant [85]. The RNA : DNA is sensitive to nutrition and as an index of the cell's metabolic intensity and closely related to protein synthesis [86]. In the present experiment, the concentration of DNA did not vary (P>0.05) among the three stocking densities of *L. rohita* however the RNA and RNA to DNA ratio was significantly decreased (P<0.05) with an increase in stocking densities. The present study was endorsed by previous studies in Japanese flounder, *Paralichthys olivaceus* [87]; rohu, *L. rohita* [88,89] and catla, *C. catla* [90].

In the present study, Pearson correlation coefficient and PCA analysis depicted that growth attributes like FBW, WG, SGR, AGR, survival, PER and FCE and biochemical parameters namely serum protein, albumin, globulin, T3, T4, and IGF1 are strongly correlated. However a negatively correlation established among serum glucose, cortisol, SGOT, SGPT, SOD and CAT. These correlated parameters are interdependent to each other and their responses are similar under crowding stress. Similar observation reported in *P. sarana* reared in cages [7]. The outcome of PCA analysis revealed that stress responses negatively influenced while, decreased in the level of digestive enzymes (amylase and protease), serum protein and thyroid hormones (T3, T4 and IGF1) are positively influence on the growth attributes. Cluster analysis depicted a separate cluster by HSD indicates the bio-markers are significantly differ in HSD comparison to LSD and MSD.

Material And Methods

Ethical statement

The study protocol and the experiment conducted was approved by the ethical committee of ICAR-Central Inland Fisheries Research Institute, Barrackpore (IAEC/2020/04). The present study is in compliance with the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines (http://arriveguidelines.org/).

Experimental facilities and animals

The present study was carried out in Salia reservoir (N19°48.3887′ and E85°03.6983′), Odisha, India (Fig. 1a and 1b) in floating rectangular cages made of high density polyethylene (HDPE) of dimension 6m x 4m x 4m with an effective volume of 84 m^{-3} for a period of 240 days. Advance fingerlings of *L. rohita* (14.25±0.19 cm and 30.35±1.08 g) was stocked at three different stocking density, namely LSD (10 m^{-3}), MSD (20 m^{-3}), and HSD (30 m^{-3}) in triplicates as per complete randomized design (CRD). Fishes were fed with commercial extruded floating feed (28% protein and 4% fat) at a rate of 3 % of body weight twice (09.00 hour and 16.00 hour). The feed rations were adjusted based on the estimated fish biomass in every week. During the study, antibiotics, chemicals and medicines were strictly avoided. The health status of fish was monitored at regular basis (Fig.1c)

Growth performance and feed efficiency

The growth performances were assessed in every week by randomly lifting 30 fishes from cages in order to adjust the feed ration however other parameters were studied at the end of the experiment. Prior to sampling, the feeding was withheld for 24 h. The parameters such as weight gain (WG), absolute growth rate (AGR), specific growth rate (SGR), total yield and survival were calculated as follows: weight gain (g) = FBW (g) – IBW (g) where IBW = Initial body weight (g), FBW = Final body weight (g), absolute growth rate = [FBW (g) – IBW (g)]/culture period (days), specific growth rate (%) = (ln FBW – ln IBW) × 100/culture period (days), yield (kgm^{-3}) = total biomass of fish harvested per m^3 volume at the end of experiment, survival (%) = (number of fish harvested / number of fish stocked) × 100. The feed efficiency was assessed by feed conversion ratio (FCR), feed conversion efficiency (FCE) and protein efficiency ratio (PER) and were calculated as follows: FCR = dry weight of diet fed (g)/wet weight gain of fish (g), FCE = wet weight gain of fish (g)/dry weight of diet fed (g), PER = wet weight gain of fish (g)/crude protein intake (g).

Serum sample collection and preservation

Five fish from each cage were randomly collected and anesthetized using clove oil @ 50 µL per liter water. The blood sample was collected using 2 mL sterile disposable syringe, by puncturing the caudal vein and kept in 1.5 mL eppendorf tube for 30 minute for coagulation. The blood samples were centrifuged @ 4000 rpm for 10 minute at 4 °C, the straw colored serum was pipetted out. The serum samples were carried to laboratory in dry ice and stored at -80 °C for further analysis.

Serum biochemical indices

The automated blood biochemistry analyzer (Transaia-Erba, EM-2000, USA) was used to measure serum parameters such as protein, albumin, glucose, serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) using necessary reagents provided by manufacturer. With additional use of ISE module in the same instrument, the electrolytic balance of fish such as Na^+, K^+ and Cl^- were estimated. Serum globulin was calculated by subtracting serum albumin from serum protein, and A/G ratio was calculated as albumin/globulin.

Superoxide dismutase (SOD) was assayed using carbonate-bicarbonate buffer (0.1 M) with epinephrine (3 mM) [91]. The change in optical density was measured at 480 nm (BioTek's Epoch™ 2). Catalase (CAT) activity was analyzed using phosphate buffer (50 mM, pH 7.0) with H_2O_2 solution [92]. The change in OD value was measured at 240 nm (BioTek's Epoch™ 2).

Cortisol, tri-iodothyronine (T3), thyroxine (T4) and insulin-like growth factor 1 (IGF1) in serum were quantified using enzyme-linked immune survey assay (ELISA) kit (BT Bio Assay, Shanghai, China) as per the manufacturer's instruction and the final OD value was taken at 450 nm (BioTek's Epoch™ 2).

Digestive enzyme assay

The fish were dissected from each treatment and the gut tissues were kept in 0.25M sucrose solution. The samples were homogenized using tissuelyser (Qiagen, Hilden, Germany), and centrifuged at 10000 rpm for 10 minutes in 4 °C and the supernatant was collected to store in -80 °C for further analysis. Amylase activity was assayed using 3,5-dinitrosalicylic acid method [93] by observing reducing sugar production by the gluco-amylase and α-amylase. Lipase activity was measured using phenolphthalein indicator based titration method [94]. The casein digestion method (triphosphate buffer- pH 7.8, trichloroacetic acid) was performed to measure protease activity [95].

Chemical composition of muscle and flesh quality

Fish flesh samples (n = 3) from all the treatments were collected. The proximate composition assessed as per [96]. Moisture content was estimated via desiccation in an oven at 105 °C for 30 minutes and then cooling and weighing to a constant weight. Crude protein (nitrogen × 6.25) was determined by the Kjeldhal method using kjeltec System (Tecater 1002 Distilling Unit), crude fat content was evaluated by using extraction with ether by soxtech system (Tecater 1043 Extraction Unit). The ash content was assessed by incineration in a muffle furnace at 550 ± 10 °C for 12 h. Muscular pH (n = 6) was measured in three different parts of the body (two dorsal sides and one caudal side), then average was calculated. The fish was dissected and insert a pH probe (Thermo orion A211) into the muscular part. Before every use, the probe was calibrated with pH 4 & pH 7 buffer. Drip loss (DL) was calculated as per [97], the sample was weighed and kept in a vacuum polythene bag and stored at 4℃ for 72 hour. After thawing and the fish fillets were taken out from the bag and wiped with a paper towel and weighed. The calculation of the drip loss was based on difference of initial sample weight and weight after 72 hr. DL = {100 × (Initial weight-weight after 72 hr)/Initial weight}**.** For FLR, the sample was weighed and kept in a vacuum polythene bag and stored at -20°C for 72 hours [98]. After thawing, the fish fillets were taken out from the bag and wiped with a paper towel and weighed. The calculation of FLR was as follows; FLR = [100*(Initial weight-weight after 72 hr)/Initial weight].

Nucleotide ratio

Fish muscle tissue were collected in RNA*later* and 70% ethanol for RNA and DNA isolation. DNA and RNA from muscle tissue were extracted using DNeasy and RNeasy kit (Qiagen Hilden, Germany) respectively as per manufacturer instruction. The concentration of DNA and RNA samples were measured by Nanodrop plate at 260/280 (BioTek's Epoch™ 2, USA). After quantification, RNA/DNA ratio was calculated.

Statistical analyses

The data generated from all the parameters were subjected to one way analysis of variance (ANOVA) and Duncan's multiple range test to determine significant differences among the means and undergone principal component analysis, using statistical software SPSS 22.0, in which $P<0.05$ considered statistically significant. Pearson correlation and hierarchical clustering analysis was carried out using PAST, 4.03 software.

Conclusion

In conclusion, the present study accentuates that stocking density has a distinctive effect on fish physio-biochemical responses and importance of evaluating density stress, towards determining optimal density to warrant the fish production.The stocking density mediated crowding stress negatively affects the growth attributes like WG, SGR and percentage survival. Poor digestive enzymes (amylase and protease) activity and flesh quality, fall of thyroid activity (T3, T4 and IGF1) and serum electrolytes imbalance has been perceived with increment in stocking density. However, elevated levels of the stress response like SOD, CAT, SGOT, SGPT, serum cortisol and glucose were encountered at higher stocking density. Based on growth attributes and multiple biomarker responses

it is suggested that the optimum stocking density of Indian major carp (*L. rohita*) for tropical inland open water cage culture is 10 m^{-3}. This is the first ever attempted in inland cage reared fish to optimize stocking density based on multiple biomarker responses.

Declarations

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Conflict of interest

Authors have no conflict of interest

Author's contribution

HSS: conceptualization, methodology, formal analysis, investigation, data curation, writing – original draft; BKD: conceptualization, validation, review and editing, supervision, funding acquisition, AU: formal analysis, data curation, writing – review and editing; MHR: formal analysis, data curation, writing – review and editing, VK: software, data curation, writing – review and editing, DKM: writing – review and editing, formal analysis, data curation, UKS: writing – review and editing, supervision, NKC: writing – review and editing, supervision, KDR: writing – review and editing, supervision.

References

1. Tolussi, C. E., Hilsdorf, A. W. S., Caneppele, D. & Moreira, R. G. The effects of stocking density in physiological parameters and growth of the endangered teleost species piabanha, *Brycon insignis* (Steindachner, 1877). *Aquaculture* **310**, 221–228 (2010).
2. Wang, Y. *et al.* Effects of stocking density on growth, serum parameters, antioxidant status, liver and intestine histology and gene expression of largemouth bass (*Micropterus salmoides*) farmed in the in-pond raceway system. *Aquac. Res.* **51**, 5228–5240 (2020).
3. Zahedi, S., Akbarzadeh, A., Mehrzad, J., Noori, A. & Harsij, M. Effect of stocking density on growth performance, plasma biochemistry and muscle gene expression in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **498**, 271–278 (2019).
4. Yousefi, M., Paktinat, M., Mahmoudi, N., Pérez-Jiménez, A. & Hoseini, S. M. Serum biochemical and non-specific immune responses of rainbow trout (*Oncorhynchus mykiss*) to dietary nucleotide and chronic stress. *Fish Physiol. Biochem.* **42**, 1417–1425 (2016).
5. Duan, Y., Dong, X., Zhang, X. & Miao, Z. Effects of dissolved oxygen concentration and stocking density on the growth, energy budget and body composition of juvenile Japanese flounder, *Paralichthys olivaceus* (Temminck et Schlegel). *Aquac. Res.* **42**, 407–416 (2011).
6. Castillo-Vargasmachuca, S. *et al.* Effect of stocking density on growth performance and yield of subadult pacific red snapper cultured in floating sea cages. *N. Am. J. Aquac.* **74**, 413–418 (2012).
7. Upadhyay, A. *et al.* Stocking density matters in open water cage culture: Influence on growth, digestive enzymes, haemato-immuno and stress responses of *Puntius sarana* (Ham, 1822). *Aquaculture* 737445 (2021).
8. Rebl, A. *et al.* The synergistic interaction of thermal stress coupled with overstocking strongly modulates the transcriptomic activity and immune capacity of rainbow trout (*Oncorhynchus mykiss*). *Sci. Rep.* **10**, 1–15 (2020).
9. Braun, N., de Lima, R. L., Baldisserotto, B., Dafre, A. L. & de Oliveira Nuñer, A. P. Growth, biochemical and physiological responses of *Salminus brasiliensis* with different stocking densities and handling. *Aquaculture* **301**, 22–30 (2010).
10. Refaey, M. M., Tian, X., Tang, R. & Li, D. Changes in physiological responses, muscular composition and flesh quality of channel catfish *Ictalurus punctatus* suffering from transport stress. *Aquaculture* **478**, 9–15 (2017).
11. Liu, G. *et al.* Influence of stocking density on growth, digestive enzyme activities, immune responses, antioxidant of *Oreochromis niloticus* fingerlings in biofloc systems. *Fish Shellfish Immunol.* **81**, 416–422 (2018).
12. Kumar, G. & Engle, C. R. Technological advances that led to growth of shrimp, salmon, and tilapia farming. *Rev. Fish. Sci. Aquac.* **24**, 136–152 (2016).
13. Sundin, L. Hypoxia and blood flow control in fish gills. *Biol. Trop. Fishes. Manaus INPA* 353–362 (1999).
14. Beveridge, M. C. M. *Cage aquaculture*. vol. 5 (John Wiley & Sons, 2008).
15. Valenti, W. C., Barros, H. P., Moraes-Valenti, P., Bueno, G. W. & Cavalli, R. O. Aquaculture in Brazil: past, present and future. *Aquac. Reports* **19**, 100611 (2021).
16. Das, A. K., Meena, D. K. & Sharma, A. P. Cage farming in an Indian Reservoir. *World Aquac.* **45**, 56–59 (2014).
17. Sarkar, U. K. *et al.* Status, prospects, threats, and the way forward for sustainable management and enhancement of the tropical Indian reservoir fisheries: an overview. *Rev. Fish. Sci. Aquac.* **26**, 155–175 (2018).
18. Singh, A. K. & Lakra, W. S. Culture of *Pangasianodon hypophthalmus* into India: impacts and present scenario. *Pakistan J. Biol. Sci.* **15**, 19 (2012).
19. Jena, J. *et al.* Evaluation of growth performance of Labeo fimbriatus (Bloch), Labeo gonius (Hamilton) and *Puntius gonionotus* (Bleeker) in polyculture with *Labeo rohita* (Hamilton) during fingerlings rearing at varied densities. *Aquaculture* **319**, 493–496 (2011).
20. Liu, B., Jia, R., Han, C., Huang, B. & Lei, J.-L. Effects of stocking density on antioxidant status, metabolism and immune response in juvenile turbot (*Scophthalmus maximus*). *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* **190**, 1–8 (2016).

21. Wu, F. *et al.* Effect of stocking density on growth performance, serum biochemical parameters, and muscle texture properties of genetically improved farm tilapia, *Oreochromis niloticus*. *Aquac. Int.* **26**, 1247–1259 (2018).
22. Andrade, T. *et al.* Evaluation of different stocking densities in a Senegalese sole (*Solea senegalensis*) farm: implications for growth, humoral immune parameters and oxidative status. *Aquaculture* **438**, 6–11 (2015).
23. Qi, C. *et al.* Effect of stocking density on growth, physiological responses, and body composition of juvenile blunt snout bream, *Megalobrama amblycephala*. *J. World Aquac. Soc.* **47**, 358–368 (2016).
24. Shao, T. *et al.* Evaluation of the effects of different stocking densities on growth and stress responses of juvenile hybrid grouper♀ *Epinephelus fuscoguttatus*×♂ *Epinephelus lanceolatus* in recirculating aquaculture systems. *J. Fish Biol.* **95**, 1022–1029 (2019).
25. Adineh, H., Naderi, M., Hamidi, M. K. & Harsij, M. Biofloc technology improves growth, innate immune responses, oxidative status, and resistance to acute stress in common carp (*Cyprinus carpio*) under high stocking density. *Fish Shellfish Immunol.* **95**, 440–448 (2019).
26. Fazelan, Z., Vatnikov, Y. A., Kulikov, E. V., Plushikov, V. G. & Yousefi, M. Effects of dietary ginger (Zingiber officinale) administration on growth performance and stress, immunological, and antioxidant responses of common carp (*Cyprinus carpio*) reared under high stocking density. *Aquaculture* **518**, 734833 (2020).
27. Hoseini, S. M., Yousefi, M., Hoseinifar, S. H. & Van Doan, H. Effects of dietary arginine supplementation on growth, biochemical, and immunological responses of common carp (*Cyprinus carpio* L.), stressed by stocking density. *Aquaculture* **503**, 452–459 (2019).
28. Adineh, H., Naderi, M., Nazer, A., Yousefi, M. & Ahmadifar, E. Interactive effects of stocking density and dietary supplementation with Nano selenium and garlic extract on growth, feed utilization, digestive enzymes, stress responses, and antioxidant capacity of grass carp, *Ctenopharyngodon idella*. *J. World Aquac. Soc.* **52**, 789–804 (2021).
29. Zhao, H. *et al.* Transcriptome and physiological analysis reveal alterations in muscle metabolisms and immune responses of grass carp (*Ctenopharyngodon idellus*) cultured at different stocking densities. *Aquaculture* **503**, 186–197 (2019).
30. Frisso, R. M., de Matos, F. T., Moro, G. V. & de Mattos, B. O. Stocking density of Amazon fish (*Colossoma macropomum*) farmed in a continental neotropical reservoir with a net cages system. *Aquaculture* **529**, 735702 (2020).
31. Tammam, M. S., Wassef, E. A., Toutou, M. M. & El-Sayed, A.-F. M. Combined effects of surface area of periphyton substrates and stocking density on growth performance, health status, and immune response of Nile tilapia (*Oreochromis niloticus*) produced in cages. *J. Appl. Phycol.* **32**, 3419–3428 (2020).
32. Zaki, M. A. A. *et al.* The impact of stocking density and dietary carbon sources on the growth, oxidative status and stress markers of Nile tilapia (*Oreochromis niloticus*) reared under biofloc conditions. *Aquac. Reports* **16**, 100282 (2020).
33. Rowland, S. J., Mifsud, C., Nixon, M. & Boyd, P. Effects of stocking density on the performance of the Australian freshwater silver perch (*Bidyanus bidyanus*) in cages. *Aquaculture* **253**, 301–308 (2006).
34. Mohler, J. W., King, M. K. & Farrell, P. R. Growth and survival of first-feeding and fingerling Atlantic sturgeon under culture conditions. *N. Am. J. Aquac.* **62**, 174–183 (2000).
35. Mirghaed, A. T., Hoseini, S. M. & Ghelichpour, M. Effects of dietary 1, 8-cineole supplementation on physiological, immunological and antioxidant responses to crowding stress in rainbow trout (*Oncorhynchus mykiss*). *Fish Shellfish Immunol.* **81**, 182–188 (2018).
36. Hoseini, S. M., Mirghaed, A. T., Iri, Y. & Ghelichpour, M. Effects of dietary cineole administration on growth performance, hematological and biochemical parameters of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **495**, 766–772 (2018).
37. Barton, B. A., Morgan, J. D. & Vijayan, M. M. Physiological and condition-related indicators of environmental stress in fish. *Biol. Indic. Aquat. Ecosyst. Stress* 111–148 (2002).
38. Varela, J. L. *et al.* Dietary administration of probiotic Pdp11 promotes growth and improves stress tolerance to high stocking density in gilthead seabream Sparus auratus. *Aquaculture* **309**, 265–271 (2010).
39. Costas, B., Aragão, C., Dias, J., Afonso, A. & Conceição, L. E. C. Interactive effects of a high-quality protein diet and high stocking density on the stress response and some innate immune parameters of Senegalese sole Solea senegalensis. *Fish Physiol. Biochem.* **39**, 1141–1151 (2013).
40. Long, L. *et al.* Effects of stocking density on growth, stress, and immune responses of juvenile Chinese sturgeon (*Acipenser sinensis*) in a recirculating aquaculture system. *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* **219**, 25–34 (2019).
41. Sadhu, N., Sharma, S. R. K., Joseph, S., Dube, P. & Philipose, K. K. Chronic stress due to high stocking density in open sea cage farming induces variation in biochemical and immunological functions in Asian seabass (*Lates calcarifer*, Bloch). *Fish Physiol. Biochem.* **40**, 1105–1113 (2014).
42. Zahran, E., Risha, E., AbdelHamid, F., Mahgoub, H. A. & Ibrahim, T. Effects of dietary Astragalus polysaccharides (APS) on growth performance, immunological parameters, digestive enzymes, and intestinal morphology of Nile tilapia (*Oreochromis niloticus*). *Fish Shellfish Immunol.* **38**, 149–157 (2014).
43. Aruoma, O. I. Free radicals, oxidative stress, and antioxidants in human health and disease. *J. Am. oil Chem. Soc.* **75**, 199–212 (1998).
44. Haridas, H. *et al.* Enhanced growth and immuno-physiological response of Genetically Improved Farmed Tilapia in indoor biofloc units at different stocking densities. *Aquac. Res.* **48**, 4346–4355 (2017).
45. Ruane, N. M., Carballo, E. C. & Komen, J. Increased stocking density influences the acute physiological stress response of common carp *Cyprinus carpio* (L.). *Aquac. Res.* **33**, 777–784 (2002).
46. Wang, X. *et al.* Effects of stocking density on growth, nonspecific immune response, and antioxidant status in African catfish (*Clarias gariepinus*). (2013).
47. Johnson, K. M. & Lema, S. C. Tissue-specific thyroid hormone regulation of gene transcripts encoding iodothyronine deiodinases and thyroid hormone receptors in striped parrotfish (*Scarus iseri*). *Gen. Comp. Endocrinol.* **172**, 505–517 (2011).

48. El-Khaldi, A. T. F. Effect of different stress factors on some physiological parameters of Nile tilapia (*Oreochromis niloticus*). *Saudi J. Biol. Sci.* **17**, 241–246 (2010).
49. Li, D., Liu, Z. & Xie, C. Effect of stocking density on growth and serum concentrations of thyroid hormones and cortisol in Amur sturgeon, *Acipenser schrenckii*. *Fish Physiol. Biochem.* **38**, 511–520 (2012).
50. Park, J.-W. *et al.* The thyroid endocrine disruptor perchlorate affects reproduction, growth, and survival of mosquitofish. *Ecotoxicol. Environ. Saf.* **63**, 343–352 (2006).
51. Refaey, M. M. *et al.* High stocking density alters growth performance, blood biochemistry, intestinal histology, and muscle quality of channel catfish Ictalurus punctatus. *Aquaculture* **492**, 73–81 (2018).
52. Reinecke, M. *et al.* Growth hormone and insulin-like growth factors in fish: where we are and where to go. *Gen. Comp. Endocrinol.* **142**, 20–24 (2005).
53. Salas-Leiton, E. *et al.* Dexamethasone modulates expression of genes involved in the innate immune system, growth and stress and increases susceptibility to bacterial disease in Senegalese sole (*Solea senegalensis* Kaup, 1858). *Fish Shellfish Immunol.* **32**, 769–778 (2012).
54. Dyer, A. R. *et al.* Correlation of plasma IGF-I concentrations and growth rate in aquacultured finfish: a tool for assessing the potential of new diets. *Aquaculture* **236**, 583–592 (2004).
55. Kajimura, S. *et al.* Dual mode of cortisol action on GH/IGF-I/IGF binding proteins in the tilapia, *Oreochromis mossambicus*. *J. Endocrinol.* **178**, 91–99 (2003).
56. Ren, Y., Wen, H., Li, Y. & Li, J. Stocking density affects the growth performance and metabolism of Amur sturgeon by regulating expression of genes in the GH/IGF axis. *J. Oceanol. Limnol.* **36**, 956–972 (2018).
57. Salas-Leiton, E. *et al.* Effects of stocking density and feed ration on growth and gene expression in the Senegalese sole (*Solea senegalensis*): potential effects on the immune response. *Fish Shellfish Immunol.* **28**, 296–302 (2010).
58. Vijayan, M. M., Aluru, N. & Leatherland, J. F. Stress response and the role of cortisol. *Fish Dis. Disord.* **2**, 182–201 (2010).
59. Hegazi, M. M., Attia, Z. I. & Ashour, O. A. Oxidative stress and antioxidant enzymes in liver and white muscle of Nile tilapia juveniles in chronic ammonia exposure. *Aquat. Toxicol.* **99**, 118–125 (2010).
60. Kpundeh, M. D., Xu, P., Yang, H., Qiang, J. & He, J. Stocking densities and chronic zero culture water exchange stress' effects on biological performances, hematological and serum biochemical indices of GIFT tilapia juveniles (*Oreochromis niloticus*). *J Aquac Res Dev.* **4**, 2 (2013).
61. Tan, C. *et al.* Effects of stocking density on growth, body composition, digestive enzyme levels and blood biochemical parameters of *Anguilla marmorata* in a recirculating aquaculture system. *Turkish J. Fish. Aquat. Sci.* **18**, 9–16 (2018).
62. Ni, M. *et al.* The physiological performance and immune responses of juvenile Amur sturgeon (*Acipenser schrenckii*) to stocking density and hypoxia stress. *Fish Shellfish Immunol.* **36**, 325–335 (2014).
63. Abdel-Tawwab, M. Effects of dietary protein levels and rearing density on growth performance and stress response of Nile tilapia, *Oreochromis niloticus* (L.). *Int. Aquat. Res.* **4**, 1–13 (2012).
64. Chatterjee, N. *et al.* Effect of stocking density and journey length on the welfare of rohu (*Labeo rohita* Hamilton) fry. *Aquac. Int.* **18**, 859–868 (2010).
65. Pakhira, C., Nagesh, T. S., Abraham, T. J., Dash, G. & Behera, S. Stress responses in rohu, *Labeo rohita* transported at different densities. *Aquac. Reports* **2**, 39–45 (2015).
66. Tahmasebi-Kohyani, A., Keyvanshokooh, S., Nematollahi, A., Mahmoudi, N. & Pasha-Zanoosi, H. Effects of dietary nucleotides supplementation on rainbow trout (*Oncorhynchus mykiss*) performance and acute stress response. *Fish Physiol. Biochem.* **38**, 431–440 (2012).
67. Montero, D. *et al.* Effect of vitamin E and C dietary supplementation on some immune parameters of gilthead seabream (*Sparus aurata*) juveniles subjected to crowding stress. *Aquaculture* **171**, 269–278 (1999).
68. Urbinati, E. C., de Abreu, J. S., da Silva Camargo, A. C. & Parra, M. A. L. Loading and transport stress of juvenile matrinxã (*Brycon cephalus*, Characidae) at various densities. *Aquaculture* **229**, 389–400 (2004).
69. Evans, D. H. Cell signaling and ion transport across the fish gill epithelium. *J. Exp. Zool.* **293**, 336–347 (2002).
70. McCormick, S. D. Endocrine control of osmoregulation in teleost fish. *Am. Zool.* **41**, 781–794 (2001).
71. Postlethwaite, E. & McDonald, D. Mechanisms of Na+ and C-regulation in freshwater-adapted rainbow trout (*Oncorhynchus mykiss*) during exercise and stress. *J. Exp. Biol.* **198**, 295–304 (1995).
72. Liu, P., Du, Y., Meng, L., Li, X. & Liu, Y. Metabolic profiling in kidneys of Atlantic salmon infected with *Aeromonas salmonicida* based on 1H NMR. *Fish Shellfish Immunol.* **58**, 292–301 (2016).
73. Hosfeld, C. D., Hammer, J., Handeland, S. O., Fivelstad, S. & Stefansson, S. O. Effects of fish density on growth and smoltification in intensive production of Atlantic salmon (*Salmo salar* L.). *Aquaculture* **294**, 236–241 (2009).
74. Wagner, E. I., Miller, S. A. & Bosakowski, T. Ammonia excretion by rainbow trout over a 24-hour period at two densities during oxygen injection. *Progress. Fish-Culturist* **57**, 199–205 (1995).
75. Trenzado, C. E. *et al.* Effect of dietary lipid content and stocking density on digestive enzymes profile and intestinal histology of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **497**, 10–16 (2018).
76. Li, X., Liu, Y. & Blancheton, J.-P. Effect of stocking density on performances of juvenile turbot (*Scophthalmus maximus*) in recirculating aquaculture systems. *Chinese J. Oceanol. Limnol.* **31**, 514–522 (2013).
77. Hoseini, S. M., Hoseinifar, S. H. & Doan, H. Van. Effect of dietary eucalyptol on stress markers, enzyme activities and immune indicators in serum and haematological characteristics of common carp (*Cyprinus carpio*) exposed to toxic concentration of ambient copper. *Aquac. Res.* **49**, 3045–3054 (2018).

78. Ni, M. *et al.* Effects of stocking density on mortality, growth and physiology of juvenile Amur sturgeon (Acipenser schrenckii). *Aquac. Res.* **47**, 1596–1604 (2016).
79. Abdel-Tawwab, M., Hagras, A. E., Elbaghdady, H. A. M. & Monier, M. N. Dissolved oxygen level and stocking density effects on growth, feed utilization, physiology, and innate immunity of Nile Tilapia, *Oreochromis niloticus*. *J. Appl. Aquac.* **26**, 340–355 (2014).
80. Toko, I., Fiogbe, E. D., Koukpode, B. & Kestemont, P. Rearing of African catfish (*Clarias gariepinus*) and vundu catfish (*Heterobranchus longifilis*) in traditional fish ponds (whedos): Effect of stocking density on growth, production and body composition. *Aquaculture* **262**, 65–72 (2007).
81. Suárez, M. D. *et al.* Influence of dietary lipids and culture density on rainbow trout (*Oncorhynchus mykiss*) flesh composition and quality parameter. *Aquac. Eng.* **63**, 16–24 (2014).
82. Santín, A., Grinyó, J., Bilan, M., Ambroso, S. & Puig, P. First report of the carnivorous sponge *Lycopodina hypogea* (Cladorhizidae) associated with marine debris, and its possible implications on deep-sea connectivity. *Mar. Pollut. Bull.* **159**, 111501 (2020).
83. Jørpeland, G., Imsland, A., Stien, L. H., Bleie, H. & Roth, B. Effects of filleting method, stress, storage and season on the quality of farmed Atlantic cod (*Gadus morhua* L.). *Aquac. Res.* **46**, 1597–1607 (2015).
84. Bulow, F. J. RNA-DNA ratios as indicators of growth in fish: a review. *Age growth fish* 45–64 (1987).
85. Regnault, M. & Luquet, P. Study by evolution of nucleic acid content of prepuberal growth in the shrimp *Crangon vulgaris*. *Mar. Biol.* **25**, 291–298 (1974).
86. Tanaka, H. K. M. *et al.* High resolution imaging in the inhomogeneous crust with cosmic-ray muon radiography: The density structure below the volcanic crater floor of Mt. Asama, Japan. *Earth Planet. Sci. Lett.* **263**, 104–113 (2007).
87. Gwak, W. S. & Tanaka, M. Developmental change in RNA: DNA ratios of fed and starved laboratory-reared Japanese flounder larvae and juveniles, and its application to assessment of nutritional condition for wild fish. *J. Fish Biol.* **59**, 902–915 (2001).
88. Akhtar, M. *et al.* Studies on wheat bran Arabinoxylan for its immunostimulatory and protective effects against avian coccidiosis. *Carbohydr. Polym.* **90**, 333–339 (2012).
89. Ali, M., Iqbal, R., Rana, S. A., Athar, M. & Iqbal, F. Effect of feed cycling on specific growth rate, condition factor and RNA/DNA ratio of *Labeo rohita African J. Biotechnol.* **5**, (2006).
90. Zehra, S. & Khan, M. A. Dietary lysine requirement of fingerling *Catla catla* (Hamilton) based on growth, protein deposition, lysine retention efficiency, RNA/DNA ratio and carcass composition. *Fish Physiol. Biochem.* **39**, 503–512 (2013).
91. Misra, H. P. & Fridovich, I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J. Biol. Chem.* **247**, 3170–3175 (1972).
92. Takahara, S. *et al.* Hypocatalasemia: a new genetic carrier state. *J. Clin. Invest.* **39**, 610–619 (1960).
93. Rick, W. & Stegbauer, H. P. α-Amylase measurement of reducing groups. in *Methods of enzymatic analysis* 885–890 (Elsevier, 1974).
94. Cherry, I. S. & Crandall Jr, L. A. The specificity of pancreatic lipase: its appearance in the blood after pancreatic injury. *Am. J. Physiol. Content* **100**, 266–273 (1932).
95. Drapeau, G. R. [38] Protease from *Staphyloccus aureus*. in *Methods in enzymology* vol. 45 469–475 (Elsevier, 1976).
96. AOAC. *Official Methods of Analysis of AOAC International*. (Association of Official Analytical Chemists Washington, DC, 2005).
97. Bosworth, B. G., Small, B. C. & Mischke, C. Effects of transport water temperature, aerator type, and oxygen level on channel catfish *Ictalurus punctatus* fillet quality. *J. World Aquac. Soc.* **35**, 412–419 (2004).
98. Ma, L. Q., Qi, C. L., Cao, J. J. & Li, D. P. Comparative study on muscle texture profile and nutritional value of channel catfish (*Ictalurus punctatus*) reared in ponds and reservoir cages. *J. Fish. China* **38**, 532–537 (2014).

Tables

Table 1. Growth performance and feed utilization efficiency of *L. rohita* stocked at three different stocking densities

Treatment	IBW (g)	FBW (g)	WG (g)	SGR (%)	AGR (g)	Survival (%)	FCR	PER	FCE	Yield (kgm^{-3}
LSD	30.35±1.08	680.69±21.30^{a}	650.34±21.30^{a}	1.29±0.01^{a}	2.70±0.08^{a}	88±0.11^{a}	1.96±0.01^{c}	1.69±0.01^{a}	0.50±0.02^{a}	5.98±(
MSD	30.35±1.08	560.52±25.98^{b}	530.17±25.98^{b}	1.21±0.01^{a}	2.20±0.10^{b}	72±0.14^{b}	2.56±0.02^{b}	1.29±0.01^{b}	0.38±0.02^{b}	8.059±
HSD	30.35±1.08	405.45±32.79^{c}	375.1±32.79^{c}	1.07±0.03^{b}	1.56±0.13^{c}	66±0.18^{c}	2.87±0.01^{a}	1.16±0.02^{c}	0.34±0.03^{c}	8.050±

Values are mean±SE. Means in the same column within each classification bearing different superscripts are significantly different at (P <0.05).

Table 2. Serum biochemical indices of *L. rohita* stocked at three different stocking densities

Treatments	SGOT (UL^{-1})	SGPT (UL^{-1})	Protein (gdL^{-1})	Albumin (gdL^{-1})	Globulin (gdL^{-1})	A:G ratio	Na^+ ($mmolL^{-1}$)	K^+ ($mmolL^{-1}$)	Cl^- ($mmolL^{-1}$)
LSD	58.16±1.72^b	3.88±0.04^b	3.29±0.01^a	0.96±0.01^a	2.33±0.01^a	0.41±0.01^a	131.13±0.54^a	2.52±0.02^a	112.50±4.04^a
MSD	60.01±2.89^b	4.00±0.04^b	2.96±0.05^b	0.85±0.01^b	2.11±0.04^b	0.40±0.01^a	129.50±0.28^b	2.58±0.07^a	102.40±3.41^{ab}
HSD	66.09±1.90^a	4.51±0.08^a	2.33±0.08^c	0.72±0.01^c	1.61±0.06^c	0.44±0.01^b	130.50±0.28^{ab}	2.12±0.05^b	98.16±1.30^b

Values are mean±SE. Means in the same column within each classification bearing different superscripts are significantly different at (P <0.05).

SGOT- Serum glutamate oxaloacetate transaminase

SGPT- Serum glutamate pyruvate transaminase

Table 3. Muscle chemical composition and flesh quality of *L. rohita* stocked at three different stocking densities

Treatments	Moisture (%)	Crude protein (%)	Crude fat (%)	Ash (%)	Muscle pH	Drip loss (%)	FLR (%)
LSD	77.67±0.74^a	13.82±0.11^b	2.03±0.05^a	3.72±0.01^a	6.5±0.11^a	2.1±0.17^b	0.7±0.05^b
MSD	77.03±0.26^a	14.45±0.52^{ab}	1.90±0.06^a	3.74±0.01^a	6.0±0.05^b	3.2±0.11^a	1.1±0.11^{ab}
HSD	75.61±1.62^a	15.28±0.22^a	1.63±0.06^b	3.72±0.02^a	5.8±0.17^b	3.8±0.40^a	1.4±0.17^a

Values are mean±SE. Means in the same column within each classification bearing different superscripts are significantly different at (P <0.05).

FLR – Frozen leakage rate

Figures

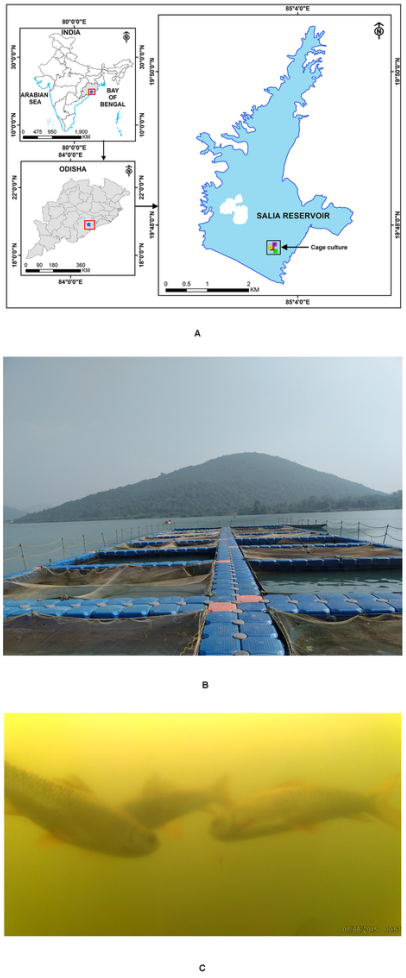

Figure 1

a GIS map of experimental site. Salia reservoir, Ganjam district, Odisha, India (N19°48.3887′ and E85°03.6983′). The reservoir area is 947.8 ha at its FRL. The present experiment was conducted in 9 cages out of 58 installed in the reservoir. b Experimental cage (HDPE) site, Salia reservoir, Ganjam district, Odisha, India c Underwater image of L. rohita in cage. The images were taken by using SONY-RX 100 VI underwater camera at daytime from 10 AM to 2 PM. The camera was operated by scuba divers at different depths with all safety features as per requirement and visibility in the water.

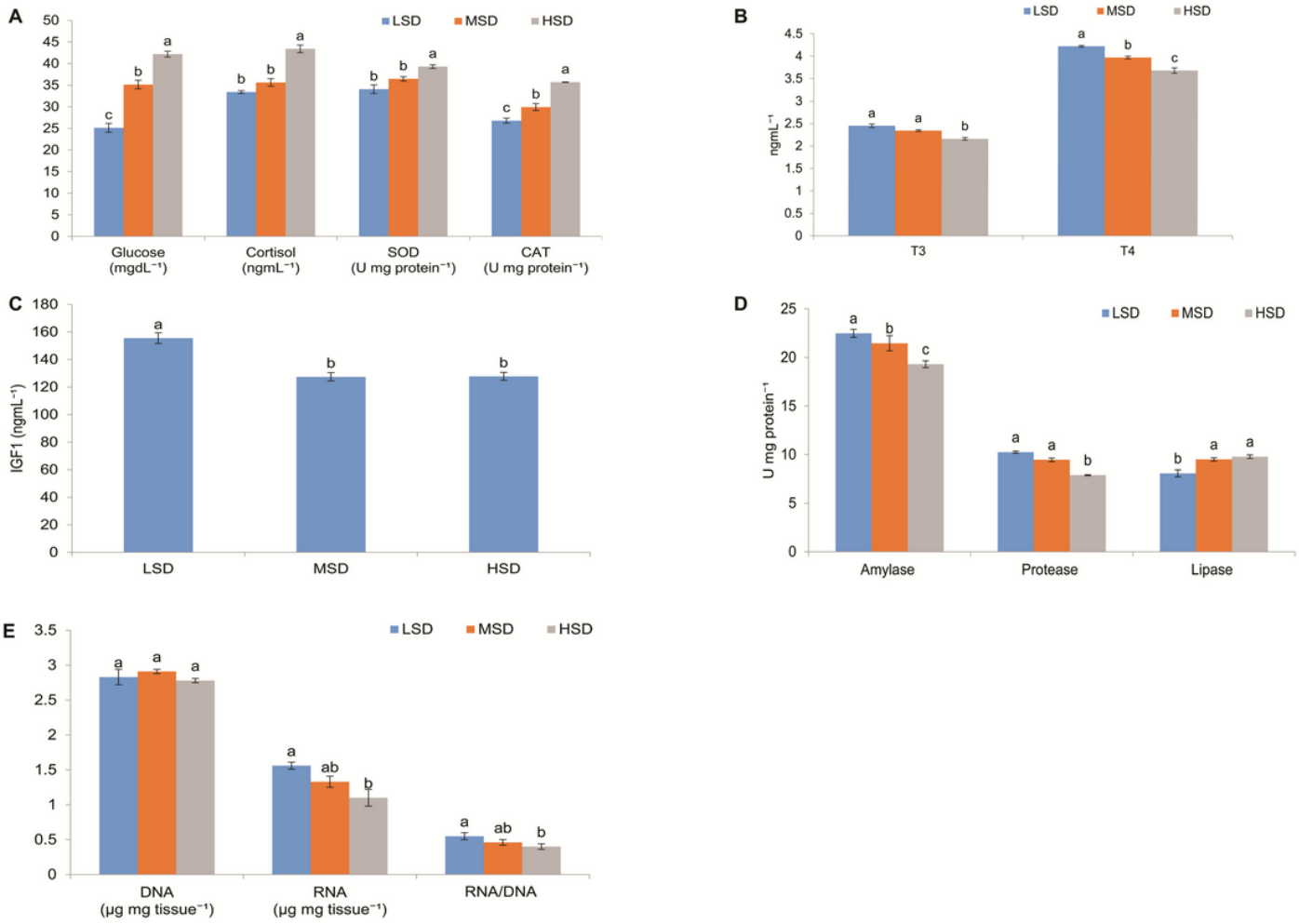

Figure 2

Activities of (A) glucose, cortisol, SOD, CAT in serum (B) T3 and T4 in serum, (C) IGF1 in serum, (D) amylase, protease and lipase in gut, and (E) concentration of DNA, RNA and RNA/DNA ratio in muscle of L. rohita stocked at three different stocking densities. Each bar represent values as mean ± SE. Mean values of each parameters having different superscripts are significantly different ($P<0.05$)

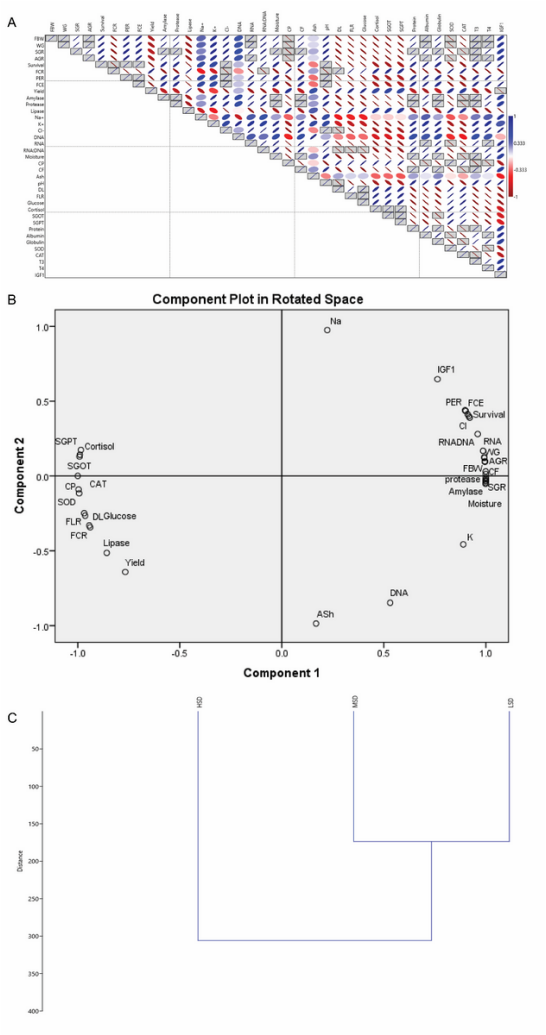

Figure 3

(A) *Pearson correlation coefficient, (B) **Principal component analysis, and (C) ***Hierarchical cluster analysis using growth attributes and multiple biomarkers of L. rohita cultured in three stocking densities in cages. *Red coloured small eclipse showed negatively correlated variables while, blue coloured small eclipse shape showed positive correlated variables, large eclipse showed less correlated variables, boxed variables showing significantly (P<0.05) correlated **Extraction method: Principal component analysis, Rotation method: Varimax with Kaiser Normalization, ***Wards method with Euclidean method