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EFFECTS OF STOCKING DENSITY ON CORTISOL AND GLUCOSE LEVELS IN BLOOD SERUM OF EUROPEAN CATFISH (*Silurus glanis* Linnaeus, 1758)

ABSTRACT

In this study, the serum cortisol and glucose levels in the European catfish (*Silurus glanis*) stocked at the different densities were investigated. For this purpose, a control (0.06kg fish/L) and D1 (0.08kg fish/L), D2 (0.10kg fish/L) and D3 (0.12kg fish/L) stoking groups were formed. Experiments were carried out in glass aquariums in triplicate. The water temperature in the aquariums was 22±2C° and the dissolved oxygen level was set to 9mg/L. In the experiment, fish with an initial weight of 64±2g were used. The daily feed amount to be given to the fish was calculated taking into account the feeding coefficient and live weights of the fish, and this amount was given to the fish for 60 days with three times. Blood samples to determine the cortisol and glucose levels in the serum were take from the fish at 1, 15, 30, 45 and 60 days of the experiment. The cortisol and glucose levels in blood serum samples was determined in ELISA using appropriate kits. During the trial no fish deaths were observed in any stocking group. However, it was determined that there was a significant increase in the cortisol (20.60-30.04mg/dl) and glucose (74.97-89.78mg/dl) levels in blood serum of fish at high stocking densities ($p<0.05$). The best results in terms of cortisol and glucose values were obtained from fish in the control and D1 stocking groups ($p>0.05$). This showed that stocking densities higher than 0.08kg/L cause stress in fingerling European catfish.

Keywords: European Catfish, Cortisol, Glucose, Stoking Density, Stress

1. INTRODUCTION

Fish respond differently to stress factors shows that these responses are species-specific. Fish species can show different levels of sensitivity to changes in water quality as a result of intensive stocking and exhibit different behaviors. Therefore, the stocking density is not suitable; It can cause many undesirable negative situations in terms of aquaculture, such as health problems in fish, reduced feed utilization, decreased growth performance and increased risk of death [1]. The sum of the physiological changes that the fish show against physical, chemical and biological threats is defined as the "stress response". Regardless of the type of stressor, all of these stages are physiologically exhibited by the fish. However, the chronic or acute nature of the stressor may differentiate the stress response. Response to stress increases the possibility of survival of the organism against the current stressors. However, this has an energy cost and can only provide protection for a certain period of

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time. Under the pressure of prolonged stressors, the available energy source is depleted. Having a good condition of the creature increases the response or resistance time. If the effect of any stressor on a system lasts for a long time; secreted cortisol regulates energy use, provides water-mineral balance, activates oxygen supply and immune system, and protects the organism against the negative effects of stress by restricting some systems. If the secreted cortisol level is too high; regression in growth, delay in reaching sexual maturity occurs and the effect on the organism is quite negative [2].

The direct measurement of stress is made by determining the amount of hormones (eg. Cortisol) secreted. In addition, by detecting the physiological (secondary) changes that occur in the blood and tissues after the stress response, information about the level of stress can be obtained. In addition, the effect of the response to the stress encountered with the detection of physiological changes; Enzymes, which are indicators of tissue or organ damage in the serum, can also be determined directly by measuring the blood glucose level (indirect indicator of hormonal activity) and the amount of C11 as a sign of ion regulation [3].

2. RESEARCH SIGNIFICANCE

Stocking density is considered to be the "limiting factor" for intensive fish farming systems. In this study, it was aimed to investigate serum cortisol and glucose levels in European catfish (*Silurus glanis*) stocked at different densities.

Highlights:

- It was determined that there was a significant increase in cortisol (20.60-30.04mg/dl) and glucose (74.97-89.78mg/dl) levels in the blood serum of European catfish at high stocking densities ($p<0.05$).
- The best results in terms of cortisol and glucose values were obtained from fish in Control and D1 stocking groups ($p>0.05$).
- This indicated that stocking densities higher than 0.08kg/L cause stress in European catfish fry.

3. MATERIALS AND METHODS

3.1. Materials

The study was acted in Research Unit of Experimental Research Center of Firat University. A total of 444 European catfish (*S. glanis*) were used in the experiment. These fish were provided from the Ministry of Agriculture and Forestry, General Directorate of State Hydraulic Works, 9th Region Keban Dam Fisheries Branch Directorate, Elazig, Turkiye. Fish in the same size (Weight:64±2.00g and total Length:23.6±0.50cm) were selected from a large population, and randomly distributed to the trial groups. Fish were acclimated to the environmental conditions for a week before starting the experiment. Fish were weighed before and after the acclimation period.

For the feeding of the fish in the research, a feed with a crude protein (HP) ratio of 45%, containing 17% crude oil and 3600kcal/kg of total energy, meeting the needs of European catfish fry in terms of other nutrients and whose open formula is given below (pellet diameter:3mm and pellet length:6mm) were prepared [4 and 5]. In the feed component; 20% anchovy meal (72% HP), 35% soybean meal (48% HP), 20% corn gluten (64% HP), 7.7% wheat starch (11.7% HP), 14.2% soybean oil, 1% anchovy oil, 0.1% antioxidant (125.000mg Butylene Hydroxy Toluene/kg), vitamin mix (as IU or mg/kg; vitamin A 250000 IU, vitamin D3 240000 IU, vitamin E 10.000 IU, vitamin K 3.000mg, vitamin B1 1000mg, vitamin B2 3000mg, vitamin B6 2000mg, vitamin B12 4mg, choline

chloride 100000mg, vitamin C 6000mg, niacin 30000mg, calcium d-pantothenate 10000mg, folic acid 600mg, d-biotin 200mg) and 1% mineral mix (mg/kg; Manganese 1300, zinc 3000, iron 6000, copper 300, iodine 110, potassium 70, phosphorus 60, selenium 30, cobalt 20, magnesium 5) were used.

3.2. Methots

The fish used in the experiment were processed in accordance with the "Fırat University Animal Experiments Local Ethics Committee Directive" (Protocol No:2016/134). Fingerling fish were kept in the stock pool for 15 days; Fish were accustomed to the environmental conditions, the food used, and their health status was observed.

Experiments were carried out in glass aquariums with a volume of 25.6L (50x25x20.48cm). Aquariums were aerated with the help of an air pump. The water temperature was adjusted to 22±2°C using a thermostatic heater. The water quality was maintained by regularly replenishing the water in the aquariums with 1/3 of fresh water of the same temperature every day. In the study, a control (0.06kg fish/L) and 3 (0.08, 0.10 and 0.12kg fish/L) experimental groups with different stock densities were formed. For these stocking densities, 25.6L aquariums, respectively; 24, 32, 40 and 48 fish were stocked. Each trial group was carried out in 3 repetitions. Fish were exposed to natural daily light regime. Daily feed amount given to fish; It was calculated by taking into account the feeding coefficient and the live weights of the fish, and this amount was given to the fish as three meals a day for 60 days. In order not to stress the fish, the feeding processes were completed in a short time (approximately 1 minute) [4].

In this study, the effects of different stocking densities on cortisol and glucose levels in European catfish were investigated. For this purpose, blood samples were taken from 12 fish at the beginning of the study, on the 15th, 30th, 45th and 60th days, for the analysis of cortisol and glucose in the blood plasma serum of the fish in 15-day periods. In each measurement period during the trial; Taking into account the possible changes that may occur in the total weight of the stocked fish as a result of using fish for blood sampling, the water volume in the aquarium will be reduced accordingly, ensuring the continuity of the stocking densities studied.

Anesthesia (100mg Benzocaine/L) was administered to the fish before weighing their weight, measuring their length, and taking blood samples [6]. The weight of the fish was determined by weighing on a digital scale with a precision of 0.1g, and the total length was determined using a measuring board (with 1mm scale).

The tail fin of the fish was cut and blood samples were taken. For this purpose, after the fish were anesthetized, the tail area of the fish was dried with an absorbent cloth to prevent the contamination of mucus and water. Then, with the help of sharp scissors, close to the tail region (peduncle region), the tail artery (caudal vein) was cut crosswise in the dorso-ventral direction. Blood samples were taken by placing a tube with EDTA at the end of the cut tail vein without allowing blood to clot [7].

3.2.1. Cortisol and Glucose Analysis

The blood samples taken into glass tubes were centrifuged at the 3000 xg at 4 °C for 15 minutes and their serum were removed. Cortisol and glucose levels in the serum samples were determined by ELISA using appropriate kits [8]. The serum samples were stored in -80°C freezer until ELISA test. Cayman cortisol ELISA kit (No: 500360) and standard (No: 4000364) were used for cortisol measurement. Cortisol measurement was made by absorbing a plate reader between 405-420nm. For glucose

measurement, Cayman glucose colorimetric assay kit (No:10009582) and standard (No:10010098) were used. The plate reader showed absorption between 500-520nm, and then glucose measurement was performed. Cortisol and glucose concentrations in biofluids were determined and accepted for statistical analysis when higher than each ELISA kit's limit of detection of 0.01-100ng/mL.

3.2.2. Chemical and Physical Analysis

Proximate composition and energy content of the diet were analysed by standard methods [9]. The moisture and ash contents were determined by drying with the dry oven and muffle furnace at 100^{0}C and 600^{0}C, respectively. The crude protein level was determined by the micro Kjeldahl, and the crude lipid by the Soxhlet extraction, and the crude fibre by the cellulose determination device. During the experiment, some physical and chemical properties of water in the trial aquariums were determined daily. Temperature (^{0}C) and pH values of the aquarium water were measured using a digital pH meter. Dissolved oxygen content (mg/L) was determined using a portable oxygen meter.

3.2.3. Statistical Analysis

The values were given as mean ±standard error (SE) of three replicates for each treatment. Data were analysed ($p<0.05$) using SPSS computer program (SPSS 21.0, Chicago, IL, USA) for Windows. The cortisol and glucose values of fish in the different stocking groups, and the water quality values were subjected to "two-way analysis of variance", and followed by "the Duncan's new multiple range test".

4. RESULTS

The blood serum cortisol and glucose levels of the fish, the temperature, dissolved oxygen and pH values of the water used in the experimental aquariums are given below. No fish death occurred in any of the experimental groups during the study.

4.1. Cortisol Values

In the measurements made at the beginning of the experiment, the cortisol levels in the blood serum of the fish belonging to the stocking groups were found to be between 19.98±0.85mg/dl and 20.08±0.99mg/dl (Table 1). In parallel with the increase in stocking density during the experiment, an increase in the cortisol values in the blood serum of the fish occurred. However, increases in cortisol levels were significant only in D2 and D3 stocking groups ($p<0.05$). At the end of the trial, the lowest cortisol value was respectively; 20.60±1.01mg/dl and 21.00±1.04mg/dl were obtained from fish in Control and D1 stocking groups ($p>0.05$). This was followed by fish in D2 and D3 stocking groups with 25.60±1.40mg/dl and 30.04±1.97mg/dl, respectively ($p<0.05$). Cortisol levels of fish in control and D1 stocking groups were found to be statistically significantly lower than those in D2 and D3 stocking groups ($p<0.05$).

Table 1. Cortisol levels in blood serum of European catfish *S. glanis* in different stocking densities

Periods (day)	Experimental Groups (mg/dl)			
	Control	D1	D2	D3
Initial Value	19.98±0.85^{a}	20.02±0.87^{a}	20.05±0.90^{a}	20.08±0.99^{a}
15	19.90±0.91^{a,A}	20.13±0.94^{a,A}	23.22±1.11^{b,A}	26.10±1.60^{c,A}
30	20.00±0.95^{a,A}	20.25±0.97^{a,A}	24.40±1.29^{b,AB}	27.95±1.78^{c,B}
45	20.25±0.99^{a,A}	20.50±0.95^{a,A}	25.03±1.31^{b,B}	29.09±1.89^{c,BC}
60	20.60±1.01^{a,A}	21.00±1,04^{a,A}	25.60±1.40^{b,B}	30.04±1.97^{c,C}
Avarage Value	20.19±0.41^{a}	20.47±0.53^{a}	24.56±1.04^{b}	28.30±1.74^{c}

$^{a, b, c}$: The difference between the mean (±SE) values indicated by different letters on the same line is statistically significant ($p<0.05$, N=12)
$^{A, B, C}$: The difference between the mean values indicated by different letters in the same column is significant ($p<0.05$, N=12)

4.2. Glucose Values

The blood serum glucose levels of fish at different stocking densities are given in Table 2. In the measurements made at beginning of the experiment, it was seen that the glucose level in the blood serum of fish belonging to the stocking groups was between 74.97±1.35mg/dl and 75.13±1.53mg/dl. During the experiment, in parallel with the increase in the stocking density, an increase in the blood serum glucose levels of the fish occurred. However, these increases were significant only in the D3 stocking group from day 45 ($p<0.05$). At the end of the experiment, the lowest glucose value, respectively; 74.50±1.11mg/dl and 77.09±1.97mg/dl were obtained from the Control and D1 stocking groups ($p>0.05$). This was followed by the D2 and D3 stocking groups with 83.27±2.33mg/dl and 89.78±2.75mg/dl, respectively ($p<0.05$). Blood serum glucose levels of fish in control and D1 stocking groups were found to be significantly lower than those in D2 and D3 stocking groups ($p<0.05$).

Table 2. Glucose levels in blood serum of European catfish (*S. glanis*) in different stocking densities

Periods (Day)	Experimental Groups (mg/dl)			
	Control	Trial 1	Trial 2	Trial 3
Initial Value	74.97±1.35^{a}	75.06±1.41^{a}	75.11±1.48^{a}	75.13±1.53^{a}
15	75.06±1.45^{a,A}	75.40±1.57^{a,A}	79.58±2.05^{b,A}	83.77±2.36^{c,A}
30	74.77±1.39^{a,A}	76.00±1.73^{a,A}	81.45±2.10^{b,A}	85.51±2.46^{c,AB}
45	74.65±1.28^{a,A}	76.62±1.88^{a,A}	82.92±2.24^{b,A}	88.93±2.61^{c,B}
60	74.50±1.11^{a,A}	77.09±1.97^{a,A}	83.27±2.33^{b,A}	89.78±2.75^{c,B}
Avarage Value	74.75±0.31^{a}	76.28±0.81^{a}	81.81±1.46^{b}	87.00±2.78^{c}

$^{a, b, c}$: The difference between the mean (±SE) values indicated by different letters on the same line is statistically significant ($p<0.05$, N=12)
$^{A, B}$: The difference between the mean values indicated by different letters in the same column is significant ($p<0.05$, N=12)

4.3. Water Quality Values

Some physical and chemical values of aquarium waters belonging to the different stocking groups were determined daily for 60 days. There were no statistically significant differences between the average temperature (22.0±2.00°C), pH (7.16±0.15ppm) and dissolved oxygen (7.10±0.14mg/L) values of the experimental groups ($p>0.05$).

5. DISCUSSION AND CONCLUSION

High stocking density cause chronic stress in a result of some physiological changes, and reduce fish welfare [1]. In the event that the effect of any stressor on a system lasts for a long time, the secreted cortisol regulates energy use, ensures water-mineral balance, activates oxygen supply and the immune system, and protects the organism against the negative effects of stress by restricting certain

systems (e.g. digestion). If the secreted cortisol level is too high, the growth will decrease, delay in reaching the maturity and the effect on the organism will be quite negative [2].

In the present study, effect of stocking density on the serum cortisol and glucose levels of *S. glanis* was investigated in the first time. The cortisol (20.60-30.04mg/dl) and glucose (74.50-89.78 mg/dl) levels increased in parallel with the increase in stocking density ($p<0.05$). The lowest values were obtained from the control and D1 stocking groups ($p>0.05$), followed by the D2 and D3 stocking groups ($p<0.05$), respectively. Serum cortisol and glucose values of the control and S1 stocking fish were found to be significantly lower than those of the D2 and D3 stocking groups ($p<0.05$).

According to Liu et al., high stocking density is significantly ($p<0.05$) increased the cortisol level in the rainbow trout (Oncorhynchus mykiss), decreased the growth performance and negatively affected the fish welfare [10]. Initially, the plasma cortisol level of fish in the high stocking density has been showed a significant decrease. Then it has increased significantly on day 180. Nieuwegiessen et al., reported that an increase in the stocking density of C. gariepinus causes acute stress and then chronic stress symptoms due to inadequate cortisol response [11]. The findings in this study are similar to the results of Liu et al., and Nieuwegiessen et al., [10 and 11].

In contrast, in the some studies has been reported that high stocking density reduces plasma cortisol levels of some fish species. Nieuwegiessen et al., have investigated the effect of stocking density at different stages throughout the growth cycle of the juvenile African catfish (Clarias gariepinus) [12]. Surprisingly, net-stress rates of plasma cortisol levels have reduced by half in the fish groups in the range of 100-300g to 1000-1500g. Similarly, the mean plasma cortisol levels of the fish in the control group have 200-300nmol/L, while the net stress rates have determined as 100-150nmol/L ($p<0.05$). The plasma glucose levels of fish in the net stress groups have seen a significantly increase compared to the fish in the control groups ($p<0.05$). In the experiment with C. gariepinus weighing between 100-300 g; the plasma glucose value of fish in the control group with 160 g fish/m^3 has 2.77mmol/L, while this value has been significantly increased (6.67mmol/L) in the net-stress group ($p<0.05$). Again in experimental group where fish weighing 1000-1500 g have used; while the plasma glucose value has 3.6 mmol/L in the control group with 500g fish/m^3, this value has almost doubled (6.58mmol/L) in the net-stress group ($p<0.05$).

However, Hasanalipour et al., showed that the different stocking densities have no significant ($p<0.05$) effect on the cortisol (7.8-13.1ng/ml) and glucose (41.4-46.8ng/ml) values in blood of the immature Siberian Sturgeon (*Acipenser baerii*) [13].

In this study, there was not found a statistically significant ($p>0.05$) difference between the average temperature (22.0-22.0°C), pH (7.15-7.20) and dissolved oxygen (7.05-7.10mg/L) values of aquarium waters belonging to the different stocking groups. Bhatnagar and Devi reported the water quality criteria for freshwater fish culture: temperature 20-30°C, pH 6.5-8.5 and dissolved oxygen <5mg/L values of water in the trial aquariums are in accordance with the water quality criteria reported by Bhatnagar and Devi for freshwater fish culture [14].

In conclusion, the high stocking density caused to stress in the European catfish (*S. glanis*), and increased their serum cortisol and glucose levels ($p<0.05$). However, it was concluded that the D1 stocking density (0.08kg fish/L) may be optimum stocking density for

the European catfish fingerlings as it does not significantly increase the stress ($p>0.05$), but increase the production efficiency ($p<0.05$). In the present study, it was seen that stocking density could be increased in European catfish culture by maintaining water quality. However, to increase of fish welfare and production efficiency in aquaculture, it is great importance to carry out new researches that will reduce the stress in high stocking densities.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

FINANCIAL DISCLOSURE

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DECLARATION OF ETHICAL STANDARDS

Ethical statement The fish used in the experiment were processed in accordance with "The Animal Experiments Local Ethics Committee Directive (Protocol No: 2016/134) of Fırat University, Republic of Türkiye.

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