BIOSCIENCES BIOTECHNOLOGY RESEARCH ASIA, March 2023. Vol. **20**(1), p. 135-145

Effect of Handling Stress on Primary and Secondary Stress Responses of the Catfish, *Clarias batrachus*

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http://dx.doi.org/10.13005/bbra/3076

(Received: 06 February 2023; accepted: 24 March 2023)

Cortisol is a major hormone directly associated with stress in fish and is a reliable physiological indicator of primary stress response in fish, whereas glucose and osmolality are the indicators of secondary stress response in fish. This study explored the stress levels in the catfish, Clarias batrachus (Magur) by measuring the cortisol, glucose, and osmolality levels in plasma by exposing the fish to three different kinds of interventions namely, non-anaesthetized, anaesthetized, and stressed. No statistically significant changes were reported in the plasma cortisol, plasma glucose, and plasma osmolality levels when the blood samples were collected after the three interventions. These results indicated that Clarias batrachus is a sturdy fish, which can withstand routine laboratory handling, and that the blood samples can be collected without anaesthetization.

Keywords: Anaesthetization; Cortisol; Glucose; Osmolality; Teleost.

Aquaculture is one of the fastest growing food sectors in the world which has prompted the desire to improve our understanding of the physiological alterations related to the stress response and their ultimate effects on fish health[1]. Stress is a state of altered homeostasis which is reestablished collectively by physiological and behavioural responses of an organism[2]. It represents homeostasis disequilibrium evoking both specific and non-specific responses which enable the animal to overcome perturbation[3]. The physiological responses to capture and handling are highly magnified in fish than in most of the higher vertebrates[4]. The stress response has been classified into three levels namely, primary, secondary, and tertiary[5]. The initial exposure of fish to a stressor leads to an immediate neuroendocrine response which is the direct result of an increase in circulating catecholamines like epinephrine (adrenaline) and norepinephrine (noradrenaline) released from chromaffin cells and corticosteroids (cortisol from the head kidney)[4].

Teleosts represent a large and diverse group of ray-finned fishes and are the most advanced group of bony fishes and include almost all commercial and game fishes. In teleosts such as *Clarias batrachus*, cortisol acts as a mineralocorticoid as well as a glucocorticoid and has long been used to measure stress response[6]. However, 1á-hydroxycorticosterone (1á-OHB)

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also plays a similar role in elasmobranchs, which represent cartilaginous fishes such as sharks, rays, and skates[4]. The secondary responses are initiated by the primary stress response and are readily evaluated by various methods such as physiological alterations in blood circulation, especially between blood and muscle tissue (peripheral), metabolic indices, and hydromineral imbalance which includes the change in plasma electrolyte content, hematocrit value, and plasma osmolality[4]. These changes have a cascading effect on the energy reserves which can be mobilized rapidly when impacted by the sudden physiological needs of fishes[4,7]. The secondary physiological responses can cause sublethal tertiary issues among organisms especially fishes and, in the process, cause a population level response in them which can compromise the development, reproduction, and immunity of organisms[4,8].

There is evidence to show that routine laboratory handling and various experimental procedures such as injection, anaesthetization, and blood draw may generate stressful conditions for teleosts which can compromise the physiological phenomena of the fish causing detrimental effects on their health[9]. Several methods have been devised to withstand the stressful conditions caused by routine laboratory and experimental methods. However, the most commonly used method to counter stress in fish during experimentation is the anaesthetization before the collection of blood samples which would otherwise lead to changes in various indicators of primary and secondary responses such as plasma cortisol, glucose, electrolyte, hematocrit value, pH, and osmolality[10,11]. Some of the commonly used anaesthetics for fish are clove oil[11,12], menthol[13], carbon dioxide[14], essential oils of *Aloysia triphylla* (EOAT)[15,16], and eugenol[17,18]. Among these, MS-222 (Tricaine Methanesulphonate) is most commonly used[18,19]. It is important and necessary to obtain blood samples only from unstressed fish, as these parameters show great variation because of the handling stress in the laboratory.

The catfish, *Clarias batrachus*, commonly known as Magur is an economically and commercially important air-breathing teleost fish that is commonly found in the freshwater habitats of India and the surrounding countries. It constitutes an important component of culture and capture fishery. Extensive research has been conducted on its osmoregulatory physiology[20-22], toxicology[23], and bioenergetics[24]. However, no study has been carried out yet to assess the effect of laboratory handling stress on *Clarias batrachus*. Therefore, the present study was carried out to investigate the effect of laboratory handling stress on the catfish, *Clarias batrachus* by analyzing the alterations in plasma cortisol, plasma glucose, and plasma osmolality levels.

MATERIALS AND METHODS

Collection and care of fish: Adult specimens of the catfish, *Clarias batrachus* were bought and brought from the local fish market, Rasalganj, Aligarh. They were maintained in glass aquaria (60×25×30 cm) containing dechlorinated tap water with light and dark cycle schedules maintained automatically at 12h of light (0800 to 2000 h) and 12 h of dark (2000 to 0800 h) cycles. Fishes (average body weight: 50 g) were acclimated to the laboratory conditions for 2 weeks before the initiation of experiments. During this acclimation period, they were fed *ad libitum* daily with Hind Lever laboratory Animal Feed (Hindustan Lever Limited, Mumbai, India), and the water of the aquaria was replenished daily with stored tap water adjusted to laboratory conditions.

Collection of blood samples: Blood was drawn from the caudal artery and collected into heparinized glass syringes using 24-gauge dispensing needles which were disposable. Post-collection, the blood was immediately centrifuged for 10 min at 3000 rpm (REMI Ltd., India, Model: RM-12C), and the plasma was separated and stored at -20! until utilized for the analysis.

Plasma cortisol: Plasma cortisol levels were estimated using a commercially available kit, Cortisol RIA kit (REF IM1841).

Plasma glucose: Plasma glucose was estimated by glucose-O-toluidine method[25].

Plasma osmolality: Plasma osmolality was estimated using a vapour pressure osmometer (Wescor 5500, Utah, USA).

Statistical analysis: Statistical comparison between experimental and control groups was performed by student's *t*-test using GraphPad Prism

5 software. The significance was accredited at *P* d" 0.05 and all the results are presented as mean ± standard error of the mean (SE).

Experimental Protocol

The fish were divided into three groups with each group containing 5 specimens. They were maintained in glass aquaria containing 20 L of tap water.

Group I: The fish were netted out gently from the aquarium and blood was collected using heparinized syringes via the caudal artery.

Group II: The blood samples were drawn after anaesthetization with MS-222 (Tricaine Methanesulphonate) at a dose of 100 mg L^{-1}, which was adequate for the immobilization of fish within 1 min.

Group III: The blood was drawn immediately following the 3 min handling stress which comprised aggressively chasing the fish with a handheld net.

RESULTS

Plasma cortisol

Significant changes were not observed in the plasma cortisol levels of non-anaesthetized, anaesthetized, and stressed groups of fish (Figure 1).

Plasma glucose

Significant changes were not observed in the plasma glucose levels of non-anaesthetized, anaesthetized, and stressed groups of fish (Figure 2).

Plasma osmolality

Significant changes were not observed in the plasma osmolality levels of non-anaesthetized, anaesthetized, and stressed groups of fish (Figure 3).

DISCUSSION

Estimation of plasma cortisol has long been used as an essential indicator to assess the effect of a given stressor on fishes[26]. Cortisol is a primary stress hormone and a dependable biomarker of stress in fish[27]. The interrenal glands and chromaffin cells are instrumental in releasing cortisol into the circulatory system of the fish. The head kidney is a unique organ prevalent in teleosts and is similar in function to the adrenal gland among mammals. Cortisol, possessing both glucocorticoid and mineralocorticoid hormonal

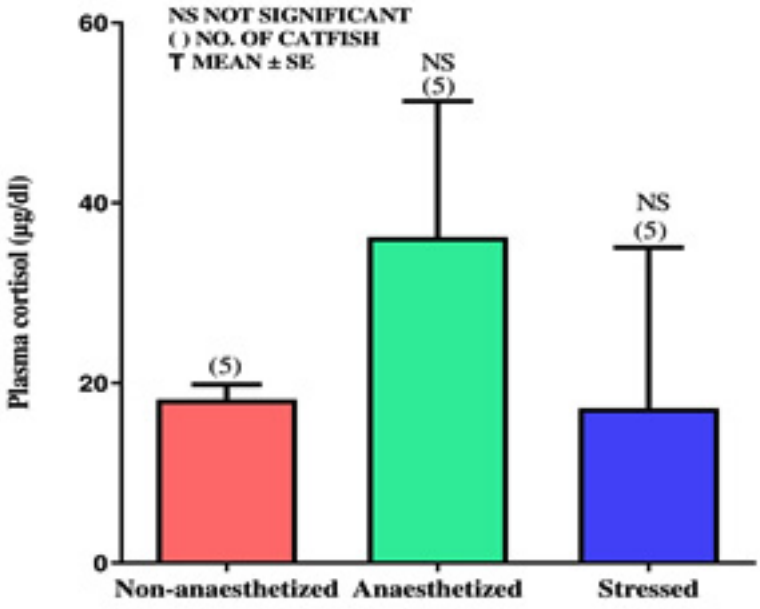

Fig. 1. Changes in plasma cortisol in non-anaesthetized, anaesthetized, and stressed catfish, *Clarias batrachus*

properties in teleosts[6], is secreted by cholesterol upon stimulation of the interrenal cells by the hormonal cascade[28] (Figure 4). Cortisol plays a key role in aerobic (presence of oxygen) and anaerobic (absence of oxygen) metabolism, increases oxygen uptake, elevates gluconeogenesis, inhibits glycogen synthesis, leading to high energy costs for the fish[29]. This physiological phenomenon has been extensively studied and applied to assess the stress levels in fishes by physiologists[19,27] and behavioural ecologists[30] alike, in both laboratory and open environmental studies[31,32].

In the present study, the experimental design and the analyses of various parameters

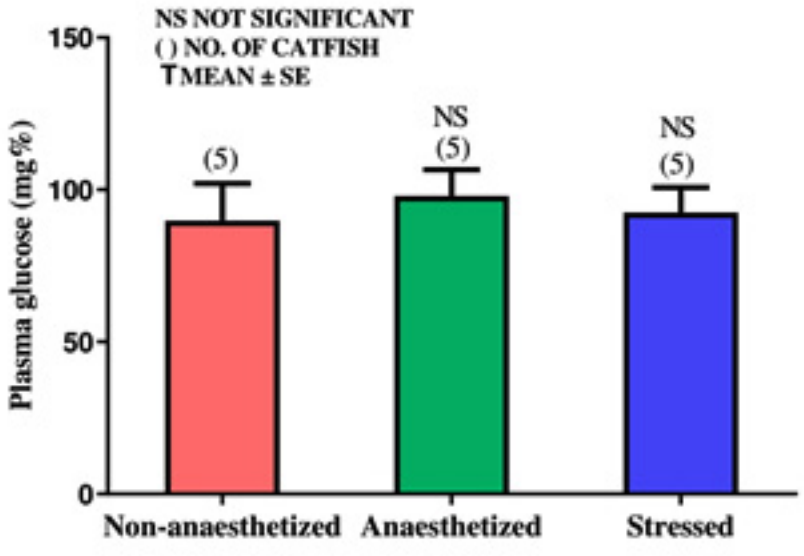

Fig. 2. Changes in the plasma glucose in non-anaesthetized, anaesthetized, and stressed catfish, *Clarias batrachus*

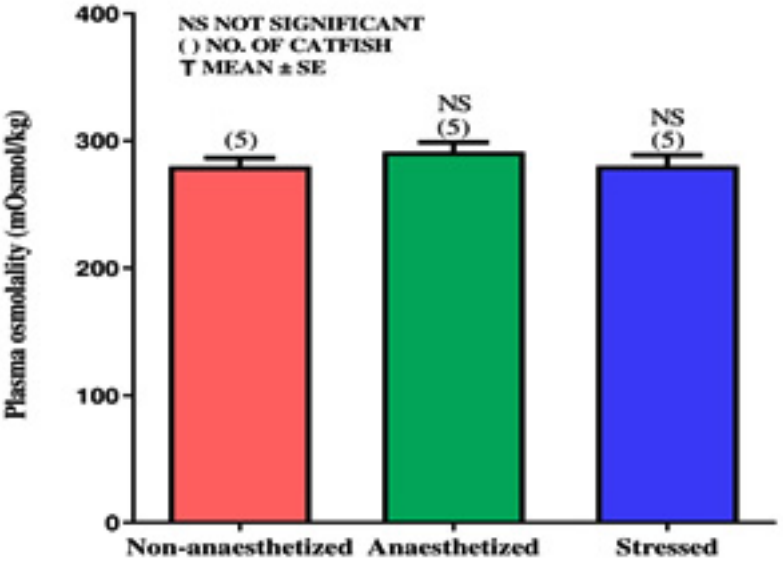

Fig. 3. Changes in the plasma osmolality in non-anaesthetized, anaesthetized, and stressed catfish, *Clarias batrachus*

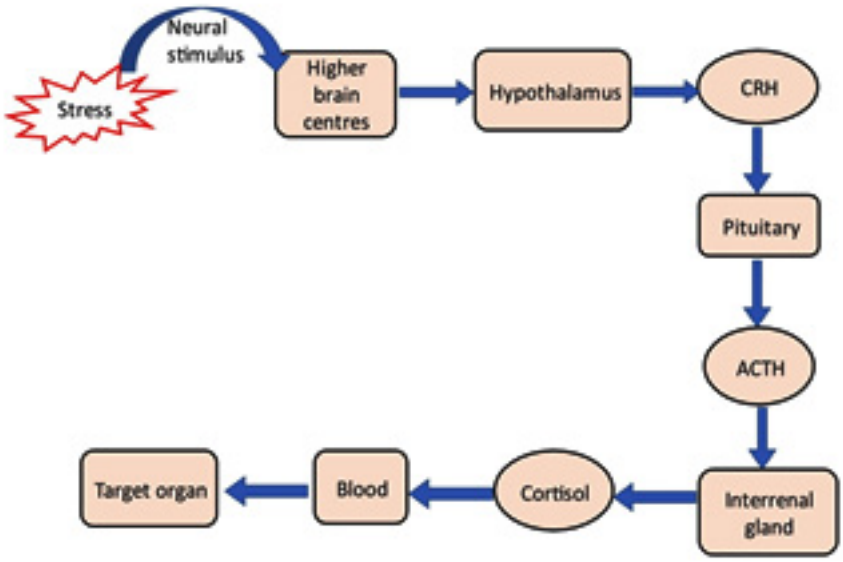

Fig. 4. Hypothalamus-pituitary-interrenal axis of fish; CRH – Corticotropin releasing hormone; ACTH – Adrenocorticotropic hormone

suggest that the handling of fish routinely in the laboratory does not cause significantly discernible stress in the catfish, *Clarias batrachus*, which belongs to the group of fishes which are hardy and not easily susceptible to minor stresses[19]. The results are in accordance with the findings on *Heteropneustes fossilis*[19], *Oreochromis niloticus*[16,33] and *Rhamdia quelen*[34,35] where no significant changes in cortisol levels were reported in anaesthetized and non-anaesthetized groups. However, an elevation of plasma cortisol concentrations was reported in juvenile Atlantic Sturgeon, *Acipenser oxyrinchus*[36] and in the silver catfish, *Rhamdia quelen* after anaesthetization[37]. These results suggest that anaesthetization itself could have been a physiological stressor in these fishes[19]. In this context, it is interesting to note that, in the present study also, a slight though not statistically significant increase in plasma cortisol levels was noticed after the anaesthetization of *Clarias batrachus* compared to non-anaesthetized fish (groups I and II, Figure 1). Decreased plasma cortisol concentrations after anaesthetization have also been reported in some fishes[17,38,39].

The handling of fish routinely in the laboratory does not cause any significant stress on *Clarias batrachus*. This can be further substantiated by the observation that significant changes in plasma cortisol levels were not observed when fish were exposed to sustained physical stress by aggressively chasing for 3 min with a handheld net as compared to control groups (groups I and III, Figure 1). Similar results were reported on another catfish, *Heteropneustes fossilis* after handling stress, suggesting the hardy nature of the fish[19]. Serial removal of cohorts from the shared aquarium led to no significant change in plasma cortisol in the residual fishes[40]. These results corroborate the findings of Milla *et al*.[41] on *Perca fluviatilis* and Easy and Ross[42] on Atlantic salmon, *Salmo salar*, who observed statistically insignificant changes in plasma cortisol levels after net handling stress at 0 h. However, Hosoya *et al*.[43] reported that long-term handling stress on *Melanogrammus aeglefinus* causes a significant increase in plasma cortisol levels. Falahatkar *et al*.[44] also recorded similar results in juvenile great sturgeon, *Huso huso* after net handling stress of 1 min. An increase in the whole-body cortisol levels in *Danio rerio was* reported after net handling stress by Ramsay *et al*.[45]. Jentoft *et al*.[46] detected a significant increase in plasma cortisol levels following the handling stress in wild *Perca fluviatilis* and domesticated *Oncorhynchus mykiss*. In contrast, decreased levels of plasma cortisol were shown in Juvenile pallid sturgeon *Scaphirhynchus albus*[47] and juvenile

gilthead sea bream, *Sparus aurata*[48] after handling stress. The decrease in cortisol concentrations can be associated with the inhibition of the transmission of sensory information to the higher brain centers, thereby blocking the series of hormone-controlled changes that lead to stress. These physiological responses are primarily stimulated in cascades and are related to the secretion of glucocorticoid, which influences the energy requirement of the fish in response to any stress[16,49].

Plasma glucose has been used as a biomarker for measuring stress levels and secondary metabolic responses in fish[50]. The level of plasma glucose in the blood depends upon the metabolic production of glucose and the rate at which it is removed from circulation. Animals obtain energy for cellular metabolism through glycolysis or the breakdown of glucose – a cytoplasmic pathway. Stressors cause alterations in glucose metabolisms which can compromise various tissues such as muscle, gill, and brain often leading to glucose intolerance and insulin resistance. The liver is the seat of glucose production via the glycogenolysis and/or gluconeogenesis pathways. It also acts as a reservoir of glucose until the animal needs it for its various energy needs. Closely related stress hormones, adrenaline (epinephrine) and cortisol can increase the plasma glucose levels in fish during stressful conditions[51].

During the present study, *Clarias batrachus* anaesthetized with MS-222, did not exhibit a significant change in plasma glucose concentrations as compared to non-anaesthetized fish. Similar results were observed after 0 h in case of Juvenile silver catfish, *Rhamdia quelen*[52] and *Oncorhynchus mykiss*[53] after anaesthetization. However, a significant increase was reported after giving anaesthesia to juvenile *Colossomam acropomum*[54] and *Rhamdia quelen*[37]. The increase in plasma glucose concentration may be correlated with hypoxia and/or higher fish activity (swimming) after anaesthetization[16,33]. There several reports which indicate decreased levels of plasma glucose concentrations after anaesthetization on some fishes such as *Cyprinus carpio*[55] and *Oncorhynchus mykiss*[56].

Hyperglycemia can result from many environmental stressors and compromise the development, health, and quality of fishes[57]. Increased levels of plasma glucose were seen in golden perch, *Macquaria ambigua* after repeated capture, aquarium transfer, and blood sampling[58]. Grutter and Pankhurst[59] reported significant increases in plasma glucose levels in *Hemigymnus melapterus* after acute handling stress. Hosoya *et al*.[43] also observed significant increases in plasma glucose levels in *Melanogrammus aeglefinus*, when exposed to daily handling stress for four weeks. However, plasma glucose levels exhibited no significant changes after handling stress in the present study. which may further highlight the sturdy nature of the fish, *Clarias batrachus*. Similar results have been reported in different species of fish[46,47,60].Plasma osmolality measures the electrolyte-water balance in animals, including fish. Plasma osmotic pressure/ionic concentration represents the resultant factor of all adaptation mechanisms. Alterations in plasma osmolality represent a secondary stress response in fish. However, the present study on the catfish, *Clarias batrachus* demonstrated no significant changes in plasma osmolality after anaesthetization as well as handling stress by chasing the fish with a handheld net for 3 minutes as compared to non-anaesthetized and non-stressed group of fish (Figure 3), which further indicates the hardy nature of fish. A previous study on another catfish, *Heteropneustes fossilis* also showed similar observations[19]. The results were also comparable with the findings of Cataldi *et al*.[61] on Adriatic sturgeon *Acipenser accarii* which did not seem to be susceptible to overcrowding and prolonged handling stress, since neither the plasma osmolality nor the other blood parameters, such as serum cortisol and glucose, were affected by these stressors. Similarly, the serial netting of immature turbot, *Scophthalmus maximas* from the tanks did not significantly modify the plasma osmolality[62]. According to these authors, it is possible to net the fish, one after the other, without the danger of inducing a physiological stressor. Likewise, no significant changes were observed in plasma osmolality and other blood parameters in cannulated immature turbot at the end of a 9 min aerial exposure[63]. Breves *et al*.[64] also observed no significant changes in plasma osmolality in *Oreochromis mossambicus* after exposure to confinement and net chasing handling stress at 1 h. Similarly, Acerete *et al*.[60] reported no significant changes in plasma osmolality in *Perca fluviatilis* after being subjected to handling stress.

However, some reports indicate increased levels of the haematological parameters (plasma osmolality, haematocrit, and plasma electrolyte) of secondary response after handling and acute stress[64,65].

Anaesthesia is often used before blood sampling to reduce the stress in fish which may otherwise lead to effects such as ionic/ osmoregulatory imbalance[66]. It is apparent from the present study, that anaesthetization of the catfish, *Clarias batrachus* with MS-222 at a dose of 100 mg L^{-1} caused no significant change in the plasma osmolality, which is evident from the similar levels of plasma osmolality in non-anaesthetized and anaesthetized groups. These results further highlight that the fish, *Clarias batrachus*, is hardy and corroborate with the observations on another catfish[19], *Heteropneustes fossilis*, which also shows insignificant differences in plasma osmolality after anaesthetization with MS-222. These results are in agreement with the findings on *Salvelinus alpines*[67], *Argyrosomus japonicas*[68], and *Centropomus parallelus*[69], wherein no significant changes in the plasma osmolality and iono-regulatory ability of the fish after anaesthetization was reported. Though, a significant increase was reported in plasma osmolality in *Ictalurus punctatus*[70] and *Paralichthys olivaceus*[11] after anaesthetization. In contrast, significant decreases in plasma osmolality were reported in *Coreius guichenoti* after exposure to anaesthesia at low temperatures[71]. Similar results were reported by Okey[72] who observed significant decreases in plasma electrolytes in *Heterobranchus bidorsalis* with increases in concentrations when clove was used for anaesthesia.

CONCLUSION

In conclusion, the catfish, *Clarias batrachus* is quite sturdy in nature and not easily influenced by regular laboratory handling procedures. Thus, blood samples can be obtained directly without exposing this fish to anaesthesia. This commercially important fish can be managed and maintained during culture, transfer, shipping, and handling due to its sturdy nature. One of the limitations of this study was the sample size and the species. As part of the future research directions, we would like to build upon the findings of this study and expand this study to include larger sample size and at least three different species of teleosts to conduct a comparative study on these parameters (plasma cortisol, plasma glucose, and plasma osmolality).

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