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The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, Jabłonna

Impact of slaughter method on stress in organic common carp (*Cyprinus carpio*)

B. Łosiewicz and H. Szudrowicz*

Warsaw University of Life Sciences, Institute of Animal Sciences, Department of Ichthyology and Biotechnology in Aquaculture, 02-786 Warsaw, Poland

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* Corresponding author:
e-mail: hubert_szudrowicz@sggw.edu.pl

ABSTRACT. Aquaculture is transforming due to global food demand. Organic carp is a popular fish farmed in ponds, mostly by small and medium enterprises. This is associated with environmental and quality benefits, but also high costs and low production density. Despite being a large sector of food industry, the ethics of aquaculture slaughter methods are perceived as inferior. Implementing the least stressful killing method would ensure better welfare of fish. This study aimed to determine the most optimal strategy among commonly used and approved fish sacrificing techniques. To assess the level of stress experienced by fish, the following biochemical blood parameters were analysed: cortisol, glucose, lactic acid and cholesterol levels, as well as the activity of alkaline phosphatase, amylase, aspartate aminotransferase and alanine aminotransferase. In conclusion, slaughter methods used in the experiment involved three main stress factors: handling time, fish body damage and stunning method. After analysis, we would recommend percussion followed by brain destruction as the least stressful method.

Introduction

The continuously increasing demand for global food production is also transforming the aquaculture sector. Due to insufficient and declining natural resources, this trend is driving the development of inland aquaculture. The most commonly farmed organisms worldwide include tilapia, shrimp and carp. Carp is a fish belonging to the family Cyprinidae, which includes such cultured species as Chinese carp (common carp, *Cyprinus carpio*), amur carp (*Cyprinus rubrofuscus*), crucian carp (*Carassius carassius),* major Indian carp (catla, *Catla catla*), rohu (*Labeo rohita*) and mrigal (*Cirrhinus mrigala*) (Miao and Wang, 2020). Common carp has a long history of aquaculture, dating back 8 000 years (Nakajima et al., 2019). In Europe, common carp is predominantly consumed in central-eastern regions, with Poland, Germany, and Romania being the main importers of live carp (Karnai and Szűcs, 2018). In some countries like Poland, its popularity is linked to the tradition of its consumption, especially during Christmas. According to the aquaculture statistics of the Food and Agriculture Organization of the United Nations (FAO), 123 countries and regions reported carp farms to the FAO in 2018. Moreover its production in the same year reached 28.9 million tons (Miao and Wang, 2020). Carp is typically raised in extensive systems in earthen ponds, either in polycultures or monocultures (Szücs et al., 2007). EUMOFA reports (EUMOFA., 2017; 2022) indicate that this type of production is most often carried out by small and medium-sized enterprises, providing a livelihood for farmers who do not focus on a large volume of product and lack the capital to engage in intensive aquaculture systems preferred by larger enterprises (Raftowicz and Le Gallic, 2020). The advantages of extensive/ecological carp farming include sustainability, fish welfare, and high-quality product, as well as environmental benefits such as water retention, self-purification and increased biodiversity. However, drawbacks include low production volumes and higher prices of the final product. Similar challenges in organic aquaculture have been described by Xie et al. (2013), Gambelli et al. (2019) and Ahmed et al. (2020).

An important aspect of carp aquaculture is the method of stunning and/or killing. According to the 2018 Report from the European Commission to the European Parliament and the Council on the possibility of introducing certain requirements regarding the protection of fish at the time of killing, recommended methods for common carp slaughter included electrical stunning and percussion, resulting in a quick death (European Commission, 2018). Although aquaculture is a significant sector of the food industry, the ethical standards for killing fish are perceived to be inferior compared to those applied for farmed mammals and birds. While stunning followed by bleeding or a rapid killing blow are standard in mammalian and bird livestock breeding, fish slaughter methods that cause unnecessary suffering and stress are still used. Unethical methods such as asphyxiation on air or ice, bleeding, gutting and decapitation without prior stunning are still practised in some parts of the world. Slightly more humane methods like ikijime without percussion are also used. The issue of insufficient standards of fish well-being in aquaculture has already been previously acknowledged and discussed by Conte (2004), Huntingford et al. (2006), Ashley (2007), Grigorakis (2010), Lines and Spence (2014), and Saraiva et al. (2019). While organic carp aquaculture aims to minimise stress levels throughout the rearing process, there is still room for slaughter process optimisation to improve fish welfare. Therefore, identifying the most effective method of killing fish in terms of welfare is necessary to standardise slaughter practises across various fish aquacultures. This is particularly important for organic aquaculture, where fish do not experience much handling or interaction with humans before slaughter (Kestemont, 1995; Adámek et al., 2019). Consequently, fish reared in organic farms may experience even more stress during slaughter compared to fish raised in conventional aquaculture. Implementing the least stressful method of killing common carp is crucial for improving fish welfare and enhancing the quality of fish meat, thereby assisting small and medium ecological aquacultures in providing superior end products. The objective of the study was to identify the method of euthanising organic carp that generates the least stress by assessing primary and secondary physiological stress responses. Blood parameters have been selected as indicators due to their well established correlation with such reactions (Wendelaar Bonga, 1997).

Material and methods

The experiment did not require the consent of the local ethics committee in accordance with the Polish Law on the Protection of Animals Used for Scientific or Educational Purposes. A total of 30 carp specimens were purchased from The Stanisław Sakowicz Inland Fisheries Institute in Żabieniec, Poland, where fish were kept under ecological conditions in accordance with European Union regulations. The facility was fed with water from the Czarna River, and sampling took place at the end of the 3-year production cycle in December, at a temperature of 6 °C, directly after catching, following 24 h of retention in a high-water flow tank located at the facility. The fish were subjected to the following six killing procedures (n = 5 for each group) according to the reports of the General Veterinary Inspectorate (Poland) and EFSA (EU): percussion (P), percussion followed by spinal cord rupture (P+SR), percussion followed by brain damage (P+BD), electrical stunning (E), electrical stunning followed by spinal cord rupture (E+SR), and electrical stunning followed by brain damage (E+BD). After death, blood samples were quickly collected from the caudal vein of each fish using heparinised vacutainer tubes. Immediately after blood collection, a portion of it was placed in capillaries for haematocrit determination using a haematocrit centrifuge. The remainder of the blood was centrifuged to isolate plasma. Fish body parameters, including total length, standard length, and mass were measured, and Fulton's condition factor was calculated using the following equation:

$$CF = \frac{w \times 100}{tl^3},$$

where: CF – Fulton's condition factor, w –fish weight (g), tl – total length (cm).

To determine the level of stress experienced by the fish, the following biochemical blood parameters were analysed: cortisol level [ng/ml] (DRG, Springfield, USA kit); glucose, lactic acid, total protein and cholesterol levels [mg/dl], as well as amylase, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities [U/l] (Spinreact, Girona, Spain kits). All measurements were performed at 37 °C, in triplicate, using a Tecan Infinite 200 PRO plate spectrometer (Tecan Austria, Grödig, Austria) according to the kit manufacturers' instructions and recommendations.

Statistical analysis

All quantitative data (body measurements, Fulton's condition factor, haematocrit and serum biochemical parameters) were attested for normal distribution using the Shapiro-Wilk test. Subsequently, depending on the result (*P*-value higher or lower than 0.05), each parameter was analysed using the appropriate statistical tests. Parameters with a normal distribution (body measurements, Fulton's condition factor, levels of cortisol, glucose, lactic acid, total protein content, and activities of ALT and AST) were checked with a one-way ANOVA followed by a post-hoc Tukey test. Parameters with a non-normal distribution (haematocrit, cholesterol level, and amylase activity) were analysed using the Kruskal-Wallis test to determine statistically significant differences. All analyses were performed using STATISTICA (v13.3).

Results

The fish body measurements used in this experiment were homogeneous among all groups and included the following parameters: body mass 1.75 ± 0.26 kg, total length 42.68 ± 2.4 cm, standard length 36.25 ± 2.21 cm and Fulton's condition factor 2.25 ± 0.17.

The data from Figure 1 show that the activity of ALT differed significantly between all groups. The highest ALT activity was observed in the E+SR group, while the lowest levels were recorded in the P, P+BD and E+BD groups (Figure 1A). The lowest cortisol levels were observed in the P group, while the highest in the E group (Figure 1B). The lowest concentrations of glucose and lactic acid occurred in the P+BD and P groups. The highest concentrations of glucose were detected in the E+BD group, while highest levels of lactic acid were noted in the E and E+SR groups, which also exhibited high levels of glucose (Figures 1C,D). Haematocrit results varied significantly between the groups, with the

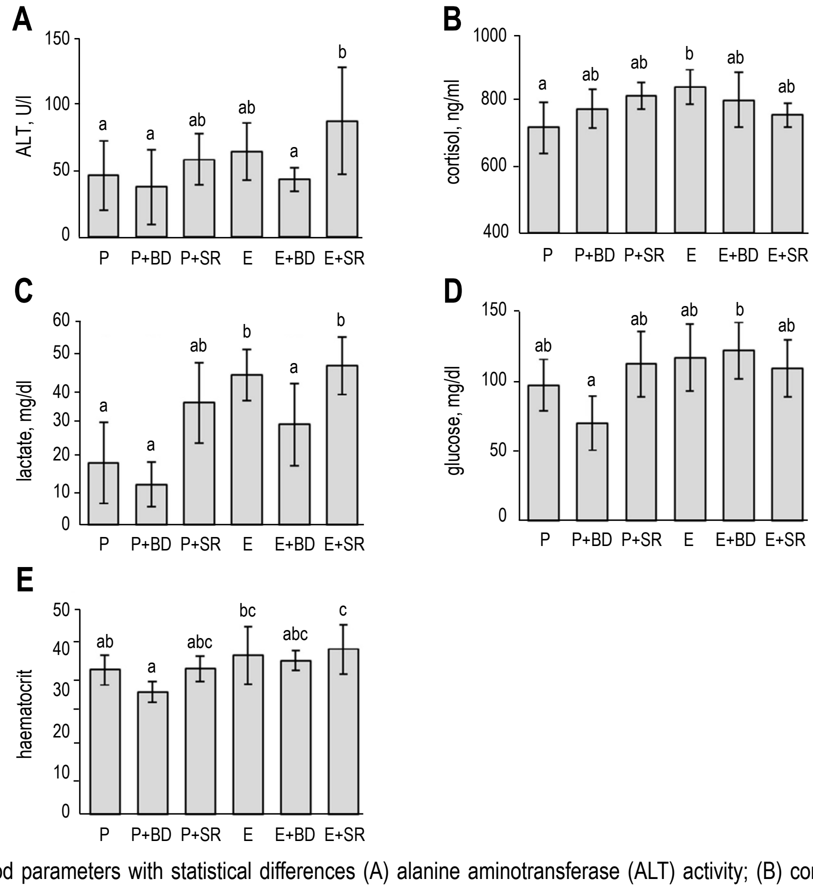

Figure 1. Selected blood parameters with statistical differences (A) alanine aminotransferase (ALT) activity; (B) coristol level; (C) lactic acid concentration; (D) glucose concentration; (E) haematocrit. Values are presented as mean ± standard deviation; lowercase letters indicate statistically significant differences between individual homogeneous groups at $P < 0.05$. (P) percussion, (P+SR) percussion followed by spinal cord rupture, (P+BD) percussion followed by brain damage, (E) electrical stunning, (E+SR) electrical stunning followed by spinal cord rupture, and (E+BD) electrical stunning followed by brain damage.

most notable differences observed in the P group (Figure 1E).

The obtained amylase activities showed no statistical differences among the groups. However, the highest amylase activity was recorded in the P group (Figure 2C). In the measurements of cholesterol and aspartate aminotransferase, the highest concentration of the steroid was found in the E+BD and E+SR groups, which also showed the lowest AST activity. Meanwhile, the highest activity of AST was detected in the P+SR group, which along with the P group, had the lowest cholesterol levels (Figures 2A,D). The concentration of total protein was relatively similar in all groups with slightly higher levels observed the P+SR group (Figure 2B).

a predator), physical (e.g., handling, transportation) and chemical (e.g., water acidification, low oxygen content pollution). Slightly stressful situations can have a positive effect (eustress), while severe stress, inducing adaptive responses of the body, can have negative consequences (distress). Stress response can also be grouped into 3 tiers (Wendelaar Bonga, 1997). The primary stress response is initiated and controlled by two hormonal pathways that lead to the production of corticosteroids (mainly cortisol) and catecholamines (Gorissen and Flik, 2016). Both of these systems regulate the secondary response to stressors that disrupt distribution of oxygen and energy sources, as well as the osmotic balance and immune system. The tertiary stress response

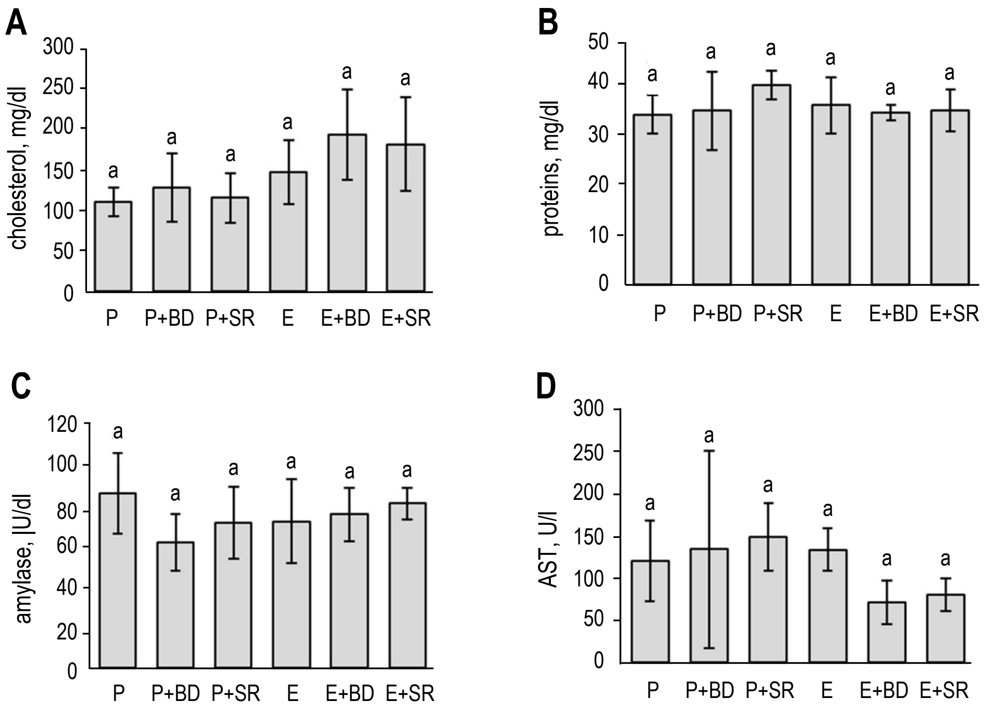

Figure 2. Selected blood parameters without statistical differences (A) cholesterol concertation; (B) total protein concentration; (C) amylase activity; (D) aspartate aminotransferase (AST) activity. Values are presented as mean ± standard deviation, lowercase letters indicate statistically significant differences between individual homogeneous groups at $P < 0.05$. (P) percussion, (P+SR) percussion followed by spinal cord rupture, (P+BD) percussion followed by brain damage, (E) electrical stunning, (E+SR) electrical stunning followed by spinal cord rupture and (E+BD) electrical stunning followed by brain damage.

Discussion

The physiological response of a fish to a threatening situation or any other noxious stimuli, as in all vertebrates, is referred to as stress. The stress response is activated almost immediately upon perception of the stressor (Rose, 2002). In the context of fish, stress factors can be divided into three main groups: perceived (e.g., presence of introduces behavioural changes, growth abnormalities, which may also lead to lowered immunity. These changes can occur as a result of primary and secondary stress responses. If the fish survives an encounter with a stressor, a pre-threat reminiscent state is restored (Barton et al., 1998, 2002; Schreck and Tort, 2016; Daskalova, 2019). The present study sought the least stressful method of killing organic carp; to this end, the research parameters were selected to comprehensively analyse the physiological primary and secondary response to stress. Physical parameters validated the representativeness of the groups in relation to the population. Cortisol levels were measured to assess differences in the intensity of the primary stress response between culling methodologies. Other biochemical parameters measured, along with haematocrit, examined the level of the secondary stress response (Akbary et al., 2016; Seibel et al., 2021). Haematocrit levels indicate changes in blood composition due to stress, as does total protein content. Glucose and lactic acid concentrations reflect alterations in metabolism resulting from stress (Velisek et al., 2011), particularly aerobic and anaerobic muscle activity. Additionally, amylase provides insights into metabolic changes, because it is involved in glycogen breakdown (Burt, 1966). During this experiment, AST and ALT were primarily utilised as indicators of cell damage, as their elevated plasma levels are often associated with tissue damage or necrosis. While mainly associated with hepatocytes, these enzymes can also be found in the kidney, heart, and skeletal muscles. Consequently, they are frequently used to assess liver damage, albeit lacking specificity, as they may also signify cardiac or skeletal muscle damage (Kim et al., 2007; Pettersson et al., 2008; Yap and Aw, 2010; Nie et al., 2011) due to metabolic changes induced by stressors (Chatterjee et al., 2006; Velisek et al., 2011; Toni et al., 2014). In this experiment, they were applied as cell damage markers and to determine whether they can signal a stress response. The measured lactic acid content provides information about the level of acidification in the fish's body resulting from the slaughter method. Cholesterol is an important substrate in many biosynthetic pathways, including its role as a precursor of steroid hormones and participation in regulatory processes of cell membranes (Tabas, 2002); variations in cholesterol levels may indicate a more acute stress response. The main differences in the killing methodologies used included the type of stunning, with or without the rupture of the fish's body. This categorised the methods into four groups: two based on the damage to the fish's body or lack thereof, and two based on the type of stunning employed – electricity or percussion. The results of the biochemical panel indicated a difference in induced stress between piercing the fish's body during killing and not compromising the fish's integrity.

Disruption of one of the fish's nerve centres affects the post-mortem behaviour of organs and muscles, as evidenced by the results of glucose and lactic acid levels, as well as ALT and AST activities. These nerve centres play a crucial role in the stress response in fish (Schreck and Tort, 2016). In the context of muscle work, brain destruction seems to be particularly effective in reducing aerobic and anaerobic processes. The E group exhibited fairly high glucose concentrations, while the P group the lowest. In addition, lactic acid levels were the lowest in both stunning types, which suggested a significant reduction in anaerobic activity. These findings were consistent with results obtained by Morzel et al. (2003). Although glucose levels indicate the onset of glycogenolysis, the P group showed the lowest intensity of this process in the whole experiment. In contrast, spinal cord interruption results indicated intensive glycogenolysis and anaerobic muscle activity in fish. This implies that brain disruption might be more beneficial for the well-being of the fish and the potential quality of the meat obtained. In addition to metabolite results, ALT and AST activities could indicate liver damage. However, due to the lack of specificity (Pettersson et al., 2008), these enzymes likely reflect the degree of muscle damage caused by body puncture, percussion, or current-induced spasms. Elevated levels of these enzymes have also been reported in studies concerning transportation stress (Dobšíková et al., 2009; Pakhira et al., 2015), which was relatively short compared to other studies examining prolonged exposure to stress factors and these enzymes (De Smet and Blust, 2001; Joseph and Raj, 2011; Yousefi et al., 2020; Nephale et al., 2024). A study by Pakhira et al. (2015) was the only one that reported elevation in enzyme activities indicating liver damage. ALT and AST have also been utilised in other studies to determine tissue injury. This was also reflected in the present results, which showed higher ALT activity in the case of spinal cord rupture. A similar relationship could be observed for AST; despite higher overall values in all percussion-stunned groups, the highest levels of AST activity was recorded for spinal cord rupture.

The stress response also occurs in unconscious fish, triggered by a purely primary cellular reaction, without fish perception (Rose, 2002), such as the observation of a threat. This type of response was also indicated by the results of the present experiment, with individuals from the P group showing the lowest plasma cortisol levels. Conversely, in the E group, the highest levels of cortisol were recorded in the entire experiment. This could be due to a further neuroendocrine stress response that caused the release of cortisol and a cellular stress response throughout the body resulting from the electric shock. Changes in cortisol levels indicate the presence of a strong cellular stress response induced by electrocution, suggesting that the electric current alone activates neuroendocrine mechanisms to release cortisol even after the fish's death. Similar conclusions have been drawn by researchers studying electrical accidents, in which patients often developed neurological problems after exposure to high voltage currents, while low voltages often required prolonged exposure to cause complications (Huan-Jui et al., 2010; Boudier-Revéret et al., 2020). Additionally, the P group had one of the lowest corticosteroid levels, suggesting a fairly effective suppression of neuroendocrine functionality by percussive stunning compared to electrocution. Conclusions reached by other scientists are similar, with methodologies involving electrocution being described as more humane than, for example, air asphyxiation (Van De Vis et al., 2003; de la Rosa et al., 2021). Electric shock is still being regarded as insufficient in terms of fish welfare (Zampacavallo et al., 2015). Although Ribas et al. (2007), Sattari et al. (2009) and Trushenski and Bowker (2012) have published promising utilisation of this methodology in temporary sedation.

Regarding the second factor that divides methodologies into groups, i.e., the type of stunning methodology, these procedures differ primarily in the handling time leading to the actual stunning in addition to the type of stimulus causing stunning. Fish stunned with electricity are exposed to additional stress due to prolonged handling procedures related to transportation, tank changes and equipment setup, while during percussion, fish are stunned immediately upon removal from the water (Morzel et al., 2003). The longer handling time results in higher stress levels before the fish's death, as shown by higher levels of cortisol, glucose, or lactic acid in the electrocuted groups compared to the percussively stunned groups. Similar conclusions have been drawn by other researchers (Skjervold et al., 1999; Mørkøre et al., 2008; Acerete et al., 2009), who found that techniques requiring long handling times or resulting in a slow death were more stressful than those causing a quick death.

The effect of sacrificing methodologies on the quality of organic carp meat is an important issue. While the primary stress response plays a lesser role in the subsequent physical properties of the meat, the secondary response involving metabolic changes significantly impacts it. Specifically, the breakdown of glycogen to glucose and the generation of lactic acid due to anaerobic muscle work is among the processes most negatively affecting meat quality. Thus, methods inducing more anaerobic motility result in inferior meat characteristics. This is evidenced by lower levels of glycogen, creatine phosphate, and ATP in muscle directly post-mortem as well as lower initial pH caused by stress. Consequently, meat has poorer water retention properties, and more visible yellow and red discoloration during prolonged cold storage time. In addition, ante-mortem stress leads to weaker meat structure, earlier onset of rigor mortis, and faster loss of freshness through faster onset of protease activities after death (Daskalova, 2019; Terlouw et al., 2021). These effects have been described in various fish species, including Atlantic cod, highlighting the significant impact of killing methods on meat quality (Poli et al., 2005, Kristoffersen et al., 2006, Hultmann et al., 2012).

Conclusions

In summary, slaughter procedures examined in the present experiment involved three main stress factors: handling time, fish body disruption and the stunning method. These stress factors can be categorised as perceived and physical, and they begin with fish removal from the tank. Physical stressors, particularly the disruption of the fish's body, contribute to stress induction even in unconscious fish, leading to cellular-level stress responses triggered by tissue damage. Considering this information and prioritising both the welfare of the fish as well as the potential quality of their products, it is advisable to administer a quick and certain death using the method of percussion followed by brain destruction. Various blood biochemical parameters were assessed in the present experiment, revealing varying degrees of usefulness in studying exposure to short-term stressors. Glucose, lactic acid and cortisol emerged as particularly informative indicators, reflecting the immediate stress response. Furthermore, the activity of amylase, cholesterol levels, haematocrit and total protein may provide additional background about the stress response. On the other hand, the activity of aspartate aminotransferase and alanine aminotransferase can signify tissue damage rather than direct response to stressors.

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Conflict of interest

The Authors declare that there is no conflict of interest.

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