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The Effects of Stress on Physiology and Meat Quality in Cultured Channel Catfish

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Stress during fish culture can impact growth, physiology and fillet quality. Maintenance of high quality seafood is important to ensure the production of a highly marketable product. The present study assessed how sequential stressors affect the physical, physiological, sensory and quality characteristics of channel catfish (*Ictalurus punctatus*) fillets. Temperature (25°C or 33°C) and dissolved oxygen (DO, ~2 mg/L or >5 mg/L) were maintained for four weeks, followed by socking and transport stress for a total of 12 treatments. After each stage of stress (environment, socking and transport), physical (length, weight and feed consumption), physiological (hematocrit, plasma cortisol, pH, glucose, lactate, total protein, osmolality and ionic composition) and fillet quality (color, texture, sensory and pathogen load) attributes of the fish and fillets were evaluated. Fillet yield decreased with increasing severity of environmental stress. Overall, increasing stress resulted in decreased feed consumption, growth and fillet yields. A cumulative stress response was identified with regard to circulating cortisol and glucose, which increased with each sequential stress event. Under low oxygen conditions there was a suppression of the stress response. Handling imposed a more pronounced

physiological response than environmental conditions. The sequential stressors resulted in a less intensely colored fillet that was less red, which suggests the quality changes imposed are beneficial to the marketability of the fillets. However, increased redness in fish reared under high temperatures and oxygen levels suggest that an increased prevalence of red fillets can be expected. Sensory analysis revealed that fillet flavor was acceptable in all treatments with the severe stress treatment preferred due to lower intensity of less favorable flavor attributes. The changes in flavor were presumably due to fasting of the stressed fish and a subsequent purging of fat stores. As fish progressed through the harvest event, cook loss decreased, tenderness increased and pH increased, indicating that stress induced positive textural changes. Proteomic analysis revealed mainly down-regulation of structural and metabolic proteins, which indicates higher proteolytic activity and an adjustment in energy metabolism in response to stress. The overall effects of chronic environmental conditions and handling highlight the importance of managing for stress in cultured channel catfish.

DEDICATION

I dedicate this to my family who have encouraged and supported me throughout my academic career and without whom this would never have been possible.

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CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

Fish represent a valuable food source throughout the world. They contain an ample supply of highly digestible protein, n-3 poly unsaturated fats and vitamins and minerals to supplement the human diet and promote healthy living (Monti et al., 2005; Tuomisto, 2008). As populations worldwide continue to increase, the demand for aquatic foods is also increasing (Rodrigues et al., 2012). This has resulted in the depletion of commercially important wild fish species and a reliance on cultured fish (NOAA, 2012). Growth in the aquaculture industry is expected to increase further (Rodrigues et al., 2012).

1.1 Catfish aquaculture

Farming of channel catfish (*Ictalurus punctatus*) represents the largest segment of aquaculture in the United States (Vilsack and Reilly, 2014). Channel catfish generally have an elongated body with a slightly compressed head, dark black skin on the dorsal side and a light white or silver ventral belly. They are native to Mexico, the states bordering the Gulf of Mexico, the Mississippi Valley and the Red River Valley. Over the past century channel catfish have been widely introduced throughout the United States and world (Hubert, 1999; Tucker and Robinson, 1990). They inhabit fresh water to brackish water environments with quick to moderate water flow but can be found in a

variety of other slow moving, turbid environments (Tucker and Robinson, 1990). The euryoecious nature of this species makes it an ideal candidate for culture in ponds, which can experience drastic fluctuations in environmental conditions throughout the year.

Pond culture represents the most economical method for large scale production compared cage and raceway culture (Tucker and Robinson, 1990). A disadvantage of pond culture is that high stocking rates and the resulting high nutrient inputs can result in drastic fluctuations in environmental conditions (Arnold et al., 2013). These fluctuations can ultimately result in stressful conditions that may have negative effects such as losses at harvest or decreased quality post-processing.

Cultured catfish are sold to processors who prepare the fish as a marketable product for distribution and sale. Procedures and techniques can vary between processors but the processing steps are generally similar. Processing consists of euthanizing, de-heading, filleting, skinning, trimming, sizing, chilling, freezing, storing and distribution (Silva et al., 2001). Procedures and policies ensure food safety, food security and product quality. However, processing techniques alone cannot control product quality. Fish quality at harvest is affected by many physiological and biochemical factors. These factors are often a direct result of environmental stimuli and culture practices.

1.2 Stress physiology

The term stress was coined by Hans Selye in 1936, when examining the effects of noxious stimuli in laboratory animals (Harper and Jeffrey, 2009). He defined the term as "the non-specific response of the body to any demand for change" (Selye, 1936). Any stimulus that causes physiological changes is considered a stressor (Sánchez et al., 2011). The stress response is an adaptation that allows fish to cope with the stressor and

maintain a normal homeostatic state (Barton, 2002). The alterations in physiology and biochemistry observed post-stress represent the stress response of an organism. In fishes, there are many environmental stimuli that will induce a stress response. The energy devoted to initiating and maintaining the stress response is no longer available for basic metabolic activity and growth. Severe and chronic stress can be detrimental to fish health and even lethal (Bosworth et al., 2007; Eva et al., 2010).

The physiological and behavioral responses of fish to stress are considered adaptive mechanisms, allowing a fish to cope with the stress and maintain a homeostatic state (Barton, 2002). If the imposed stress is severe or chronic it may become maladaptive, leading to a whole body response that can be detrimental to fish health (Barton, 2002). The stress response can be separated into three levels (Small et al., 2008). The first is a cellular endocrine response signaling the induction of a stress event (Iwama et al., 1998). This primary response results in elevation of stress hormones in the blood including catecholamines and cortisol (Wang et al., 2004). Cortisol represents the principle corticosteroid stress hormone that is commonly used to quantify stress in fish (Barton, 2002)**.** The secondary response consists of the biochemical and physiological changes that result from the increased hormone concentrations in circulation. Some of the hematological changes observed can include reallocation of energy reserves, alterations in ionic composition and increased hormonal secretions (Wendelaar Bonga, 1997). For example, during stress, circulating cortisol concentrations increase and can result in glycogenolysis in the liver, where glycogen storage is greatest, and subsequent increase in blood glucose concentrations (Iwama et al., 1998). This glycemic response has been observed following handling stress in juvenile salmon and striped bass (Sharpe et al.,

1998; Wang et al., 2004). The increasing blood glucose concentration is a product of mobilized energy from stored reserves to cope with the changing energy demands induced by the stress event (Iwama et al., 1998). The final tertiary phase of the stress response represents the population or whole animal response to the stressor. These can include a decreased ionic and osmotic stability, immune function, growth and tolerance for additional stressors (Wendelaar Bonga, 1997). Re-allocation of energy reserves can have significant effects on somatic and reproductive growth (Iwama et al., 1998). Different stress events will elicit various physiological stress responses, which can vary depending on genetics, developmental stage, environment and frequency of the stress (Barton, 2002). These changes may translate into biochemical changes in the muscle, which could ultimately result in deleterious effects on meat quality. Decreased meat quality post stress has been observed in many aquatic (Bosworth et al., 2004; Bosworth et al., 2007; Bjørnevik and Solbakken, 2010; Eva et al., 2006; Skjervold et al., 1999) and terrestrial vertebrates (Imik et al., 2012; Zeferino et al., 2012; Zhang et al., 2012). This can have significant impacts on cultured fish as stress is inherent in aquaculture practices (Barton, 1997; Conte, 2004).

1.3 Stress in aquaculture

Various aspects of aquaculture, including: handling, sorting, grading, transport and poor water quality, can elicit a stress response in fish and have negative impacts on their overall health and well-being (Barton, 1997; Conte, 2004). Common stressors observed in cultured species include: temperature (Bosworth et al., 2004), oxygen content (Bosworth et al., 2004), stocking density (Eva et al., 2010), handling and transport (Bosworth et al., 2004; Bjørnevik and Solbakken, 2010). Pond water temperatures in the

Southern United States can exceed 35°C (Arnold et al., 2013; Davis and Simco, 2001; Wax and Pote, 1990) in the summer months. Thermally-induced stress responses have been documented in channel catfish (Davis and Simco, 2001) and rainbow trout (Sánchez et al., 2011). Catfish in culture ponds also experience diurnal changes in temperatures (Davis and Simco, 2001). High summer temperatures (~30 °C) prior to harvest have been shown to deteriorate catfish muscle quality (Bosworth et al., 2007). Changes in dissolved oxygen (Kayim et al., 2011) can also induce stress. Oxygen is considered one of the most limiting factors affecting fish production (Hargreaves and Steeby, 1999). Oxygen concentrations below 1 mg/L have been recorded in catfish ponds (Hargreaves and Steeby, 1999), especially during the summer months when water temperatures are high. The high feeding rates during grow-out ultimately result in poor water quality (Tucker and Robinson, 1990).

Handling and transport also impose significant stress on cultured species (Harper and Jeffrey, 2009; Poli et al., 2005). During harvest catfish are held at high densities in socks prior to transfer into hauling trucks (Bosworth et al., 2004). Fish to water ratios are even higher during transport.

Chronic stress in a semi-extensive culture system such as catfish ponds is usually due to environmental conditions, which are sometimes difficult to manage (Culberson and Piedrahita, 1996). One of the most readily observed effects of chronic stress is its impact on fish growth. The negative impacts of high water temperatures (Andrews et al., 1973; Arnold et al., 2013; Buentello et al., 2000; Stewart et al., 2014) and low oxygen concentration (Green and Rawles, 2011; Torrans, 2008) on fish growth and survival have been well documented. Changes in growth can be associated with alterations in feed

intake due to a variety of different stressors (Bernier and Peter, 2001; Buentello et al., 2000; Green and Rawles, 2011). Cultured catfish experience drastic fluctuations in temperature and dissolved oxygen (DO) concentrations (Davis and Simco, 2001; Hargreaves and Steeby, 1999; Wax and Pote, 1990).

These two stressors represent the two most important factors affecting growth, reproduction and survival in production ponds (Arnold et al., 2013; Buentello et al., 2000; Hargreaves and Steeby, 1999; Torrans, 2005; Torrans et al., 2012 Tucker and Robinson, 1990). Buentello et al. (2000) examined the interactive effects of temperature and DO on juvenile catfish and found that they interact to affect consumption, feed utilization and growth. Thus, understanding the interaction between these two stressors and their effects on growth and feed consumption is vital to developing effective management strategies.

Stress in fish results in many physiological changes. Acute stress can elicit changes in plasma glucose and lactate (Poli et al., 2005), ionic composition and osmotic balance (Wendelaar Bonga, 1997) and total protein concentrations (Coeurdacier et al., 2011). Increased blood lactate, glucose and cortisol and decreased pH and oxygen content represent some of the commonly observed changes in fish experiencing stress (Wendelaar Bonga, 1997). These changes in blood chemistry are commonly observed in commercially-important species following low-water confinement, crowding and low DO (Bosworth et al., 2004; Bosworth et al., 2007; Minchew et al., 2007; Small, 2004; Small et al., 2008; Strange, 1980). Following acute and long-term handling and transport stress, salmonids exhibited an endocrine and hematological stress response (Barton, 2000; Fast et al., 2008). In sea bass (*Dicentrarchus labrax*), total serum protein changes were

reported in response to high stocking densities, sequential transfer, hypoxia, hypercapnia and nodavirus injection (Coeurdacier et al., 2011). Temperature dependent alterations in stress related hematological parameters were observed in striped bass (*Morone saxatilis*) following confinement stress (Davis and Parker, 1990). An increased circulating cortisol and glucose concentration in response to stress is well documented in many species (Martínez-Porchas et al., 2009).

Prior acclimation conditions can affect a fish's response to a particular stress event. Temperature-dependent differences in physiological stress responses have been reported in channel catfish (Bosworth et al., 2007; Strange, 1980), longjaw mudsucker (*Gillichthys mirabilis*) (Logan and Somero, 2011), striped bass (Davis and Parker, 1990) and Atlantic cod (*Gadus morhua*) (Methling et al., 2010). Exposure to multiple stressors can elicit a cumulative stress response (Barton, 2002) while acute stress events likely don't have lasting effects on the fish (Davis and Small, 2006). This is especially important since pre-harvest stress events affect post-harvest and processing quality attributes of fish meat (Bosworth et al., 2007; Bjørnevik and Solbakken, 2010; Digre et al., 2011; Erikson et al., 1997; Ginés et al., 2004; Hansen et al., 2012; Johnston et al., 2006; Morzel et al., 2006; Silva et al., 2012).

1.4 Stress and fillet quality

Quality in fish products encompasses a variety of different physical, chemical, biochemical and biological characteristics. "Quality" is a very subjective term that is commonly used but often lacks a specific definition as to the components used to address it (Bremner, 2002). Thus, the physical, chemical, biochemical and biological characteristics of the fish product that will be used to address quality must be defined. For

the purposes of this study, quality will refer to the physical, chemical and biochemical characteristics that affect overall consumer acceptance and preference. These characteristics will include color, texture, water holding capacity (WHC), pH and proteome expression.

The effects of stress-induced changes on meat quality have been widely studied in a variety of meat products. The effects of temperature stress on meat quality characteristics can include changes in color, texture and protein degradation. These changes have been documented in many terrestrial (Imik et al., 2012; Zeferino et al., 2012; Zhang et al., 2012) and aquatic (Bosworth et al., 2007; Ginés et al., 2004; Johnston et al., 2006) species. The alterations observed are typically attributed to changes in consumption of food and water and alterations in metabolic processes to cope with the stressor (Imik et al., 2012; Small et al., 2008).

There are many physiological and biochemical factors that will affect muscle quality in fish. Significant alterations in pH, protein expression, texture, cook loss/water holding capacity, color and susceptibility to microbial spoilage have been previously reported. Pre-slaughter activity and handling have been shown to alter fillet quality characteristics such as protein structure and integrity (Morzel et al., 2006; Silva et al., 2012), color (Bjørnevik and Solbakken, 2010; Desai et al., 2014; Digre et al., 2011), water holding capacity and texture (Bjørnevik and Solbakken, 2010) in many fish species. Dissolved oxygen concentrations during transport of channel catfish were shown to affect fillet color and water holding capacity (Bosworth et al., 2004). Ante-mortem handling stress resulted in a decreased fillet pH in Atlantic salmon (Erikson et al., 1997; Hansen et al., 2012). Pre-harvest activity depletes energy reserves and limits the scope of

aerobic respiration post processing, leading to a rapid decrease in pH due to lactic acid build up through anaerobic respiration (Huss and Gill, 1995). The rapid decrease in pH results in a more rapid onset of rigor and can ultimately affect protein structure and function, altering the textural characteristics of the meat (Sikorski and Pan, 1994)

Changes in protein expression following stress are caused by enzymatic processes that control the production and break down of energy molecules such as adenosine triphosphate (ATP) and those involved in coping with oxidative stress (Silva et al., 2012). Pre-slaughter stressors increase proteolytic processes in the muscle resulting in alterations in protein expression through fragmentation (Eva et al., 2010; Morzel et al., 2006; Silva et al., 2012). This can cause both textural and flavor changes (Rodrigues et al., 2012). Pre-slaughter chilling and crowding stress caused a decrease in firmness of salmon fillets (Lerfall et al., 2015). Similar trends in softening of salmon fillets have been reported following short term (20 min) crowding stress (Hansen et al., 2012). Hansen et al. (2012) also evaluated the effects of long-term stress and found that firmer fillets were produced when fish were exposed to long-term crowding. Similar to what was observed following short-term crowding stress, ante-mortem handling stress resulted in a softening of Arctic char (Jittinandana et al., 2003) and Atlantic salmon (*Salmo salar*) (Erikson et al., 1997) fillets. Arctic char reared at 10°C produced softer fillets when compared to those reared at 15°C (Ginés et al., 2004)**.** Textural changes result from alterations in protein folding and structure, which have been observed due to post-mortem proteolysis of myofibrillar proteins (Rodrigues et al., 2012; Silva et al., 2012). Textural changes may also be brought about by changes in water holding capacity due to protein degradation (Rehnein, 2002).

Thus, enhanced textural quality of fish meats could be achieved through reduction in pre-harvest and slaughter stress (Erikson et al., 1997).

Rigor mortis, which occurs post-mortem in muscle tissues, can have a profound effect on muscle quality. During rigor mortis, the last of muscle energy reserves are depleted and the tissues resort to anaerobic respiration to meet their energy requirements (Haard, 2002). Lactic acid begins to build up in the muscle tissues, which decreases pH to an extent that ATP synthesis is inhibited (Robb, 2002). The extent of lactic acid build up depends greatly on the amount of ATP and glycogen present at the time of death. In addition, the energy available post-mortem will vary as a result of pre-slaughter stress. Stressors during harvest induce a stress response, which alters the use of energy reserves to elicit the physiological changes necessary to cope with the stress event and maintain a homeostatic physiological state. The more severe the stress event the more energy is used pre-slaughter, resulting in less available energy during rigor when flesh is converted to meat through post-mortem physiological changes. Alterations in pH and onset of rigor have been observed in many fish species post stress (Bosworth et al., 2004, 2007; Bjørnevik and Solbakken, 2010; Elisabete et al., 2010; Silva et al., 2012). The majority of quality changes that occur post-rigor are due to a loss in enzymatic regulation and function due to decreased pH, caused by anaerobic metabolism (Haard, 2002).

In addition to chemical and textural changes, stress can alter fillet sensory attributes, especially flavor, which is important for product quality (Johnsen and Kelly, 1990) and for ensuring marketability of the product. When assessing quality in fish there are two main methods available. The first includes sensory evaluations, which are subjective as they rely on human senses to evaluate quality. The second includes

objective non-sensory methods, which are based on analytical techniques that use instruments or laboratory techniques to quantify particular characteristics of fillets. Some commonly measured sensory characteristics of quality include appearance, aroma, texture, flavor, tenderness, water holding capacity and nutritive composition (Alasalvar et al., 2002; Kayim et al., 2011). Some commonly used procedures include: proteomic evaluation, texture analyzers, color analyzers, microbial analysis and quantitative analysis of muscle components (Alasalvar et al., 2002; Bosworth et al., 2007). Changes in the non-sensory quality characteristics of a product will often result in alterations of the sensory characteristics. These alterations in the sensory characteristics of a product can result in the production of off-color, odor and flavors, which make products undesirable to consumers.

1.5 Color

When purchasing meat and seafood products, consumers consider color closely as a means of assessing freshness and quality (Maciel et al., 2014). Alterations in the color of whitefish fillets is due to a variety of different factors including residual blood (Roth et al., 2007), lipid oxidation (Ruff et al., 2002), storage time (Guillerm-Regost et al., 2006), the food consumed (Li et al., 2007), storage temperature and method of slaughter (Stien et al., 2005). Color is especially important with regard to catfish as any deviation from the typical white flesh color results in decreased marketability of product (Lovell, 1984; Shahidi and Brown, 1998).

The effects of ante-mortem stress on color are well documented in muscle meat (Akşit, 2006; Bosworth et al., 2004, 2007; Erikson and Misimi, 2008; Ginés et al., 2004; Robb et al., 2000). Ante-mortem rearing and crating temperatures altered color in broiler

meat, with high temperature rearing leading to a higher percentage of lighter and redder meat (Akşit, 2006). Increased prevalence of pale soft exudative meat (PSE) was reported in swine after exposure to temperature, transport and loading stressors (Santos et al., 1997). Alterations in fillet color, including lightness (L*), redness (a*), yellowness (b*), hue and chroma values were documented in salmonids when exposed to stress and varied depending on the type of stress experienced (Erikson and Misimi, 2008; Robb et al., 2000). In rainbow trout (*Oncorhynchus mykiss*), high ante-mortem activity, simulated through electro-stimulation immediately after slaughter resulted in lighter, less red fillets with higher chroma values (Robb et al., 2000). Bosworth et al. (2007) demonstrated that fish that were rested prior to harvest experienced a minimal stress response, resulting in higher quality fillets when compared to the stressed fish. Temperature dependent changes in fillet color were reported in arctic char (*Salvelinus alpinus*), with higher redness, yellowness and chroma intensity in fish reared at a colder temperature (10°C), resulting in a more intense orange color when compared to fish reared at a warmer temperature (15°C) (Ginés et al., 2004). In channel catfish, low pond dissolved oxygen (DO) concentrations resulted in lighter fillets when compared to fish reared with high DO (Bosworth et al., 2004).

Yellow and red fillet discoloration represent the most common off-colors observed in processing facilities (Desai et al., 2014; Li et al., 2007, 2013; Lovell, 1984). Yellow fillets are caused by increased pigmentation in the muscle that is incorporated through consumption of natural foods present in ponds (Li et al., 2007). The increased red coloration in catfish fillets has been correlated with increased hemoglobin in the muscle (Desai et al., 2014) and is believed to be caused by ante-mortem stress. Similarly,

in Atlantic salmon, slaughter procedures used at harvest were correlated to changing hemoglobin concentrations in the white muscle (Olsen et al., 2006). Hemoglobin is located in red blood cells (RBC) and is the primary respiratory pigment modulating the RBC's oxygen carrying capacity. The transport of oxygen by RBC's is integral for respiration and the production of energy in living organisms.

1.6 Sensory evaluation

Sensory evaluation of food products is commonly used throughout the food processing industry for monitoring quality and freshness of a variety of food products. A combination of many different water and fat-soluble compounds results in basic flavors and taste (Kayim et al., 2011). Alterations in the concentrations of these various flavor compounds can result in changes in flavor and preference of meat products. Changes in flavor compounds result from the treatment of the meat including handling, storage and cooking. The ability to assess alterations in sensory characteristics and the average consumer's ability to detect such changes are integral to the development of quality products and represent the basis of sensory testing. The science of sensory evaluation incorporates many different sciences including psychology, anatomy, physiology and psychophysics (FDA, 2013). Sensory testing involves human subjects and focuses on their abilities to see, taste, feel, smell and even hear (Meilgaard et al., 2007a). The human subjects used for sensory tests are considered instruments that are highly variable and prone to bias (Meilgaard et al., 2007a), requiring large sample sizes to produce significant results. Some common sensory tests rely on number scales that consumers can use to rate food products on various product attributes (Peryam and Pilgrim, 1957). The four attributes tested in the order they are perceived include appearance, aroma, texture and

flavor (Meilgaard et al., 2007a). These attributes, in addition to overall acceptance of a product, are typically assessed on a 9-point hedonic scale (Peryam and Pilgrim, 1957), which has value ratings as follows: dislike extremely (1), dislike very much (2), dislike moderately (3) dislike slightly (4), neither like nor dislike (5), like slightly (6), like moderately (7), like very much (8) and like extremely (9) (Peryam and Pilgrim, 1957). Consumer data collected in this manner represent preference for products tested and allows for comparison of similar products subjected to various treatments or recipe changes.

The Food and Drug Administration (FDA) commonly uses sensory evaluation techniques to protect consumers by monitoring quality and freshness of seafood products (FDA, 2013). They are often used to evaluate the prevalence of off-flavors associated with algal populations in the culture systems that produce the secondary metabolites methylisoborneol and geosmin (Dionigi et al., 1998). In addition, studies have been conducted to evaluate the effects of stress on the sensory characteristics of fish meat. Deterioration of sensory attributes of salmon fillets were observed following long-term crowding stress (Hansen et al., 2012). Handling stress in Atlantic salmon was reported to receive lower odor and texture scores when compared to the unstressed control fish (Sigholt et al., 1997).

1.7 Proteomics

Proteins make up a large portion all cells and are important to the physiology and structure of biological systems (Damodaran, 2008). The quantitative and qualitative evaluation of proteins expressed under various conditions is known as proteomics, which is studied as a means of understanding cellular mechanisms and biological processes

(Anderson and Anderson, 1996). The proteome can be studied to better understand many biological functions in an organism (Rodrigues et al., 2012). In food products, proteomics can be used to evaluate how various physiological changes in the living organism can affect the final quality of the products they produce. Proteomics can also aid in understanding the effects that specific processing techniques have on food quality and safety (Carbonaro, 2004).

Proteomics has proven to be a very useful tool in understanding the biology and physiology of aquatic organisms by providing information on the physiological state and production issues in aquaculture (Rodrigues et al., 2012). A better understanding of the physiological and biological changes that aquaculture practices have on fish aids the industry in maintaining efficient productivity of a high quality product (Rodrigues et al., 2012). Proteomic techniques show great promise for the aquaculture industry in helping to understand the mechanisms behind changing fillet quality (Rodrigues et al., 2012). Correlating alterations in protein expression with pre- slaughter environmental and culture stressors and fillet quality will allow for the identification of stress response pathways and their specific effects on quality. The muscle proteome of fish is important not only for its nutritional benefits but as an integral component of the structural, textural and sensory properties of the meat (Qixing et al., 2014). Thus, proteomic techniques can be utilized to monitor quality changes and relate them to the physiological changes that cause them. Visualization of changes in protein expression will also aid in the identification of the stress response pathways that contribute to changes in the sensory characteristics of meat products. Proteomic techniques have been used to evaluate meat color in beef (Joseph et al., 2012), pork (Sayd et al., 2006) and turkey (Joseph et al.,

2011). In catfish, proteomics has been used to identify the cause of red color defect (Desai et al., 2014), which is becoming more prevalent in cultured catfish. Tenderness has been evaluated using proteomic analysis in beef (D'Allessandro et al., 2012; Laville et al., 2009) and pork (Lametsch et al., 2003: Morzel et al., 2004).

Alterations in protein expression and function are expected in proteins that are involved in energy metabolism (Eva et al., 2010; Morzel et al., 2006; Silva et al., 2012). Some proteins of interest that may exhibit stress induced changes in concentration have been identified as adenylate kinase (AK), nucleoside diphosphate kinase (NDPK), carbonic anhydrase (CA), phosphatidylethanolamine binding protein (PEBP), glycogen phosphorylase, peroxiredoxin 5, Cu/Zn superoxide dismutase, transferrin, DJ-1 protein (Silva et al., 2012), triosephosphate isomerase, sarcalumenin and enolase (Morzel et al., 2006). In Atlantic salmon (*Salmo salar*), the proteins enolate 3 and phosphoglycerate kinase 1, related to energy metabolism, were over expressed when compared to controls following crowding stress (>200kg/m^3, 40 m) (Veiseth-Kent et al., 2010). In the same study, expression of methylmalonate-semialdehyde dehydrogenase, a protein involved in amino acid metabolism, expression was decreased. In rainbow trout (*Oncorhynchus mykiss*), the protein triosephosphate isomerase, which is involved in glycolysis, increased while pyruvate dehydrogenase, which is integral in aerobic metabolic processes, decreased after experiencing a crowding stress (>50kg/ m^3, 15 min) (Morzel et al., 2006). Several proteins involved in energy homeostasis and signaling, including creatine kinase, adenylate kinase, nucleoside diphosphate kinase, carbonic anhydrase, glycogen phosphorylase and phosphatidylethanolamine binding protein increased in gilthead seabream (*Sparus aurata*) subjected to crowding stress (~140 kg/m^3, 20 m) (Silva et al.,

2012). Repeated handling and high stocking densities caused an increase in fatty acid binding protein and a decrease in triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase and enolase, which represent liver proteins involved in energy metabolism. Pyruvate dehydrogenase increased in gilthead seabream subjected to chronic handling stress and decreased following chronic density stress (Alves et al., 2010).

In addition to the changes in proteins related to metabolic processes, fragmentation of structural proteins was observed in crowded Atlantic salmon, suggesting an increase in muscle degradation and proteolytic activity (Veiseth-Kent et al., 2010). In rainbow trout, the structural proteins desmin and cap-Z decreased in crowded fish, likely due to increased proteolytic activity and muscle degradation (Morzel et al., 2006). Similarly, increased protein fragmentation was observed in gilthead seabream exposed to crowding stress prior to slaughter. Concentrations of the myofibrillar proteins actin and myosin were lower in stressed fish, with higher incidence of fragmentation, further suggesting higher proteolytic activity during stress (Silva et al., 2012). Degradative processes in the muscle are species specific and represent an important factor affecting fish quality (Kjærsgård and Jessen, 2003).

CHAPTER II

PHYSICAL EFFECTS OF STRESS ON GROWTH, FEEDING AND FILLET YIELD IN CULTURED CHANNEL CATFISH (*ICTALURUS PUNCTATUS*)

2.1 Introduction

Global population rise, depletion of natural aquatic food stocks, increased urbanization, heightened awareness of the health benefits of eating aquatic food products and advances in culture technologies have all contributed to aquaculture growing faster than any other food sector worldwide (FAO, 2014). A better understanding of growth and survival of a species will enable the implementation of good management practices that optimize growth rate, feed conversion, survival and fillet yield. Such knowledge is integral to the advancement of aquaculture practices. In 2012, 66.6 million tons of food fish were produced through aquaculture, with production expected to increase annually (FAO, 2014). Aquaculture makes up nearly half of the total food fish supply and represents an important sector of food production to ensure food security (Cunningham, 2005; FAO, 2014).

Pond culture of channel catfish, *Ictalurus punctatus*, represents the largest segment of aquaculture in the United States (Bastola and Engle, 2012; Harvey, 2012; Vilsack and Reilly, 2014). Therefore, it is important to understand the dynamics involved in their growth and survival. Pond culture conditions can fluctuate daily and annually. In addition to the natural changes inherent in the culture environment, traditional harvest

practices can impose additional stressors on the fish, which can have negative impacts on production (Ashley, 2007) and quality.

The shallow nature and high stocking densities employed during pond culture result in significant alterations in environmental conditions, including low dissolved oxygen (DO) levels (Hargreaves and Steeby, 1999) and summer temperatures in excess of 35°C (Davis and Simco, 2001; Wax and Pote, 1990). Low levels of DO are considered one of the most limiting factors affecting fish production (Hargreaves and Steeby, 1999). Low DO conditions have been shown to elicit a stress response in channel catfish (Small, 2004) and rainbow trout, *Oncorhynchus mykiss*, (Sánchez et al., 2011). The high temperatures observed in the summer months have also been shown to elicit a stress response in channel catfish (Davis and Simco, 2001) and rainbow trout (Sánchez et al., 2011), which negatively impacts growth and survival (Andrews et al., 1973; Arnold et al., 2013; Buentello et al., 2000).

Alterations in feed intake, on account of a variety of different stressors, is well documented (Bernier and Peter, 2001; Buentello et al., 2000; Green and Rawles, 2011). Environmental changes in temperature (Arnold et al., 2013) and DO levels (Buentello et al., 2000; Torrans, 2005; Torrans et al., 2012) alter feed intake in catfish, with the more stressful conditions resulting in decreased consumption and ultimately growth. The effects of environmental stressors including temperature (Stewart et al., 2014) and DO levels (Green and Rawles, 2011; Torrans, 2008) on growth are also well documented for catfish. Common stressors that pond reared catfish experience include daily (2-4°C) and seasonal (1-35°C) fluctuations in temperature and DO concentrations dropping as low as 1 mg/L (Davis and Simco, 2001; Hargreaves and Steeby, 1999; Wax and Pote, 1990).

Temperature and DO represent notably the two most important factors affecting growth, reproduction and survival in production ponds (Hargreaves and Steeby, 1999; Tucker and Robinson, 1990). Buentello et al. (2000) examined these two variables in juvenile catfish and found that they interacted to affect feed consumption, feed utilization and growth. Thus, the interaction between these two stressors and their effects on growth, feed consumption and yield are important aspects of understanding and managing the culture system. However, the response of juveniles to a particular stressor, including temperature tolerance, can sometimes vary when compared to that of an adult (Brett, 1979). This study evaluated the effects of temperature and DO on growth, condition, survival, feed intake and fillet yield for cultured food size channel catfish. Data will aid in identifying the effects of environmental stress and their interaction effects on the growth and survival of channel catfish during culture. These data are important for the management of culture ponds to promote fast and efficient growth with maximum product yield after harvest.

2.2 Materials and Methods

2.2.1 Fish acquisition and rearing conditions

Channel catfish (*Ictalurus punctatus*) were acquired as fingerlings from the Mississippi State University Thad Cochran National Warmwater Aquaculture Center in Stoneville, Mississippi, USA. Fish were grown to just under market size or larger in flow-through well water culture systems ranging from 400 to 4,000 L for 12-24 months. The systems were equipped with forced air aerators set to maintain DO levels ≥5 mg/L with temperatures fluctuating with an ambient well water thermal profile (22-27°C). During grow-out, fish were fed commercial 36% protein fingerling feed (Land O'Lakes Purina Feed LLC, Arden Hills, MN, USA) by hand once daily to satiation. Fish were

switched to a commercial 32% protein floating finishing feed (Purina Animal Nutrition LLC, Gray Summit, MO, USA) when they approached market size. A 32% protein feed is typically employed during grow out in commercial pond culture (Robinson et al., 1998).

2.2.2 Experimental conditions

The stress trial was repeated three times with fish increasing in average size in each progressive trial. Fish were anesthetized in a CO_2 bath with 3 g/L NaCl and water from rearing tanks, weighed to the nearest g and measured to the nearest mm. Each fish was randomly distributed among four 4,000 L treatment tanks until 80 fish/tank was achieved (mean weight 704 ± 484 g). Treatment tanks were individual recirculating aquaculture systems (RAS) (~1.3 ± 0.3 L/s) supplied with a slow exchange of well water (~0.15 ± 0.08 L/s) to maintain adequate water quality. Each RAS system contained a biofilter with plastic floating bio beads (Model DF3 or 6; Aquaculture Systems Technologies, New Orleans, LA, USA), two high output ultra violet sterilizers (120 V SMART HO, Emperor Aquatics; Pottstown, PA, USA), a pump (SHE2.4 or SHE1.7, Aquatic Ecosystems, Inc., Apopka, FL, USA) and a sump tank. After stocking, fish were slowly acclimated for 1 week to experimental temperatures (25°C and 33°C; 1°C/day) and DO levels (~2.5 mg/L, [L] and >5 mg/L, [H]). The low DO conditions were achieved by turning off diffuser stones connected to the forced air supply and decreasing the oxygen regulator system set point by 10% daily for approximately 5 days until the experimental concentration was reached (~2.5 mg/L). The high temperatures were maintained with direct immersion heaters, with one 4,000 watt, 200-250 V and two 1,700 watt, 100-140 V controlled with single stage temperature controllers (Smart One; Process

Technology, Mentor, OH, USA) placed in each system. The 25°C treatments were maintained with immersion chillers with single stage temperature controllers (AquaLogic Inc., San Diego, CA, USA). Oxygen levels in the low DO treatments were monitored using a galvanic oxygen probe (MINI-DO, Loligo® Systems, Tjele, Denmark or CellOx 325, WTW GmbH, Weilheim, Germany) attached to a Loligo® Systems oxygen analyzer and regulator system (Version 2.4; Loligo® Systems, Tjele, Denmark) or a dissolved oxygen pocket meter (Oxi 330i, WTW GmbH, Weilheim, Germany) with a custom designed solenoid valve system (Petersen and Gamperl, 2011). The solenoid valves were attached to compressed oxygen cylinders and were set to open when the DO levels dropped below 2 mg/L and close when 2 mg/L was reached. Four environmental treatments were evaluated representing control (25-H), moderate oxygen (25-L), moderate temperature (33-H) and severe stress (33-L). The environmental treatments were maintained for four weeks after the initial one week acclimation.

During acclimation and stress trials, fish were fed once daily to satiation to evaluate the effects of treatment on feed consumption starting with 1% total initial body weight and adding additional feed as needed for 30 min. Any feed remaining at the surface after 30 min was collected and quantified by counting pellets or drying larger amounts in a drying oven at 35°C overnight. Pilot studies on pre-weighed portions of feed (n = 3) were conducted in treatment tanks without fish to determine the percent loss during the drying process. Percent weight loss was determined to be 31%, thus dried feed values were adjusted to reflect this loss (weight of dried feed + weight of dried feed *0.31). Counted pellets were converted to weights using the mean pellet dry weight of

0.17 g/pellet (number of pellets * 0.17 g), which was determined by averaging the individual pellet weights (n = 60).

During the stress trials, temperature and DO were monitored three times daily with a DO meter (YSI ProODO, YSI Inc., Yellow Springs, OH, USA). Temperature was also monitored at 30 min intervals using data loggers (Hobo water temp pro V2, Onset ®; Bourne, MA, USA) that were placed in each of the tanks. Ammonia and nitrite were measured with a colorimeter (DR/850, HACH Co., Loveland, CO, USA) and pH was measured with an EcoSense pH10A pH pen (YSI Inc., Yellow Springs, OH, USA) three days a week for the duration of the study.

2.2.3 Fish harvest

After four weeks of exposure to treatment conditions, 20-27 fish per tank were anesthetized (5-10 min) in a CO_2 bath containing 3 g/L NaCl, using well water and a diffuser stone to bubble in CO_2. Once fish were docile and easy to handle they were weighed to the nearest g and measured to the nearest mm. After weight and length were measured, fish were killed by a sharp blow to the head and placed on ice for further processing. Fish on ice were sent to the Ammerman Hearnsberger Processing Plant in the Department of Food Science, Nutrition and Health Promotion at Mississippi State University. The fish were filleted by hand and the yield (g) of fillet was recorded for each fish then percent yield for each treatment was calculated as:

$$100 * \left(\frac{Sum\ fillet\ yield\ /treatment}{\left(\frac{\#\ of\ fish\ filleted}{\#\ of\ fish\ weighed\ at\ harvest} \right) Sum\ fish\ weight/treatment} \right) = Percent\ (\%) Yield \quad (2.1)$$

The ratio of number of fish filleted and number of fish harvested was used as in some treatments only the fish necessary for quality analysis were filleted and yield recorded. Physiological response to the environmental stress conditions and subsequent handling stressors are evaluated in chapter 3.

Condition factor (K) was calculated according to Moyle and Cech (2004):

$$K = \frac{W(100)}{L^3} \tag{2.2}$$

where W represents the weight of the fish (g) and L represents the length of the fish (cm). Feed conversion ratio (FCR) was calculated on a mean individual fish basis for each of the three trials as high mortalities in the sock during trial three made FCR difficult to compute by treatment. The FCR was calculated for each trial and averaged (n=3), according to:

$$FCR = \frac{FC}{HW - SW} \tag{2.3}$$

where HW represents the average individual weight of the first 20 fish harvested from each tank following the environmental stress event, SW represents the mean individual stocking weight of the 80 fish stocked per treatment tank and FC represents the estimate of feed consumption per individual fish (total feed eaten over acclimation period and 4 week trial/number of fish). The procedures used herein were reviewed and approved by the Mississippi State University institutional animal care and use committee, and conducted in accordance to the approved protocols (no. 13-041).

2.2.4 Statistical Analysis

The experiment was set up as a randomized complete block design with a 2x2 factorial arrangement of treatments and subsampling. Stocking data was analyzed using a two way analysis of variance (ANOVA) with four temperature-DO combinations as the treatments. Harvest data were analyzed as a two way analysis of covariance (ANCOVA) with 2x2 factorial arrangement of temperature and DO concentrations. Fish were considered subsamples and stocking weight, length or condition were used as the covariate since mean stocking weights between trials ranged from 0.38 to 1.24 kg/fish. All analyses were carried out using least square means. Mean separation was carried out using Fisher's protected least significant difference (LSD) comparisons with statistical significance determined at $p < 0.05$. Due to small sample size (n=3), FCR and percent yield were analyzed using the non-parametric Wilcoxon rank-sum test. All statistical analyses were performed with SAS statistical software version 9.3 (SAS Institute Inc., Cary, NC, USA).

2.3 Results

Water chemistry was maintained at treatment levels for temperature and DO and varied slightly among treatments for other parameters (Table 2.1). Oxygen levels in the low DO treatments were maintained as close to 2 mg/ml as possible (mean 2.5 mg/L), but varied several times due to brief equipment malfunctions. The primary malfunctions experienced were tubing rupture and depletion of compressed oxygen in tanks. The low DO levels were on average maintained at least half the high level (>5 mg/L) treatments. Oxygen levels for the two high DO treatments were both >5 mg/L, but differed due to

solubility differences at different temperatures, with the higher solubility of oxygen at 25°C resulting in higher average DO (Table 2.1).

The pH varied between treatments ($F = 105.24$, $p = <0.0001$) with no interaction effects ($F = 0.41$, $p = 0.524$) and values between 7.1 and 7.8 (Table 2.1). The tank pH was, on average, ~0.2 units higher ($F = 34.18$, $p = <0.0001$) in the 33°C treatments and about 0.4 units higher ($F = 103.18$, $p = <0.0001$) when DO levels were held above 5 mg/L. The higher pH at higher DO levels and higher temperatures is likely a result of the increased food intake and thus, higher metabolic activity (Yang and Somero, 1993). Although differences in pH existed between treatments, the pH was never outside the optimum range, 6.5 - 9, for catfish ponds (Tucker, 1996).

Table 2.1 Water quality parameters.

Trt[a]		Temp[b]	DO (mg/L)[c]	DO (%)[d]	pH	NO_2[e]	TAN[f]	NH_3[g]
25-H		25.3[d]	6.3[a]	75.9[a]	7.57[b]	0.61[a]	0.37	0.02[b]
	SE	0.1	0.1	1.2	0.07	0.07	0.04	0.00
25-L		25.6[c]	2.5[c]	30.7[c]	7.16[d]	0.24[b]	0.33	0.01[b]
	SE	0.1	0.1	1.1	0.06	0.03	0.04	0.00
33-H		33.2[a]	5.4[b]	74.7[a]	7.77[a]	0.58[a]	0.35	0.04[a]
	SE	0.1	0.1	1.6	0.08	0.07	0.07	0.01
33-L		33.0[b]	2.5[c]	35.3[b]	7.41[c]	0.29[b]	0.0	0.02[b]
	SE	0.1	0.2	2.1	0.08	0.06	0.07	0.01

Water chemistry for each of the four environmental treatments represent mean values for the 4 week trial. Temperature and dissolved oxygen (DO) were measured daily and all other parameters three times weekly (two-way ANOVA, LSD). Different letters represent significant differences between treatments at $p< 0.05$.

[a]Treatment = temperature (°C) – high (H, >5 mg/L) or low (L, ~2.5 mg/L) DO

[b]Temperature (°C)

[cd]Dissolved oxygen

[e]Nitrite (mg/L)

[f]Total Ammonia Nitrogen (mg/L)

[g]Unionized ammonia (mg/L)

Total ammonia concentrations did not differ among treatments (Table 2.1). Un-ionized ammonia levels varied ($F = 11.74$, $p = <0.0001$) from 0.01 to 0.04 mg/L, with no interaction affects ($F = 1.25$, $p = 0.266$). The un-ionized ammonia concentrations were, on average, about 3 times higher in the 33 °C treatment ($F = 8.28$, $p = 0.005$) and two times higher when DO levels were maintained above 5 mg/L ($F = 4.92$, $p = 0.028$). Nitrite levels varied significantly between treatments ($F = 6.82$, $p = <0.0001$) with no significant temperature x DO interaction effects ($F = 0.57$, $p = 0.4502$). On average, nitrite levels were two times higher ($F = 30.48$, $p = <0.0001$) in the high DO treatments, but were not significantly affected by temperature ($F = 0.01$, $p = 0.9116$). The higher nitrite and ammonia levels observed in the 25-H and 33-H treatments were likely the result of increased feed intake (Table 2.2). This is consistent with that observed by Torrans (2008) where higher nitrite levels were observed in the high DO treatments, suggesting higher nitrification rates due to higher DO levels. The average ammonia and nitrite levels were below the levels identified to effect growth in a pond system (Hargreaves and Kucuk, 2001).

Oxygen concentrations had the greatest effect ($F = 20.91$, $p = 0.0038$) on feed consumption with, on average, a ~200 g (120%) decrease in total feed consumed per fish at low DO when compared to high DO treatments. The low DO treatments showed no difference in feed consumption when temperatures were manipulated (Table 2.2). When DO levels were maintained above 5 mg/L feed consumption, on average, more than doubled. Additionally, fish reared under control conditions (25-H) consumed more feed than fish reared under moderate temperature stress (33-H; Table 2.2). Total feed consumed over the course of the five weeks was higher in the control treatment (25-H)

than the low DO treatments (Figure 2.1). In addition, fish reared at higher DO levels consumed more feed than fish in the severe stress treatment (33-L, Figure 2.1).

Table 2.2 Physical data for stressed channel catfish (*Ictalurus punctatus*).

	Treatment			
	25-H	**25-L**	**33-H**	**33-L**
Stocking Length (mm)	398	398	396	396
SE	5	5	5	5
Harvest Length (mm)	437	420	418	418
SE	5	5	5	5
Stocking Weight (g)	703	715	693	706
SE	31	30	30	33
Harvest Weight (g)	928[a]	781[b]	727[b]	720[b]
SE	37	36	33	34
Stocking Condition Factor	0.97	1.00	1.07	0.99
SE	0.01	0.01	0.10	0.01
Harvest Condition Factor (K)	1.07[a]	0.94[ab]	0.88[b]	0.87[b]
SE	0.07	0.03	0.01	0.01
Daily Consumption per Fish (g)	11.8[a]	5.2[c]	9.2[b]	4.3[c]
SE	0.5	0.3	0.5	0.3
Feed Conversion Ratio	1.90	2.95	2.04	1.60
SE	0.25	0.47	3.32	3.68
Mean Fillet Yield (g)	197	183	161	165
SE	14	14	12	13
Mean Fillet Yield (%)	22.9	20.3	19.7	20.8
SE	2.2	1.2	0.6	0.3
Mortality (%)	0.0	1.3	5.6	15.6

The data represent means (± standard error) for stocking weight and length; harvest weight and length; condition at harvest; fillet yield and daily feed intake with standard error (SE). Treatment represents temperature (°C) - Dissolved oxygen at high (H, >5 mg/L) and Low (L, ~2.5 mg/L) levels. Statistical significance is denoted by different letters within a row using least square means (two-way ANCOVA, LSD, $p < 0.05$).

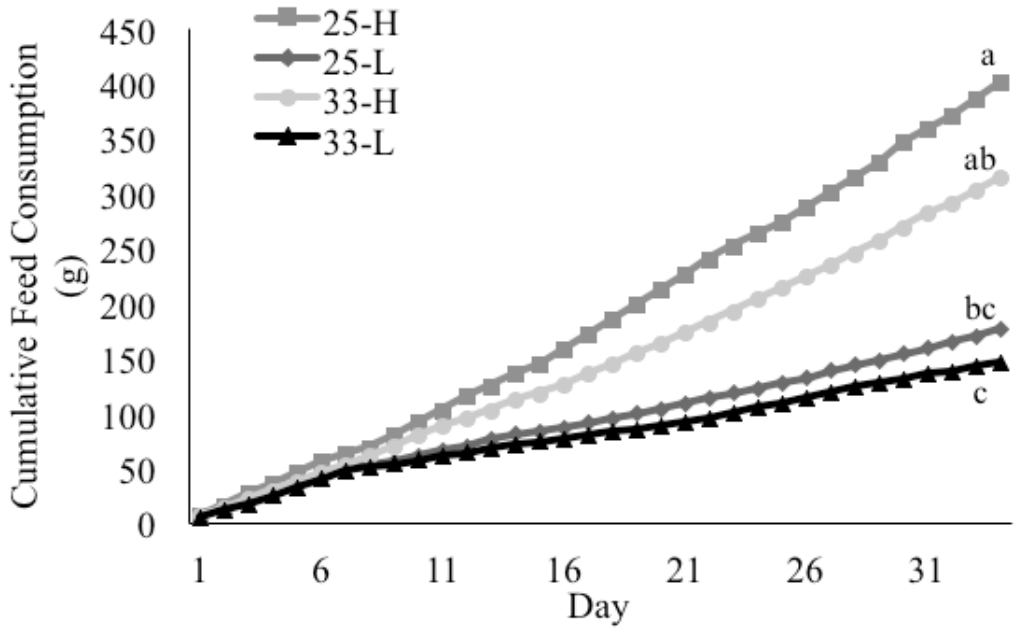

Figure 2.1 Cumulative feed consumption of channel catfish (*Ictalurus punctatus*) exposed to environmental stress.

The average cumulative amount of feed consumed by individual fish in each of the different environmental temperatures (25°C and 33°C) and high (H, >5 mg/L) and low (L, ~2.5 mg/L) dissolved oxygen concentrations. Significant differences between final feed consumed per treatment is designated with different letters (two-way ANOVA, LSD, $p < 0.05$).

Temperature and DO levels showed no significant interaction effects with regard to harvest weight. Each variable significantly affected the end weight of the fish separately. Fish held at 25°C weighed, on average, >100 g (~19%) ($F = 12.52$, $p = 0.0123$) more than fish held at 33°C (Figure 2.2). Fish held at high DO (>5 mg/L) weighed, on average, ~100 g (~15%) ($F= 8.58$, $p = 0.0263$) more than fish held at low DO concentrations (~2.5 mg/L, Figure 2.2). There were no significant treatment differences in fish length over the course of the four week trial. Condition factor showed no significant interaction effects between temperature and DO level. There was a main effect of temperature, with fish reared at 25°C having a higher condition factor when

compared to fish reared at 33°C (F= 8.73, p = 0.0255). Fish in the control treatment (25-H) had a higher condition factor when compared to fish reared at high temperatures (Table 2.2).

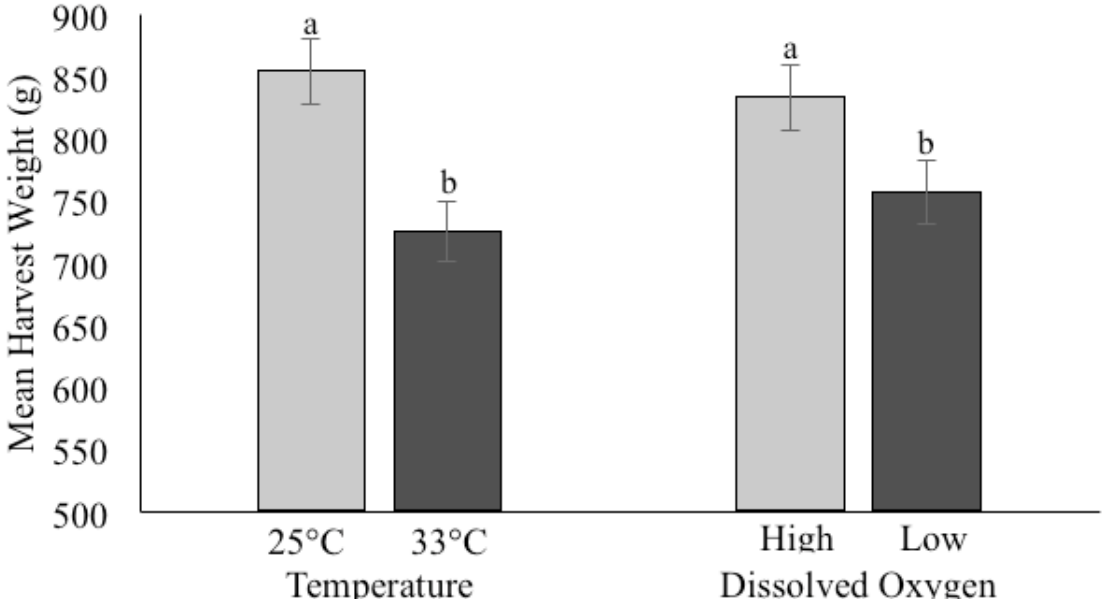

Figure 2.2 Main effects of temperature and dissolved oxygen on channel catfish (*Ictalurus punctatus*) harvest weight.

Least square means for harvest weight of fish from different temperature and dissolved oxygen treatments. Significant differences between factors are indicated by different letters (two-way ANCOVA, LSD, $p<0.05$).

There was no interaction between temperature and DO with respect to fillet yield (F = 0.48, p = 0.5123). Fish reared at 25°C yielded, on average (F = 7.26, p = 0.0358), about 30 g (19%) more than fish reared at 33°C (Figure 2.3). Percent yield was not affected by environmental stress (Table 2.2).

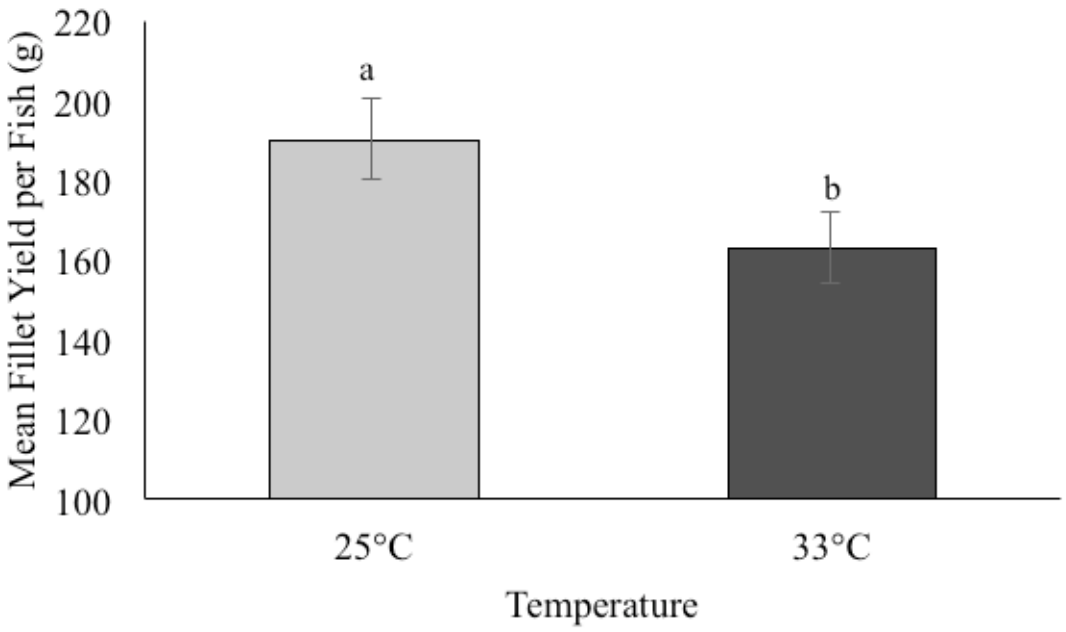

Figure 2.3 Main effects of temperature on mean channel catfish (*Ictalurus punctatus*) fillet yield.

Least square means for mean fillet yield per fish reared at high and low temperatures. Significant differences between factors are indicated by different letters (two-way ANCOVA, LSD, $p<0.05$).

It was difficult to calculate overall feed conversion ratios due to the higher mortalities during trial 1 and the exact cause and effect of mortality on weights was unknown (Table 2.2). Thus, feed conversion ratio (FCR) was calculated on an average per fish basis for each treatment in each trial. This allowed statistical analysis to be conducted on three points (n=3). There were no differences in FCR ($F = 2.24$, $p = 0.1773$), which may be due to the large variability between replicates.

2.4 Discussion

Environmental stress affected growth and condition of channel catfish. In this study, DO and temperature affected growth, food consumption and survival. The negative

effects of high temperature and low DO were clearly demonstrated in food size channel catfish produced under temperature and low DO regimes commonly experienced in commercial aquaculture ponds (Davis and Simco, 2001; Hargreaves and Steeby, 1999; Wax and Pote, 1990). A better understanding of the negative impacts that specific environmental variables have on channel catfish is integral for proper management and efficient production.

Water quality parameters were tightly monitored and well within the optimal range for growth. Additionally, the chemical characteristics of the RAS were within the observed ranges of traditional culture ponds. Thus, the changes in growth and condition reported are likely due to treatment effects. Exposure of channel catfish to stressful environmental conditions (low DO [2.5 mg/L] and high temperature [33°C]) resulted in decreased feed consumption and growth of channel catfish when compared to higher DO (> 5 mg/L) and lower temp (25°C). As stress increased (Ch. 3), feed consumption decreased, with DO levels identified as the most important factor moderating consumption in this study. Decreased feed consumption ultimately resulted in decreased growth as observed in the smaller sized fish harvested from the low DO and high temperature treatments when compared to the non-stressed conditions. The decrease in size resulted in decreased fillet yield.

Oxygen has been identified as a factor that effects feed consumption and ultimately growth in fish (Buentello et al., 2000; Lakani et al., 2012; Torrans, 2005; Torrans et al., 2012; Tran-Duy et al., 2008). In great sturgeon, *Huso huso* L., feed consumption and growth increased with increasing DO levels, with large sturgeon showing little growth over the course of 8 weeks under hypoxic conditions (Lakani et al.,

2012). Nile tilapia, *Oreochromis niloticus* L., consumed less feed and grew less when reared at low DO (<3.5 mg/L) for one month compared to fish reared under high DO (>5 mg/L) conditions (Tran-Duy et al., 2008). In catfish ponds, when DO levels decrease below 3 mg/L, feed consumption begins to decrease (Torrans, 2011). Buentello et al. (2000) reported that temperature and DO concentrations had interactive effects, eliciting alterations in feed consumption and growth in juvenile channel catfish (15 ± 0.2 g). In the present study, the temperature and DO interaction did not affect feed consumption in food size channel catfish (704 ± 484 g). The lack of interaction effects suggests that temperature and DO affect physical condition and growth separately in food-size channel catfish contrary to what was observed in juveniles (Buentello et al., 2000). Thus, the effects of temperature and DO can be examined and managed separately in food-size catfish. This highlights the need for size dependent alterations in management techniques to ensure the most efficient growth and survival.

Although the effects of DO in the present study are similar to what was observed by Buentello et al. (2000), the inverse was observed with regard to temperature. The fish held at the higher temperature regardless of DO level showed lower daily feed intake. In the present study, the high temperature (33°C) was higher than the maximum temperature examined in the Buentello et al. (2000) study (30°C) and exceeds the optimal temperature range (25-30°C) for growth in channel catfish (Tucker, 1996). Additionally, the temperatures in the present study were maintained steadily for four weeks as opposed to gradually adjusted over the course of 12 weeks in the Buentello et al. (2000) study. This suggests that in channel catfish there is a temperature level at which feed consumption will decrease. Once temperatures exceed approximately 32°C, the potential for growth

begins to decrease (Tucker and Robinson, 1990). A decrease in growth and feeding performance in juvenile catfish has been identified with cycling thermal regimes with minimum temperatures above 31°C (Arnold et al., 2013; Stewart et al., 2014). The decrease in consumption likely occurs as temperatures approach the thermal lethal maximum, which has been identified as ~39°C for juvenile channel catfish (Stewart and Allen, 2014). In Atlantic salmon, *Salmo salar,* feed consumption increased with increasing temperatures from 6°C to 14°C (Handeland et al., 2008). However, these temperatures did not exceed those identified as the upper temperature limit for this species (Handeland et al., 2000). Decreases in growth and yields at high temperatures could have negative implications for the future as temperatures rise due to global climate change (Ficke et al., 2007), potentially resulting in decreased yields in production ponds. According to the Intergovernmental Panel on Climate Change, mean global temperatures are expected to increase 0.3-0.7°C annually (IPCC, 2014). While low DO resulted in a 120% decrease in feed consumption, it only resulted in a 15% decrease in weight. Rearing at high temperature (33°C) caused a 19% decrease in fish weight at harvest. This highlights the importance of thermal regulation in culture ponds. As oxygen management has received much attention over the past several years (Torrans, 2011; Torrans et al., 2012) and is more easily manipulated in a pond system, future research on management techniques for providing thermal stability in culture ponds may be integral for enhancing and maintaining efficient production systems.

Condition factor was affected by DO concentration and temperature with a decrease in condition when fish were reared under moderate to severe stress (high

temperature or low DO). This is consistent with the decrease in growth that was observed with increased temperature.

Previous studies demonstrated an increase in FCR's when fish were exposed to temperatures above or below the optimal range (Arnold et al., 2013) and when DO levels decreased (Andrews et al., 1973; Torrans et al., 2012), indicating less efficient feed conversion when stressed. In the current study, calculations of the FCR's on an average per fish basis resulted in a very small sample size and large variations between trials, thus statistical differences were undetectable. The variations between trials could be partially explained by the increasing size of fish in subsequent trials as FCR is known to increase with increasing fish size (Robinson and Li, 2010). However, all the ratios were within the range of FCR's reported for channel catfish of a similar size range grown in culture ponds (Robinson and Li, 2010).

The mortalities observed throughout rearing at environmental conditions were primarily attributed to a hypoxic event that occurred in the severe stress (33-L) treatment during the first trial. Feeding rates were adjusted based on weights of fish removed and uneaten feed was removed after 30 min for all trials, thus the mortalities should not have had a significant effect on the growth of the catfish in the severe stress treatment. It should also be kept in mind that these tank studies with fish reared at somewhat constant temperature and DO concentrations are not mimicking that of a culture pond where diurnal fluctuations occur and differences in response and interaction effects could be observed (Torrans, 2008). This warrants further evaluation of the effects of combined environmental stressors on food-size channel catfish.

2.5 Conclusions

Temperature and DO are both important environmental stressors that can affect growth, food consumption and food conversion in channel catfish. While oxygen management techniques have already been implemented in commercial systems, increasing global temperatures cause concern for catfish culture, which could be affected by decreased growth and FCR. This highlights the importance of evaluating methods for maintaining thermal stability in the pond system. Similar studies should also be conducted on hybrid (channel x blue) catfish as there is a shift toward hybrid culture (APHIS, 2011) and the effect of stressors on the growth of hybrids may be expressed differently (Stewart et al., 2014).

CHAPTER III

PHYSIOLOGICAL RESPONSE TO SEQUENTIAL STRESS EVENTS IN CULTURED CHANNEL CATFISH (*ICTALURUS PUNCTATUS*)

3.1 Introduction

Rearing conditions and harvest procedures cause stress (Wang et al., 2004), which elicits physiological changes to cope with the stress events (Small et al., 2008). The stress response has been defined as an interruption in the dynamic equilibrium of an organism due to stimuli (Wendelaar Bonga, 1997). The response of a fish to a particular stressor is considered an adaptive mechanism, allowing the fish to cope with the stressor and maintain a homeostatic state (Barton, 2002). If the stressor is severe or chronic the stress response may become maladaptive. Maladaptive stress responses typically result in whole body responses that can be detrimental to fish health (Barton, 2002). The physiological response of fish to stress can include: changes in plasma glucose and lactate (Poli et al., 2005), ionic composition, hormonal secretions and osmotic balance (Wendelaar Bonga, 1997) and total protein concentrations (Coeurdacier et al., 2011). These responses vary depending on species, genetics, developmental stage, environment and frequency of the stress (Barton, 2002).

Many aspects of aquaculture including handling, sorting, grading, transport and poor water quality can elicit a stress response and impact fish health (Barton, 1997; Coeurdacier et al., 2011; Conte, 2004). Acute and long-term handling and transport stress

typically elicit an endocrine and hematological response in fish (Barton, 2000; Fast et al., 2008). Crowding and confinement stress also alters stress related hematological parameters in many species (Davis and Parker, 1990; Minchew et al., 2007; Small, 2004; Strange, 1980). The stress response can vary depending on environmental conditions leading up to the stress event, including acclimation temperature (Davis and Parker, 1990) and dissolved oxygen (DO) concentrations (Lakani et al., 2013; Petersen and Gamperl, 2011).

In the United States, pond culture of channel catfish, *Ictalurus punctatus,* represents the largest segment of aquaculture (Vilsack and Reilly, 2014). Due to traditional culture practices, channel catfish experience stress throughout rearing and harvest. These stressors include drastic fluctuations in water temperatures and DO levels (Arnold et al., 2013; Davis and Simco, 2001; Hargreaves and Steeby, 1999; Small, 2004; Wax and Pote, 1990), crowding (Minchew et al., 2007; Small, 2004; Strange, 1980), socking/grading and transport stress (Davis et al., 1993). In the summer, DO in culture ponds can drop below 1 mg/L (Hargreaves and Steeby, 1999) and water temperatures can exceed 35°C (Arnold et al., 2013; Davis and Simco, 2001; Wax and Pote, 1990). At harvest, fish are held at high densities (242 Kg/m^3 or ~0.24 Kg/L) in socks (net cages with specific mesh size) used for sorting prior to loading (Pearson and Beecham, 2007). After sorting, fish are transferred to hauling trucks where they are loaded at even higher densities (0.7 to 1.0 Kg/L) (Bosworth et al., 2004).

A fish's response to a particular stressor can depend on prior acclimation conditions. Temperature-dependent alterations in the physiological stress response of fish are well documented (Bosworth et al., 2007; Davis and Parker, 1990; Logan and Somero,

2011; Methling et al., 2010; Strange, 1980). Pre-exposure to hypoxic conditions has been shown to affect feeding, growth, feed conversion efficiency and physiology in fish following subsequent hypoxic stressors (Dan et al., 2014; Petersen and Gamperl, 2011; Yang et al., 2013). Thus, the daily and seasonal fluctuations in pond temperature and DO levels represent an important aspect of understanding a fish's response to further stress events.

It is important to understand the effects of potential environmental stressors on the ultimate stress response and how they interact and accumulate to alter the fish's physiology. During typical culture practices, it is common for fish to experience multiple sequential stress events in a short period of time. Exposure to multiple stressors can elicit a cumulative stress response where physiological changes increase to a greater extent after each subsequent stressor (Barton, 2002). The cumulative nature of stress in fish has been previously documented (Barton, 2002; Schreck, 2000) and may differ from acute stress responses, including cortisol clearance rates (Davis and Small, 2006).

Understanding the physiological response of catfish to different culture conditions and practices is integral to the successful management and production of cultured species. Such knowledge will allow for a better insight into how each aspect of culture will affect the fish physiologically. Better control over the cortisol response during periods of stress is desirable in an aquaculture setting (Davis and Small, 2006), and may have impacts on fish health, survival and fillet quality (Poli et al., 2005). The purpose of this study was to quantify the physiological stress response of channel catfish to sequential environmental and harvest stressors in order to guide refinement of future management and production practices.

3.2 Materials and Methods

3.2.1 Fish acquisition and rearing

Fingerling channel catfish were supplied by the Mississippi State University Thad Cochran National Warmwater Aquaculture Center in Stoneville, MS. Fingerlings were grown out in flow-through culture systems ranging from 400 to 4,000 L for up to two years. Water was maintained at ambient well-water temperatures (22 - 27°C) and oxygenated with constant forced air aeration. Fish were fed a 36% protein commercial fingerling pelleted feed once daily to satiation (Land O'Lakes Purina Feed LLC, Arden Hills, MN). After approximately one year, prior to transfer to treatment tanks, the fish were switched to a 32% protein finishing feed (Purina Animal Nutrition) commonly used during commercial grow-out (Robinson et al., 1998).

3.2.2 Stress trials

Channel catfish with a mean weight (± standard error) of 704 ± 484 g were anesthetized in a CO_2 bath containing 3 g/L NaCl. Each fish was individually weighed to the nearest g, measured to the nearest mm and randomly distributed between four 4,000 L treatment tanks, with a total of 80 fish/tank. The treatment tanks were recirculating systems (~1.3 ± 0.3 L/s) supplemented with a constant supply of makeup unchlorinated well water (~0.15 ± 0.08 L/s) at ambient temperature (~27°C). Tanks were fitted with expandable granular biofilters filled with plastic floating biobeads (Polygeyser Models DF3 or 6; Aquaculture Systems Technologies, New Orleans, LA) that provided mechanical and biological filtration. Each system had two in-line high-output ultra violet sterilizers (120 Watt SMART HO, Emperor Aquatics; Pottstown, PA), a pump (SHE2.4 or SHE1.7, Aquatic Ecosystems, Inc., Apopka, FL) and a sump tank. Fish were

acclimated over the course of one week to experimental temperatures (25°C and 33°C) at a rate of change of 1°C/day, and high (H) and low (L) DO levels (>5 mg/L and ~2.5 mg/L, altered at a rate of 10% saturation/day) and then maintained under the experimental conditions for 4 weeks. The DO levels were decreased through natural respiration and maintained by continuous monitoring with a galvanic oxygen probe (MINI-DO, Loligo® Systems, Denmark or CellOx 325, WTW GmbH, Weilheim, Germany) attached to a Loligo® Systems oxygen analyzer and regulator system (Version 2.4; Loligo® Systems, Denmark) or a DO pocket meter (Oxi 330i, WTW GmbH, Weilheim, Germany) with a custom designed solenoid valve system (Petersen and Gamperl, 2011). The system was set to open a solenoid and bubble in oxygen when the DO levels dropped below 2 mg/L and turn off as soon as 2 mg/L was reached. High DO was maintained with constant aeration with forced air aerators. Throughout the acclimation period and stress trial, fish were fed a commercial 32% protein finishing feed (Purina Animal Nutrition LLC., Gray Summit, MO) by hand once daily to satiation, starting with 1% total initial body weight and adding feed as needed so that it was available for 30 min.

3.2.3 Water quality analysis

Temperature and DO were monitored three times daily with a DO meter (YSI ProODO, YSI Inc., Yellow Springs, OH), and temperature was recorded every 30 min using data loggers (Hobo water temp pro V2 Onset ®; Bourne, MA). Ammonia, nitrite and pH were measured three days per week for the duration of the study, using a portable colorimeter (DR/850, HACH Co., Loveland, CO) to measure ammonia and nitrite, and a pH meter (EcoSense pH10A, YSI Inc., Yellow Springs, OH). Unionized ammonia was

calculated based on pH, temperature, salinity and total ammonia nitrogen (Emerson et al., 1975).

3.2.4 Fish harvest

Eighty fish per tank were held at the experimental temperature (25°C and 33°C) and DO concentration (>5 mg/L and mean ~2.5 mg/L) for four weeks. After four weeks, 1/3 (n= ~27) of the fish were harvested. Blood was collected (<5 min after removal) for the first ten fish in each treatment using a 22 gauge 2.5 cm needle attached to a blood collection tube coated with K_2 EDTA (BD Vacutainer™, Becton, Dickinson and Company; Franklin Lakes, NJ) as an anticoagulant. The remaining fish (n= ~53/treatment) were transferred by netting to a 1.82 m^3 (0.60 m L x 0.60 m W x 0. 46 m H) cube constructed out of polyvinyl chloride piping and a plastic 1.25 cm mesh netting to mimic a commercial sock. The socks were constructed based on initial stocking weights of fish in the first trial to attain a density of ~0.17 Kg/L, which allowed for some growth without exceeding commercial socking densities (~0.24 Kg/L) (Pearson and Beecham, 2007). Fish were held in the sock in the experimental tanks for 15 h representing the maximum time fish are held in a sock for sorting under commercial settings (Avery, 2013). Afterwards, half of the fish (n= ~27/treatment tank) were anesthetized in a CO_2 bath, containing 3 mg/L NaCl, and blood was collected through caudal puncture within 5 min of removal. The remaining fish (n= ~26/treatment) were transferred to transport tanks at a density of 0.72 Kg/L supplied with well-oxygenated water (>5 mg/L, compressed oxygen) directly from the treatment tanks and held for 3 h, mimicking typical transport times for commercially reared catfish (Bosworth et al., 2004). Transported fish were then harvested as previously described. The procedures

were conducted in accordance with the approved protocols reviewed and approved by the Mississippi State University institutional animal care and use committee (no. 13-041).

3.2.5 Physiological analysis of plasma

Blood samples were placed on ice and plasma was collected by centrifugation (7,000 rpm, 5 min) within 10 min of collection. Plasma was transferred to two pre-labeled 1.5 ml microfuge tubes and flash frozen in liquid nitrogen prior to storage at -80°C until analysis. Plasma cortisol was measured with an enzyme immunoassay cortisol test kit (EA65; Oxford Biomedical Research, Oxford, MI). Plasma glucose was measured with a glucose assay test kit (DIGL-100; BioAssay Systems, Hayward, CA) and plasma lactate was measured with an L-lactate assay kit (A-108L; Biomedical Research Service Center, Buffalo, NY). Total protein was determined with a Bradford protein assay (quickstart^{TM}, BioRad; Hercules, CA). Plasma pH was measured with an accumet® AB15 pH meter with a micro pH electrode (Fisher Scientific; Pittsburg, PA). Osmolality was measured with a vapor pressure osmometer (Vapro 5520, Westcor Inc.; Logan, UT). Anions and cations were measured in the plasma samples using a Dionex 500 ion chromatography system (ThermoFisher, Waltham, MA). Anions (Cl^-, F^-, PO_4^{3-} and SO_4^{2-}) were evaluated with an anion column (AS4A-SC; ThermoFisher; Waltham, MA) with 180 mM carbonate/170 mM bicarbonate as the eluent at 2 ml/min with a 25 µl sample loop and an electrolytically regenerated suppressor (AERS 500; ThermoFisher; Waltham, MA). Cations (Na^+, K^+) were analyzed with a cation column and guard (CS4A-SC, ThermoFisher; Waltham, MA) using 20 mM methane sulfonic acid (MSA) eluent at 1 mL/min, 100 µl sample loop and an electrolytically regenerated suppressor (CERS 500; ThermoFisher; Waltham, MA). An electrochemical detector (ED40, Dionex Corporation,

Sunnyvale, CA) was used to measure ion concentrations for comparison with standard ion curves that were generated with each sample set and run in duplicate.

3.2.6 Statistical analysis

A randomized complete block design with 2x2 factorial arrangement of treatments and subsampling was used to evaluate the effects of temperature, DO and handling on the stress response of channel catfish. Each trial represented a replicate block with temperature and DO concentration as the factorial variables. Plasma samples from fish (n=10) in each treatment were considered subsamples of each of the three replicate blocks. A two way analysis of variance (ANOVA) was used to detect differences among treatments for all physiological parameters. Mean separation was carried out using Fisher's protected least significant difference (LSD) comparisons and all statistical significance was stated at $p < 0.05$. All analyses were carried out as least square means using SAS statistical software version 9.3 (SAS Institute Inc., Cary, NC) and reported as means with their standard error.

3.3 Results

3.3.1 Water quality

Environmental temperature and DO levels were maintained over the course of the four week trial (Table 3.1). Significant differences in temperature ($F = 3322.55$, $p = <0.0001$) and DO ($F = 206.94$, $p = <0.0001$) among all treatments were identified with low variation. Differences in water chemistry were also observed for pH ($F = 134.74$, $p = <0.0001$), unionized ammonia ($F = 23.32$, $p = <0.0001$) and nitrite ($F = 7.00$, $p = <0.0001$) (Table 3.1). Although there were slight variations in the water chemistry among

treatments, likely due to the different experimental temperature and DO levels, pH, ammonia and nitrite were maintained below levels known to cause stress and affect growth in cultured catfish (Hargreaves and Kucuk, 2001; Tucker, 1996).

Table 3.1 Treatment tanks water quality.

Treatment	25-H	25-L	33-H	33-L
DO mg/L	6.28 ± 0.09^{a}	2.51 ± 0.09^{b}	4.76 ± 0.09^{c}	2.54 ± 0.15^{b}
DO% sat	75.9 ± 1.2^{a}	30.7 ± 1.2^{b}	65.7 ± 1.3^{c}	35.3 ± 2.1^{d}
Temperature (°C)	25.3 ± 0.1^{a}	25.6 ± 0.1^{b}	32.8 ± 0.1^{c}	33.0 ± 0.1^{d}
pH	7.53 ± 0.07^{a}	7.08 ± 0.06^{b}	7.48 ± 0.09^{a}	7.36 ± 0.07^{c}
Nitrite (mg/L)	0.61 ± 0.07^{a}	0.24 ± 0.03^{b}	0.46 ± 0.07^{ac}	0.29 ± 0.06^{bc}
Ammonia (mg/L)	0.36 ± 0.04	0.33 ± 0.04	0.32 ± 0.07	0.30 ± 0.07
Unionized Ammonia (mg/L)	0.01 ± 0.00^{a}	0.00 ± 0.00^{a}	0.03 ± 0.01^{b}	0.02 ± 0.01^{ab}

Mean water quality parameters for each of the treatment tanks. Treatments represent the two temperature (25°C or 33°C) and high (H, >5mg/L) and low (L, 2.5 mg/L) dissolved oxygen levels maintained for the 4 week trial period. Handling stressors include socking (S) and transport (T). Different letters represent significant differences between treatments at $p < 0.05$.

3.3.2 Physiological analysis

3.3.2.1 Cortisol

Differences in circulating plasma cortisol were observed among treatments ($F = 21.53$, $p < 0.0001$), with no differences among fish reared under different environmental conditions prior to the harvesting events (Table 3.2). Cortisol increased with socking and transport compared to fish sampled directly out of the environmental treatment ($F = 23.75$, $p = 0.0001$; Figure 3.1). A suppression of the cortisol response was observed when fish were exposed to handling stressors with DO levels maintained at ~2.5 mg/L ($F = 6.85$, $p = 0.0157$; Figure 3.1).

3.3.2.2 Glucose

As fish progressed through harvest practices, glucose increased ($F = 24.36$, $p = 0.0001$; Figure 3.2) in fish subjected to handling when compared to those reared under the different environmental conditions alone. Glucose also increased following the combined socking and transport when compared to socking alone (Table 3.2). Fish maintained at 25°C and 2.5 mg/L DO had lower circulating glucose following four weeks under environmental conditions when compared to control fish at 25°C and >5 mg/L DO (Table 3.2).

3.3.2.3 Lactate

Circulating plasma lactate differed among treatments ($F = 7.69$, $p = <0.0001$), with significant DO x handling interaction effects ($F = 4.74$, $p = 0.0194$). Circulating plasma lactate showed a significant increase in fish following transport only when reared under moderate temperature stress (33-H, Table 3.2).

Table 3.2 Biochemical analysis of channel catfish (*Ictalurus punctatus*) plasma following sequential stress events.

Treatment	pH	Cortisol (ng/ml)	Glucose	Lactate (mM)	Total Protein (mg/ml)	Osmolality LSMean (mOSM)
25-H	6.9 ± 0.1^{a}	4.9 ± 5.4^{e}	98.1 ± 4.7^{cd}	13.2 ± 3.4^{bc}	33.0 ± 4.7	295 ± 5.6^{ab}
25-H-S	7.0 ± 0.1^{ab}	23.1 ± 5.4^{bcd}	122.5 ± 4.9^{bcd}	12.9 ± 3.4^{bc}	38.0 ± 4.7	288 ± 5.6^{ab}
25-H-ST	7.0 ± 0.1^{a}	43.7 ± 5.4^{a}	204.2 ± 10.4^{a}	19.5 ± 3.4^{ab}	41.2 ± 4.7	292 ± 5.6^{ab}
25-L	6.9 ± 0.1^{a}	8.9 ± 5.4^{de}	83.2 ± 3.8^{d}	13.9 ± 3.4^{abc}	36.8 ± 4.7	283 ± 5.6^{b}
25-L-S	7.0 ± 0.1^{ab}	18.2 ± 5.4^{cde}	135.9 ± 7.0^{cd}	20.0 ± 3.4^{ab}	43.0 ± 4.7	285 ± 5.6^{ab}
25-L-ST	7.1 ± 0.1^{ab}	23.3 ± 5.4^{bcd}	161.9 ± 7.1^{ab}	14.3 ± 3.4^{abc}	40.3 ± 4.7	281 ± 5.6^{b}
33-H	7.0 ± 0.1^{ab}	5.8 ± 5.4^{e}	100.9 ± 4.5^{cd}	10.3 ± 3.4^{bc}	40.2 ± 4.7	292 ± 5.6^{ab}
33-H-S	7.1 ± 0.1^{ab}	30.6 ± 5.4^{abc}	104.2 ± 5.1^{cd}	9.3 ± 3.4^{c}	44.5 ± 4.7	289 ± 5.6^{ab}
33-H-ST	7.0 ± 0.1^{ab}	36.6 ± 5.4^{ab}	161.9 ± 10.8^{ab}	23.6 ± 3.4^{a}	37.8 ± 4.7	305 ± 5.6^{a}
33-L	7.0 ± 0.1^{ab}	4.1 ± 5.4^{e}	90.1 ± 4.7^{d}	14.0 ± 3.4^{abc}	34.8 ± 4.7	289 ± 5.6^{ab}
33-L-S	7.1 ± 0.1^{b}	16.4 ± 5.4^{cde}	122.1 ± 7.3^{bcd}	13.1 ± 3.4^{bc}	38.9 ± 4.8	284 ± 5.6^{b}
33-L-ST	7.1 ± 0.1^{ab}	24.6 ± 5.8^{bcd}	144.9 ± 10.9^{bc}	10.9 ± 3.7^{bc}	38.3 ± 5.1	281 ± 5.6^{b}

Least square mean of plasma cortisol, glucose, lactate, total protein, pH and osmolality for fish subjected to different stressors. Treatments represent temperature (25°C or 33°C) – dissolved oxygen (2.5 mg/L or >5mg/L) – handling. Handling stressors include socking (S) and transport (T). Different letters represent significant differences between treatments at $p < 0$.

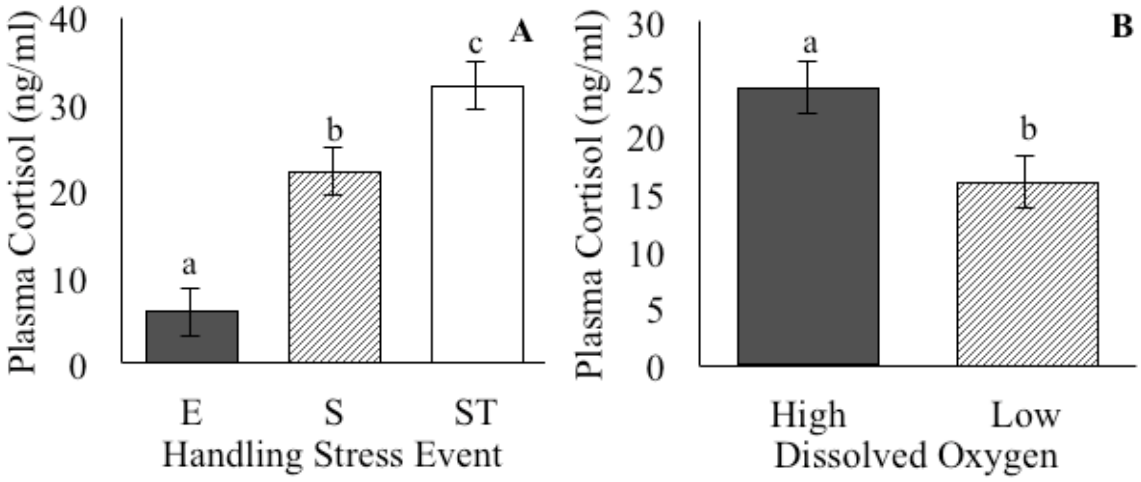

Figure 3.1 Main effects of stress on plasma cortisol in channel catfish (*Ictalurus punctatus*).

Mean (± SE) plasma cortisol concentrations in fish subjected to high (H, >5 mg/L) and low (L, ~2.5 mg/L) dissolved oxygen concentrations and rearing at environmental conditions (E), socking (S) and both socking and transport (ST) stress. Different letters represent significant differences at $p < 0.05$ (ANOVA, n = ~30).

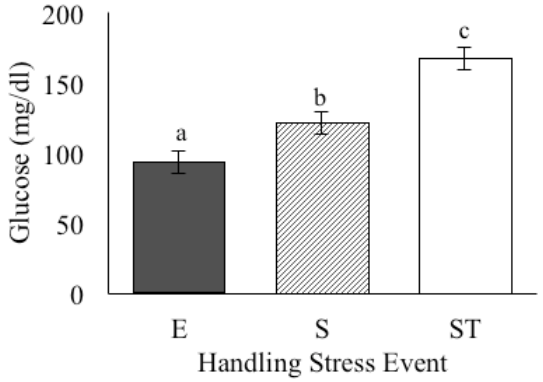

Figure 3.2 Main effects of handling on plasma glucose in channel catfish (*Ictalurus punctatus*).

Mean glucose (± SE) for fish subjected to rearing at environmental conditions (E), socking (S) and both socking and transport (ST) stress. Different letters represent significant differences at $p < 0.05$.

3.3.2.4 Total protein

No differences in circulating total protein concentrations ($p > 0.05$) were observed among treatments.

3.3.2.5 pH

The plasma pH of fish reared at 33°C was, on average, 0.1 unit greater ($F = 17.09$, <0.0001) than fish reared at 25°C ($F = 5.56$, $p = 0.0276$). Fish subject to high temperature, low DO and socking stress alone (33-L-S) had higher plasma pH than fish exposed to the control (25-H) or the socking and transport stress combined with control conditions (25-H-ST, Table 3.2).

3.3.2.6 Osmolality

On average, plasma osmolality was ~10 mOsm greater ($F = 12.96$, $p = <0.0001$; Table 3.2), in fish reared with DO levels maintained > 5 mg/L ($F = 8.36$, $p = 0.0085$). Fish reared under moderate temperature stress and subject to the socking and transport stress (33-H-ST) had greater osmolality than fish experiencing the same handling stressors when DO levels were held low regardless of temperature (Table 3.2).

3.3.2.7 Ionic composition

Fluoride concentrations differed among treatments ($F = 12.72$, $p = <0.0001$) with DO x handling interaction effects ($F = 6.03$, $p = 0.0081$). On average, fluoride concentrations increased when subjected to both handling and transport stress ($F = 3.48$, $p = 0.0485$; Figure 3.3). Plasma chloride decreased ($F = 23.94$, $p = <0.0001$) as fish experienced socking and transport stress (Table 3.3). On average, chloride levels decreased as fish progressed through the harvest stressors ($F = 42.85$, $p = <0.0001$; Figure

3.3). Phosphate levels increased ($F = 9.85$, $p = <0.0001$) in the socked and transported fish when compared to environmentally stressed and socked only fish when reared at DO levels >5 mg/L (Table 3.3). On average, phosphate levels increased in fish that were subjected to socking and transport stress ($F = 16.52$, $p = <0.0001$, Figure 3.3). Plasma sulfate differed among treatments ($F = 37.6$, $p = <0.0001$), with an increasing trend in plasma sulfate observed as fish progressed through the harvest event (Table 3.3). On average, high temperature ($F = 8.2$, $p = 0.009$), low DO ($F = 7.09$, $p = 0.0142$) and sequential handling stress ($F = 3.63$, $p = 0.0433$) decreased plasma sulfate levels (Figure 3.4). Differences among treatments were observed in plasma sodium concentrations ($F = 13.2$, $p = <0.0001$) with temperature x DO interaction effects ($F = 8.35$, $p = 0.0085$). Fish reared under the severe stress conditions (33-L) showed a decrease in plasma sodium content (Table 3.3). Plasma potassium levels differed among treatments ($F = 5.42$, $p = <0.0001$), with fish reared under severe stress (33-L) showing the only increase in plasma potassium following the combined socking and transport stress (Table 3.3). On average, fish reared at 33°C had higher plasma potassium than fish reared at 25°C ($F = 35.24$, $p = <0.0001$; Figure 3.3).

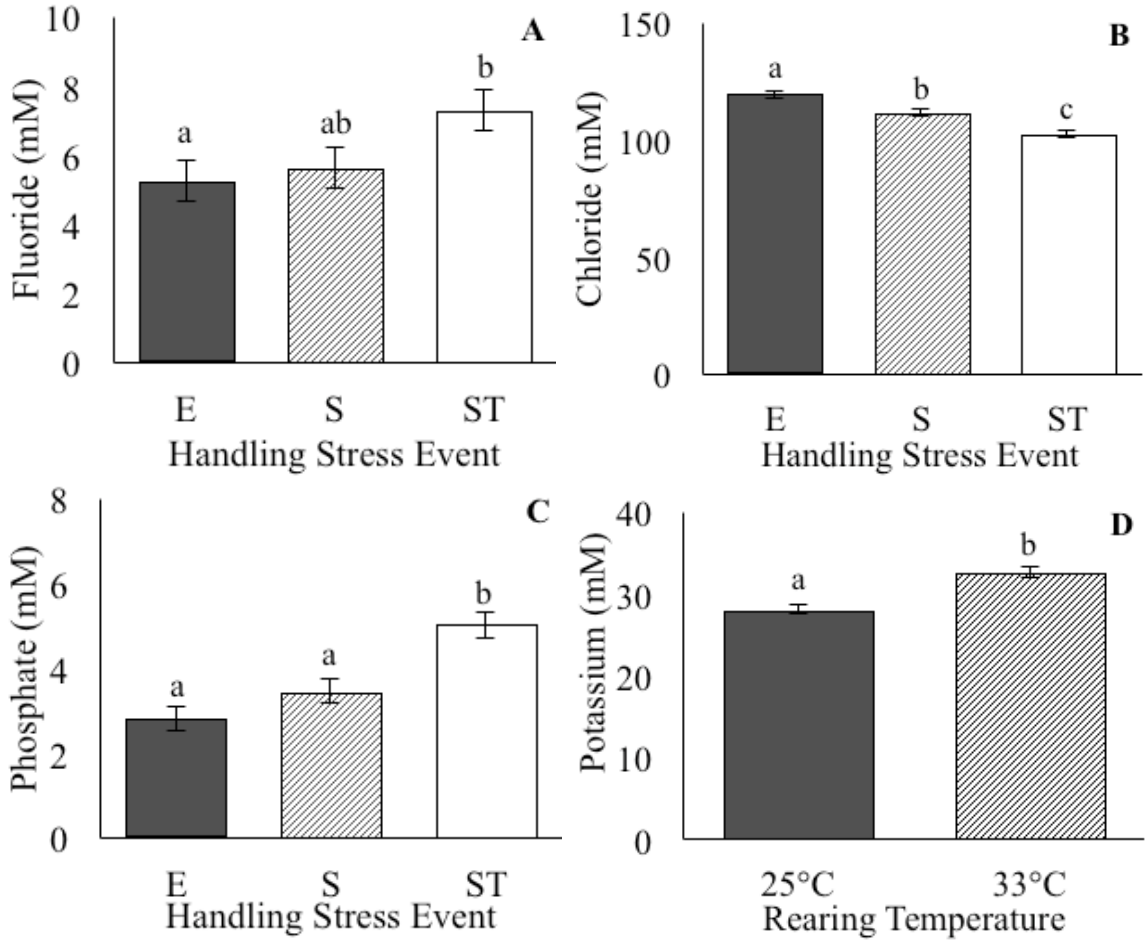

Figure 3.3 Main effects of stress on plasma ionic composition in channel catfish (*Ictalurus punctatus*).

Values represent means with standard error for A) fluoride B) chloride and C) phosphate concentrations after rearing at environmental conditions (E), socking (S) and both socking and transport (ST) stress. D) Main effects of high (33°C) and low (25°C) temperature on potassium concentrations. Different letters represent significant differences at $p < 0.05$ (ANOVA, LSD, n = ~30).

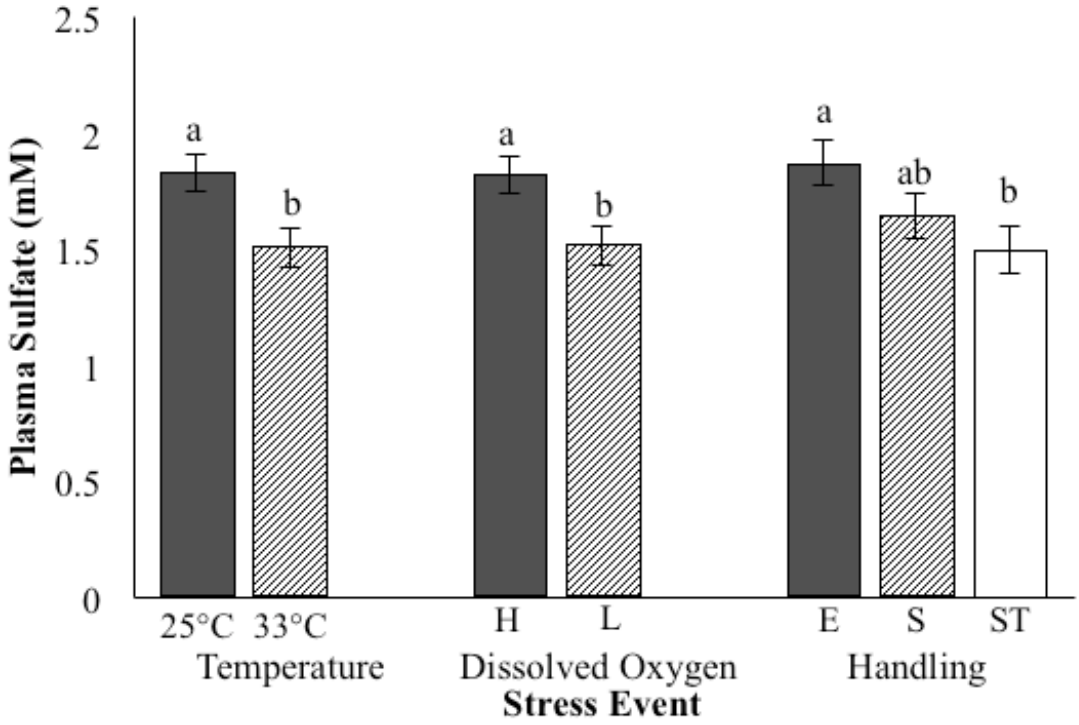

Figure 3.4 Main effects of temperature, dissolved oxygen and handling on plasma sulfate concentrations in channel catfish (*Ictalurus punctatus*).

Mean (± SE) plasma sulfate concentrations in fish reared at two different temperatures, high (>5 mg/L) and low (~2.5 mg/L) dissolved oxygen levels, and fish subject to rearing at environmental conditions only (E), socking (S) and both socking and transport (ST) stress. Different letters represent significant differences at $p < 0.05$ (ANOVA, LSD, n = ~30).

Table 3.3 Plasma ion concentrations in channel catfish (*Ictalurus punctatus*) following sequential stress events.

Treatment	Fl^- (mM/L)	Cl^- (mM/L)	PO_4^- (mM/L)	SO_4^- (mM/L)	Na^+ (mM/L)	K^+ (mM/L)
25-H	5.6 ± 1.1^{bc}	118.5 ± 2.5^{ab}	3.4 ± 0.5^{cd}	2.3 ± 0.2^{a}	142.5 ± 3.3^{ab}	26.4 ± 1.3^{e}
25-H-S	5.5 ± 1.1^{bc}	113.1 ± 2.5^{abc}	3.4 ± 0.5^{cd}	2.0 ± 0.2^{ab}	141.2 ± 3.3^{abc}	26.3 ± 1.3^{e}
25-H-ST	8.4 ± 1.1^{ab}	101.8 ± 2.5^{ef}	5.2 ± 0.5^{ab}	1.9 ± 0.2^{abc}	138.3 ± 3.3^{bc}	28.0 ± 1.3^{de}
25-L	5.1 ± 1.2^{bc}	119.5 ± 2.7^{ab}	2.6 ± 0.6^{cd}	1.7 ± 0.2^{bcd}	142.9 ± 3.3^{ab}	28.2 ± 1.3^{cde}
25-L-S	7.0 ± 1.2^{abc}	111.5 ± 2.7^{bcd}	3.1 ± 0.6^{cd}	1.6 ± 0.2^{bcd}	143.4 ± 3.3^{ab}	30.5 ± 1.3^{bcd}
25-L-ST	5.3 ± 1.2^{bc}	105.3 ± 2.7^{def}	4.2 ± 0.6^{bc}	1.4 ± 0.2^{cd}	142.1 ± 3.3^{abc}	28.0 ± 1.3^{cde}
33-H	4.6 ± 1.2^{c}	120.1 ± 2.7^{a}	2.6 ± 0.6^{cd}	1.7 ± 0.2^{bcd}	149.0 ± 3.3^{a}	31.9 ± 1.3^{abc}
33-H-S	4.3 ± 1.2^{c}	111.9 ± 2.7^{bcd}	3.2 ± 0.6^{cd}	1.6 ± 0.2^{bcd}	146.0 ± 3.3^{ab}	31.1 ± 1.3^{bcd}
33-H-ST	10.2 ± 1.2^{a}	100.3 ± 2.7^{f}	6.5 ± 0.6^{a}	1.4 ± 0.2^{cd}	145.9 ± 3.3^{ab}	33.7 ± 1.3^{ab}
33-L	5.4 ± 1.2^{bc}	119.0 ± 2.7^{ab}	2.4 ± 0.6^{d}	1.7 ± 0.2^{bcd}	142.9 ± 3.3^{ab}	31.3 ± 1.3^{bcd}
33-L-S	5.6 ± 1.2^{bc}	108.3 ± 2.7^{cde}	4.0 ± 0.6^{bcd}	1.4 ± 0.2^{cd}	138.8 ± 3.3^{bc}	31.3 ± 1.3^{bcd}
33-L-ST	5.1 ± 1.3^{bc}	99.6 ± 2.9^{f}	4.1 ± 0.6^{bcd}	1.3 ± 0.2^{d}	131.8 ± 3.7^{c}	35.6 ± 1.5^{a}

Least square mean of plasma anion and cation concentrations for fish subject to different stressors. Treatments represent temperature (25°C or 33°C) – dissolved oxygen (2.5 mg/L or >5 mg/L) – handling. Handling stressors include socking (S) and transport (T). Different letters represent significant differences between treatments at $p < 0.05$.

3.4 Discussion

Environmental and sequential handling stressors resulted in cumulative stress responses identified by increases in circulating cortisol and glucose concentrations, as well as alterations in electrolyte composition. Handling was identified as the most severe stressor based on number and magnitude of physiological changes observed. Prior exposure to low DO was found to have a suppressing effect on stress responses of channel catfish following harvest stressors.

Circulating cortisol and glucose represent two widely used hematological indicators of stress in fish, which are typically observed to increase in response to a stressor (Barton, 2002; Cnaani et al., 2014; Frisch and Anderson, 2000; Mommsen et al., 1999). Cortisol and glucose have been found to increase following netting (Wang et al., 2004), low-water stress (Frisch and Anderson, 2000), confinement (Small, 2004; Strange, 1980) and handling (Sharpe et al., 1998). Although cortisol levels increased with the addition of each sequential handling stressor, rearing conditions did not affect the resting levels of plasma cortisol. The resting cortisol levels for each environmental treatment were below the 10 ng/mL reported for resting cortisol levels in Small et al. (2008). The increasing concentrations of cortisol and glucose in circulation with subsequent socking and transport demonstrate the cumulative nature of the stress response in channel catfish. As fish in culture often experience multiple stressors, the cumulative effects of such events are important to identify in order to properly manage a population (Bevelhimer and Bennett, 2000) and counteract or avoid any negative effects of cumulative stress.

The cortisol response was suppressed following handling and transport. This supports previous findings that exposure to stressful environmental conditions influences

the response to a subsequent stressor (Barton, 2002; Davis et al., 1993). In Atlantic cod, a cold water species, acclimated to high temperatures (15°C), plasma cortisol was suppressed following an acute hypoxic event (Methling et al., 2010). In striped bass acclimated to water temperatures below 16°C, cortisol levels were suppressed with nearly two fold higher levels observed post-confinement stress at temperatures above 21°C (Davis and Parker, 1990). The effects of environmental conditions prior to stress have also been reported in other physiological parameters. Circulating glucose increased following acute hypoxic stress when reared under hypoxic conditions (2-3 mg/L), but decreased when reared under normoxic and hyperoxic conditions prior to the hypoxic stress event in great sturgeon (*Huso huso*) (Lakani et al., 2013). It is important to understand the factors that affect the stress response, as it will be necessary to consider their effects on the response when interpreting the physiological significance (Barton, 2002). In addition, a suppression of the physiological stress response could have deleterious effects on a fish's ability to cope with a stress event and more drastic consequences on reproduction and survival (Quabius et al., 1997; Wingfield and Sapolsky, 2003)

In fish, stressful events have been associated with alterations in circulating lactate, glucose and pH levels (Donaldson et al., 2014; Urbinati et al., 2004; Wood, 1991). Channel catfish stressed by socking and transport had higher circulating glucose concentrations, presumably in response to higher metabolic rates associated with increased energy expenditures inherent during stress (Wendelaar Bonga, 1997). Fish reared at low DO (2.5 mg/L) had lower resting glucose than fish reared under control conditions (25-H), possibly indicating an increased energy requirement for coping with

the chronic low DO environment or reflecting a decrease in metabolic activity due to decreased feed consumption (Ch. 2). Bosworth et al. (2007) observed increased plasma lactate, glucose and cortisol and decreased pH in juvenile channel catfish following low water stress when compared to rested harvested fish. In the present study, average plasma pH increased slightly in fish reared at 33°C with no changes after handling stress. All pH values were lower than those observed by Bosworth et al. (2007) for juveniles (mean weight 81.5 g) after low-water stress, which could be explained by the higher plasma lactate levels in the fish from this study. The higher lactate levels reported by Bosworth et al. (2007) for the larger (mean weight 300 g) catfish experiencing low-water stress could explain why pH values were slightly lower in most treatments in the present study. Plasma pH among treatments remained relatively stable even in treatments exhibiting changes in lactate concentrations, possibly due to the use of CO_2 narcosis to immobilize fish prior to blood collection and slaughter (Bosworth et al., 2007; Fivelstad et al., 2003).

Plasma lactate concentration increases due to increased anaerobic respiration (Wood, 1991), which occurs when oxygen is limited and is often observed in exhausted fish and after periods of high activity (Donaldson et al., 2014; Wood, 1991). In this study, plasma lactate concentrations remained fairly stable among treatments, with the exception of increases in the moderate temperature stress (33-H) where a more than two fold difference was observed between the fish subjected to the environmental treatment alone and those that experienced both socking and transport stress. The higher temperature (33°C) combined with high DO (>5 mg/L) could have promoted greater activity during the transport stage of the study and thus, a switch to anaerobic respiration

(Currey et al., 2013; Donaldson et al., 2014; He et al., 2013; Wood, 1991), although this was not quantified.

Proteins have been shown to play a significant remedial role during stress (Iwama et al., 1998) and their concentrations have been identified as potential indicators of stress in fish (Coeurdacier et al., 2011). In the present study, total protein concentrations in channel catfish were regulated tightly with no observable trends in fish reared under different environmental conditions or following socking and transport stress. This suggests that the function and importance of proteins to the stress response is species specific (Iwama et al., 1998; Martínez-Porchas et al., 2009) and protein is not a good indicator of catfish stress and welfare.

Altered plasma ionic composition and osmolarity, due to stress is well documented in fish and attributed to increased diffusion at the gills, high ion loss in the urine, hemodilution due to increased water uptake or a combination of these mechanisms (McDonald and Milligan, 1997). Wang et al. (2004) reported that plasma chloride and osmolality decreased in striped bass following netting stress while an increase was reported in juvenile matrinxã (*Brycon cephalus*) following transport stress (Urbinati et al., 2004). The increase in osmolarity was suggested to be due to increased protein concentrations since the fish had resumed feeding. In the present study, the relatively stable plasma osmolality suggests tight regulation, likely due to upregulation of other osmolites, some of which may not have been measured in the present study such as nitrogen compounds (Yancey, 2001).

Ionic composition in blood is an important contributor to physiological processes and can be affected by stress (Marshall and Grosell, 2005; Urbinati et al., 2004).

Decreased plasma chloride following handling stress under all environmental conditions, and sodium levels in the severe stress treatment (33-L), are likely due to increased ion loss and water uptake at the gills. Alterations in gill structure occur during environmental rearing to help promote branchial gas exchange through increased respiratory surface area along with increased blood flow, enhanced rate of water osmosis and diffusion of ions (Cnaani et al., 2014; Wendelaar Bonga, 1997). These changes result in increased ion loss and water uptake during subsequent handling stressors. The changes observed in the gill structure can include dilation of the blood sinuses, hypertrophy and hyperplasia of respiratory and chloride cells, increased mucus secretion, infiltration of leukocytes into intracellular spaces and higher rates of necrosis and apoptosis (Wendelaar Bonga, 1997). The main effects of environmental DO did not affect plasma chloride concentrations, similar to what was observed in yellow perch (Cnaani et al., 2014).

Lower plasma sodium content, which is attributed to increased ion loss at the gills, was reported in yellow perch exposed to acute hypoxic conditions when compared to fish held under normoxic conditions (Cnaani et al., 2014). In the present study, a decrease in plasma sodium content was observed in the severe stress treatment, suggesting that catfish are highly capable of maintaining sodium balance following handling when environmental stressors are moderate to low prior to harvest. Similarly, potassium levels increased in the severe stress treatment following the socking and transport stress combined, but not following exposure to any of the moderate stress or control conditions. The increased potassium in the plasma suggests increased cellular lysis and release of internal potassium stores (Marshall and Grosell, 2005). In support, visual evaluation of the plasma samples as fish progressed through the harvest process revealed an increase in

red pigmentation of the plasma, suggesting increased cellular lysis and release of hemoglobin. However, a quantitative measure of plasma color was not taken.

Increased phosphate concentrations following any kind of stress event likely represents increased energy expenditures and release of free phosphates (Nelson and Cox, 2008). When fish were subjected to both socking and transport, the increase in plasma phosphates was likely caused by cellular lysis (McCay, 1931) and the release of intracellular inorganic phosphate (Nelson and Cox, 2008c).

The physiological function of fluoride in fish is not well documented. In the present study, plasma fluoride levels nearly doubled following socking and transport in fish reared under moderate temperature stress. While it does contribute to bone formation and mineralization (Lall and Lewis-McCrea, 2007), it is not likely that there was significant bone de-mineralization occurring during acute stress. Thus fluoride may be an important part of the physiological stress response in catfish that should be considered in the future.

Physiologically, sulfates play an important role in the activation and detoxification of many compounds (Markovich, 2001). Physiological analysis of euryhaline eels revealed that plasma sulfate could play a role in maintaining osmotic balance in freshwater (Nakada et al., 2005). However, sulfate levels in the plasma were low (<2 mM) and changes, although statistically significant, never exceeded 0.5 mM, making it unlikely that sulfates contribute to osmotic balance in channel catfish. The slight decreases in sulfate concentrations that occurred due to a stress event could also be the result of decreased food consumption, which has been observed in mammals (Markovich, 2001).

It is important to note that fish in this study were subjected to constant temperatures. Fish respond differently to stress under the fluctuating thermal regimes which occur naturally in culture ponds (Davis and Simco, 2001). This should be taken into consideration for future evaluations of stress during culture. The physiological response of catfish to high temperature could be integral to future management of catfish pond culture since global temperatures are predicted to rise (Karl et al., 2009) and environmental conditions in culture ponds are highly correlated to air temperatures (Wax and Pote, 1990). In addition, a better understanding of how the different culture stressors affect fish physiologically can be important for determining what conditions and stressors should be managed or monitored more closely to optimize health and survival of the fish and quality of the flesh produced (Frisch and Anderson, 2000).

3.5 Conclusions

The data demonstrate the physiological effects of environmental conditions, socking and transport stress on catfish. Socking and transport represent the most severe stressors experienced in cultured catfish and the fishes' response to them depends on environmental conditions. The suppression of the stress response when fish are pre-exposed to high environmental temperatures and/or low DO highlights the importance of monitoring and potentially controlling environmental rearing conditions to manage stress that is imparted on a population as this could have deleterious effects on fish survival and fillet quality. Future research focusing on correlations between physiological response of fish at harvest and pond temperature and DO for nearly a month preceding harvest could be used to evaluate how fish will respond physiologically to a harvest event. Such physiological changes can be important in the ultimate welfare, survival and quality of

fish at harvest. Additionally, this information could be used to develop protocols to monitor environmental conditions leading up to harvest events as indicators of survival and fillet quality.

CHAPTER IV

EVALUATION OF THE ROLE STRESS PLAYS IN THE FORMATION OF RED FILLETS IN CULTURED CHANNEL CATFISH (*Ictalurus punctatus*)

4.1 Introduction

Pond culture of catfish is the largest sector of aquaculture in the United States (Silverstein, 2013). Since 2000, catfish producers have placed an emphasis on modifying culture practices in an attempt to enhance product quality rather than rely solely on post-mortem processing procedures (Minchew et al., 2007). In order to implement such changes, a detailed understanding of how culture conditions and practices affect fish physiology and fillet quality is necessary.

Pond culture inherently results in daily and seasonal fluctuations in environmental conditions, with dissolved oxygen (DO) levels decreasing below 1 mg/l (Hargreaves and Steeby, 1999) and summer temperatures exceeding 35°C (Davis and Simco, 2001; Wax and Pote, 1990). Low DO (Small, 2004) and temperatures greater than 35ºC have been shown to elicit a physiological stress response in channel catfish (Davis and Simco, 2001).

During harvest, fish density and handling during socking, a method of sorting fish confined to a net cage with a specific mesh size to allow smaller fish to escape, and transport represent additional stress events that further elicit a stress response (Poli et al., 2005). When harvesting catfish, the sequential socking and transport events, elicit a stress

response due to confinement and handling (Minchew et al., 2007; Small, 2004; Strange, 1980). In addition to the physical stressor of confinement, high fish density can result in hypoxic conditions and buildup of ammonia, further contributing to stress (Small, 2004).

Ante-mortem stress events and product treatment impact meat quality (Dave and Ghaly, 2011), especially color (Akşit, 2006; Bosworth et al., 2004, 2007; Erikson and Misimi, 2008; Ginés et al., 2004; Robb et al., 2000). In salmonids, ante-mortem handling stress produced darker, less red and more yellow fillets with lower hue angles and higher chroma values (Erikson and Misimi, 2008). In addition, high ante-mortem activity produced lighter, less red fillets with higher hue angles and chroma values in rainbow trout (*Oncorhynchus mykiss*) (Robb et al., 2000). Bosworth et al. (2007) demonstrated that catfish that were rested, using an anesthetic bath, prior to harvest experienced a minimal stress response and produced darker fillets when compared to non-rested transports. In Arctic char (*Salvelinus alpinus*), fillets from fish reared at a colder temperature (10°C) exhibited higher redness, yellowness and chroma intensity resulting in more intense orange colored fillets when compared to fish reared at a warmer temperature (15°C) (Ginés et al., 2004). Low DO concentrations during transport of channel catfish resulted in lighter fillets (Bosworth et al. 2004).

Fillet color is an important factor that consumers consider when purchasing meat and seafood products (Maciel et al., 2014). This is especially important with regard to catfish as any deviation from the typical white flesh color results in decreased marketability of the product (Lovell, 1984; Shahidi and Brown, 1998). The causes of discoloration in whitefish fillets can result from many different factors including residual blood (Roth et al., 2007), lipid oxidation (Ruff et al., 2002), storage time (Guillerm-

Regost et al., 2006), food consumed (Li et al. 2007), storage temperature and method of slaughter (Stien et al., 2005). Annually, in a Mississippi commercial catfish processing facility, approximately 0.5% of fillets are deemed unmarketable due to red discoloration, while approximately 0.05% are discarded due to yellow discoloration (Miller, 2015). Yellow fillets are caused by increased pigmentation in the muscle that is incorporated through consumption of natural foods present in ponds (Li et al., 2007). Red fillets are believed to occur due to ante-mortem stress events but to date, the specific cause and mechanisms involved in red fillet formation are poorly understood. The increased red coloration in catfish fillets has been correlated with increased hemoglobin in the muscle (Desai et al., 2014).

The current study examined the physiological changes in hematocrit and hemoglobin concentrations in channel catfish following various sequential harvest stress events to determine their effects on fillet color. These data offer a better understanding of the stressors involved in the development of off-color in catfish fillets and offer insight into particular culture conditions and practices that could be monitored and altered to reduce the prevalence of red fillets in cultured channel catfish.

4.2 Materials and Methods

4.2.1 Fish acquisition and rearing

Channel catfish were acquired as fingerlings from the Mississippi State University Thad Cochran National Warmwater Aquaculture Center in Stoneville, MS. Fingerlings were grown for up to two years in flow-through culture systems ranging from 400 to 4,000 L at ambient well-water temperatures (22°C - 27°C) and oxygenated with constant forced air aeration. Water quality was monitored weekly and maintained within the

optimal range for growth and survival of channel catfish (Hargreaves and Kucuk, 2001; Tucker, 1996). Fish were fed a 36% protein fingerling feed once daily (Land O'Lakes Purina Feed LLC, Arden Hills, MN). Prior to transfer to treatment tanks (~1 yr), the fish were switched to a 32% protein finishing feed (Purina Animal Nutrition), which is commercially utilized in grow-out ponds (Robinson et al., 1998).

4.2.2 Live stress trial

Fish were stocked (mean weight 704 ± 484 g) into experimental tanks for final grow-out and application of stress treatments. Catfish were anesthetized in a CO_2 bath, weighed to the nearest g, measured to the nearest mm and randomly distributed between four 4,000 L treatment tanks (n = 80/tank). Treatment tanks were separate recirculating aquaculture systems operated at ambient temperature (~27°C) with a constant supply of makeup water (~0.15 ± 0.08 L/s). Tanks were fitted with expandable granular biofilters filled with plastic floating biobeads (Polygeyser Models DF3 or 6; Aquaculture Systems Technologies, New Orleans, LA) that provided mechanical and biological filtration. Each system had two in-line high-output ultra violet sterilizers (120 Watt SMART HO, Emperor Aquatics; Pottstown, PA), a pump (SHE2.4 or SHE1.7, Aquatic Ecosystems, Inc., Apopka, FL) and a sump tank. After stocking, fish were slowly acclimated (1°C/day and 10% DO saturation/day) for 1 week to one of two experimental temperatures (25°C and 33°C) and one of two DO levels (>5 mg/L and ~2.5 mg/L). The lower temperature (25°C) served as a control as it reflects the approximate average grow-out/rearing temperature in our facilities, while the high temperature (33°C) approaches the thermal lethal maximum temperature for channel catfish (Currie et al., 1998). Oxygen levels >5 mg/L were considered high (H) and DO levels of 2.5 mg/L were considered low (L). The

treatments included the control (25-H), moderate temperature stress (33-H), moderate oxygen stress (25-L) and severe stress (33-L). Temperature and DO levels were monitored three times daily and pH, ammonia and nitrite were measured three times per week. Following the 1 week acclimation period, fish were held for four weeks and fed a 32% protein finishing feed (Purina Animal Nutrition) once daily to satiation. Food was withheld for two days before harvest at which point 1/3 of the fish from each treatment were harvested (n=27/tank) and the remaining fish were transferred to a 1.82 m^3 (0.60 m L x 0.60 m W x 0.46 m H) sock constructed of polyvinyl chloride tubing and 1.27 cm plastic netting. Fish were held in the sock in the experimental tanks for 15 h, representing the maximum time fish are held in commercial socks (unpublished data), before half of the fish (n=27/tank) were harvested. Fish that were subjected to socking stress are designated with an S. The remaining fish (n=26/tank) were transferred to transport tanks at a density of ~0.72 kg/L, commonly experienced during commercial transport (Bosworth et al., 2004). Transport tanks were filled with water from the respective treatment tanks and were supplied with oxygen via diffuser stones and compressed oxygen cylinders to maintain high DO conditions (>5 mg/L). Fish were held under these conditions for the maximum transport time experienced by commercially reared catfish (3 h) prior to harvesting (Bosworth et al., 2004). Fish subjected to transport stress following socking are designated as ST. The procedures used were conducted in accordance to the approved protocols reviewed and approved by the Mississippi State University Institutional Animal Care and Use Committee (IACUC) (no. 13-041).

4.2.3 Physiological analysis

Fish were anesthetized in a CO_2 bath with 3 g/L NaCl. Blood was drawn from 10 fish per treatment with a 22 G needle and a 3.0 ml Monoject™ EDTA coated blood collection tube (Fisher Scientific; Pittsburg, PA). Three micro hematocrit capillary tubes per fish were filled with blood and centrifuged in a microcapillary centrifuge (Model CHT; Lourdes Instrument Corp., Brooklyn, NY) at 7,000 rpm for 5 min. Hematocrit was read on a micro-hematocrit capillary tube reader (Leica Microsystems, Buffalo Grove, IL). Whole blood hemoglobin concentration was quantified at harvest using a Quantichrom™ hemoglobin assay test kit (DIHB-250; BioAssay Systems, Hayward, CA). Data on hemoglobin in the blood was only collected during the second and third trials in the environmentally stressed fish.

4.2.4 Evaluation of fillet color

Anesthetized fish were killed by a sharp blow to the head and put on ice for transport (5 km) to the Mississippi State University Department of Food Science, Nutrition and Health Promotion's Pilot Plant where fish were filleted within 2 h. Instrumental fillet color was evaluated on the first 8 fish from each treatment using a handheld colorimeter (CR-400, Konica Minolta Inc. Chiyoda-ku, Tokyo, Japan) with an 8 mm port size, 2 degree standard observer and illuminant D65. Calibration was performed with a standard white calibration plate (Minolta Model No 20933026, Japan). Color values were taken on three points on the vertebral side of the two fillets produced per fish; two on the first fillet and one from the second (Figure 4.1). Color was reported as the least square means of CIE L* (lightness), a* (redness-greenness) and b* (yellowness-blueness) (Kin et al., 2009, 2010), after averaging the three color readings for each fish. Color was measured at

0 h post mortem and after 24 h of cold storage (4°C). The hue angle and chroma values were calculated for each color measure as described by Erikson and Misimi (2008). The hue angle was calculated as hue angle = arctan(b*/a*) when a* > 0 and b* > 0, hue angle = 180 + arctan(b*/a*) when a* < 0 and hue angle = 360 + arctan(b*/a*) when a*> 0 and b* < 0. Chroma was calculated as chroma = $(a^{*2} + b^{*2})^{1/2}$.

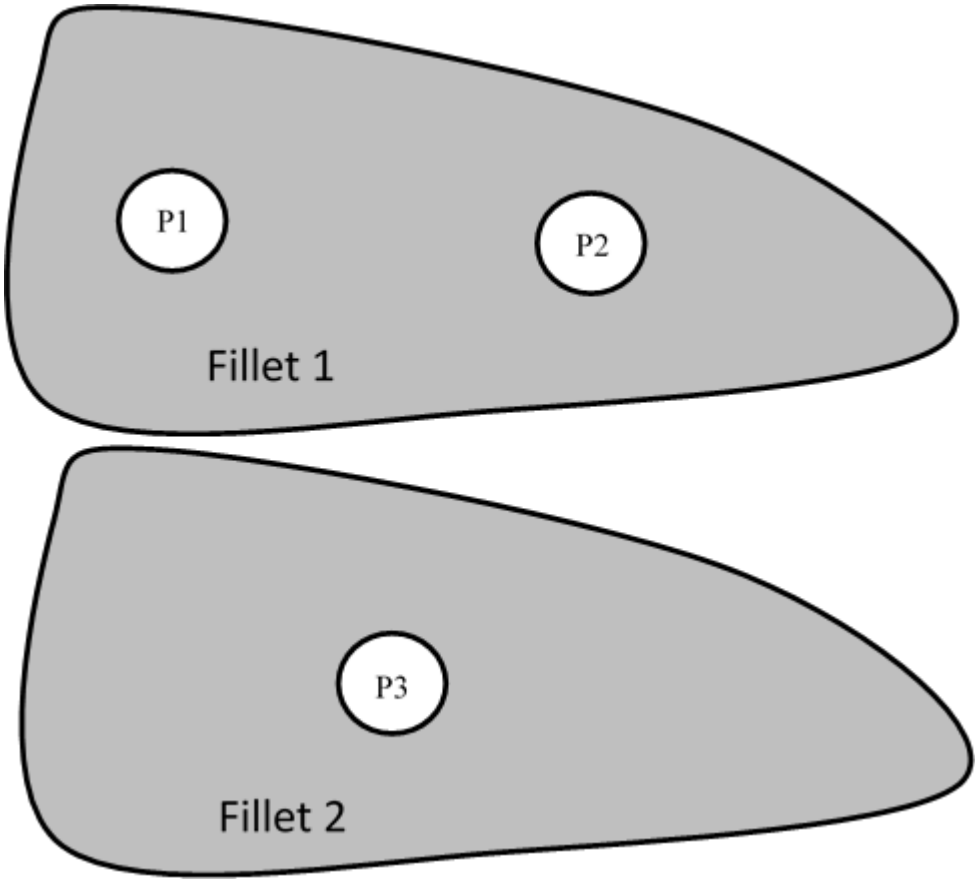

Figure 4.1 Diagram of color analysis.

The image depicts the points on the vertebral side of the catfish fillet where the color readings were taken. Both fillets from a single fish were used and each circle represents one of the three color readings taken (P1-3).

4.2.5 Commercially raised catfish

Catfish fillets were collected from Superior Catfish Products in Macon, MS in July and August of 2014 when the highest prevalence of red fillets were reported. A minimum of nine red and nine normal fillets were collected from fish reared in a single pond at each incidence (n=22) for color evaluation as described above.

4.2.6 Statistical analysis

Data were analyzed as a 2 x 2 factorial arrangement of treatments in a randomized complete block design with subsampling. Each of the three trials represented the blocks. When differences were observed among treatments, mean separation was conducted with Fisher's protected least significant difference (LSD) using SAS Statistical software version 9.3 (SAS Institute Inc., Cary, NC) at a significance level of $p < 0.05$. Due to the small sample size, hemoglobin data were analyzed non-parametrically for the environmental treatments using the Kruskall-Wallis test with means separation carried out with the Friedman's test. Due to non-normality, data for commercial catfish fillets were analyzed using the Wilcoxin Rank Sum test to evaluate differences in medians between color attributes of fillets deemed normal vs. red by commercial catfish processors.

4.3 Results

It is important to note that during the first stress trial the fish from the 33-H treatment escaped the sock and thus, only data for environmentally stressed fish were collected. Therefore, the whole treatment was repeated during the third trial. This resulted in two of the replications for this treatment being carried out with a larger size class (~1 kg), since fish in holding continued to grow between each trial period, which resulted in an increase in sock density with each subsequent replication.

4.3.1 Physiology

Blood hematocrit increased in fillets from the 33-H treatment after socking and transport, with no other significant alterations observed among stressors within an

environmental group (Figure 4.2). Fish reared under severe environmental conditions (33-L) had higher hematocrit than fish reared under control conditions (25-H), regardless of additional handling stress. Interaction effects between temperature, DO and handling stress were not significant ($F = 3.05$, $p = 0.0676$) and evaluation of the main effects revealed that DO was the only variable that affected hematocrit ($F = 4.99$, $p = 0.036$), with hematocrit levels in fish reared under high DO conditions being approximately two units lower than those reared under low DO.

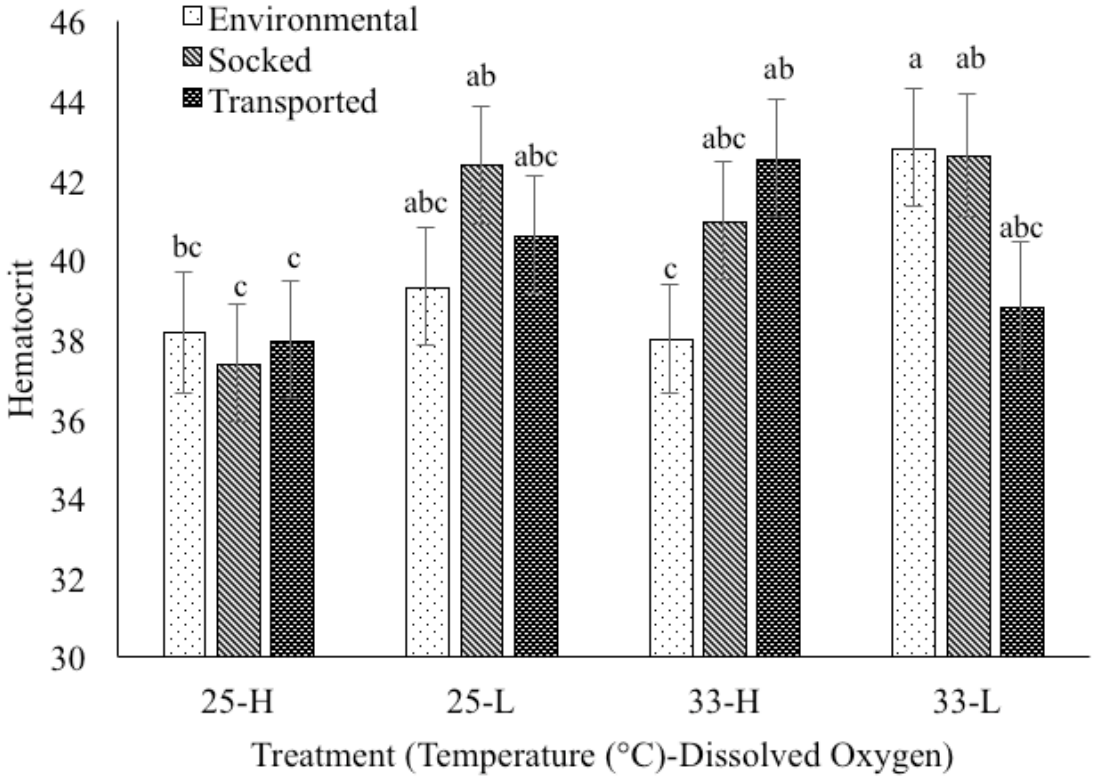

Figure 4.2 Blood hematocrit ± standard error in stressed channel catfish (*Ictalurus punctatus*).

The graph shows least square means for hematocrit at each stage of harvest following a one month stress trial at two temperatures (25 and 33°C) and high (H, >5 mg/L) and low (L, ~2.5 mg/L) dissolved oxygen concentrations. Different letters represent significant difference among treatments at $p < 0.05$ (ANOVA, n = ~30/trt).

Differences in hemoglobin content between the two temperatures examined were observed when fish were reared at low DO levels ($\chi^2 = 10.7874$, $p = 0.0129$) with fish reared under severe environmental stress (33-L) having a higher hemoglobin content than those reared under moderate oxygen stress (25-L, Table 4.1).

Table 4.1 Blood hemoglobin in environmentally stressed channel catfish (*Ictalurus punctatus*).

Treatment	Hemoglobin (g/dl)
25-H	17.8 ± 0.44^{ab}
25-L	16.7 ± 0.63^{b}
33-H	17.7 ± 0.58^{ab}
33-L	19.4 ± 0.59^{a}

Mean hemoglobin content in channel catfish (*Ictalurus punctatus*) blood samples following harvest from each of the environmental treatments (temperature-dissolved oxygen). Different letters represent significant differences between medians of normal and red fillets (n=22) at $p<0.05$ using Kruskall-Wallis test.

4.3.2 Fillet color

Minor variations in fillet luminescence (L*, black-white) or lightness were observed at 0 h after filleting. Fish from the 33-H-S treatment exhibited darker fillets compared to the 25-L-S, 25-L-ST and 33-L-ST treatments. Redness (a*, green-red) decreased in fillets from catfish reared under controlled environmental conditions after experiencing socking and transport stress (25-H-ST) when compared to those experiencing just socking stress (25-H-S) at 0 h. Yellowness (b*, blue-yellow) decreased at 0 h in fillets from the severely stressed fish (33-L-ST) when compared to controls (25-H) (Table 4.2). Hue angle tended to increase ($F = 3.42$, $p = 0.0517$) as fish progressed through the handling stressors, with the exception of fish reared under moderate temperature stress (33-H). Although these trends were observed, they were not statistically different, likely due to high variation in hue angles among fish. A lower hue angle was observed in control fillets (25-H) when compared to fillets from fish reared under low DO conditions and subjected to handling stress (25-L-ST and 33-L-ST) (Table 4.2). Minor variations in chroma intensity were recorded with fillets from the 25-H-ST treatment exhibiting greater intensity than fish from the 33-H treatment (Table 4.2).

Following 24 h of cold storage, fillets from fish reared under moderate temperature stress (33-H) were darker after experiencing handling stress (33-H-S and 33-H-ST) (Table 4.2). In addition, these fillets were darker than fillets from all fish experiencing socking and transport stress under all other environmental conditions, with the exception of the 25-H-S and 33-L-S treatments (Table 4.2). In several treatments (25-H-ST, 25-L-S, 33-H-S and 33-L), fillet lightness increased from 0 to 24 h. The main effects on lightness revealed that fillets from fish reared under low DO ($F= 5.17$, $p = 0.0331$) conditions were, on average, approximately one unit lighter than those from fish reared under high DO conditions (Figure 4.3).

Redness in fillets from fish reared at 25°C with high DO concentrations decreased after 24 h of cold storage when subjected to socking and transport stress (25-H-ST). After 24 h at 4°C, redness in fish from all environmental treatments except the 33-H treatment was lower following both socking and transport stress (ST) (Table 4.2). Redness in fillets from fish reared under moderate temperature stress (33-H) was higher than all those from fish reared at 25°C after experiencing the combined socking and transport stress (Table 4.2). On average, fillets from fish reared at 25°C were less red ($F = 6.61$, $p = 0.0174$; Figure 4.3) than fillets from fish reared at 33ºC. In addition, the combination of socking and transport stress led to the production of less red fillets ($F = 12.52$ $p = 0.0002$; Figure 4.3).

An increase in yellowness from 0 to 24 h of cold storage was observed in fillets from fish reared at 33°C and high DO (33-H) and those reared under these conditions and subjected to the combined socking and transport stress (33-H-ST) (Table 4.2). Yellowness decreased in fillets from all four environmental conditions after experiencing

the combined socking and transport stress (Table 4.2). Fillets from the 33-H-ST treatment were more yellow than fillets from fish subjected to the combined socking and transport stress and reared at 25°C (25-H-ST and 22-L-ST) (Table 4.2). On average, yellowness decreased in fillets from fish reared at 25°C ($F = 10.15$, $p = 0.0043$; Figure 4.4) and following each handling stress ($F = 27.29$, $p = 0.0001$; Figure 4.4).

Hue angle in fillets from fish reared at 25°C with high DO and 33°C with low DO were higher after fish experienced combined socking and transport stress (Table 4.2). A lower hue angle was observed in the 33-H-ST treatment compared to the 33-L-ST treatment (Table 4.2). On average, fish reared at 33°C ($F = 5.04$, $p = 0.0351$; Figure 4) and those reared with high DO ($F = 4.4$, $p = 0.0477$; Figure 4.5) yielded fillets with lower hue angles than fish reared at 25°C or low DO, respectively. Fillets from fish that experienced a handling stress ($F = 11.99$, $p = 0.0003$; Figure 4.5) had a higher hue angle than those that experienced no handling stress.

Chroma in fillets from fish reared under moderate environmental stress and the combined socking and transport stress (25-L-ST and 33-H-ST) decreased after 24 h of cold storage (Table 4.2). Chroma was lower in fillets from the control and severely stressed fish after the fish experienced the combined socking and transport stress (Table 4.2). On average, fillets from fish subjected to socking and/or transport stress had lower chroma values ($F = 11.61$, $p = 0.0004$; Figure 4.4) than those that did not experience any handling stress.

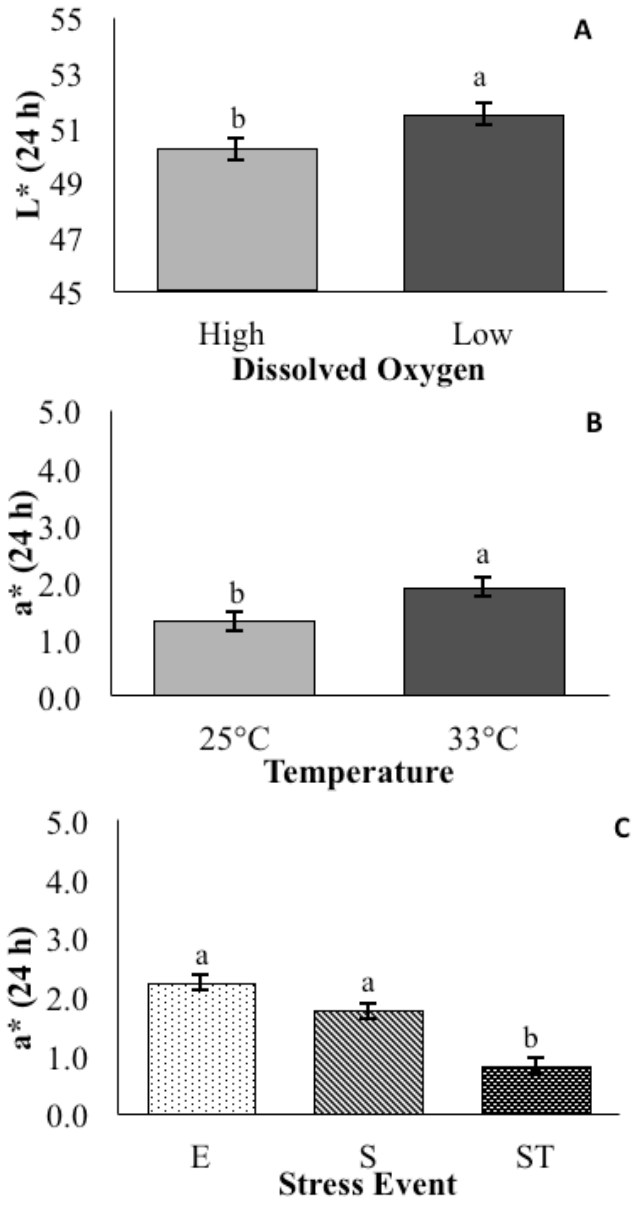

Figure 4.3 Main effects of stressors on lightness (L*) and redness (a*) in channel catfish (*Ictalurus punctatus*) fillets.

Data represent least square means for color values ± standard error after 24 h in cold storage following environmental (E), socking (S) and socking/transport (ST) stress. A) Main effect of high (>5 mg/L) and low (~2.5 mg/L) dissolved oxygen (DO) levels on L* values after 4 weeks. B) Main effects of high (33°C) and low (25°C) temperatures on a* values after 4 weeks. C) Main effects of sequential handling stressors on a* values averaged over temperature and DO levels. Different letters represent significant differences between treatments at $p < 0.05$ (ANOVA, n = ~24).

Table 4.2 Alterations in fillet color following sequential stress events in channel catfish (*Ictalurus punctatus*).

		Treatment											
Time	**Color Attribute**	**25-H**	**25-H-S**	**25-H-ST**	**25-L**	**25-L-S**	**25-L-ST**	**33-H**	**33-H-S**	**33-H-ST**	**33-L**	**33-L-S**	**33-L-ST**
0 h	**L***	47.7^{ab}	46.7^{ab}	47.6^{abB}	47.8^{ab}	49.0^{aB}	49.4^{a}	48.2^{ab}	45.8^{bB}	46.9^{ab}	48.2^{abB}	48.3^{ab}	49.3^{a}
	±SE	1.0	1.0	1.0	1.0	1.0	1.0	0.9	1.0	1.0	1.0	1.0	1.2
	a*	2.7^{ab}	3.1^{a}	1.2^{bA}	2.2^{ab}	2.7^{ab}	1.8^{ab}	1.6^{ab}	2.8^{ab}	2.5^{ab}	2.6^{ab}	2.5^{ab}	1.7^{ab}
	±SE	0.6	0.6	0.6	0.6	0.6	0.6	0.5	0.6	0.6	0.6	0.6	0.7
	b*	0.9^{a}	0.5^{ab}	-0.1^{ab}	0.8^{ab}	0.4^{ab}	-0.0^{ab}	0.4^{abB}	0.6^{ab}	0.2^{abB}	0.7^{ab}	0.2^{ab}	-0.3^{b}
	±SE	0.4	0.4	0.4	0.4	0.4	0.4	0.3	0.4	0.4	0.4	0.4	0.4
	Hue	47.5^{b}	106.0^{ab}	172.0^{ab}	93.5^{ab}	169.0^{ab}	176.7^{a}	154.6^{ab}	89.5^{ab}	155.5^{ab}	99.3^{ab}	169.3^{ab}	211.8^{a}
	±SE	43.0	43.0	43.0	43.0	43.0	43.0	39.3	43.0	43.0	43.0	43.0	49.6
	Chroma	2.9^{ab}	3.3^{a}	2.0^{ab}	2.5^{ab}	2.9^{abA}	2.3^{abA}	1.9^{b}	3.0^{ab}	2.7^{abA}	2.9^{ab}	2.7^{ab}	1.9^{ab}
	±SE	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.6
24 h	**L***	50.5^{ab}	50.1^{ab}	51.6^{aA}	50.7^{ab}	51.6^{aA}	51.6^{a}	52.0^{a}	48.4^{bA}	48.3^{b}	51.9^{aA}	51.1^{ab}	51.9^{a}
	±SE	1.0	1.0	1.0	1.0	1.0	1.0	0.9	1.0	1.0	1.0	1.0	1.1
	a*	2.4^{a}	1.7^{abc}	0.0^{dB}	1.8^{ab}	1.3^{abc}	0.5^{cd}	2.2^{a}	2.1^{ab}	1.7^{ab}	2.5^{a}	1.9^{ab}	0.9^{bcd}
	±SE	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.5
	b*	1.4^{abc}	0.6^{def}	-0.2^{fg}	0.9^{cd}	0.2^{defg}	-0.3^{g}	1.7^{abA}	0.9^{bcd}	0.6^{deA}	1.8^{a}	0.7^{cde}	-0.1^{efg}
	±SE	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.3	0.3	0.3	0.3	0.3
	Hue	56.6^{d}	96.4^{cd}	154.4^{abc}	120.3^{abcd}	113.8^{abcd}	183.2^{ab}	40.5^{d}	53.7^{d}	101.6^{bcd}	39.4^{d}	56.8^{d}	203.6^{a}
	±SE	29.3	29.3	29.3	29.3	29.3	29.3	26.7	29.3	29.3	29.3	29.3	33.8
	Chroma	2.9^{ab}	2.0^{abc}	1.5^{c}	2.1^{abc}	1.6^{cB}	1.3^{cB}	2.9^{ab}	2.4^{abc}	2.0^{bcB}	3.1^{a}	2.1^{abc}	1.3^{c}
	±SE	0.4	0.4	0.4	0.4	0.4	0.4	0.3	0.4	0.4	0.4	0.4	0.4

Color values represent least square means for lightness (L*), green-redness (a*) and blue-yellowness (b*) at time 0 and 24-h of cold storage (n = 8). Different lower case letters represent significant differences (ANOVA, n = ~24) among treatments and capital letters represent differences between 0 and 24 h at $p < 0.05$. Statistical analysis performed as General Linear Model 2x2 FAT RCB with subsampling. The treatments represent environmental temperatures (25 or 33 °C) and dissolved oxygen (High (H), > 5 mg/L or low (L), ~2.5 mg/L) levels followed by handling stressors: socked (S) and transported (T).

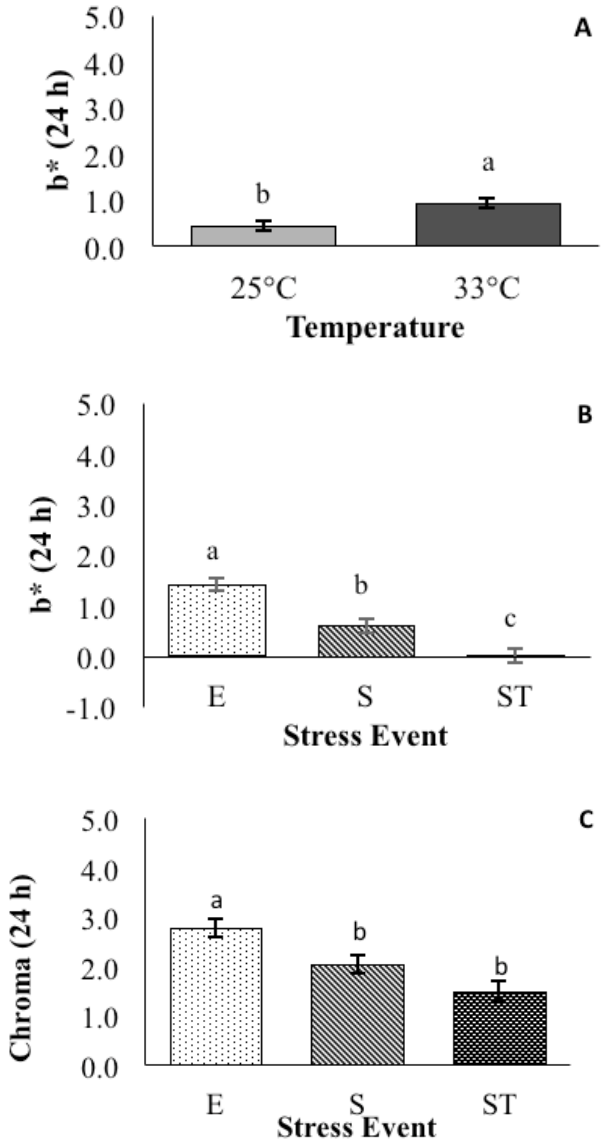

Figure 4.4 Main effects of temperature on yellowness (b*) and sequential handling on yellowness and chroma values in channel catfish (*Ictalurus punctatus*) fillets.

Data represents least square means for b* values ± standard error after 24 h of cold storage (4°C) following environmental (E), socking (S) and socking/transport (ST) stress. A) Main effects of rearing at high (33°C) and low (25°C) temperatures for 4 weeks. B) Main effects of sequential handling stressors averaged over temperature and dissolved oxygen. C) The main effects of handling stress on chroma values. Different letters represent significant differences between treatments at $p < 0.05$ (ANOVA, p = ~24).

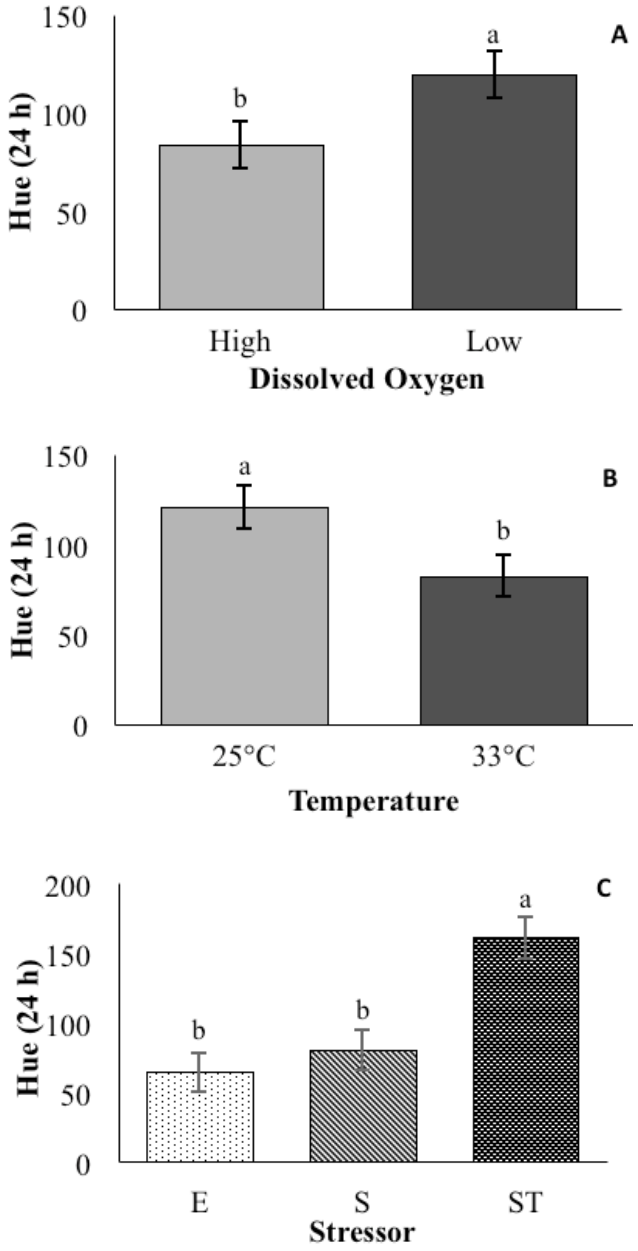

Figure 4.5 Main effects of stress treatments on hue angle in channel catfish (*Ictalurus punctatus*) fillets.

Data represents least square means for hue angle ± standard error in fillets after 24 h of cold storage (4°C). A) Main effects of high (>5 mg/L) and low (~2.5 mg/L) dissolved oxygen concentrations. B) Main effects of high (33°C) and low (25°C) temperatures. C) Maine effects of sequential handling stressors. Different letters represent significant difference between treatments at $p < 0.05$ (ANOVA, n = ~24).

4.3.3 Commercially raised catfish

Fillets that were deemed as inferior quality by the commercial processor due to red discoloration had slightly higher pH (Table 4.3). Lightness and hue angle were lower in fillets deemed as red fillets compared to those considered normal (Table 4.3). Redness (a*; Z = 5.3177, p = <0.0001), yellowness (b*; Z = 4.9648, p = <0.0001) and chroma (Z = 4.7534, p = <0.0001) values were higher in red fillets when compared to the normal fillets (Table 4.3).

Table 4.3 Color attributes for commercially collected channel catfish (*Ictalurus punctatus*) fillets.

	Normal	Red
pH	6.3 ± 0.0^{b}	6.4 ± 0.1^{a}
L*	57.9 ± 0.9^{a}	55.5 ± 0.6^{b}
a*	0.5 ± 0.1^{b}	4.4 ± 0.6^{a}
b*	-0.5 ± 0.2^{b}	1.8 ± 0.3^{a}
Hue	225.5 ± 23.9^{a}	68.2 ± 25.0^{b}
Chroma	1.1 ± 0.1^{b}	4.8 ± 0.6^{a}

Mean color value attributes for normal and red catfish fillets acquired from a commercial processing plant in Macon, MS. Different letters represent significant differences between medians of normal and red fillets (n=22) at $p<0.05$ using Kruskall-Wallis test.

4.4 Discussion

Sequential stressors elicited a stress response in channel catfish that varied depending on environmental conditions leading up to the stress events (Ch. 3). A physiological response was observed through evaluation of hematocrit values and was inversely related to DO concentration. Temperature and DO dependent changes in fillet color affected the ultimate quality of the fillets produced.

The different trends in hematocrit at 33°C when compared to the 25°C treatments suggest that temperature plays an important role in the stress response, with higher

temperatures eliciting changes in hematocrit following handling stress. The lack of change in hematocrit when channel catfish were reared at 25°C is consistent with previous studies that examined the effect of DO levels on physiology and growth in channel catfish**.** Andrews et al. (1973) noted that catfish maintained at 26.6°C showed no changes in hematocrit when reared for 6 weeks at oxygen levels of 36, 60 and 100% air saturation. The increased hematocrit in catfish held at 33°C and high DO levels reflects the expected trend of increased hematocrit to cope with the increased oxygen demand when temperatures are high (Davis, 2004; Radoslav et al., 2013), since hematocrit relates to the blood's capacity to carry oxygen (Allen et al., 2014; Brill and Jones, 1994). Hematocrit has been shown to increase in Yellow Perch, *Perca flavescens*, when exposed to low DO conditions (Cnaani et al., 2014). The inverse was observed in the severe stress (33 °C, 2.5 mg/L) treatment and could be attributed to a variety of factors: 1) increased permeability at the gills, caused by stress (Wendelaar Bonga, 1997) resulting in an influx of water diluting the blood constituents, 2) alterations in erythrocyte volume (Nikinmaa, 1983; Radoslav et al., 2013) and 3) stress induced hemolysis (Vickers et al., 2010). Although not quantified, an increase in red pigmentation of the plasma as the fish progressed through the socking and transport stressors was noted and was likely the result of increased hemoglobin released by hemolysis (Sowemimo-Coker, 2002). The unexpected decrease in hematocrit during severe stress, where hematocrit levels were expected to be highest, may be due to the severity of the stress. The 33°C temperature is approaching the thermal limit for channel catfish (Bennett et al., 1998; Stewart and Allen, 2014) and represents a chronic thermal stress. Previous reports of increased hematocrit following thermal stress were due to acute thermal regimes (Davis, 2004; Radoslav et al.,

2013), and the combined effects of high temperature and low DO on hematocrit are not well documented. The different trends could also reflect species specific hematological changes (Radoslav et al., 2013). Water content, metabolic capacity, erythrocyte volume and cellular lysis were not quantified in the present study and thus cannot be solely attributed to the observed trends in hematocrit. However, it is likely that a combination of these factors contributed to the observed trends, warranting a more comprehensive evaluation of the physiological mechanisms involved in temperature and DO dependent changes in catfish hematocrit. This would help explain the observed decrease in hematocrit values when reared under severe stress conditions and handling stress.

Fillets harvested from fish that were subjected to moderate temperature stress (33-H) were the only fillets that became darker when handling stress was applied. A darkening of the fillets was observed previously in catfish reared under high DO concentrations (Bosworth et al., 2004). In contrast, rainbow trout subjected to ante-mortem stress simulations exhibited a lightening in fillet color (Robb et al., 2000). Cod fillets from fish experiencing low DO stress were lighter when compared to unstressed fish (Stien et al., 2005). This suggests that differences observed between cold and warm water species may not be comparable. The differences are likely due to differences in the biological mechanisms and physiology of cold versus warm water species since temperature plays an important regulatory role in the biological processes of ectotherms (Johnston and Dunn, 1987).

Arctic char, a cold water species, had more intense orange flesh color when reared at lower temperatures (10°C vs 15°C) with higher redness, yellowness and chroma intensity (Ginés et al., 2004). This is inverse to what was observed in channel catfish in

the present study where lower temperatures resulted in decreased redness and yellowness. Robb et al. (2000) reported that high ante-mortem muscle activity in rainbow trout led to a less red and more intense color in the fillets. These differences further highlight the need for understanding the effects that stress and physiology have on fillet color on an individual species basis.

Hue angle increased with sequential stress events moving away from red/orange coloration to a more green-yellow color. This was combined with a decrease in chroma values similar to what was observed in cod fillets when exposed to an aerial stress (Stien et al., 2005). Although the hue angle increased as the fish experienced socking and transport, the fillets from fish reared under moderate temperature stress (33-H) showed a less pronounced increase when compared to the other treatments. This resulted in a range of hue angles that extended further into the red range, suggesting an increased prevalence of red fillets. In addition, though the intensity of the color decreased with sequential stressors, they did not decrease as much in the 33-H treatment, suggesting a higher prevalence of more intensely colored fillets when fish are reared under these conditions.

Overall, these data suggest that although fillets with a red coloration comparable to those found in industry were not produced, catfish reared in ponds at high temperature (33°C) with ideal DO levels (>5 mg/L) will likely exhibit a higher prevalence of red fillets at harvest. The increased prevalence is supported by the higher a* values and low range of hue angles observed in the 33-H treatment. This is unexpected since red fillets have been associated with increased hemoglobin content and thus its prevalence was expected to be higher in the severely stressed fish (33-L) where the highest hemoglobin concentrations were observed. The red color in the fillets is likely due to increased

hemoglobin concentrations (Desai et al., 2014), although they were not quantified for fish that experienced handling stress in the present study. In cod fillets, higher concentrations of heme pigments were reported in the muscle following ante-mortem crowding stress (Olsen et al., 2008). The increase was attributed to increased pre-slaughter activity and the redirection of blood flow to white muscles in response to an increased energy demand (Soldatov, 2006). The higher hemoglobin levels observed when fish were reared under severe environmental conditions did not correlate to an increased redness in fillets. The higher redness observed in catfish in the 33-H treatment suggests that there are other physiological or cellular mechanisms involved in hemoglobin deposition in the muscle. In addition, there could be more severe acute stressors during socking (anoxic pockets) that exaggerate the formation of red fillet in commercial aquaculture. These conditions are difficult to imitate outside of commercial harvest practices.

The increased hemoglobin production could be in response to the stress events (Houston and DeWilde, 1972). Further evaluation of the role of hematocrit in the formation of red fillet is warranted since even though the hematocrit values in the 33-H-ST treatment were higher than the similarly stressed control fish (25-H-ST), they did not differ from the hematocrit levels observed in fillets from all other stress treatments. Thus, further evaluation of the relationship between hematocrit and hemoglobin regulation during stress could help better understand their role in the formation of red fillets in channel catfish. Future research on hematological changes in hemoglobin content and correlations to accumulation in the muscle would be beneficial in evaluating the role of sequential stressors and their effects on fillet color. In addition, closer evaluation of the

effects that fish size and sock density have on blood physiology and fillet color are warranted.

4.5 Conclusions

As catfish experienced the sequential harvest stressors, hue angle increased, moving from more red to a yellowish color, and fillet color intensity decreased, which is preferred in catfish fillets. Thus, handling stress can have beneficial effects on catfish fillet color. However, environmental conditions leading up to a stress event can alter the beneficial effects of handling. While hue angle and redness of the fillets from fish reared under moderate temperature stress (33-H) still decreased, it was to a lesser extent than in all other environmental conditions. This resulted in a range of color values extending further into those associated with a red color, indicating that a higher prevalence of red fillet may be observed when harvesting under these conditions. Future research should evaluate the correlations between hematocrit and hemoglobin in the blood and plasma to better understand its relationship and involvement in the formation of red fillets. Additional studies on the biochemical and cellular mechanisms involved in hemoglobin deposition in the muscle would also help to better understand and control for red fillet defect in channel catfish.

CHAPTER V

THE EFFECTS OF SEQUENTIAL ENVIRONMENTAL AND HARVEST STRESSORS ON THE SENSORY CHARACTERISTICS OF CULTURED CHANNEL CATFISH (*ICTALURUS PUNCTATUS*) FILLETS

5.1 Introduction

As wild fisheries are currently fully exploited, the increasing needs for food fish must be filled with cultured products, resulting in aquaculture becoming the fastest growing food sector globally (FAO, 2014). The increased demand has led to more intensive culture of food fish (FAO, 2014). More intense culture practices can result in greater stress by intensifying the stressors inherent in aquaculture. Stressors commonly affecting cultured fish include crowding, changes in environmental temperature and dissolved oxygen (DO) levels, light intensity, water quality, handling and transport (Ashley, 2007).

Pre-harvest stress events affect post-harvest and processing quality attributes of fish fillets. Ante-mortem handling stress resulted in a decrease in the ultimate fillet pH of Atlantic salmon (Erikson et al., 1997; Hansen et al., 2012). Pre-harvest activity depletes energy reserves and limits aerobic respiration post processing. This switch to anaerobic respiration leads to lactic acid build up and a rapid decrease in muscle pH (Huss and Gill, 1995). The rapid decrease in pH results in a quicker onset of rigor and can ultimately

affect protein structure and function, which alters the textural characteristics of the fillet (Sikorski and Pan, 1994).

Studies on chilling and short term crowding stress in salmon have shown that pre-slaughter stress can decrease the firmness of fillets (Hansen et al., 2012; Lerfall et al., 2015). Hansen et al. (2012) also evaluated the effects of long-term stress and found the opposite to be true with a firmer fillet produced. Similar to what was observed following short-term crowding stress, ante-mortem handling resulted in a softening of Arctic char (Jittinandana et al., 2003) and Atlantic salmon (*Salmo salar*) (Erikson et al., 1997) fillets. Temperature affected texture in raw Arctic char fillets, which were softer when fish were reared at 10°C compared to those reared at 15°C (Ginés et al., 2004)**.** While tenderization in post-mortem muscle of mammalian meats enhances the ultimate quality of the meat, such changes in fish are undesirable (Hultmann et al., 2012; Tsuchiya et al., 1992). Reduction in pre-harvest and slaughter stress can result in enhanced textural quality of fish meats (Erikson et al., 1997)

In addition to chemical and textural changes, stress can alter fillet sensory attributes, especially flavor, which is important for product quality (Johnson and Kelly, 1990) and ensuring marketability of the product. Deterioration of sensory attributes of salmon fillets were observed following long-term crowding stress (Hansen et al., 2012). Cultured catfish (*Ictalurus punctatus*) can frequently exhibit off flavors that make them poorly marketable (Dionigi et al., 1998). In the United States, catfish represents the primary aquaculture sector with over 73 million kilograms sold in 2013 (Harvey, 2014). Production of high quality products from pond raised catfish is integral to maintaining the support of consumers (Bosworth et al., 2004; Cline, 2011; Johnson et al., 1987).

This study evaluated the effects of sequential environmental and handling stressors, commonly experienced in aquaculture, on fillet quality and sensory characteristics. Results will offer insight into the potential effects that specific rearing conditions and harvest practices can have on the final product. A better understanding of these effects will allow for alterations in management strategies to enhance fillet quality and ensure consistency.

5.2 Materials and Methods

5.2.1 Fish acquisition and grow-out

Fingerling channel catfish were acquired from the Mississippi State University Thad Cochran National Warmwater Aquaculture Center in Stoneville, MS. Fingerlings were grown to a minimum of 300 g, to achieve food fish size by the end of the trial (Harvey, 2014). Fish were cultured in 400 – 4,000 L flow-through systems with dechlorinated well water at ambient temperature (22-27°C) for a maximum of two years. During grow out (6-12 months), fish were fed once daily to satiation using a commercial, 36% protein pelleted feed (Land O'Lakes Purina Feed LLC, Arden Hills, MN). Prior to the stress trials, fish were gradually switched to a 32% protein finishing feed (Purina Animal Nutrition LLC, Gray Summit, MO), which is used during commercial grow-out (Robinson et al., 1998).

5.2.2 Stress trials

At the start of each trial (n=3), four 4,000 L recirculating aquaculture systems (RAS) with slow exchange of well water were stocked with 80 catfish each (704 ± 484 g, mean ± standard error). Each system was fitted with a biofilter with plastic floating

biobeads (Model DF3 or 6; Aquaculture Systems Technologies, New Orleans, LA), two high output ultra violet sterilizers (120 V SMART HO, Emperor Aquatics; Pottstown, PA), a pump (SHE2.4 or SHE1.7, Aquatic Ecosystems, Inc., Apopka, FL) and a sump tank. Throughout the trial, fish were fed once daily to satiation a 32% protein finishing feed (Purina Animal Nutrition LLC, Gray Summit, MO). Fish were acclimated over the course of one week to four different environmental conditions with temperature and DO levels manipulated at a rate of 1°C/day or 10% saturation/day until experimental levels were reached. A control temperature of 25°C was maintained with immersion chillers with single stage temperature controllers (AquaLogic Inc., San Diego, CA). The high temperature treatments (33°C) were achieved with direct immersion heaters, one 4,000 watt, 200-250 V and two 1,700 watt, 100-140 V in each system, controlled with single stage temperature controllers (Smart one; Process Technology, Mentor, OH). The high DO (H) treatments were maintained by constant forced air aeration throughout the trial. Low DO (L) levels were achieved through natural respiration for depletion of DO and maintained by continuous monitoring with a galvanic oxygen probe (MINI-DO, Loligo® Systems, Denmark or CellOx 325, WTW GmbH, Weilheim, Germany) attached to a Loligo® Systems oxygen analyzer and regulator system (Version 2.4; Loligo® Systems, Denmark) or a DO pocket meter (Oxi 330i, WTW GmbH, Weilheim, Germany) with a custom designed solenoid valve system (Petersen and Gamperl, 2011). The system was set to open a solenoid and bubble in oxygen when the DO levels dropped below 2 mg/L and turn off as soon as 2 mg/L was reached.

After four weeks at the four different environmental conditions (25-H, 25-L, 33-H and 33-L), 1/3 of the fish (n~26/tank) were harvested and the remaining fish (n=

~53/tank) were transferred to a 1.82 m^3 (0.60 m L x 0.60 m W x 0.46 m H) sock constructed of PVC and a rigid plastic 1.25 cm mesh netting to prevent escape. Sock size was determined during the first trial using the average weight of fish stocked into the tanks to achieve an approximate sock density of 175 kg/m^3 (Avery, 2013). Fish were held in the sock within the experimental tanks for 15 h, which represents the maximum time fish are held in socks during commercial culture (Avery, 2013). At the end of socking, half of the fish (n ~27/tank) were anesthetized (5-10 min) in a CO_2 bath with 3 g/L NaCl and harvested, representing the fish subjected to socking (S) stress (25-H-S, 25-L-S, 33-H-S and 33-L-S). The rest of the fish (n~26) were subjected to transport (T) stress by stocking in well oxygenated (>5 mg/L) tanks at a density of 0.72 Kg/L and held for 3 h, representing the maximum time of transport for commercial catfish aquaculture (Bosworth et al., 2004), before harvesting as the last four treatments (25-H-ST, 25-L-ST, 33-H-ST and 33-L-ST).

At harvest, fish were anesthetized in CO_2 as described above, weighed to the nearest g and measured to the nearest mm. After weight and length were measured, fish were killed by a sharp blow to the head and transported on ice to the Ammerman Hearnsberger Processing Plant in the Department of Food Science, Nutrition and Health Promotion at Mississippi State University. Within 3 h of arrival, the fish were manually filleted and fillet yield per fish were recorded to the nearest g. Fillet pH was measured using a pH meter (Model Accumet 61a, Fisher Scientific, Hampton, NH) with a meat penetrating pH electrode attachment (Model Flexihet SS Penetration tip, Cole Palmer, Vernon Hills, IL) inserted at the anterior end of one fillet per fish. The pH was measured for eight fish per treatment for each of the three trials (n=24) immediately after filleting

(0 h). The same eight fillets per treatment were set aside for pH measurement following storage at 4°C for 24 h. Another eight fillets per treatment were sectioned into 25-50 g pieces, individually bagged and frozen (-20°C) for determination of cook loss and tenderness. All remaining fillets were stored overnight (4°C) before freezing and storing (-20°C) for sensory analyses. Fillets were held in storage for a maximum of 9 months before sensory analyses were conducted. The procedures used herein were reviewed and approved by the Mississippi State University institutional animal care and use committee, and conducted in accordance to the approved protocols (no. 13-041).

5.2.3 Cook loss and tenderness evaluation

A 25 g square section was cut from the center portion of the fillets that were frozen for tenderness evaluation. The thawed 25 g sections were baked in individual foil pouches at 177°C for 25 min, using a cooking method that was previously determined to fully cook fillets in pilot studies. Fillets were allowed to cool to room temperature (~45 min), patted dry with a paper towel and re-weighed to determine cook loss. Cooked fillets were cut into 2.5 cm x 2.5 cm squares for instrumental texture analysis. An Instron Universal Testing Center (Model 3300, Instron, Norwood, MA) with an Allo Kramer shear compression cell (CS-2) attachment was used to determine shear force and total energy according to Kin et al. (2010).

5.2.4 Consumer evaluation of fried catfish fillets

Catfish fillets from each of the four environmental treatments were thawed under refrigeration for 24 h and then cut into 10 g pieces from the center of the fillets. Pre-cut pieces were marinated in equal parts (w:w) of a salt (1%) and phosphate (0.5%) solution

overnight. The fillets were coated in a commercial catfish cornmeal breading (Young's fry products LLC, Little Rock, AR) then fried in Crisco pure vegetable oil (J.M. Smucker Company, Orrville, OH) at 160°C for 3-5 min depending on fillet thickness. Fillets from each treatment were served to panelists (n= 180) and rated for acceptability using a 9-point hedonic scale, with 1 = dislike extremely, 2 = dislike very much, 3 = dislike moderately, 4 = dislike slightly, 5 = neither like nor dislike, 6 = like slightly, 7 = like moderately, 8 = like very much and 9 = like extremely (Peryam and Girardot, 1952). The panelist score sheet was designed and acceptance scores were recorded using CompuSense version 5.2 (Compusense Inc., Guelph, Ontario).

5.2.5 Descriptive evaluation of baked catfish fillets

Descriptive panels consisted of a minimum of 7 trained panelists and each treatment was tasted in duplicate for each of the three stress trials. A 10 g piece from the center portion of fillets was marinated overnight in 1% salt and 0.5% phosphate solution and then placed into a foil pouch, baked (177°C, 25 min) and served to panelists. Panelists rated intensity on a 15 cm line scale (Meilgard et al., 2007b) for appearance (color and redness), aroma (fishy, sulfury and grassy), flavor (sweet, salty, bitter, umami, earthy, grassy, metallic, fishy, buttery and off-flavor) and texture/mouthfeel (Flaky and Astringent). The panelist score sheet was designed and attribute ratings were recorded with CompuSense version 5.2 (Compusense Inc., Guelph, Ontario).

5.2.6 Statistical analysis

Data were analyzed as a split plot randomized complete block with 2x2 factorial arrangement of treatments and subsampling. Temperature and DO levels represent the

factorial variables and fish represented subsamples of the three replicate trials. All data were analyzed as least square means to account for missing data points and reported as means plus or minus standard error, herein. The data were analyzed using two-way analysis of variance (ANOVA) with SAS statistical software version 9.3 (SAS Institute Inc., Cary, NC). Mean separation was carried out with Fisher's protected least significant difference test and significance is reported at $p < 0.05$.

5.3 Results and Discussion

Oxygen levels significantly altered fillet yield ($p = 0.0003$) with fish reared under low DO (~2.5 mg/L) yielding approximately 50 g less fillet than fish from high DO (>5 mg/L) treatments (Figure 5.1). This correlates well to the decreased growth observed in channel catfish (Ch. 2). Stressful environmental temperatures and DO concentrations are well documented to hinder growth in fish (Arnold et al., 2013; Buentello et al., 2000; Handeland et al., 2008; Lakani et al., 2012; Torrans, 2005; Torrans et al., 2012; Tran-Duy et al., 2008; Tucker and Robinson, 1990). Decreased fish growth will ultimately translate into decreased fillet yields and income.

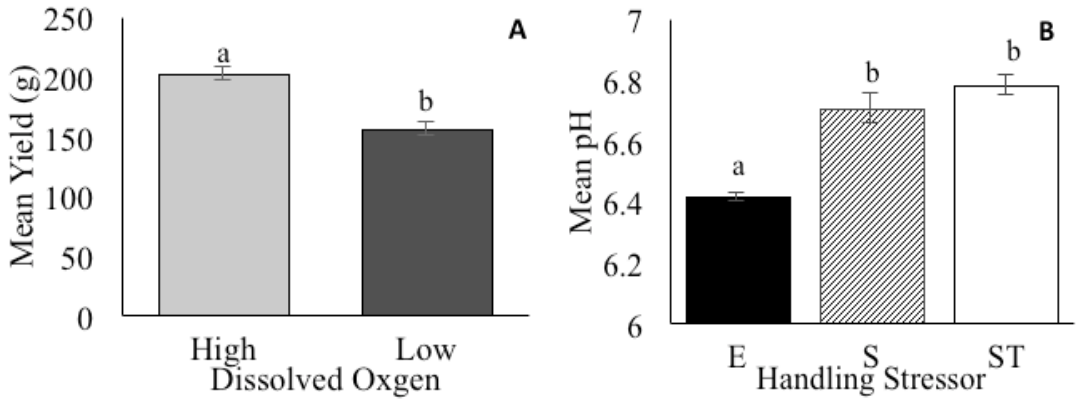

Figure 5.1 Main effect of dissolved oxygen on fillet yield and handling on fillet pH in channel catfish (*Ictalurus punctatus*).

A) Yields represent the average weight (± SE) of both fillets from an individual fish averaged over the three experimental trials, temperature and handling stress for the high (H, >5 mg/L) and low (L, ~2.5 mg/L) dissolved oxygen levels. B) Fillet pH (± SE) after 24 h of cold storage averaged over environmental stressors. Different letters represent significant differences among treatments (ANOVA, LSD, $p < 0.05$).

Temperature x handling stress ($p = 0.0083$) affected fillet pH in channel catfish at 0 h post processing, with higher pH observed in fish experiencing handling stress (socking and transport) (Data not shown). These interaction effects were no longer observed after 24 h of cold storage (2-4°C). Environmental rearing conditions did not affect fillet pH (Table 5.1). Following 24 h of cold storage, handling ($p = 0.001$) was the only variable affecting fillet pH, with fillet pH increasing, on average, as fish advanced from rearing, through the socking and transport stress (Figure 5.1). The higher pH is most likely explained by the increased energy expenditure required to regain a homeostatic state in response to the environmental stress and the subsequent depletion of energy reserves (Thomas et al., 1999). Decreased glycogen reserves limited anaerobic respiration post mortem, thus limiting lactic acid production and pH decline resulting in the higher pH observed (Strasburg et al., 2008). The pH values in the control fish (25-H treatment)

were similar to those observed in channel catfish by Kin et al. (2010). Fillet pH plays an important role in the ultimate textural (Hansen et al., 2012; Kristoffersen et al., 2006; Sigholt et al., 1997) quality of fish and stress prior to slaughter can result in significant alterations in fillet pH. Fillet pH is reported to decrease following chasing (Erikson et al., 1997; Hultmann et al., 2012), aerial exposure (Stien et al., 2005), confinement (Hultmann et al., 2012) and crowding stress (Bagni et al., 2007). Rapid pH decline at high storage temperatures post-processing, typically results from slow decline in internal temperature during storage. This decline results in protein denaturation and ultimately decreased water binding, which negatively impacts raw meat quality in terrestrial vertebrates (Huff-Lonergan and Lonergan, 2005). Although, sequential measurements of pH changes were not measured in the present study, pre harvest stress results in a more rapid pH decline in fish (Bagni et al., 2007; Robb et al., 2000; Stien et al., 2005; Thomas et al., 1999). The decrease in cook loss (Figure 5.2) could be due to this change in water content. Kin et al. (2010) reported that an increase in pH was accompanied by higher yields and decreased cook loss. A higher water content in the moderate temperature stress treatment (33-H) would also explain the lower L* values in this treatment (Ch. 4). Higher water content causes greater separation of myofibrils and reduced light scatter, which results in the darkening of the meat (Allen et al., 1998; Kin et al., 2010; Strasburg et al., 2008; Qiao et al., 2001).

Table 5.1 Fillet pH following sequential environmental and handling stressors in channel catfish (*Ictalurus punctatus*).

Treatment	0 h pH	0 h SE	24 h pH	24 h SE
25-H	6.69^{abc}	0.09	6.39^{a}	0.12
25-H-S	6.85^{abde}	0.09	6.49^{abc}	0.12
25-H-ST	6.94^{ade}	0.09	6.66^{abcd}	0.12
25-L	6.81^{abce}	0.09	6.45^{abc}	0.12
25-L-S	6.90^{ade}	0.09	6.78^{bcd}	0.12
25-L-ST	7.01^{def}	0.09	6.76^{bcd}	0.12
33-H	6.65^{bc}	0.08	6.44^{ac}	0.11
33-H-S	7.25^{f}	0.09	6.86^{d}	0.12
33-H-ST	7.00^{def}	0.09	6.87^{d}	0.12
33-L	6.57^{c}	0.09	6.39^{a}	0.12
33-L-S	7.07^{df}	0.09	6.70^{abcd}	0.12
33-L-ST	7.10^{df}	0.10	6.83^{bd}	0.14

Mean pH at 0 h post processing and following 24 h of cold storage. Treatments represent temperature (25°C or 33°C) – high (>5mg/L) or low (~2.5 mg/L) dissolved oxygen – and socking (S) and transport (T) handling stress. Statistical differences between treatments are indicated by different letters (ANOVA, $p < 0.05$, LSD, n = ~24).

5.3.1 Cook loss and tenderness

No differences ($p > 0.05$) in cook loss were observed among treatments. However, in all treatments where an environmental stressor was imposed (25-L, 33-H and 33-L) there was an average decrease of nearly 4% when fish were subjected to both socking and transport stress (Figure 5.2). This could be explained by the lower 24 h pH (Figure 5.1) observed in fish that were not subjected to handling stress, which would likely result in greater protein denaturation and water loss (Strasburg, et al., 2008) as was seen in Atlantic cod (Kristoffersen et al., 2006). The high cook loss following socking and transport stress, in fish reared under control environmental conditions (25-H) is inverse to the trends observed in fish from the environmentally stressed treatments (25-L,

33-H and 33-L) (Figure 5.2). The high cook loss following stress in the control is likely a result of the lower muscle pH. This alters protein water interactions and results in greater water loss upon cooking, which was observed in Atlantic cod (Hultmann et al., 2012) following combined confinement and chasing stress. The different trends observed in cook loss (control versus environmentally stressed) could reflect environmental effects on drip loss in the fillets prior to cooking, which was not evaluated in the present study. In Atlantic salmon stressed by crowding and harvest methods, drip loss was significantly greater than rested harvest fish (Roth et al., 2006). Thus, future evaluations of the effects of environmental stress on fillet drip loss are warranted. The interaction between rearing temperature, DO and handling stress affected total energy required to shear the fillets ($p = 0.0489$). On average, total energy was lower by about 0.02 J/g when fish were reared at high temperatures ($p = 0.0124$) and was 0.03-0.04 J/g lower when fish were subjected to a socking and/or transport stress ($p = 0.008$, Figure 5.3). Shear force was lower when any handling stress ($p = 0.0013$) was employed (Figure 5.3) and was about 5 units lower in fillets from fish reared at 33°C ($p = 0.0064$).

Tenderness increased as fish progressed through the socking and transport stressors when pre-exposed to any type of environmental stress (0). An increase in tenderness following stress has been previously documented in Atlantic cod (Stien et al., 2005) and salmon (Lerfall et al., 2015). These changes have been associated with increased protease activity since protein denaturation allows better access for proteolytic enzymes to muscle proteins, which contributes to muscle softening (Hultmann et al., 2012). Changes in tenderness following 24 h of cold storage have been associated with the detachment of individual myofibers from myofiber bundles, which resulted in

increased tenderness of salmon fillets (Taylor et al., 2002). Although decreasing tenderness post processing is typically seen as a reduction in fish fillet quality (Hultmann et al. , 2012; Tsuchiya et al., 1992), the lowest shear force and total energy values measured in the present study were similar to those of untreated control fish reported by Kin et al. (2010). The control fish from Kin et al. (2010) study represented normal high quality fillets. The addition of phosphates to those fillets increased tenderness to levels lower than those observed here. Since those fillets were considered higher quality, it can be assumed that the handling stressors implemented in the present study are enhancing the textural properties of catfish fillets. Slightly higher shear force values were observed in the present study when compared to the control fillets (17.2-17.7 N/g) in Kin et al. (2010) and total energy (0.24 J/g) measurements were similar (Kin et al., 2009, 2010). If more severe stress is imposed pre-harvest, more severe decreases in tenderness could occur and negatively impact the quality (Hultmann et al., 2012; Tsuchiya et al., 1992), especially if additional post-harvest treatments are employed (Kin et al., 2010).

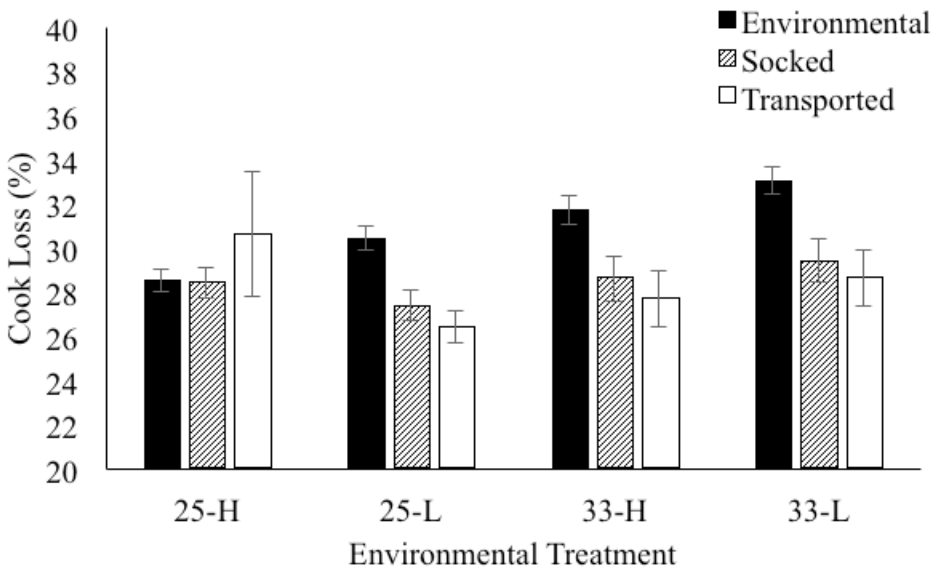

Figure 5.2 Mean cook loss in channel catfish (*Ictalurus punctatus*) fillets following sequential environmental and handling stressors.

Data represents the average cook loss (±SE) of a 25g piece of fillet after baking 25 min at 177°C**.** Fish from the environmental group were processed immediately following rearing at the two environmental temperatures (25°C and 33°C) and the high (H, >5mg/L) and low (L, ~2.5mg/L) dissolved oxygen levels. Socked fish were held in a 1.8m^3 sock for 15 h and transported fish were held at a density of 0.7 kg/L for 3 h after socking.

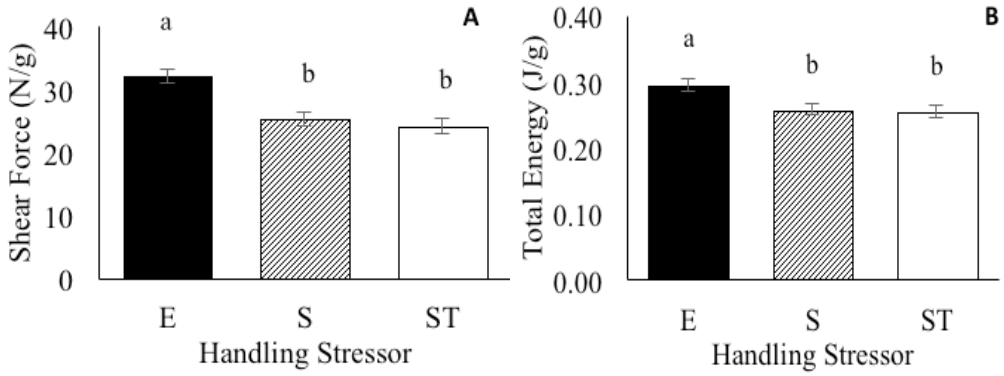

Figure 5.3 Main effect of handling stress on channel catfish (*Ictalurus punctatus*) fillet texture characteristics.

The data represents the average A) force and B) total energy required (± SE) to shear fillets for each of the handling stress events averaged over environmental conditions. Fish subjected to environmental manipulation only (E) served as the control. Socking (S) and socking in addition to transport stress (ST) were imposed on fish prior to harvest and processing when shear force was evaluated. Different letters represent significant differences among the stress events (ANOVA, LSD, $p<0.05$).

Table 5.2 Fillet shear force and total energy of cooked channel catfish (*Ictaulurus punctatus*) fillets.

Treatment	Shear Force (N/g)	SE	Total Energy (J/g)	SE
25-H	31.1[ab]	2.1	0.26[ab]	0.02
25-H-S	30.8[ab]	1.8	0.29[ac]	0.02
25-H-ST	32.6[ad]	2.5	0.32[c]	0.02
25-L	32.8[ad]	1.9	0.32[c]	0.02
25-L-S	27.8[abc]	1.9	0.27[abc]	0.01
25-L-ST	23.4[bc]	1.7	0.24[ab]	0.01
33-H	30.9[ab]	2.0	0.31[c]	0.02
33-H-S	20.1[c]	1.6	0.23[ab]	0.02
33-H-ST	18.8[c]	1.2	0.22[b]	0.01
33-L	34.1[a]	2.6	0.28[ac]	0.02
33-L-S	20.6[c]	2.0	0.23[b]	0.02
33-L-ST	20.6[bcd]	2.2	0.23[ab]	0.02

The mean shear force and total energy values for catfish fillets subject to sequential environmental and handling stress events. Treatments represent temperature (25°C or 33°C) – high (>5mg/L) or low (~2.5 mg/L) dissolved oxygen – and socking (S) and transport (T) handling stress. Statistical differences between treatments are indicated by different letters (ANOVA, $p < 0.05$, LSD, n = ~24).

5.3.2 Consumer evaluation of fried catfish fillets

Acceptance scores increased as fish were subjected to environmental stress, with fish reared under severe stress conditions rated higher than controls with regard to flavor ($F = 6.62$, $p = 0.0002$) and overall acceptability ($F = 6.22$, $p = 0.0004$) (Table 5.3). No differences ($P>0.05$) in the acceptability of appearance, aroma and texture were observed among treatments. This suggests that flavor was the driving factor in the consumer acceptance of catfish fillets in this study. Flavor and freshness were the two major factors identified that influenced fish choice for southern flounder fillets (Drake et al., 2006). The increased preference for stressed fish is likely due to decreased feed consumption (Ch 2). The decreased consumption could have resulted in greater lipid metabolism

essentially purging the stressed fish. Flavors accumulate in fats and thus are removed from the muscle with fat during purging (Howgate, 2004). Stress induced increases in proteolytic activity and an altered metabolic state (Morzel et al., 2004; Silva et al., 2012; Veiseth-Kent et al., 2010) could also result in alterations in peptides and ribonucleotide metabolite concentrations, which contribute to meat and seafood flavor (Jones, 1969; Kayim et al., 2011).

Table 5.3 Consumer acceptability scores for fried channel catfish (*Ictalurus punctatus*) fillets.

	Treatment			
Attribute	**25-H**	**25-L**	**33-H**	**33-L**
Appearance	7.2	7.2	7.2	7.3
Aroma	6.9	7.0	7.1	7.1
Flavor	6.4^{a}	6.6^{b}	6.8^{bc}	6.8^{c}
Texture	7.0	7.1	6.8	6.9
Overall	6.7^{a}	6.9^{b}	6.9^{b}	7.0^{c}
S.E.	0.1	0.1	0.1	0.1

Data represents mean values for consumer acceptability scores form a 9-point hedonic scale (1 = Dislike Extremely, 5 = Neither like nor dislike and 9 = Like Extremely). The panelists were general consumers of catfish over the age of 18 (n=180). Statistical differences between treatments are indicated by different letters (ANOVA, LSD, $p < 0.05$, n = ~24).

Consumer panels were run on the environmentally stressed fish only, in order to limit the numbers of samples consumers were expected to taste and since off flavors are often associated with rearing conditions (Grimm et al., 2004). Off flavor accumulation is commonly observed in cultured fish (Davidson et al., 2014; Dionigi et al., 1998) and is typically associated with intense algal blooms and the microbial load of culture systems (Smith et al., 2008). The most offensive off-flavors that affect cultured catfish include those caused by the secondary metabolites methylisoborneol (MIB) and geosmin (Dionigi

et al., 1998). The cyanobacteria associated with these metabolites are also present in biosolids and microbial biofilms in recirculating aquaculture systems (Davidson et al., 2014). Prevention of off-flavor is integral to ensuring product quality and poses a serious threat to the US catfish industry, with an estimated loss of 30% revenue from off flavor contamination (Smith et al., 2008). On average, all treatments were rated between 6.4 (Like slightly) and 7.0 (Like moderately) for flavor and overall acceptability on a 9-point hedonic scale. The statistically significant alterations in acceptability are likely due to the large sample size as fish from all treatments were liked by consumers. The small numerical differences in acceptability were likely due to increased ketogenic metabolism and fat loss in stressed fish due to decreased feed consumption.

5.3.3 Descriptive evaluation of baked catfish fillets

Descriptive analysis of catfish fillets from fish subjected to different environmental and handling stress events revealed small but statistically significant changes in sensory attributes. There were slight changes in appearance with regard to color and redness. On average, fillets were lighter (1.7 intensity score) following socking and transport stressors when compared to fish that did not experience any handling stress (2.1). This correlates well with the decreases in color intensity observed through fillet color analysis (Ch 4). More intense sulfury and grassy aromas were observed in the moderate temperature stress treatment (33-H) after handling stressors were employed (Table 5.4). On average, fillets from fish reared at high temperatures received higher intensity scores for sweetness, umami and buttery. The most intense favorable flavor attributes detected were umami and salty, with intensity scores above 2.0 (Table 5.4). Saltiness was higher in the 33-L-S treatment compared to the 25-L-S treatment with no

other differences observed and ratings of approximately 2.0. Fish reared at 33°C received intensity scores close to 3, while fish reared at 25°C were rated approximately 2.5 for umami flavor (Table 5.4). This suggests that fish reared at higher temperatures will exhibit slightly better flavor characteristics with higher intensity scores for favorable flavor attributes (Johnsen et al., 1987; Phan and Nguyen, 2012; Van der Ploeg, 1991). On average, there were less earthy and off-flavors in fillets from fish reared at high temperature (Table 5.4). Fish reared at 25°C yielded fillets that received intensity scores for grassy flavor of approximately 2.0 or slightly greater, while fillets from fish reared at 33°C were closer to 1.5 on the 15 cm intensity scale (Table 5.4). Fishy flavor was less intense in fish reared under moderate temperature stress (33-H) and following socking and transport (33-H-ST) compared to the moderate oxygen stressed fish (25-L) (Table 5.4). Off-flavor, which was characterized as rancid or oxidized, was highest in fish reared at 25°C with the lowest intensity scores observed in the moderate temperature stress (33-H) and moderate temperature stress with combined socking and transport stress (33-H-ST) (Table 5.4). On average, fish reared at 25°C yielded fillets that were less flaky than fish that were reared at 33°C. Fillets from fish reared under moderate oxygen stress (25-L) and moderate oxygen stress combined with socking and transport (25-L-ST) were less flaky than fish reared under moderate temperature stress after experiencing socking (33-H-S) and transport (33-H-ST) stressors (Table 5.4).

The data collected herein demonstrate that stress and decreased feed consumption likely results in an increase in ketogenic metabolisms and a purging of lipid soluble flavor compounds. Although differences in consumer preference were identified, fillets from all treatments were liked slightly to moderately. Descriptive analysis results

indicated that fillets reared at 25°C exhibited a higher intensity of less favorable flavors commonly associated with catfish fillets (Johnson et al., 1987; Phan and Nguyen, 2012) However, none of these attributes exceeded 3.5 on a 15 cm intensity scale.

Table 5.4 Descriptive evaluation of baked channel catfish (*Ictalurus punctatus*) fillets.

Category	Attribute		Treatment											
			25-H	25-H-S	25-H-ST	25-L	25-L-S	25-L-ST	33-H	33-H-S	33-H-ST	33-L	33-L-S	33-L-ST
Appearance	Color		2.5^{a}	2.1^{ab}	1.7^{b}	1.9^{ab}	2.0^{ab}	1.5^{b}	2.0^{ab}	1.9^{ab}	1.6^{b}	2.1^{ab}	2.1^{ab}	2.1^{ab}
		SE	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
	Redness		1.4^{ab}	1.5^{a}	1.1^{ab}	1.2^{ab}	1.1^{ab}	0.9^{b}	1.2^{b}	1.4^{ab}	1.3^{ab}	1.3^{b}	1.5^{a}	1.6^{a}
		SE	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Aroma	Fishy		1.7^{ab}	1.9^{ac}	1.8^{abc}	2.2^{c}	2.0^{ac}	1.9^{abc}	1.5^{b}	1.7^{ab}	1.7^{ab}	1.9^{ac}	2.0^{ac}	2.0^{ac}
		SE	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
	Sulfury		1.3^{ab}	1.4^{ab}	1.6^{a}	1.3^{ab}	1.3^{ab}	1.3^{ab}	1.0^{b}	1.5^{a}	1.4^{a}	1.4^{ab}	1.6^{a}	1.5^{ab}
		SE	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
	Grassy		1.0^{ab}	1.1^{a}	1.2^{a}	1.2^{a}	1.2^{a}	1.0^{ab}	0.8^{b}	1.3^{a}	1.2^{a}	1.1^{ab}	1.1^{a}	1.0^{ab}
		SE	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Flavor	Sweet		1.0^{c}	1.1^{abc}	1.2^{abc}	1.2^{abc}	1.0^{c}	1.0^{bc}	1.5^{a}	1.3^{abc}	1.4^{ab}	1.2^{abc}	1.1^{bc}	1.3^{abc}
		SE	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
	Salty		2.2^{ab}	2.2^{ab}	2.1^{ab}	2.2^{ab}	2.0^{b}	2.0^{b}	2.2^{ab}	2.1^{ab}	2.0^{b}	2.1^{ab}	2.4^{a}	2.1^{ab}
		SE	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
	Bitter		1.1^{a}	1.0^{abc}	0.9^{abc}	0.8^{abc}	1.0^{abc}	0.8^{abc}	0.8^{abc}	0.8^{bc}	0.8^{abc}	0.8^{abc}	1.0^{ab}	0.7^{c}
		SE	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
	Umami		2.5^{a}	2.7^{abc}	2.8^{bcd}	2.8^{bcd}	2.8^{abcd}	2.6^{ac}	3.1^{d}	2.9^{bd}	3.0^{bd}	2.8^{abcd}	2.9^{bcd}	2.9^{bd}
		SE	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
	Earthy		2.4^{a}	2.3^{ab}	2.1^{abc}	2.1^{abc}	2.1^{abc}	1.8^{abcd}	1.4^{d}	1.6^{cd}	1.4^{d}	1.6^{cd}	1.7^{bcd}	1.5^{cd}
		SE	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
	Grassy		1.3	1.5	1.4	1.2	1.3	1.2	1	1.4	1.1	1.2	1.3	1
		SE	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
	Metallic		1.3^{ab}	1.5^{ab}	1.6^{a}	1.5^{ab}	1.2^{ab}	1.2^{ab}	1.1^{ab}	1.3^{ab}	1.0^{b}	1.2^{ab}	1.5^{ab}	1.4^{ab}
		SE	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
	Fishy		2.1^{ab}	2.2^{ab}	2.0^{ab}	2.3^{a}	2.2^{ab}	1.9^{ab}	1.5^{b}	2.0^{ab}	1.6^{b}	2.0^{ab}	2.1^{ab}	1.9^{ab}
		SE	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
	Buttery		1.0^{a}	1.4^{bcd}	1.3^{abcd}	1.2^{acd}	1.2^{ad}	1.3^{abcd}	1.6^{be}	1.5^{be}	1.7^{e}	1.5^{bce}	1.3^{abcd}	1.5^{bce}
		SE	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
	Off		1.7^{a}	1.7^{a}	1.5^{abc}	1.4^{abcd}	1.6^{ab}	1.4^{abcd}	0.9^{d}	1.3^{abcd}	0.8^{d}	1.0^{cd}	1.0^{bcd}	1.1^{abcd}
		SE	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Texture/ Mouthfeel	Flaky		9.5^{bc}	10.1^{abc}	9.8^{abc}	9.1^{c}	9.6^{abc}	9.3^{c}	10.1^{abc}	10.8^{ab}	11.0^{a}	9.8^{abc}	10.1^{abc}	10.0^{abc}
		SE	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	Astringent		2.0^{a}	1.7^{abc}	1.8^{ab}	1.6^{abc}	1.7^{abc}	1.2^{c}	1.5^{bc}	1.5^{abc}	1.3^{bc}	1.7^{abc}	1.7^{abc}	1.5^{bc}
		SE	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2

The data represent mean intensity scores for major descriptive attributes evaluated in catfish fillets. The intensity scores are based off of a 15 point intensity scale. Different letters represent statistical significance between treatments (ANOVA, LSD, $p < 0.05$, n = ~42).

5.4 Conclusions

Environmental conditions and sequential handling stressors affect physical, chemical and sensory quality characteristics of channel catfish fillets. The environmental and handling stressors employed in this study are frequently observed and/or utilized during pond culture of channel catfish, and impact the quality of the fillets produced. The quality changes observed were primarily in textural characteristics. The effects of harvest stressors on fillet tenderness indicate that environmental conditions do not have a significant impact. However, handling stress causes an increase in tenderness and thus may enhance the quality and marketability of the product. Environmental conditions and handling stress resulted in minor alterations in the sensory quality of channel catfish fillets. Sensory descriptive and consumer acceptability data suggests that environmental conditions alone will have minimal effects on fillet flavor attributes in cultured channel catfish.

CHAPTER VI

ALTERATIONS IN PROTEIN EXPRESSION OF CULTURED CHANNEL CATFISH (*ICTALURUS PUNCTATUS*) FILLETS DUE TO ANTE-MORTEM ENVIRONMENTAL AND HARVEST STRESSORS AND IMPLICATIONS ON QUALITY ATTRIBUTES

6.1 Introduction

Proteins are abundant in all cells and play an important physiological and structural role in biological systems (Damodaran, 2008). Proteomics employs the quantitative and qualitative evaluation of all proteins expressed under various conditions, as a means of understanding cellular mechanisms and biological processes (Anderson and Anderson, 1996). Studies of the proteome allow for a better understanding of an organism's biology (Rodrigues et al., 2012). When applying proteomic techniques to food products, various physiological changes that affect food quality can be elucidated. Proteomics can also aid in the evaluation of processing techniques and their ultimate effects on quality (Carbonaro, 2004).

In addition to their integral role in biological systems, proteins are an important component of the human diet and fish meat represents a significant source of high quality protein (Qixing et al., 2014). In recent years, proteomics has been being utilized to provide insight into the physiological and biological changes that different culture practices have on fish (Rodrigues et al. 2012). This has helped the industry achieve more

efficient productivity while maintaining product quality (Rodrigues et al., 2012). The proteome of fish muscle is important not only for its nutritional benefits but as a key factor mediating the structural, textural and sensory properties of the fillet (Qixing et al., 2014). Thus, proteomic techniques can be utilized to monitor quality changes and relate them to the physiological changes that cause them. Stress induced physiological changes in fish and the implications that such changes have on fillet quality are well documented (Jittinandana et al., 2003; Paterson et al., 1997; Robb et al., 2000). The majority of proteomic studies on fish species have focused on quality changes, often with regard to stress (Rodrigues et al., 2012). Such techniques show promise for the aquaculture industry in helping to understand the mechanisms involved in quality changes (Rodrigues et al., 2012). This knowledge can then be used to implement management practices for the optimization of product quality.

In the United States, pond culture of catfish represents the largest sector of the aquaculture industry (Vilsack and Reilly, 2014). Traditional culture practices, environmental conditions and fish harvest (netting, socking and transport) can elicit a stress response. Such stress events have been well documented and result in physiological and quality changes in fish muscle (Ch. 5) (Bosworth et al., 2007; Erikson and Misimi, 2008; Ginés et al., 2004; Hansen et al., 2012; Jittinandana et al., 2003; Lerfall et al., 2015). The most common quality changes documented are in color and texture (Ch. 4 and 5), which represent two of the most important quality attributes with respect to consumer purchasing decisions (Gobert et al., 2014; Maciel et al., 2014) and eating satisfaction (Rasmussen, 2001), respectively. When purchasing seafood products, fillet color plays an important role in a consumer's willingness to buy (Maciel et al., 2014). This is important

in catfish since fillets that deviate from the typical white flesh color are less marketable (Kin et al., 2010; Lovell, 1984). Proteomic techniques have been employed in the evaluation of meat color in beef (Joseph et al., 2012), pork (Sayd et al., 2006) and turkey (Joseph et al., 2011). In catfish, proteomic evaluation was used to identify the cause of the red color defect, which is becoming more prevalent in cultured catfish (Desai et al., 2014). Tenderness has been evaluated using proteomic analysis in beef (D'Allessandro et al., 2012; Laville et al., 2009) and pork (Lametsch et al., 2003; Morzel et al., 2004).

When examining the effects of stress on protein expression in fish, two main alterations are commonly observed across species: proteins involved in metabolic processes including energy production/allocation and changes indicating increased proteolytic activity. The majority of these changes have been observed following crowding and handling stressors with alterations in protein expression reported in Atlantic salmon (*Salmo salar*) (Veiseth-Kent et al., 2010), rainbow trout (*Oncorhynchus mykiss*) (Morzel et al., 2006) and gilthead seabream (*Sparus aurata*) (Alves et al., 2010; Silva et al., 2012). Rearing temperature can have significant impacts on fish growth (Buentello et al., 2000; Stewart et al., 2014; Tucker, 1996; Tucker and Robinson, 1990), thus affecting protein accretion. While studies on changing muscle proteomes of fish have been carried out with regard to storage temperatures (Kjærsgård et al., 2006; Kjærsgård and Jessen, 2003; Terova et al., 2011), to our knowledge no one has evaluated the effects of rearing temperature on the fish muscle proteome.

Changes in protein expression were evaluated along with alterations in instrumental quality characteristics. The primary goal of this study was to identify the specific stressors and biological mechanisms involved in red fillet formation and textural

changes in catfish fillets. The study will offer insight into the physiological changes induced by stress and the potential effects such changes will have on the quality of the fillets produced.

6.2 Materials and Methods

6.2.1 Fish acquisition and rearing

The procedures used herein were reviewed and approved by the Mississippi State University institutional animal care and use committee, and conducted in accordance to the approved protocols (no. 13-041). Fingerling channel catfish (*Ictalurus punctatus*) were supplied by the Mississippi State University Thad Cochran National Warmwater Aquaculture Center in Stoneville, MS. Fingerlings were grown out in flow-through culture systems ranging from 400 to 4,000 L for up to two years. Water was maintained at ambient well-water temperatures (22°C - 27°C) and oxygenated with constant aeration via forced air. Fish were fed once daily to satiation with a commercial pelleted fingerling feed, 36% protein (Land O'Lakes Purina Feed LLC). The fish were switched to a 32% protein finishing feed (Purina Animal Nutrition) after approximately one year prior to transfer to treatment tanks. Protein requirements for larger catfish decrease and a 32% finishing feed is commonly used for grow-out in commercial ponds (Robinson et al., 1998).

6.2.2 Environmental stress

Channel catfish with a mean weight among all three trials of 704 ± 484 g were anesthetized in a CO_2 bath containing 3 g/L NaCl, generated by bubbling in compressed gas, prior to being individually weighed to the nearest g and measured to the nearest mm.

Eighty fish were randomly distributed into each treatment tank, which were recirculating systems (~1.3 ± 0.3 L/s) supplemented with a constant supply of makeup unchlorinated well water (~0.15 ± 0.08 L/s) at ambient temperature (~27°C). Each tank was fitted with expandable granular biofilters that were filled with plastic floating biobeads (Polygeyser Models DF3 or 6; Aquaculture Systems Technologies, New Orleans, LA) providing mechanical and biological filtration. Each system had two in-line high-output ultra violet sterilizers (120 Watt SMART HO, Emperor Aquatics; Pottstown, PA), a pump (SHE2.4 or SHE1.7, Aquatic Ecosystems, Inc., Apopka, FL) and a sump tank. During a one week acclimation period, experimental temperatures (25°C and 33°C, 1°C/day) and high (H) and low (L) dissolved oxygen (DO) levels (>5 mg/L and ~2.5 mg/L, respectively altered at a rate of 10% saturation/day) were manipulated to achieve four environmental treatments representing a control (25-H), oxygen stress (25-L), temperature stress (33-H) and severe stress (33-L). Dissolved oxygen (DO) levels in the oxygen stressed treatments were decreased through natural respiration and maintained by continuous monitoring with a galvanic oxygen probe (MINI-DO, Loligo® Systems, Denmark or CellOx 325, WTW GmbH, Weilheim, Germany) attached to a Loligo® Systems oxygen analyzer and regulator system (Version 2.4; Loligo® Systems, Denmark) or a dissolved oxygen pocket meter (Oxi 330i, WTW GmbH, Weilheim, Germany) with a custom designed solenoid valve system (Petersen and Gamperl, 2011). The Loligo® system and solenoid valve system were set to bubble in oxygen when the DO levels dropped below 2 mg/ml and turn off as soon as 2 mg/L was reached. High DO was maintained with constant aeration with forced air aerators. During the acclimation and the four week trial period, fish were fed a commercial 32% protein finishing feed (Purina Animal Nutrition) once daily to

satiation. Each treatment received a minimum of 1% total initial body weight, with additional feed provided as needed for 30 min.

6.2.3 Harvest stress

After four weeks under experimental environmental conditions, 1/3 (n = ~27) of the fish from the control environmental conditions (25°C, >5 mg/L DO; 25-H) were harvested and processed to be used as control fish. At harvest, fish were anesthetized in a CO_2 bath containing 3 mg/L NaCl, weighed to the nearest g and measured to the nearest mm before being killed by a blow to the head and stored on ice. Two-thirds of the fish (n = ~53/treatment) from each treatment were transferred by netting to a 1.82 m^3 (0.60 m L x 0.60 m W x 0. 46 m H) sock constructed out of polyvinyl chloride piping and a plastic 1.25 cm plastic mesh netting to mimic a commercial sock. Sock size was determined by the average weight at the first trial to achieve an approximate sock density of 175 kg/m^3 (Avery, 2013). The socks were suspended in the same experimental tank for 15 h, representing the maximum time fish are held in a sock during commercial harvest (Avery, 2013). Afterwards, half of the fish (n= ~26/treatment tank) were transferred to transport tanks at a density of 0.72 kg/L supplied with well-oxygenated water directly from the treatment tanks and held for 3 h, mimicking typical transport times for commercially reared catfish (Bosworth et al., 2004). Transported fish were then harvested as described above prior to processing at the Ammerman Hearnsberger Processing Plant in the Department of Food Science, Nutrition and Health Promotion at Mississippi State University. Fish were hand filleted, pH and color were measured and eight fillets per treatment were each sectioned into two 25-50 g pieces and individually bagged. One set

of 8 were frozen (-20°C) for determination of cook loss and tenderness evaluation and the other 8 were frozen (-80°C) for proteomic evaluation.

6.2.4 Fillet pH measurement

Fillet pH was measured using a pH meter (Model Accumet 61a, Fisher Scientific, Hampton, NH) with a meat penetrating pH electrode attachment (Model Flexihet SS Penetration tip, Cole Palmer, Vernon Hills, IL) inserted at the anterior end of one fillet per fish. The pH was measured for eight fish per treatment for each of the three trials (n=24) after 24 h of cold storage (4°C).

6.2.5 Instrumental color analysis

Instrumental color was evaluated on 8 fish per treatment using a handheld colorimeter CR-400 (Konica Minolta Inc. Chiyoda-ku, Tokyo, Japan) with an 8 mm port size, 2° standard observer and illuminant D65 immediately after harvest. Calibration was performed with a standard Minolta white calibration plate (Model No 20933026, Japan). Color values were taken on three points on the vertebral side of the two fillets produced per fish. Two readings, one on the anterior and one on the posterior end of the first fillet and one reading in the center of the second fillet were recorded (Ciaramella et al., 2015, Ch. 4). Color was reported as mean CIE L* (lightness), a* (redness-greenness) and b* (yellowness-blueness) (Kin et al., 2009, 2010). The hue angle and chroma values were calculated for each color measure as described in (Erikson and Misimi, 2008). The hue angle was calculated as hue angle = arctan(b*/a*) when a* > 0 and b* > 0, hue angle = 180 + arctan(b*/a*) when a* < 0 and hue angle = 360 + arctan(b*/a*) when a*> 0 and b* < 0. Chroma was calculated as chroma = $(a^{*2} + b^{*2})^{1/2}$.

6.2.6 Instrumental texture evaluation

A 25 g section was cut from the center of 8 catfish fillets and frozen (-20°C) during processing. Samples were thawed overnight (4°C) prior to baking in individual foil pouches at 177°C for 25 min. Fillets were allowed to cool to room temperature (~22°C, ~45 min), patted dry with a paper towel and re-weighed to determine cook loss. Cooked fillets were cut into 2.5 cm x 2.5 cm squares and re-weighed before instrumental texture analysis. An Instron Universal Testing Center (Model 3300, Instron, Norwood, MA) with an attached Allo Kramer shear compression cell (CS-2) was used to determine shear force and total energy according to Kin et al. (2010).

6.2.7 Protein isolation and extraction

The muscle proteome was extracted from approximately 2 g (n = 6/treatment) of thawed (4°C) and minced fillet. Tissue was suspended in 8 ml (0.25 g/ml) of rehydration buffer (7M urea, 2M thiourea, 3% CHAPS, 60 mM DTT, 0.3% carrier ampholyte and bromophenol blue) and homogenized (Polytron Brinkmann, Westbury, NY, USA) at low speed (30 s on and 30 s off, x2). The homogenate was incubated in ice for 2 h on an orbital shaker (260 rpm). Samples were then centrifuged (Sorvall RC-5C Plus Super Speed Centrifuge, Newton, Connecticut) at 10,000g for 30 min at 4°C. Supernatant was reserved for protein quantification using the Bradford assay (Bio-Rad Laboratories Inc., Hercules, CA), compared against a standard curve of bovine serum albumin (Thermo-Fisher Scientific Inc., Waltham, MA). Protein concentrations determined for each treatment were used to calculate the volume of supernatant that contained 300 μg of protein to be prepped for gel electrophoresis using a commercial protein cleanup kit (Bio-Rad Laboratories Inc., Hercules, CA).

6.2.8 Two-dimensional gel electrophoresis

Two dimensional electrophoresis and mass spectrometry analysis were used to evaluate changes in muscle protein expression in channel catfish fillets following rearing under environmental and handling (socking and transport) stressors typical of commercial harvest. Clean proteins (300 µg) were rehydrated in 225 µl of rehydration buffer and used for passive rehydration (17 h) on an 11 cm-immobilized pH gradient (IPG) strip with a pH range of 3-7 (Bio-Rad Laboratories Inc., Hercules, CA). Isoelectric focusing was performed on a Protean EF cell system (Bio-Rad Laboratories Inc., Hercules, CA). Focusing conditions started with a rapid increase to 250 V (15 m), followed by 8000 volts until 25,000 volt-hours was achieved (~4 h). Following isoelectric focusing, IPG strips were equilibrated in two separate buffers for 15 min each on an orbital shaker (160 rpm). Equilibration buffer 1 contained 6 M urea, 30% glycerol, 2% SDS, 50 mM Tris HCl and 2% DTT and buffer 2 contained 6 M urea, 30% glycerol, 2% SDS, 50 mM Tris HCl and 2.5% iodoacetamide. The second dimension was resolved based on molecular weight on 12% SDS-polyacrylamide gel using a Criterion™ gel electrophoresis system (Bio-Rad Laboratories Inc., Hercules, CA) set to a constant 200 V for 55 min. Gels were then washed in 200 ml of de-ionized water for 15 min (160rpm) and stained (18 h) with 130 ml coomasie blue (Gelcode™ Blue Safe Protein Stain, Thermo Scientific, Rockford, IL). Gels were then destained in de-ionized water 6 times for approximately 1 h until the background stain was removed. Gel photographs were taken with Foto/Analyst® LuminaryFX Workstation (Camera model XL16 (FX/2.0); Fotodyne Inc., Hartland, WI). Images were analyzed with PDQuest imaging software (Version 7.3.1) and spots of interest were identified as having 1.5 fold difference in concentration or greater and

significantly different between treatments at 95% confidence, using a pairwise students t-test. The selected spots were excised and sent for digestion and mass spectrometry (MS) analysis.

6.2.9 Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) analysis

The protein gel spots were excised and subjected to dithiothreitol reduction, iodoacetamide alkylation and in-gel trypsin digestion according to (Desai et al., 2014). The resulting tryptic peptides were extracted, concentrated and injected for nano-LC-MS/MS analysis using an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) coupled with an Eksigent Nanoflex cHiPLC™ system (Eksigent , Dublin, CA) through a nano-electrospray ionization source. The peptide samples were separated with a reversed phase cHiPLC column (75 μm x 150 mm) at a flow rate of 300 nL/min. Mobile phase A was water with 0.1% (v/v) formic acid while B was acetonitrile with 0.1% (v/v) formic acid. A 50 min gradient condition was applied: initial 3% mobile phase B was increased linearly to 50% in 24 min and further to 85% and 95% for 5 min each before it was decreased to 3% and re-equilibrated. The mass analysis method consisted of one segment with eight scan events. The 1st scan event was an Orbitrap MS scan (100−1600 m/z) with 60,000 resolution for parent ions followed by data dependent MS/MS for fragmentation of the 7 most intense ions through collision induced dissociation (CID).

6.2.10 MS/MS Protein identification

The LC-MS/MS data were submitted to a local mascot server for MS/MS protein identification via Proteome Discoverer (version 1.3, Thermo Fisher Scientific, Waltham,

MA) against a custom database based on the fasta file of *Ictalurus punctatus* (Channel Catfish) downloaded from NCBI on May 5, 2015. Parameters used in the MASCOT MS/MS ion search were: trypsin digest with a maximum of two miscleavages, cysteine carbamidomethylation, methionine oxidation, a maximum of 10 mg/L MS error tolerance and a maximum of 0.8 Da MS/MS error tolerance. A decoy database was built and searched. Filter settings that determine false discovery rates (FDR) are used to distribute the confidence indicators for the peptide matches. Peptide matches that pass the filter associated with the strict FDR (target setting of 0.01) are assigned as high confidence. For MS/MS ion search, proteins with two or more high confidence peptides were considered unambiguous identifications without manual inspection. Proteins identified with one high confidence peptide were manually inspected and confirmed.

6.2.11 Statistical analysis

Analysis of color, pH and texture data was run as a randomized complete block design with subsampling. Trials represented blocks with individual fish representing subsamples within each trial. Analysis of variance (ANOVA) were carried out using SAS statistical software version 9.3 (SAS Institute Inc., Cary, NC) using least square means, but reported herein as mean values with their standard error. Mean separation was carried out using Fisher's protected least significant differences (LSD) test and all statistical significance is stated at $p < 0.05$. Proteomic analysis was carried out on PDQuest software (Version 7.3.1, Bio-Rad Laboratories, Inc., Hercules, CA).

6.3 Results and Discussion

All stressed fish showed differentially expressed protein spots following 2D electrophoresis of the muscle proteomes when compared to control fish (25-H). A total of 126 protein spots were identified in fillets from fish experiencing handling stressors alone (25-H-ST) when compared to the controls and 7 were found to be differentially expressed. A total of 147 spots were detected in fillets from fish reared under oxygen stress combined with handling (25-L-ST), and 13 spots were differentially expressed from controls. In fillets from fish reared in the thermal stress treatment with handling (33-H-ST), 86 spots were detected with 9 differentially expressed from controls. Fillets from fish subjected to severe environmental stress and handling (33-L-ST) had 108 spots total with 6 differentially expressed from controls. A total of 35 differentially expressed protein spots were identified among all treatments compared to the control fillets and sent for LC-MS/MS analysis and identification (Figure 6.1). Of the 35 spots sequenced 21 unique proteins were identified (Table 6.1).

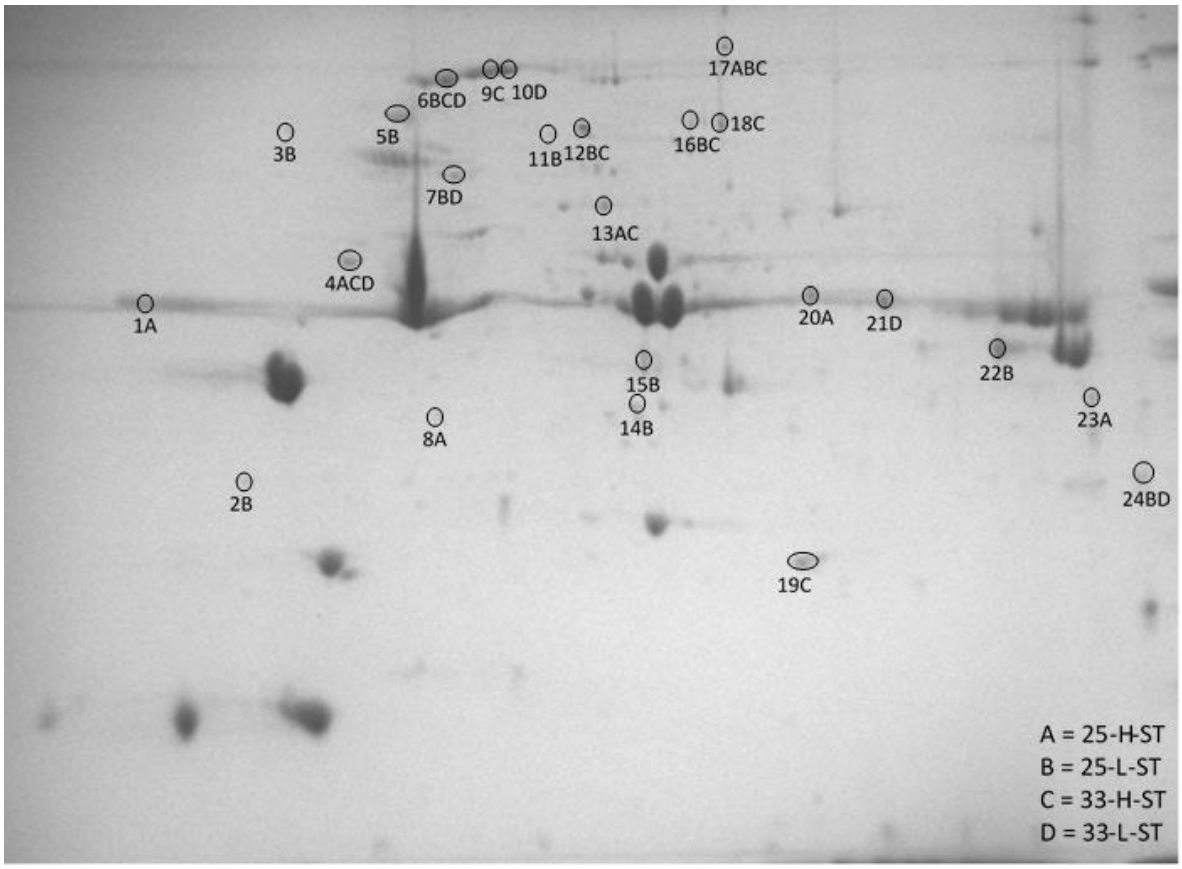

Figure 6.1 Representative protein gel from a control channel catfish (*Ictalurus punctatus*) fillet.

The gel image represents one of the control (25-H) gels that were used for comparison against all treatments. The 24 unique differentially expressed protein spots are circled with letters indicating the stress treatments in which the spots were differentially expressed. The treatments represent temperature (25°C or 33°C) – high (H) or low (L) dissolved oxygen – combined socking and transport (ST) handling stress.

Table 6.1 Differentially expressed proteins in channel catfish (*Ictalurus punctatus*) fillets experiencing temperature and dissolved oxygen stress in combination with handling stress.

Spot #	Protein	Accession No.	Species	ProtScore/ Matched Peptides	Sequence Coverage (%)	MW/pI
1A	Alpha actin sarcomeric/cardiac	317725965	*Ictalurus punctatus*	3131.37/33	72.94	42.0/5.39
2B	Elongation factor 1-beta	318037488	*Ictalurus punctatus*	414.54/24	62.28	25.1/4.61
3B	Tropomyosin alpha-1 chain	318065131	*Ictalurus punctatus*	835.90/32	78.93	28.0/4.75
4A	Actin alpha sarcomeric/cardiac	317725965	*Ictalurus punctatus*	1118.85/25	69.76	42.0/5.39
4C	Actin alpha sarcomeric/cardiac	317725965	*Ictalurus punctatus*	325.99/22	68.97	42.0/5.39
4D	Actin alpha sarcomeric/cardiac	317725965	*Ictalurus punctatus*	1077.69/27	69.76	42.0/5.39
5B	Alpha-actinin-3	576893086	*Ictalurus punctatus*	6055.06/83	79.93	103.8/5.25
6B	Alpha-actinin-3	576893086	*Ictalurus punctatus*	1417.99/42	59.31	103.8/5.25
6C	Alpha-actinin-3	576893086	*Ictalurus punctatus*	826.26/36	47.6	103.8/5.25
6D	Alpha-actinin-3	576893086	*Ictalurus punctatus*	2485.46/62	69.34	103.8/5.25
7B	Annexin A6	263202000	*Ictalurus punctatus*	2642.73/82	78.7	74.5/5.50
7D	Annexin A6	263202000	*Ictalurus punctatus*	2027.27/65	72.05	74.5/5.50
8A	F-actin capping protein subunit beta	576888456	*Ictalurus punctatus*	1337.81/24	61.68	30.8/6.27
	Annexin A4	318054624	*Ictalurus punctatus*	666.18/25	71.34	35.5/5.47
9C	M-protein, striated muscle	576893130	*Ictalurus punctatus*	375.71/17	19.52	107.1/6.32
10D	M-protein, striated muscle	576893130	*Ictalurus punctatus*	1304.73/45	36.53	165.2/6.61
11B	Programmed cell death 6-interacting protein	576892093	*Ictalurus punctatus*	565.45/33	39.98	96.7/6.09
12B	Fetuin-B	576887301	*Ictalurus punctatus*	565.21/16	39.08	55.9/6.74
12C	Fetuin-B	576887301	*Ictalurus punctatus*	391.59/15	30.06	55.9/6.74
13A	Phosphoglucomutase-1	576887217	*Ictalurus punctatus*	5204.53/54	89.3	61.2/6.55
13C	Phosphoglucomutase-1	576887217	*Ictalurus punctatus*	3602.19/52	86.63	61.2/6.55
14B	Creatine kinase	27883549	*Ictalurus punctatus*	1135.22/33	68.95	42.9/6.77
15B	Glycerol-3-phosphate dehydrogenase [NAD(+)] cytoplasmic	576887209	*Ictalurus punctatus*	1118.47/24	82.29	38.0/6.83
	Myozenin-1	576893056	*Ictalurus punctatus*	908.37/13	81.70	33.6/6.81
16B	AMP deaminase 1	576888583	*Ictalurus punctatus*	699.45/32	43.24	82.8/7.09
16C	AMP deaminase 1	576888583	*Ictalurus punctatus*	865.82/41	52.02	82.8/7.09
17A	L-lactate dehydrogenase A chain	576893138	*Ictalurus punctatus*	1162.11/24	63.36	36.2/7.25
17B	L-lactate dehydrogenase A chain	576893138	*Ictalurus punctatus*	1541.47/72	76.88	36.2/7.25
17C	L-lactate dehydrogenase A chain	576893138	*Ictalurus punctatus*	1309.85/26	76.28	36.2/7.25
18C	L-lactate dehydrogenase A chain	576893138	*Ictalurus punctatus*	689.21/21	66.07	36.2/7.25

Table 6.1 (Continued)

Spot #	Protein	Accession No.	Species	ProtScore/ Matched Peptides	Sequence Coverage (%)	MW/pI
19C	Adenylate kinase isoenzyme 1	576893067	*Ictalurus punctatus*	3349.41/30	92.97	21.5/8.0
20A	Creatine kinase	27883547	*Ictalurus punctatus*	730.28/27	64.3	42.7/6.80
21D	Fructose bisphosphate aldolase A	576892984	*Ictalurus punctatus*	1043.61/35	74.45	39.7/8.32
	Mitochondrial creatine kinase s-type	318010617	*Ictalurus punctatus*	1241.59/33	62.50	46.7/7.91
22B	Glyceraldehyde-3-phosphate dehydrogenase	308321716	*Ictalurus furcatus*	5277.69/28	78.08	35.9/8.19
23A	Troponin T, fast skeletal muscle isoforms	576892733	*Ictalurus punctatus*	360.30/12	23.53	28.6/8.90
24B	Proteasome subunit alpha type 7, partial	89266489	*Ictalurus punctatus*	190.77/8	48.70	17.1/8.87
24D	Proteasome subunit alpha type 7, partial	89266489	*Ictalurus punctatus*	97.75/3	30.52	17.1/8.87

The data represent the spot numbers corresponding to the gel image in figure 6.1 along with LCMS results for protein identification.

6.3.1 Effect of handling

Socking and transport stress decreased fillet redness, yellowness and color intensity, while hue angle and shear force increased (Table 6.2). Proteomic analysis revealed changes in the expression of a number of proteins that are important for muscle structure including actin alpha sarcomeric/cardiac, F-actin capping protein subunit A and Troponin T, which were down regulated with respect to the unstressed control fish (Table 6.3). Actin alpha sarcomeric/cardiac is important for muscle ATP binding and muscle contraction (Sveinsdóttir et al., 2008). F-actin capping protein subunit A is a calcium-sensitive protein that binds the end of actin filaments and may be important for structural integrity (Witke et al., 2001). Troponin T is a structural protein and component of the troponin complex that binds tropomyosin in muscle (Brown and Cohen, 2005). The lower levels of structural proteins in the muscle are indicative of increased proteolytic activity and muscle degradation, which have been documented following crowding stress in fish (Silva et al., 2012; Veiseth-Kent et al., 2010). Increased protein degradation will decrease water holding capacity of the meat (Bond and Warner, 2007) and affect meat color (Reppond and Babbit, 1997; Strasburg et al., 2008). A positive correlation between redness and creatine kinase was reported in beef (Joseph et al., 2012) and could also associated with fish as a decrease in creatine kinase and redness was observed in fish reared at 25°C. This indicates that similar trends may be present in catfish but they are temperature dependent. Annexin A4 was also identified in protein spot 8A and down-regulated with respect to the control. Annexin A4 has been associated with the regulation of membrane proteins, exocytosis and ion channels (Kaetzel et al., 1994; Piljić and Schultz, 2006; Yeh and Klesius, 2010).

In addition, decreased expression of proteins involved in energy metabolism including: phosphoglucomutase-1, L-lactate dehydrogenase A chain and creatine kinase were also observed. Phosphoglucomutase-1 is the enzyme involved in generating glucose-6-phosphate from glycogen stores to be used for glycolysis and energy production (Nelson and Cox, 2008a). L-lactate dehydrogenase A chain is involved in the reduction of pyruvate into lactic acid during anaerobic energy production (Nelson and Cox, 2008b). Creatine kinase is a part of the phosphotransfer network, which is integral to ATP cycling in cells (Silva et al., 2012). This contradicts the up-regulation of enzymes involved in metabolic processes post stress observed in other fish following acute stress (Morzel et al., 2006; Silva et al., 2012; Veiseth-Kent et al., 2010) and could reflect a down-regulation of metabolic processes for energy conservation (Smith et al., 1996). This could also be on account of the severity of the stress experienced since the studies above examined acute (40 min maximum) stressors, while in the present study socking and transport together lasted at least 18 h. Chronic stress that consisted of repeated handling (1 min aerial exposure 2x daily, 31 days) and high stocking densities (46.15 kg/m^3, 31 days) altered the concentrations of liver proteins involved in energy metabolism, with increases in fatty acid binding protein and decreases in triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase and enolase (Alves et al., 2010). The decreased expression of triose-phosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase was attributed to long term adaptation to redirect surplus glycerol to glucose instead of pyruvate (Alves et al., 2010). These changes in combination with the decreased pyruvate dehydrogenase and enolase following stress indicate a shift in metabolism to a more ketogenic pathway (Alves et al., 2010).

Table 6.2 Instrumental quality analysis of channel catfish (*Ictalurus punctatus*) fillets from control and stressed fish.

Value	25-H	25-H-ST	25-L-ST	33-H-ST	33-L-ST
pH	6.39 ± 0.02	6.66 ± 0.05	6.76 ± 0.05*	6.87 ± 0.07*	6.86 ± 0.07*
L*	50.5 ± 0.5	51.6 ± 0.6	51.6 ± 0.5	48.3 ± 0.7	51.2 ± 0.7
a*	2.4 ± 0.4	0.0 ± 0.3*	0.5 ± 0.2*	1.7 ± 0.3	1.0 ± 0.3*
b*	1.4 ± 0.2	-0.2 ± 0.2*	-0.3 ± 0.2*	0.6 ± 0.1*	-0.1 ± 0.2*
Hue	56.6 ± 15.3	154.4 ± 24.7*	183.2 ± 26.1*	101.6 ± 26.5	197.0 ± 30.8*
Chroma	2.9 ± 0.4	1.5 ± 0.2*	1.3 ± 0.2*	1.9 ± 0.3	1.3 ± 0.3*
SF	31.1 ± 2.1	32.6 ± 2.5	23.4 ± 1.7	18.8 ± 1.2*	20.6 ± 2.2
TE	0.26 ± 0.02	0.32 ± 0.02*	0.24 ± 0.01	0.22 ± 0.01	0.23 ± 0.02
CL	28.5 ± 0.5	30.6 ± 2.8	26.4 ± 0.7	27.7 ± 1.3	28.6 ± 1.3

Mean values are reported (± standard error). Color and pH were measured 24 h after processing and cold storage (4°C) and texture analyses were performed on frozen (-20°C) thawed (4°C) fillets. The treatments represent temperature (25°C or 33°C) – high (H) or low (L) dissolved oxygen – combined socking and transport (ST) handling stress. Texture data represent shear force (SF), total energy (TE) and cook loss (CL). Asterisks represent values that are significantly different from the control (25-H) at $p < 0.05$ (ANOVA, LSD, n = ~24).

Table 6.3 Effect of handling stress (25-H-ST) on protein expression in channel catfish (*Ictalurus punctatus*) fillets.

	Spot #	Protein	Functional Category	Spot Ratio (25-H-ST/25-H)
Down-Regulated	1A	Actin alpha sarcomeric/cardiac	Structural	0.18
	4A	Actin alpha sarcomeric/cardiac	Structural	0.61
	8A	F-actin capping protein subunit beta	Structural	0.55
		Annexin A4	Protein Regulation	0.55
	13A	Phosphoglucomutase-1	Energy metabolism	0.59
	17A	L-Lactate dehydrogenase A chain	Energy metabolism	0.59
	20A	Creatine Kinase	Energy metabolism	0.64
	23A	Troponin T, fast skeletal muscle isoforms	Muscle contraction	0.62

The data reflect the protein spots as they correlate to the sample control gel in figure 6.1 with protein identification following LC-MS/MS and MASCOT data base search. Protein functional group and spot ratios (treatment concentration/control concentration) are included for differentially expressed spots following socking and transport stress.

6.3.2 Effect of oxygen stress

When fish were reared under low DO conditions prior to socking and transport stress, pH and hue angle increased, redness, yellowness and chroma values decreased, and no changes in texture with respect to control fillets were observed (Table 6.2). Down-regulation of structural proteins including alpha-actinin-3 and tropomyosin alpha-1 chains were observed with the addition of oxygen stress (Table 6.4). Alpha-actinins are considered glue proteins and are required for the integrity and maintenance of the sarcomere (Zhang et al., 2011) by linking titin and actin filaments (Atkinson et al., 2000). The tropomyosin alpha-1 chain is integral in the regulation of muscle contraction (Galińska-Rakoczy et al., 2008). Down-regulation of creatine kinase and L-lactate dehydrogenase occurred as a means of energy conservation during hypoxic stress (Smith et al., 1996). In addition, several proteins involved in protein turnover and cellular lysis including elongation factor, proteasome subunit alpha, programmed cell death 6-interacting protein and fetuin B were down-regulated. Elongation factor 1-beta, a component of protein synthesis in channel catfish, was down-regulated (Ju et al., 2000) (Table 6.4). Proteasome subunit A alpha represents a portion of a proteolytic system associated with degradation of myofibrillar proteins (Strasburg et al., 2008). Programmed cell death 6-interacting protein is proposed to be involved in protein transport and cellular apoptosis (Biales et al., 2011). Fetuin-B inhibits calcium phosphate precipitation and protease activity in mammals (Denecke et al., 2003; Olivier et al., 2000) but its role in fish is not well understood (Nynca et al., 2011). The changes in these proteins could indicate down regulation of energy requiring activity when DO is low.

Fillets from fish exposed to oxygen stress exhibited increased prevalence of several proteins involved in metabolic processes including glycerol-3-phosphate dehydrogenase, AMP deaminase and glyceraldehyde-3-phosphate dehydrogenase. Glycerol-3-phosphate dehydrogenase is integral in the conversion of triglyceride derived glycerol into glyceraldehyde-3-phospate, which then enters the glycolytic pathway (Nelson and Cox, 2008c). AMP deaminase is important for energy metabolism since it catalyzes the conversion of AMP to IMP during anaerobic metabolism (Dobson and Hochachka, 1987). Redistribution and increases in myofibrillar bound AMP deaminase has been reported in sea scorpion (*Scorpaena porcus*) following hypoxia stress for 90 min to regulate adenylate metabolism (Lushchak et al., 1998). Glyceraldehyde-3-phosphate dehydrogenase is integral in the glycolytic pathway and production of ATP (Nelson and Cox, 2008b). The up-regulation in glycolytic enzymes including glyceraldehyde-3-phosphate is consistent with previous reports in various species following stress (Morzel et al., 2006; Silva et al., 2012; Veiseth-Kent et al., 2010) and allow for increased anaerobic production of ATP in response to the hypoxic and handling stressors.

Alpha actinin-3 was upregulated in this treatment and isolated from a different spot than the one identified to decrease likely due to different isoforms being present, post-translational modification or fragmentation through muscle degradation (Kumar and Klein, 2004; Morzel et al., 2004; Veiseth-Kent et al., 2010; Wulff et al., 2012). Myozenin 1 was up-regulated and identified in the same spot as glycerol-3-phosphate dehydrogenase. Myozenin 1 is a z-line protein that binds α-actinin and γ-filamin (Takada et al., 2001). Annexin A6 increased in fillets from fish reared under hypoxic conditions.

Annexins are calcium-dependent phospholipid binding proteins that are important for cell signaling and cellular processes including proliferation, differentiation and apoptosis (Grewal et al., 2005; Yeh and Klesius, 2010). Alterations in protein expression have been observed following exposure to anoxic conditions in the brain of crucian carp (*Carassius carassius*) (Smith et al., 2009), rainbow trout hypodermal fibroblast cells (Wulff et al., 2008), zebrafish (*Danio rerio*) embryos (Mendelsohn et al., 2009) and following chronic hypoxia in rainbow trout muscle (Wulff et al., 2012) and zebrafish muscle (Bosworth et al., 2005).

Table 6.4 Effect of low dissolved oxygen combined with handling stress (25-L-ST) on protein expression in channel catfish (*Ictalurus punctatus*) fillets.

	Spot #	Protein	Functional Category	Spot Ratio (25-L-ST/25-H)
Down-Regulated	2B	Elongation factor 1-beta	Protein biosynthesis	0.33
	3B	Tropomyosin alpha-1 chain	Structural	0.67
	6B	Alpha-actinin-3	Structural	0.35
	11B	Programmed cell death 6-interacting protein	Apoptosis	0.55
	12B	Fetuin-B	Protease inhibitor	0.52
	14B	Creatine kinase	Energy metabolism	0.46
	17B	L-Lactate dehydrogenase A chain	Energy metabolism	0.62
	24B	Proteasome subunit alpha type 7, partial	Protein regulation and turnover	0.34
Up-Regulated	5B	Alpha-actinin-3	Structural	1.54
	7B	Annexin A6	Muscle contraction	1.97
	15B	Glycerol-3-phosphate dehydrogenase [NAD(+)] cytoplasmic	Energy metabolism	1.54
		Myozenin 1	Structural	1.54
	16B	AMP Deaminase	Energy metabolism	2.67
	22B	Glyceraldehyde-3-phosphate dehydrogenase	Energy metabolism	2.03

The data reflect protein spots as they correlate to the sample control gel in figure 6.1 with protein identification following LC-MS/MS and MASCOT data base search. Protein functional group and spot ratios (treatment concentration/control concentration) are included for differentially expressed spots following rearing at low dissolved oxygen (~2.5 mg/L) and socking and transport stress.

6.3.3 Effect of temperature stress

High temperature stress prior to socking and transport increased fillet pH and decreased shear force and yellowness (Table 6.2). Down-regulation of the structural proteins actin alpha sarcomeric/cardiac and alpha actinin-3 in addition to M-protein were identified in fish subjected to high temperature (33°C) prior to handling when compared to control fish (Table 6.5). M-protein is integral to the structure of striated muscle and is an anchor for titin (Pfuhl and Pastore, 1995). The down regulation in structural proteins would ultimately affect muscle tenderness (Paredi et al., 2012; Godiksen et al., 2009). Although changes in structural proteins were identified in all treatments, the temperature stress was the only condition that resulted in a statistical decrease in shear force. Fetuin-B decreased following thermal stress as well (Table 6.5).

Phosphoglucomutase-1 and L-lactate dehydrogenase are important enzymes in the glycolytic pathway (Nelson and Cox, 2008b) and adenylate kinase isoenzyme-1 is important for the regulation of cellular ADP concentrations and the replenishment of ATP (Nelson and Cox, 2008d). These enzymes decreased when fish were reared at chronic high temperatures (Table 6.5), likely as a means of energy conservation. The identification of L-lactate dehydrogenase in two different protein spots further supports the effects of stress on post-translational modifications, such as phosphorylation (Canto et al., 2015), and muscle degradation. Similar to the oxygen stressed fish, AMP deaminase was up regulated in thermally stressed fish (Table 6.5), which is indicative of the increased energy expenditures that are associated with stress (Silva, et al., 2012; Veiseth-Kent et al., 2010).

Table 6.5 Effect of high temperature combined with handling stress (33-H-ST) on protein expression in channel catfish (*Ictalurus punctatus*) fillets.

	Spot #	Protein	Functional Category	Spot Ratio (33-H-ST/25-H)
Down-Regulated	4C	Actin alpha sarcomeric/cardiac	Structural	0.45
	6C	Alpha-actinin-3	Structural	0.29
	9C	M-protein, striated muscle	Structural	0.17
	12C	Fetuin-B	Protease inhibitor	0.46
	13C	Phosphoglucomutase-1	Energy metabolism	0.64
	17C	L-Lactate dehydrogenase A chain	Energy metabolism	0.62
	18C	L-Lactate dehydrogenase A chain	Energy metabolism	0.59
	19C	Adenylate kinase isoenzyme 1	Energy metabolism	0.62
Up	16C	AMP Deaminase	Energy metabolism	1.55

The data reflect protein spots as they correlate to the sample control gel in figure 6.1 with protein identification following LC-MS/MS and MASCOT data base search. Protein functional group and spot ratios (treatment concentration/control concentration) are included for differentially expressed spots following rearing at high temperature (33°C) and socking and transport stress.

6.3.4 Effect of severe stress

Severe stress resulted in increased pH and hue angle, decreased redness, yellowness and chroma values but did not differ from the control in lightness and texture (Table 6.2). When low DO and high temperature rearing were combined, actin alpha sarcomeric/cardiac, alpha-actinin-3 and M-protein striated muscle were all down regulated (Table 6.6) as observed when exposed to each individual stressor. Under severe stress, fructose bisphosphate aldolase A, mitochondrial creatine kinase and a partial sequence of a proteasome subunit were also down-regulated and annexin A6 was upregulated (Table 6.6). Increases in annexins including A6 have been associated with disease in channel catfish (Yeh and Klesius, 2010) and thus could also be an indicator of hypoxia stress as they were upregulated in all fish experiencing hypoxia in the present study. Fructose bisphosphate aldolase is an enzyme integral to the preparatory phase of glycolysis (Nelson and Cox, 2008b). Proteasomes are proteolytic enzyme complexes involved in protein turnover and regulation during many basic cellular processes (Ciechanover, 1998), and the partial sequence identified is indicative of increased proteolytic activity. The fewest differentially expressed proteins were identified following severe stress, which is likely a result of the suppressed physiological stress response that is observed when fish are pre-exposed to stressors (Barton, 2002; Davis and Parker, 1990; Methling et al., 2010).

Table 6.6 Combined effects of low dissolved oxygen, high temperature and handling stress (33-L-ST) on protein expression in channel catfish (*Ictalurus punctatus*) fillets.

	Spot #	Protein	Functional Category	Spot Ratio (33-L-ST/25-H)
Down-Reg.	4D	Actin alpha sarcomeric/cardiac	Structural	0.48
	6D	Alpha-actinin-3	Structural	0.47
	10D	M-protein, striated muscle	Structural	0.16
	21D	Fructose bisphosphate aldolase A	Energy metabolism	0.63
		Mitochondrial creatine kinase s-type	Energy metabolism	0.63
	24D	Proteasome subunit alpha type 7, partial	Protein regulation and turnover	0.32
Up	7D	Annexin A6	Muscle contraction	2.36

The data reflect protein spots as they correlate to the sample control gel in figure 6.1 with protein identification following LC-MS/MS and MASCOT data base search. Protein functional group and spot ratios (treatment concentration/control concentration) are included for differentially expressed spots following rearing at high temperature and low dissolved oxygen (33°C, ~2.5 mg/L) in addition to socking and transport stress.

6.4 Conclusions

Evidence of increased proteolytic activity likely caused the decrease in fillet color intensity. The most pronounced changes were observed in fish reared under low DO conditions, with the most differentially expressed proteins identified and up-regulation of several proteins involved in metabolic processes. Contrary to what was expected the severe stress produced the fewest changes in the muscle proteome. This suggests that severe environmental stress prior to handling suppresses the fish's ability to respond to subsequent stress events.

CHAPTER VII

SUMMARY AND CONCLUSIONS

Stress during pond culture of catfish is inherent and has a great impact on the physical characteristics and physiology of the fish and the quality of the fillets produced. Environmental conditions impact growth and condition in catfish and play an important role in mediating the physiological stress response. A cumulative stress response was observed in channel catfish following the sequential stressors that are associated with harvest events. The specific physiological response of channel catfish to such stressors and the impacts on fillet quality are dependent on the environmental conditions leading up to the harvest event.

Rearing catfish under stressful temperature and oxygen regimes has negative effects on feed consumption, growth and condition that can be managed separately in food-size catfish. Concerns for management of environmental conditions in culture ponds will increase as global temperatures continue to rise and demand pushes for more intensive fish culture practices. While methods of dissolved oxygen (DO) management have received much attention little has been done with regard to temperature control in culture ponds. Future research on thermal stability of culture ponds may be integral for the future success of the catfish industry. It is important to note that diurnal fluctuations in temperature and DO occur in culture ponds while in the tank studies these variables remained constant. Future research in the area should examine the interaction effects of

temperature and DO on feed efficiency and growth in natural culture systems. While changes in growth were not accompanied by altered physiological state in fish reared under different environmental regimes environmental stress affected the physiological stress response following additional handling stress events.

The physical stress incurred by socking and transport were identified as major stressors in catfish aquaculture, with a cumulative response observed as the fish progressed through the sequential harvest stressors. The data identified a suppression of the stress response when fish are pre-exposed to high environmental temperatures and/or low DO levels, highlighting the importance of environmental rearing conditions on physiological stress response.

The stressors also induced changes in the physical appearance of the fillets after processing. The sequential harvest stressors had beneficial effects on the color of the fillets, with a decrease in redness and color intensity. Thus, stress produced the typical opaque whitefish fillets consumers look for when purchasing catfish. The beneficial effects of the socking and transport stress were dependent on the environmental conditions leading up to harvest. When fish are pre-exposed to high temperature with high DO levels the range of color values reported extended further into those associated with a red discoloration. Thus, a higher prevalence of red fillet might be expected when harvesting under these conditions. The only observed increases in hematocrit were under these conditions as well, further indicating it could play a role in the formation of red fillet, however the highest levels recorded were not higher than those from fish in other treatments. Future research should evaluate the correlations between hematocrit and hemoglobin in the blood and plasma to better understand its relationship and involvement

in the formation of red fillets. The increased size and socking densities of fish in this treatment also warrants further evaluation.

In addition to the changes in physical appearance, instrumental quality and sensory attributes of the catfish fillets were also affected by stress. Texture and flavor represent two major aspects of fillet quality in fish and both were affected by stress. While environmental rearing conditions did not impact fillet tenderness, handling stress causes an increase in tenderness and thus enhances the quality and marketability of the fillets. With regard to flavor, slight increases in the intensity of the more unfavorable flavor attributes commonly associated with catfish were observed. The decreases were likely due to the decreased feed consumption and purging of fat stores in the environmentally stressed fish. The intensities, although higher in control fish, were still low (<3.5) on the 15 cm intensity scale and fillets from all treatments were liked slightly to moderately by consumers. This suggests that the minor changes in flavor attributed to environmental stress alone are not likely to significantly affect marketability of catfish fillets.

Overall, changes in protein expression of channel catfish fillets were similar to those observed in many other fish and species. Proteomic analysis identified change in the expression of structural proteins, which was likely caused by increased proteolytic activity and the reason for the decreases in color intensity identified. Several proteins involved in metabolic processes and energy production were also commonly observed among treatments. The most pronounced changes were observed in fish reared under low DO conditions, with the most differentially expressed proteins identified and up-regulation of several proteins involved in metabolic processes indicating a switch to a

ketogenic pathway for the production of energy. The stability in color values in thermally stressed fish could indicate a higher prevalence of red fillet, thus further evaluation of the severity of stress should be conducted to exaggerate the color changes and induce more color specific changes in the muscle proteome such as increased hemoglobin content.

In summary, quality of catfish fillets can be affected both positively and negatively by pre-harvest conditions and stress events. A better understanding of the specific effects of different conditions and stressors on physiology and ultimate quality will allow farmers to manage populations to ensure the highest quality fillets are produce. Future studies focused on variations in the physiology and quality of fish produced from ponds exhibiting different environmental regimes could allow for the development of methods for determining survival and fillet quality based on environmental conditions leading up to harvest events and handling stressors employed.

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