Role of autolytic enzymes in muscle softening and resultant physico-chemical changes during post-mortem storage of selected freshwater fishes

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by

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Certificate

This is to certify that the Doctoral Thesis entitled **"Role of autolytic enzymes in muscle softening and resultant physico-chemical changes during post-mortem storage of selected freshwater fishes"** is an authentic record of the research work carried out by **Ms. Treesa Varghese**, under my supervision and guidance at the School of Industrial Fisheries, Cochin University of Science and Technology, in partial fulfilment of the requirements for the degree of Doctor of Philosophy and no part thereof has been submitted for any other degree at any other institution.

Kochi-16
12-12-2017

Prof. (Dr.) Saleena Mathew
Supervising Guide

Declaration

I Ms. Treesa Varghese do hereby declare that the Doctoral Thesis entitled "**Role of autolytic enzymes in muscle softening and resultant physico-chemical changes during post-mortem storage of selected freshwater fishes**" is an authentic record of the original research work carried out by me under the guidance and supervision of **Prof. (Dr.) Saleena Mathew, Professor**, School of Industrial Fisheries, Cochin University of Science and Technology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Cochin university of Science and Technology and that no part has been submitted earlier for award of any other degree, diploma or other similar title of this in any University or Institution.

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Abbreviations

%	:	Percentage
AOAC	:	Association of Official Analytical Chemists
ADP	:	Adenosine Diphosphate
AMP	:	Adenosine Monophosphate
ANOVA	:	Analysis of Variance
ASC	:	Acid Soluble Collagen
ASP	:	Alkali soluble protein
ATP	:	Adenosine triphosphate
C	:	Celsius
Ca^{2+}	:	Calcium
$CaCl_2$	:	Calcium Chloride
CL	:	Cook Loss
cm	:	Centimeter
Cu	:	Copper
$CuSO_4$	:	Copper Sulphate
EDTA	:	Ethylene diamine tetraacetic Acid
EO	:	Essential Oils
EU	:	Enzyme unit
EWC	:	Expressible Water Content
FAO	:	Food and Agriculture Organization
Fe	:	Ferrous / Iron
FSIN	:	Food Security and information network
g	:	Gram
h	:	Hour
H_2SO_4	:	Sulphuric Acid
HCl	:	Hydrochloric Acid
HPLC	:	High-Performance Liquid Chromatography
Hx	:	Hypoxanthine
HxR	:	Inosine
ie	:	That is
IMP	:	Inosine Monophosphate

ISC	:	Insoluble collagen
K_2HPO_4	:	Dibasic Potassium Phosphate
K_2SO_4	:	Potassium Sulphate
KCI	:	Potassium Chloride
Kg	:	Kilogram
Kgf	:	Kilogram force
KH_2PO_4	:	Potassium dihydrogen phosphate
M	:	Molar
mg	:	milligram
$MgCl_2$	:	Magnesium Chloride
min	:	minutes
ml	:	Milliliter
mm	:	milli meter
mM	:	milli molar
MMP	:	Matrix Metallo Proteinase
Mn^{2+}	:	Manganese
MP	:	Myofibrillar protein
Na_2HPO_4	:	Disodium Hydrogen Phosphate
NaH_2P0_4	:	Sodium Phosphate Monobasic
NAM	:	Natural Actomyosin
NaOH	:	Sodium Hydroxide
nm	:	Nano meter
per cent	:	Percentage
pH	:	Potential of Hydrogen
Pi	:	inorganic phosphate
PMSF	:	phenylmethane sulfonyl fluoride
p-nitrophenol	:	para-nitrophenol
PSC	:	Pepsin soluble collagen
QIM	:	Quality Index Method
rpm	:	Rotations per Minute
SDS	:	Sensory Demerit Score
SP	:	Sarcoplasmic protein
SPSS	:	Statistical Package for the Social Sciences

TC	:	Total collagen
TCA	:	Trichloro acetic acid
TIMP	:	Tissue Inhibitors of Metallo Proteinases
TPA	:	Textural Profile Analysis
UN	:	United Nation
viz.	:	Videlicet
w/v	:	weight/ volume
WHC	:	Water Holding Capacity
X	:	Xanthine
Zn^{2+}	:	Zinc
μmoles	:	micromole

Chapter 1

Chapter 1:
Introduction

Contents

1.1: General Introduction

Food security is one of the important challenges of modern administrators. Even though special emphasis is given globally in this regard, the problems like wide-spread persisting hunger, poverty and malnutrition remains across the world. Food and Agriculture Organization of the United Nation (2017) reports that in 2016, about 108 million people were facing disaster level food insecurity all over the world. The major hurdle for the food security includes decline in production due to ecological problems, poor harvesting practices and wastage of food materials. According to FAO (2016), the hunger population will reach 9.7 million by 2050. The UN report also reveals one in each nine people is undernourished. Therefore, the world food-producing sector needs to ensure food and nutrition for the growing population through increased production and reduction of wastage. The reduction of wastage of materials could be accomplished by developing scientific methods in planning, developing preservation and processing techniques, enhancing storage mechanism and establishing well managed supply chain system. The special emphasis to be done in the preservation and processing of the harvest.

Fish is considered as an important source of high-quality proteins, essential amino acids, essential fatty acids, vitamins and minerals necessary for human body. It remains as the supreme traded food commodities worldwide because of its nutritional value. It is reported that the global fishery production for human consumption increased by approximately 74% to 85% during the period from 2000 to 2009. Although there was a decline in production during 2010- 2011, it remained constant at 75% until 2014. The per capita human consumption reached a record high of 20 kg in the year of 2014 from an average of 9.9 Kg in 1960 and is projected to reach 21.6 kg in live weight

equivalent by 2026, up from an average of 20.3 kg in 2014-16. It is projected to increase by 19% (29 million tonnes) by 2026 than that of base period (2014-2016), contributes 6.5% of total protein intake in both developed and developing countries (Food and Agriculture Organization, 2017a). This remarkable development has been driven by a combination of population growth, urbanization and rising domestic income and wealth along with increased awareness about fish and fishery products as a healthy alternative source to meat from farmed animals. Approximately, 150 g of fish can fulfill nearly 50- 60 % of an adult person's daily protein requirement and contributes to about 18% of animal proteins consumed (FAO, 2009). Fish provides more than 3.1 billion people with almost 20% of their average per capita animal protein intake. In 2016, 87% (146 million tons) of the world fish production was utilized for direct human consumption and was in the form of live, fresh or chilled condition and value added forms (FAO, 2016).

Even though water covers more than 70% of the total earth surface, the inland water cover is only 1%. It was estimated that 40% of 28,000 known fish species have freshwater habitat (Helfman *et al.,* 2009) and their production is from less than 0.01% of total volume of earth's water (Lynch *et al*., 2016). According to FAO (2014), marine catches is seven times higher than inland catches. Several studies report that most of the harvest of inland fisheries are often unrecorded (FAO, 2010; Welcomme *et al*., 2010; FAO, 2012; Bartley *et al*., 2015).

The FAO (2007) report states that 94% of whole freshwater fisheries are from developing countries. It also states that freshwater fish contributes above 6% of the annual animal protein supplies for human. In addition to this, they contribute an important step in overall economic wellbeing in terms of export commodity trades, tourism and recreation (World Fish Centre, 2003). Together with aquaculture, the fisheries sector provides the livelihoods of 10-12 % of the total population all around the world (FAO, 2014).

About 940 freshwater fish have been identified from inland waters of India from its estuaries, rivers and lakes of which approximately 210 are found in the inland waters of Kerala. Majority of these fish have received high economic demand due to their high nutritional quality, farming and ornamental purpose. Climbing perch (*Anabas*

testudineus), banded snakehead (*Channa striata*) and stinging catfish (*Heteropneustes fossilis*) are small indigenous fresh water fishes and belong to different diverse genus. These species have great acceptance in terms of daily food because of its high nutritional quality and availability throughout the year in fresh condition even in live condition. Prices of these fish species are considerably cheaper than the larger fish species available in market. Therefore, these fish can play significant role in satisfying the nutrient demand of malnourishment and on that basis, these three species were selected for the present study.

1.2: Morphology

1.2.1: Climbing Perch

Climbing perch (*Anabas testudineus*) (Bloch, 1792), is a fish of demersal fresh waters of Asia. Climbing perch (Plate 1.1) belongs to the family *Anabantidae*. They are generally found in the rivers, canals, lakes and ponds (Menon, 1999; Vidthayanon, 2002), frequently found in areas with dense vegetation (Rainboth, 1996) and can survive extremely unfavorable water conditions and are mainly associated with turbid, stagnant waters (Pethiyagoda, 1991). Its name is derived from a faulty observation, i.e. they can climb on trees. In natural habitat, it has a walking tendency over the dry land for a distance using its operculum and pectoral fins.

Climbing perch is an obligatory air breathing fish with four pairs of gills. Also have an accessory air breathing organ, that is one pair of labyrinthine and respiratory membranes within the supra-branchial chamber and if kept moist, they are able to survive several days to weeks out of water (Rahman, 1989).

Perch has an elongated body with broad anterior head, while the posterior part is compressed and of total length 2.4 cm. It has relatively pointed head with almost terminal mouth with fringed lower lip and dull reddish scales on the sides. Laterally, a compressed mouth was noticed and the lower jaw slightly longer with villiform teeth on jaw. Length of lower and upper jaws is 1.51cm and 1.71 cm respectively. The dorsal side and dorsal and caudal fins are greenish to dark gray in colour, while the belly, pectoral and anal fins are pale yellow coloured in nature. Total length of caudal fin is 4.0 cm. At the base of the caudal fin a dark spot is present. The body is more linear is covered with dark scales on upper part, whereas lower body and the

belly with brown colored scales. Body colour is dark to pale green, fading to pale on belly. The dorsal and caudal fins are dark gray in nature, anal and pectoral fins are yellow and pelvic fins light in colour. Dorsal, pelvic and anal rays are modified to spins. The dorsal fins have about 15 stiff spins (Alam *et al*., 2007). Bigger the size of the fish, the taster it is. It is very hardy fish and is of considerable fisheries interest for consumption.

Plate 1.1: Climbing perch (*Anabas testudineus*)

1.2.2: Banded snakehead

Banded snakehead Banded snakehead (*Channa striata*) (Bloch, 1793) (Plate 1.2) is a fresh water fish, often found in ponds, weedy streams, paddy fields and rivers. They live in bentho-pelagic environments. Natural populations of banded snakehead are distributed across Southern Asia, Southern China, Indonesia and Islands. (Lee and Ng, 1994; Hossain *et al*., 2008; Song *et al*., 2013). It grows to a maximum of 0.5 kg in weight and in length of 35 cm.

Banded snakehead belongs to the family of *Channidae* and is a hardy fish. It has a large scaled head and its name originated from its physiological appearance, i.e. head of this fish resembles that of a snake. It is a carnivorous species, locally known as Haruan. Due to its air – breathing capabilities assisted with a supra-branchial chamber, the fish is able to tolerate the adverse environments and also can tolerate slight brackish

water. It can survive a number of months with devoid of water if its skin and breathing organ remain moist.

Banded snakehead has long cylindrical body with depressed head. It has a deep gigantic mouth with full of sharp teeth. Lower jaw contains 4-7 canines, situated behind a single row of villi form teeth. The body colour is gray green to dark green in nature, sides are yellowish while below are pale in colour. Scales are organized in lateral manner. But scales on the top of the head are large in nature and arranged as rosette, scale present on front region of head forming central plate of rosette (Talwar & Jhingran, 1991). The sides and dorsal surface of the fish are dark in colour, spotted with combination of black and yellowish-brown. Dorsal and anal fins are darker in nature with dark patches and its belly is white in colour. An average of 37-46 rays are found on dorsal fin, while an anal fin 23-29 rays present. Pectoral fin account for about half of head length with 15-17 rays. Six rays are present on pelvic fin. Caudal fins are rounded in nature. It has dark round caudal and two ventral bands on its base. Banded snakehead flesh is firm and white, and is bone less with good flavor.

Plate 1.2: Banded snakehead (*Channa striata)*

1.2.3: Stinging catfish

Stinging catfish or fossil cat, *Heteropneustes fossilis* (*Bloch*, 1794) (Plate 1.3) is a fresh water fish with air sac. Often found in muddy rivers, lakes, ditches, swamps marshes, and in tropical, subtropical and temperature waters. They can tolerate slightly brackish water. They are seen in India, Pakistan, Nepal, Srilanka, Thailand and Myanmar (Berra, 2007; Hossain *et al*., 2013). Stinging catfish are omnivorous bottom feeders. It has 'stings' behind to the fins, so its name stinging catfish and are omnivorous bottom feeders.

Stinging catfish belongs to a family of *Heteropneustidae*. It has gray brown to black coloured elongated and compressed body with a depressed head, which is covered with osseous plate at the top and sides of the head and eyes are normally small in size (Hossain *et al*., 2013). Usually four pairs of barbells that is nasal, maxillary (on each sides of mouth), and two pairs of chin barbells are seen. About 6 to 7 rays are present in dorsal fin with an average length of 7.54%, whereas, in anal fin 60-79 rays are present (average length 60.89%) (Hossain *et al*., 2013). It also has external, strong hollow spine-like ray that are situated near to their dorsal and pectoral fins and can be used for defense mechanism.

Catfish have no scales, the mucus covered skin makes its body slippery in nature and used for cutaneous respiration. In addition, they also have chemo receptors across their entire body. Catfish have soft white boneless flesh with a good flavor.

Plate 1.3: Stinging catfish (*Heteropneustes fossilis)*

1.3: Relevance of the study

From the current trends, the world per capita of fish consumption reaches new record day by day. The requirement for fish and fisheries products could be managed only by effective processing and preservation of catch and by aquaculture. In this point of view, need for processing and preservation of freshwater fishes along with marine water fish is getting importance. Freshwater fish comprises about 40% of the global fish species. Unfortunately, captured freshwater fishes contribute only 7% of total Southeast Asian fishery production. Additionally, according to Southeast Asian Fisheries Development Center (2017) the region's production of Asian red tail catfish had the highest price (2,253 US$ /MT), followed by the banded snakehead (2,081 US$ /MT), climbing perch (1,665 US$ /MT), and Nile tilapia (1,534 US$ /MT). In this context, need for processing and preservation of these fish for consumption is significant.

Spoilage of food products may be due to chemical, enzymatic or microbial activity. Because of inadequate onsite storage, lots of harvested fish are lost every year due to microbial and autolytic spoilage. It is equally important to develop proper techniques for processing of fishery resources along with development of fisheries. The quality attributes of the fish muscle is influenced by its organoleptic features, sensory score, nutritional properties etc. and act as an important factor for the final acceptance and consumption of fish as a food item. In post-mortem fish spoilage, breakdown of various complex components results in the formation of compounds that are responsible for changes in odour, flavor and texture of the fish meat. Textural quality of fish is the most important rate-limiting factor in consumer acceptance as well as market price. Muscle softening or development of mushiness is the common problem occurring during storage and its distribution, representing foremost focusing area of the freshness of saleable products. Depending on the anatomical and physiological properties, constituents of the fish muscle may also vary. The physicochemical characteristics are significantly influenced by both intrinsic and extrinsic factors. This variation leads to different functional properties and thereby the process-ability.

Low temperature storage and chemical techniques for controlling microbial and autolytic spoilage are the most common methods in the industry today (Akinola *et al*., 2006; Berkel *et al*., 2004). Storage and preservation of fish using ice or other methods of chilling are the most widely preferred methods to keep the fish from all times in a cool condition and it has limited effects on sensorial quality (Mohan *et al*., 2016). The functions of ice include: (a) maintaining uniform low temperature, (b) reducing autolysis and bacterial degradation and (c) providing a gentle washing/cleaning effect during melting (Rand & Pivarnik, 1992). The fresh water fishes are mainly vended to common people in fish markets without following proper icing procedures. In this context, studies of post mortem changes in fish muscle stored at ambient temperature as well as in ice are necessary to fulfill the need of these procedures. Action of autolytic enzymes on post-mortem fish muscle induces a number of unfavorable biochemical changes. Autolytic enzymatic activity along with microbial spoilage prevent long time storage of the food item using normal preservation techniques such as refrigeration or icing. To overcome this circumstance, investigating alternative strategies such as the role of natural herbs and spices in shelf life extension of fish is attempted so as to satisfy the final consumer and to enrich the product value.

1.4: Objectives of the study

The main objectives of the study as follows:

- To study the biochemical composition of Climbing perch (*Anabas testudineus*), Banded snakehead (*Channa striata*) and Asian stinging catfish (*Heteropneustes fossilis*) with emphasis on seasonal variation.
- To study physicochemical changes in fish muscle stored at ambient temperature and iced condition.
- To study the textural parameters and sensory qualities of fish during storage at ambient temperature and iced condition.
- To study the musculature differences in the three species by histochemical analysis on storage for 18 days under ice.

- To correlate the progress of rigor and rigor resolution to the various autolytic enzymes and collagenase activity.
- To study the effect of commonly used herbs and spices in altering the activity of lysosomal acid phosphatase and collagenase enzymes.

Chapter 2

Chapter 2

Proximate composition of three freshwater fish muscle tissue with emphasis on seasonal variations

Contents

2.1: Introduction

Indigenous fish species are valuable resources of macro and micronutrients and play an important role in providing essential nutrients that are required for the human body. Fish muscle comprises of protein, fat and moisture as major nutritional components and carbohydrates, vitamins and minerals as minor components. Due to its special nutritional qualities, awareness about healthy food and fish is gaining importance. In this context, a proper understanding about biochemical constituents of fish has become a crucial necessity for nutritionists and dieticians. In addition, its assessment is important for its better processing and preservation. The composition of biochemical constituents of any organism varies with environmental changes. In order to emphasize the nutritional importance of fish as food item and its quality deterioration during various processing methods, their food chemistry has to be analyzed. Proximate compositions of fish meat differ by season and muscle positions. Additionally, total lipid content in wild fish is less than that of cultured one.

Proximate analysis of a food sample determines the total protein, fat, ash, and moisture, reported as the percentage composition of the product (Dewan *et al*., 2015). In addition, carbohydrates, vitamins, nucleotides and non-protein nitrogenous constituents are present as minor constituents. This biochemical composition depends on season, habitat, size, age and eating habitat, migratory swimming, sexual difference, starvation condition, species and even individuals (Pawar & Sonawane, 2013). Analysis of proximate composition traditionally used as good indicator of nutritional value of

food items (Rasmussen, 2001; Suleiman & Abdullahi, 2009). Additionally, the data regarding the changes in the proximate composition of the selected species at various seasonal conditions are also lacking. Hence, the objective of the present study is the seasonal variations in the chemical composition of the fishes from a minor freshwater fishery in Kerala, Tiruvalla.

2.2: Review of Literature

Fish is an important source of animal protein in the human diet and are called complete food in the nutritional value, because they fulfill all the human dietary requirements (Hoffman & Falvo, 2004). Importance of fish as source of high quality, balanced and easily digestible protein, vitamins and polyunsaturated fatty acids are well studied. The major constituents in the edible portion of fish are water, protein, lipid and ash (minerals), accounting for about 96-98% of total tissue constituents in fish (Love, 1988; Nowsad, 2007). In addition, carbohydrates, vitamins, nucleotides, other non-protein nitrogenous compounds etc. are also present in small quantities.

Water is the major constituent of fish flesh, containing about 70-80% and it varies widely. Water in the tissue exists in two forms, free form and bound to proteins. These forms have well defined biological roles. Water is lost from the tissue in many ways during processing and this may affect the quality, especially the texture of the processed products. Water acts as the medium for the transport of nutrients and metabolites, and is required for the normal functioning of many biological molecules. The sum of the percentages of lipid and water frequently spans a range of 75 to 85 percent. The lipid content increases gradually with increase in the size of fish, whereas the moisture content decreases with increasing lipid content of different size (Dewan *et al*., 2015). Lipid constitutes about 1-20 % of total tissue mass and its variations are much wider than that in protein. Lipids vary in different parts of fish body and they show enormous variation in different seasons of the year (Zafar & Ashraf, 2011). Compared to mammals, the fish protein is rich in methionine, lysine and low in tryptophan (Nowsad, 2007). Protein in fish muscle constitute about 10-22 % of total tissue composition. Protein content varies with the type of fish muscle, dark muscle usually contains low levels of protein and moisture than light muscle. Nutritionally fish provides primarily protein and 80 to 90% of food energy is derived from this and so they can be safely used in food to supplement

protein (Pawar & Sonawane, 2013). Ash is defined as the total mineral content of a food, constituting about 0.5 - 5% in fish muscle.

Variations in the biochemical compositions of fish muscle tissue are group specific and species - specific. The biochemical composition depends on season and to a great extent on reaction to size, age, sex, reproductory cycle, breeding season and region of catch (Pawar & Sonawane, 2014). The protein content decreases and fat increases with age in herbivore species, while vice versa is true carnivorous fish (Zafar & Ashraf, 2011). Reproductive cycle greatly influences the protein-glycogen-lipid content in the Oyster tissue (Mitra *et al*., 2008). The spawning and seasonal variations also greatly influence the protein content and water content of the tissue. A number of researchers reported the proximate composition of fresh and processed fish species of fresh, marine, brackish waters (Gopakumar, 1997; Chand *et al*., 2001; Sankar and Ramachandran, 2001; Sankar *et al*., 2013; Begum *et al*., 2014; Rao *et al.*, 2014; Shafakatullah and Krishnamoorthy, 2014). Zworykin (2012) reported reproductive and spawning behavior of the climbing perch (*Anabas testudineus*) in an aquarium. Monalisa *et al*. (2013) carried out a comparative study on nutrient contents of native and hybrid Koi (*Anabas testudineus*) fish in Bangladesh and they revealed the hybrid Koi has good source of proximate composition and are lower in mineral content than native one.

2.3: Materials and Methods

2.3.1: Sampling of fish

The three species of fish selected for analysis were caught from the streams and paddy fields near Tiruvalla, Kerala and brought to the laboratory live. The fish were slaughtered without delay by a blow on the head and sampling of the tissue was done. The fish used for the study were of uniform size having weight and size range of 10-± 2 cm: 100 ± 10 g, 25-± 5 cm: 350 ± 20 g and 20 ± 5 cm: 250 ± 20 g for perch, snakehead and catfish respectively. Muscle tissues from the dorsal side of the fish between the gills and the dorsal fins were used for proximate analysis. All analyses were done in triplicates. Similarly, the samples were collected every month for seasonal variation studies.

2.3.2: Determination of Moisture

Moisture content was estimated by the method of AOAC (2000). The moisture content was determined by drying 10 g sample at 103°C in a thermostatically controlled hot air oven. The samples were taken in a pre - weighed glass dish with cover and kept in oven till the weight became constant. The weight was checked for constant weight by repeatedly heating and then cooling the sample in desiccator. The percentage solid was determined from the above experiment by using the formula

$$\text{Percentage solid} = \frac{\text{Weight of dry sample}}{\text{Weight of wet sample}} \times 100$$

The percentage moisture was calculated by subtracting solid weight % from 100.

2.3.3: Determination of Crude Protein

About 1 g homogenized fish muscle sample was used for determining the crude protein content using Micro Kjeldahl method of AOAC (2000). To the sample taken in the digestion tube, 2 g of digestion mixture ($CuSO_4$ and K_2SO_4 as catalyst in the ratio of 1: 8) and 10 mL of concentrated H_2SO_4 were added to the sample taken in the digestion tube. The samples were digested to a clear solution in a Kjeldahl digestion unit. 50 ml of distilled water was added to the cooled tube slowly till no heat was generated on adding water. The solution was made up to 100 ml. Pipetted out 5 ml of the prepared sample into the Kjeldahl Micro Distillation Apparatus. The bottom end of the condenser was fitted to a delivery tube immersed in 10 ml of 2% boric acid with Tachiro's indicator. 40% NaOH was added to the sample in the distillation unit to make it alkaline. The ammonia, produced on steam distillation was absorbed into the boric acid solution. The distillate collected was back titrated against N/70 H_2SO_4 and using the titer value, nitrogen content was estimated. Crude protein content in the sample was calculated by multiplying the nitrogen content by the factor of 6.25.

$$\text{Percentage of Protein} = \frac{V \times 1 \times 100 \times 100 \times 6.25}{5 \times 5 \times \text{Weight of the sample}}$$

2.3.4: Determination of Crude Lipid

Fat content of the moisture free sample was determined by extracting the fat by Soxhlet extraction method (AOAC, 2000). About 2 g of moisture free sample was

accurately weighed into an extraction thimble (Whatman No.1) and placed in the extractor. The extractor was connected to a pre - weighed dry receiving flask and a water condenser. Petroleum ether (B. P. 40 - 60°C) was used as solvent. The unit was heated in a water bath and temperature was maintained at 40°C - 60°C so that solvent boiled continuously and siphoned at a rate of 5 - 6 times/ h. The extraction was continued till the solvent in the extractor became colorless and fat free. The solvent in the receiving flask was evaporated completely and weighed for fat content.

$$\text{Percentage of Crude Lipid} = \frac{\text{Weigh of fat}}{\text{Weight of sample}} \times 100$$

2.3.5: Determination of Ash

The ash content was estimated by the incineration of the sample according to AOAC (2000). 2 g of moisture free sample taken in a pre - weighed clean dry silica crucible was charred on low heat. Then it was kept at 550°C in a muffle furnace to get a white ash, cooled in the desiccator and weighed.

$$\text{Percentage of Ash} = \frac{\text{Weigh of ash}}{\text{Weight of sample}} \times 100$$

2.3.6: Statistical Analysis

All statistical calculations were performed using IBM SPSS Statistics 20.0 Software. Data analysis was performed using one way analysis of variance (ANOVA) with post-hoc with multiple comparison analysis performed using Duncan test. p values less than or equal to 0.05 were considered as significant. Data are represented as mean ± standard deviation. Correlation analysis between parameters analyzed was done using Pearson Correlation.

2.4: Results

Proximate composition of the three fish species for twelve months were analyzed in order to get an idea regarding influence of seasonal variation on nutritive status of fish muscle tissue in terms of protein, lipid, moisture and ash. The results obtained for twelve months (June 2010 to May 2011) are depicted in the Table 2.1 to 2.3 for the three species Climbing perch, Banded snakehead, and Asian stinging catfish respectively. Data obtained in this study was statistically analyzed using SPSS. One way ANOVA was performed to compare difference in means of crude protein content, lipid content, moisture and ash. This was followed by analysis of Duncan-post hoc test to determine in

more detail the progress of rigor mortis in response to storage time. Pearson Correlation coefficient with level of significance < 0.01 also was tested to analyze relationship between different biochemical compounds.

Data for one way ANOVA shows that the proximate composition of protein, lipid ash and moisture content of the muscle tissue of climbing perch, banded snakehead, and stinging catfish are differ significantly between them ($p< 0.01$) (Appendix 2.4).

Table 2.1: Seasonal changes in proximate composition of climbing perch muscle tissue

Month	Protein (%)	Lipid (%)	Moisture (%)	Ash (%)	Total
June	15.06 ^{c} ±0.20	13.84 $^{c, e}$ ±0.29	69.09 $^{d, e}$ ±0.49	0.91 ^{a} ±0.12	98.90
July	13.02 ^{a} ±0.35	11.08 ^{a} ±0.22	73.37 ^{h} ±0.20	1.22 ^{e} ±0.01	98.69
August	13.60 $^{a, b}$ ±0.26	11.66 ^{a} ±0.19	71.98 ^{g} ±0.28	1.07 $^{b, c, d}$ ±0.05	98.31
September	14.11 ^{b} ±0.26	12.71 ^{b} ±0.13	70.41 ^{f} ±0.23	1.29 $^{e, f}$ ±0.06	98.52
October	15.46 ^{c} ±0.37	12.85 ^{b} ±0.12	69.37 $^{, f}$ ±0.54	1.48 ^{g} ±0.03	99.16
November	16.63 ^{e} ±0.62	13.25 $^{b, c,d}$ ±0.11	67.41 $^{a, b}$ ±0.31	1.40 ^{g} ±0.01	98.69
December	16.37 ^{d,e} ±0.77	12.53 ^{b} ±0.48	68.10 $^{b, c, d}$ ±0.52	1.09 $^{c, d}$ ±0.04	98.09
January	15.21 ^{c} ±0.46	12.77 ^{b} ±0.24	67.83 $^{a, b, c}$ ±0.73	1.18 $^{d, e}$ ±0.03	96.99
February	16.53 ^{e} ±0.19	13.72 $^{c,d, e}$ ±0.13	68.36 $^{b, c, d, e,}$ ±0.13	0.95 $^{a, b}$ ±0.02	99.56
March	17.02 ^{e} ±0.44	12.93 ^{b} ±0.23	66.91 ^{a} ±0.56	1.75 ^{i} ±0.04	98.61
April	17.01 ^{e} ±0.13	14.06 ^{e} ±0.02	66.71 ^{a} ±0.52	1.60 ^{h} ±0.05	99.38
May	16.53 ^{e} ±0.13	13.07 $^{b, c}$ ±0.74	68.79 $^{c, d, e}$ ±0.63	1.03 $^{b, c}$ ±0.06	99.42
Average	15.46	12.87	68.86	1.25	98.44

All values are expressed as mean ± standard deviation, n=3

Different superscripts in the same column indicates significant difference ($p< 0.01$)

Data obtained for the analysis of the seasonal variation in the proximate composition in climbing perch muscle tissue are depicted in Table 2.1 and Appendix 2.1. Compared to stinging catfish and banded snakehead muscle lipid, climbing perch muscle tissue had high lipid content, constituting about 12.87%. The mean value of

protein, moisture and ash were found to be 15.46%, 68.86%, 1.25% respectively. Highest values were observed in the month of March and April for protein (17.02%) and lipid (14.06%) respectively, while lowest values were in the month of July for both protein (13.02%) and lipid (11.08%). Highest and lowest values for moisture content were noticed in the month of July (73.37%) and March (66.91%). Protein ($p < 0.01$) and lipid ($p < 0.01$) content showed a negative correlation with moisture content within the muscle tissue. Additionally, between protein and lipid a positive relation was observed ($p < 0.01$). Ash content showed no significant correlation with protein, lipid or moisture content in the muscle tissue and its maximum value was noticed in the month of March (1.75%). ANOVA results showed that there was a significant variation between months ($p < 0.01$).

Table 2.2: Seasonal changes in proximate composition of banded snakehead muscle tissue

Month	Protein (%)	Lipid (%)	Moisture (%)	Ash (%)	Total
June	16.44 ^{a,b} ±0.50	3.40 ^{a} ±0.56	76.84 ^{b} ±0.58	1.30 ^{a} ±0.04	97.98
July	15.94 ^{a} ±0.23	3.50 ^{a} ±0.26	78.19 ^{c} ±0.81	1.46 ^{a,b} ±0.12	99.09
August	16.09 ^{a,b} ±0.39	3.49 ^{a} ±0.45	77.74 ^{b} ±0.64	1.40 ^{a,b} ±0.18	98.72
September	18.57 ^{f} ±0. 27	4.79 ^{b} ±0.47	73.43 ^{a} ±0.86	1.37 ^{a,b} ±0.01	98.16
October	18.40 ^{e,f} ±0.33	5.08 ^{b} ±0.50	73.77 ^{a} ±0.52	1.33 ^{a,b} ±0.02	98.58
November	17.74 ^{d,e} ±0.46	4.50 ^{b} ±0.33	72.84 ^{a} ±0.96	1.84 ^{a,b} ±0.03	96.92
December	16.79 ^{b,c} ±0.19	3.70 ^{a} ±0.51	75.99 ^{b} ±0.65	1.83 ^{a,b} ±0.10	98.31
January	17.27 ^{c,d} ±0.29	4.91 ^{b} ±0.25	73.07 ^{a} ±0.54	1.84 ^{a,b} ±0.08	97.09
February	18.93 ^{f,g} ±0.30	5.49 ^{b} ±0.20	73.19 ^{a} ±0.98	1.89 ^{b} ±0.16	99.50
March	18.92 ^{f,g} ±0.11	5.43 ^{b} ±0.43	72.84 ^{a} ±0.95	1.50 ^{a,b} ±0.04	98.69
April	19. 45 ^{g} ±0.34	5.03 ^{b} ±0.21	73.05 ^{a} ±0.60	1.39 ^{a,b} ±0.03	98.50
May	19. 03 ^{f,g} ±0.29	4.95 ^{b} ±0.63	72.82 ^{a} ±0.98	1.77 ^{a,b} ±0.69	98.59
Average	17.99	4.65	74.41	1.54	98.59

All values are expressed as mean ± standard deviation, n=3
Different superscripts in the same column indicates significant difference ($p < 0.01$)

Table 2.2 and Appendix 2.2 show data of proximate composition in banded snakehead fish muscle from June to May. The average value of protein, lipid, moisture and ash content in muscle tissue were found to be 17.99%, 4.65%, 74.41% and 1.54%, respectively. The result showed that the protein content had slight fluctuations with the highest value in the month of April (19.45%). Lipid content also showed similar pattern of fluctuation and maximum lipid content was observed in the month of February (5.49%). A strong negative correlation between lipid and water content was observed ($p < 0.01$) within the muscle tissue. Also muscle protein showed an inverse relation with water content in it ($p < 0.01$), while it had a good positive co-relation with that of lipid content ($p < 0.01$). The lowest value for moisture content was noted in the month of May (72.82%) while highest was in the month of July (78.19%). Ash content showed no significant correlation with other constituents within the muscle tissue analyzed. The highest and lowest value for ash content were noticed in the month of February (1.89%) and June (1.30%) respectively.

Table 2.3: Seasonal changes in proximate composition of stinging catfish muscle tissue

Month	Protein (%)	Lipid (%)	Moisture (%)	Ash (%)	Total
June	14.30 ^{b} ±0.52	*1.87* ^{a} ±0.26	82.01 ^{e} ±1.00	1.35 ^{a} ±0.37	99.53
July	13.87 ^{b} ±0.23	2.27 ^{a,b} ±0.26	81.67 ^{c,d} ±1.28	1.18 ^{a} ±0.22	98.99
August	13.01 ^{a} ±0.39	2.22 ^{a} ±0.05	80.56 ^{c,d} ±0.64	1.63 ^{a,b} ±0.19	97.42
September	14.48 ^{b,c} ±0.26	2.08 ^{a} ±0.55	79.36 ^{b,c,d} ±0.75	1.90 ^{a,b} ±0.04	97.82
October	15.22 ^{c,d} ±0.33	2.13 ^{a} ±0.15	78.78 ^{b,c} ±0.51	1.20 ^{a} ±0.23	97.42
November	17.46 ^{f} ±0.41	2.86 ^{c} ±0.12	77.06 ^{a,b} ±0. 95	1.89 ^{a,b} ±0.28	99.27
December	16.28 ^{e} ±0.33	2.80 ^{c} ±0.11	77.65 ^{b} ±0.97	2.91 ^{c} ±0.10	99.64
January	15.97 ^{d,e} ±0.29	2.76 ^{b,c} ±0.02	79.47 ^{b,c,d} ±1.45	1.22 ^{a} ±0.08	99.42
February	16.05 ^{d,e} ±0.43	2.01 ^{a} ±0.20	79.04 ^{b,c} ±0. 98	1.32 ^{a} ±0.16	98.44
March	18.32 ^{f} ±0.11	2.6 ^{c} ±0.13	77.01 $^{a, b}$ ±0. 95	1.27 ^{a} ±0.74	99.20
April	18.23 ^{f} ±0.53	3.12 ^{c} ±0.12	75.01 ^{a} ±0.60	1.17 ^{a} ±0.26	97.53
May	17.97 ^{f} ±0.26	2.89 ^{c} ±0.16	75.07 ^{a} ±0.98	2.10 ^{b} ±0.07	98.03
Average	16.00	2.42	78.72	1.59	98.73

All values are expressed as mean ± standard deviation, n=3
Different superscripts in the same column indicates significant difference ($p < 0.01$)

Table 2.3 and Appendix 2.3 shows data for the monthly variation in the proximate composition of stinging catfish muscle tissue. The mean protein, lipid, moisture and ash content in the muscle tissue were found to be 16.00%, 2.42%, 78.72%, and 1.59% respectively. The protein content in muscle had slight fluctuation with highest value noticed in the month of March (18.32%) and the lowest in the month of August (13.01%). The lipid content also showed similar pattern of fluctuation, lowest and highest value observed in the month of June (1.87%), April (3.12%) respectively. Moisture content in muscle tissue showed an inverse relation with that of lipid ($p < 0.01$) and protein values ($p < 0.01$)and the lowest value was noted in the month of April (75.01%) and highest value in the month of June (82.01%). A positive relation was observed between protein and lipid content ($p < 0.01$). The highest value for total ash content was observed in December (2.91%) and a second peak was observed in the month of May (2.10%). No significant correlations were observed with other constituents within the muscle tissue analyzed. ANOVA results showed that there was a significant variation between months ($p< 0.01$).

2.5: Discussion

Influence of seasonal variation on proximate composition of fish and fishery products are equally important as the nutritional changes that occur in each component during various processing techniques used. Marimuthu *et al.* (2012) analyzed the proximate composition of striped snakehead fish muscle and the average value of ash, fat, protein and moisture was found to be 0.77, 5.9, 13.9 and 77.2% respectively. Similar work was undertake by Begum *et al.* (2014) to analyze the proximate composition of Koi (*Anabas testudineus*) collected from a rice field from Mymensingh, Bangladesh and the mean content of moisture, protein, lipid and ash of raw fish in wet basis was found to be 70.07, 16.97, 13.01, and 0.95 % respectively. Also Monalisa *et al*. (2013) analyzed the proximate composition in *Anabas testudineus* muscle in Bangladesh and the mean values were found to be 70.26, 18.05, 8.64, 1.30 % for moisture, protein, fat and ash respectively. Nargis (2006) noticed that in *Anabas testudineus* the protein content was higher in medium sized fishes and gradually decreased with the increase of age. Lipid content was higher in large-sized male than that of females. Moreover Banded snakehead has highest average protein content in

their muscle tissue and Climbing perch has least protein content proving that the protein content in the fish muscle varies based on their size and species.

Results show that the maximum protein content was observed in summer season (March-May) which is in agreement with previous reports of Jan *et al.* (2012). A slight decrease in protein content was observed in winter season (December-February) and it may be due to unavailability of food resulting in poor growth. An increase in food consumption in the month of February and March may be advantageous for building up of energy reserve in the form of protein which could be used in gonadal maturation. During late summer and early monsoon (May–July) season, the muscle protein content started to decline gradually and this could be due to its translocation into ovaries to meet the energy requirement of fish. Peak spawning in all three fish species, analyzed coincided with peak rainfall, i.e. from June to July. Jyotsna *et al*. (1995) has reported that the change in the protein content during spawning season was due to change in the endocrine system that monitored supply of nutrients to gonads from all parts of body including liver and muscles. When fish matures, proteins accumulated in gonads and at the time of breeding, these were released either as eggs or milt that carry the protein along with eggs resulting in reduction in protein level. After spawning period, a marked increase in protein content was observed due to recovery to normal life.

The muscle tissue from climbing perch has high lipid content than banded snakehead and stinging catfish. Peak value of the muscle lipid content was observed in early summer season (March-April). Second highest value for lipid content was also observed in post monsoon season. Rani *et al*. (2016) reports that in the case of *Euthynnus affinis*, the high mean concentration of fat was found in post-monsoon season. According to Khitouni *et al.* (2014) the highest fat contents were recorded during September for both golden grey mullet *Liza aurata* males and females. This could be due to optimum availability of food and active feeding. Sharma (2005) reported that algal blooms and plankton acquired maxima during the post monsoon period. Sharp reductions in the lipid content were observed during early monsoon season and this season was noticed as spawning period. This might be due to utilization of stored lipid as energy source to compensate the high energy demands, during the ovulation and spawning period. Langer *et al*. (2013) stated that decline in fat content

might be due to low feeding intensity and low availability of food items in *Paratelphusa masoniana*. Diana & Mackay (1979), John and Hameed (1995), Jonsson & Jonsson (2005), Langer *et al.* (2008) and Samyal *et al*. (2011) have discussed well about reduction in the muscles lipid content for the development and maturation of gonads. Shamsan & Ansari (2010) also observed a rapid fall in lipid content in *Sillago sihama* (Forsskal) during spawning season. Studies of Oduor-Odote *et al*. (2008) reported that the lipid content in fish fluctuate greatly and is associated to feed intake, migratory swimming or sexual changes in connection with spawning.

Furthermore, Shreni (1980), Ravichandran *et al*.(2012), Rodrigues *et al*.(2013) conveyed that the seasonal cycles of various biochemical constituents in the tissues of the fish were dependent on feeding and the cycle of maturation and depletion of gonad. Shaikh & Prakash (2011) reported that the gonadosomatic index (GSI) values correlated well with increased amount of protein and lipids in both pre-breeding and breeding seasons of three major carps due to probable augmented vitellogenesis in ovary and spermatogenesis in testes that required large amount of lipoproteins. Kalay *et al*. (2008) reported that protein contents decreased with age and fat contents increased accordingly, while no effect on other elements like Cu, Zn and Fe. They also reported a negative relationship between protein and lipid levels with age and size. On the contrary, Zafar & Ashraf (2011) reported that the proteins kept on increasing with age/size while lipids declined accordingly.

The average moisture content was found to be high in stinging catfish and least in climbing perch. It was also noticed that the mean water content in all three fish species muscle reached its maximum in the month of June to August, and a second maximum observed during winter season. The moisture content also showed an inverse relationship with fat and protein content in all the three fish species. This inverse relation might be due to low atmospheric temperature, low food intake, unavailability of food, and high energy demands to homeostasis the body temperature during monsoon and winter season. Merayo, 1996; Vida & Bogdanovic, 2012, earlier propounded similar inverse relations.

Ash content showed no significant correlation with other constituents in the muscle tissue of three freshwater fishes, proving there is any direct relationship

between the ash with protein, lipid and moisture content in response to their feeding or spawning activities. This result was in good agreement with Jafri & Khawaja (1968) who performed studies in freshwater murrel. On the contrary, Langer *et al.* (2013) reported an increase in the ash content in the female crabs during the post spawning period.

2.6: Conclusion

The results suggested that the proximate composition in fish muscle significantly varied during different seasons. High protein and lipid content were detected during non-spawning period and minimum during spawning months, which was inversely related to the moisture content. The present studies provide useful information on variation in proximate composition of the three fish species with seasons which provides a useful data base for the processing industry. The data can also be considered for the evaluation of their physiological and nutritional requirements at different periods of their lifespan. In addition, this study gives necessary information for adopting suitable processing technology for development of fish and fishery products from these freshwater fishes, thus improving the economic value of these freshwater fish species which are considerably lower than the larger marine fish species. This also can enhance the chances for aqua-culturing these fish species so as to satisfy the nutrient demand of malnourishment.

Chapter 3

Chapter 3

Changes in physico-chemical parameters of fish muscle tissue during storage at ambient temperature and iced condition

Contents

3.1: Introduction

Fish muscle undergoes different stages of post-mortem physico-chemical changes from the point of catch to the processing site and further during storage. Other than the microbial action, enzymatic proteolysis of muscular components adversely affects the total quality in terms of nutritional profile and functional properties. These quality problems are directly related to the type of fish, the part of the body of the fish selected and other physico-chemical properties of the fish. Information on processing and preservation of freshwater fish and fishery products is an indispensable prerequisite for future growth of fisheries industry. Hence, studies on physical and biochemical changes in fish muscle during post-mortem storage is very much helpful for standardizing optimum processing and preservation condition in order to assure the total quality of the selected fishes. Additionally, storage of fish in ice is the normal procedure to preserve the fresh fish by common people. But most of the time the fish may be subjected to temperature abuse for a limited period from harvesting to the final consumer. In this context, the present study attempts to evaluate the following factors in the three freshwater fish species selected.

1. To analyze the progress of rigor mortis with time at ambient temperature and in iced storage condition
2. To analyze the effect of storage temperature and time on changes in pH, water holding capacity, cooking loss and expressible water content in the fishes
3. To analyze adenosine nucleotide degradation pattern in fish muscle stored at ambient temperature and iced condition

3.2: Review of Literature

Soon after the death of an animal, the circulatory system ceases to supply the muscle with oxygen and metabolites. Since no oxygen is available for normal cellular respiration, the mitochondrial oxidative phosphorylation system stops to function (Hultin, 1985). Anaerobic oxidation of glycogen is the only possible way for the production of energy once the circulatory system has been disturbed and to replenish ATP, which leads to build up of lactic acid (Watabe *et al*., 1989). The initial level of muscle glycogen has a large impact on the muscle pH (Roth *et al*., 2009; Black and Love 1988). Kelly *et al*. (1966) specified that the fish with a high pH has a soft texture, while fish with low pH has a firmer texture. A number of investigations have been carried out on the pH changes in fish muscle at ambient temperature and during chilled storage condition (Haque *et al*., 1997; Pacheco-Aguilar *et al*., 2003; Hossain *et al*., 2005a; Massa *et al*., 2005; Ocano-Higuera *et al*., 2009; Susanto *et al*., 2011; Rammouz *et al*., 2013; Makawa *et al*., 2014; Viji *et al*., 2015). Variation in the pH of fish muscle mainly depends on the species; which in turn vary even in same fish species (Pedrosa-Menabrito and Regenstein, 1988). It is also reported that the rested-harvest fish has higher muscle and blood pH and lower blood and muscle lactate than the stressed fishes that greatly influence the degree and extent of post-mortem autolytic changes. These physiological parameters subsequently influence post-mortem pH (Hiltz and Dyer, 1971). pH fall in post-mortem fish muscle significantly affected by sex (Varga et al., 2010; Zhang *et al*., 2010; Rammouz *et al*., 2013). Degree of post-mortem pH reduction is higher in fish than mammals, while lower than birds (Varga *et al.*, 2010).

Post-mortem fish muscle undergoes a sequence of rigor mortis process. Immediately after death, the fish muscle is in fully relaxed condition. In this pre-rigor stage, fish muscle is very soft and pliable and texture is firm and elastic to touch. Both ATP and ADP act as plasticizers for actin and myosin, thus preventing their interaction and actomyosin cross bridge formation and keeping the muscle in state of relaxation (Louovois & Kyrana, 2005). After some time, the fish muscle begins to stiffen, whole muscle become inflexible, cannot be stretched significantly without breaking, and fish reaches in-rigor stage. Eventually, after hours or days, fish muscle gradually begins to become softer and in this state, the fish is said to have reached post-rigor condition.

Production of nitrogenous compounds as a result of degradation of complex molecules leads to an increase in post rigor muscle pH.

Water content in edible part of the fish varies widely between 65-90%. About 4 to 5% of water in tissue is directly bound to the polar surface of protein molecule viz., carboxyl and amino acid sulphydryl group while remainder being loosely bound and immobilized to varying extent due to different forces acting upon them (Hamm, 1960). The pH of a biological system has a remarkable impact on its ability to bind with water. Water holding capacity is the common term that defines the amount of water that is bound or retained by a protein under highly defined conditions (Hultin, 1985), while expressible moisture content is the actual loss of the moisture from a sample during the application of pressure. Thus, the water holding capacity and expressible water content provides different information about physical properties of fish muscle. The sensory properties of the fish muscle are influenced by the water content and its distribution. During the process of cooking, a decrease in the levels of moisture and fat were observed (Dhanapal *et al*., 2012; Aberoumand, 2014). Fishes are usually cooked before its consumption. Cooking will also lead to some irreversible changes in tissue components.

Degradation of adenosine nucleotides and their catabolism products in post-mortem fish muscle is widely considered as chemical indices of freshness of fish. The endogenous enzymes of fish muscle degrade ATP at the initial stages of the storage period and in later stage are mediated through microbial metabolism (Surette *et al*., 1988; Boyle *et al*., 1991). In post-mortem fish muscle, the degradation of adenosine triphosphate (ATP) takes place according to the following sequence: adenosine triphosphate (ATP) → adenosine diphosphate (ADP) → adenosine monophosphate (AMP) → Inosine monophosphate (IMP) → Inosine (HxR) → Hypoxanthine (Hx) → Xanthine (X) (Kassemsarn *et al*. 1963).

Immediately after death, ATP synthesis in fish muscle through phosphorylation process is catalyzed by creatine kinase from creatine phosphate. Iwamoto *et al*. (1988) reported that creatine phosphate is degraded prior to the breakdown of ATP. ATP level drop down when creatine phosphate and ATP reaches in same, ATP content begins to decrease (Watabe *et al*., 1991), which is a fast process. Accumulation of hypoxanthine is

responsible for the progressive loss of desirable fresh meat flavor (Fletcher & Statham, 1988). A number of investigations have been made in different fish species to analyze the post mortem degradation of ATP and its metabolites (Suwetja *et al.,* 1989; Greene *et al.*, 1990; Sakaguchi *et al.,* 1990**;** Mattio *et al*., 1992**;** Mattio *et al*., 2001; Massa *et al.*, 2003; Pacheco-Aguilar *et al.,* 2003; Massa *et al*., 2005; Ocaño-Higuera *et al.*, 2006; Castillo- Yanez *et al.,* 2014; Vilas *et al.,* 2017). The degree and pattern of changes in ATP and their catabolism products significantly differ with fish species (Ryder, 1985), muscle type (Obatake *et al*., 1988), and factors related to handling and storage conditions (Surette *et al.*, 1988). Morkore *et al*. (2010) studied the relevance of season and nucleotide catabolism on changes in fillet quality during chilled storage of raw Atlantic salmon (*Salmo salar L.*), and reported that conversion of hypoxanthine (Hx) showed the highest seasonal variation among the nucleotide metabolites.

Post-mortem accumulation of inosine or hypoxanthine reveals the poor quality. K value serve as an index of freshness of many of freshwater fish. The index of freshness is inspected based on the concentration of different adenosine nucleotides in terms of 'K and is expressed in percentage (Saito *et al*., 1959). The rates and patterns of changes in ATP and their catabolic products much significantly differ with fish species; muscle type and factors related to handling and storage conditions (Ryder, 1985; Surette *et al*., 1988; Mishima *et al*., 2005; Sun *et al.,* 2015). It is designated that very fresh fishery products with K value lower than 20 % is considered as "suchi" grade (optimal grade of freshness), moderately fresh with value 40% and not fresh with value higher than 60% (Saito *et al*., 1959, Ehira and Uchiyama, 1987). As a function of peculiar bitter taste of spoiled fish, estimation of H value is considered as a good indicator of fish freshness with respect to physiological and sensory aspects.

3.3: Materials and Methods

3.3.1: Sampling of Fish

Fish were caught in live condition using cast net from the nearby paddy fields of Tiruvalla, Kerala, India by fishermen and transported immediately to the laboratory in a container with sufficient water to immerse the live fish. The fish were slaughtered after a blow on the head.

All samples were divided into two groups. One set of fish was kept in plastic insulated boxes with ice: fish ratio 0.5:1 and stored in a refrigerated chamber (4 ^{O}C) for 18 days. During storage, ice was periodically added and replenished every 12 hours. The day of slaughter was defined as day 0. Fish were sampled in triplicate at 0, 1, 3, 5, 7, 9, 12, 15 and 18 days of storage and used for analysis. Second set of fish was kept at ambient temperature (25^{O}C) and was stored for 18 hours. The time of slaughter was defined as zero hour. The fish were sampled at 0, 1, 2, 3, 4, 5, 6, 7, 8, 12, 15 and18 hours after death and used for analysis. Sampling of fish was done in triplicate.

The muscle tissue between the gills and the dorsal fins were used for analyses. Precautions were taken to maintain the tissue temperature below 4 ^{O}C wherever necessary. All the analysis was done in triplicate.

3.3.2: Determination of Rigor index

Rigor index of the whole fish was determined according to the method of Bito *et al*. (1983).

The fish was placed on a horizontal table protruding half of its body (tail part) from the edge of the table. Distance from the horizontal line to tail of fish was measured at one-hour interval and rigor index was calculated using the following equation:

$$\text{Rigor-index (\%)} = \frac{(D_0 - D)}{D_0} \times 100$$

where,

D_0 - Distance of the base of the caudal fin from horizontal line of the table at the start of the experiment (that is in pre-rigor).

D - Distance of the base of the caudal fin from the horizontal line of the table at subsequent storage period respectively.

Duration of onset and resolution of rigor was measured according to Azam *et al.* (1990).

On-set of rigor: The time when tail started bending slightly.

On-set of full rigor: The time when the whole fish became very rigid.

Duration of full rigor: The time when the whole fish remained very rigid.

Resolution of rigor: The time when tail becomes almost flaccid after rigor was resolved.

Duration of total rigor: The differences in time between resolution of rigor and onset of rigor.

3.3.3: Measurement of pH

One gram of fish muscle was homogenized in 10 mL of distilled water, and the pH values were determined with a pH meter (ELICO pH Meter LI 127).

3.3.4: Moisture content

Moisture content of homogenized sample was determined by drying the sample in an oven at 105^{O}C till the weight became constant. The water content was determined as the weight loss after drying (AOAC, 2000).

3.3.5: Water holding capacity

Water holding capacity (WHC) was determined according to the method described by Borresen (1980) with slight modifications. 2 g of filleted sample was centrifuged at 4500 rpm for 15 min by keeping a filter paper (Whatman filter paper No 1) at the bottom of the centrifuge tube to remove the water content from the fillet. After centrifugation, the fillet was weighed again, and the difference between before and after centrifugation was calculated. The moisture content determined used to calculate the water holding capacity.

The water holding capacity was calculated as the ratio of water remaining compared to the water content in the sample before centrifugation by using the formula:

$$WHC = \frac{W - \Delta r}{W} \times 100$$

where:

W is the water content of the sample before centrifugation (%).

Δr is the weight lost by centrifugation (%) and is calculated by the formula:

$$\Delta r = \frac{W_1 - W_2}{W_1} \times 100$$

where:

W_1 is the weight of the original sample (g)

W_2 is the weight of the sample after centrifugation (g)

3.3.6: Expressible water content

Expressible water content (EWC) was determined according to the method of Benjakul (2003). 2 g of sample was placed between 6 filter papers (Whatman filter paper 1) and a standard weight of 5 Kg was placed on top of the sample and maintained exactly for 2 minutes. Removed the sample and weighed again. EWC was calculated as percentage of sample weight according to the following formula:

$$\text{Expressible water (\%)} = \frac{Pre-pressed\ weight\ (g) - after-\ pressed\ weight\ (g)}{\text{Pre-pressed weight (g)}} \times 100$$

3.3.7: Cook loss

Cook loss was determined according to a modified version of the method described by Borresen (1980). Homogenised sample (2 g) was put in container with a filter, incubated at 100 ^{O}C for 15 minutes and then left to thoroughly cool down at room temperature. Removed the sample and weight out. Cook loss was calculated as the percentage of reduction of the cooked sample compared with the raw sample and is presented as % lost sample.

The cooking loss was calculated as: $= \frac{W_{raw} - W_{cooked}}{W_{raw}} \times 100$

where:

W-cooked is the weight of cooked sample.

W-raw is the weight of raw sample before cooking

3.3.8: Nucleotide Analysis

Two grams of muscle tissue was taken from the mid dorsal side of the fish. Sampling was done in a cold room. Flesh was accurately weighed and homogenized with 10 ml of 0.6M cold (0–4 ^{O}C) perchloric acid for 10 minutes. The suspension was centrifuged for 10 min at 12,000 rpm at 2–4 ^{O}C (Eppendorf Centrifuge- 5430 R, Hamburg, Germany). The supernatant was immediately neutralized to pH 6.5–6.8 with 1M potassium hydroxide. The extract was stored in ice for 30 min. The precipitate was removed by filtering the solution through Whatman No. 1 filter paper disc. The filtrate was diluted to 20 ml with distilled water and stored at -20 ^{O}C until analysis. Prior to the High-performance liquid chromatography (HPLC) analysis, each sample was filtered through a 0.45µm filter (Nylon filter).

The determination of nucleotides and related compounds was done using HPLC according to the procedure of Ryder (1985). The HPLC system used in this study was Waters 2487 model HPLC system equipped with binary pump model M515, Gradient mixer solvent delivery system, Column Oven and a manual injector. The separations were performed on a Symmetry C 18 column (5 µm pore size, 4.6mm width x 250 mm length) maintained at 30 ^{O}C. The mobile phase consisted of 0.04 M K_2HPO_4 + 0.06 M KH_2PO_4, pH 7 (solution A) and acetonitrile (solution B) as follows: isocratic elution 100% A, 0–20 min. The eluate was monitored by UV absorption at 256 nm.

The chromatographic peaks of ATP and its breakdown products were determined by comparing the retention time of HPLC peak between samples with authentic external standard calibration using known concentrations of the following standards: ATP, ADP, AMP, IMP, Inosine and hypoxanthine procured from Sigma Aldrich. Data analysis was performed using EMPOWER 2 Chromatography software.

3.3.9: Determination of K and H value

The K value is used as a commercial index for estimating fish freshness. K value was determined by calculating the ratio of the sum of inosine (HxR) and hypoxanthine (Hx) to the sum of ATP and the catabolism products (ADP, AMP, IMP, HxR and Hx) expressed as a percentage (Haard, 1992; Huss, 1995). K-values were calculated according to Saito *et al.* (1959), who used the formula:

$$\text{K Value} = \frac{(\text{HxR} + \text{Hx})}{(\text{ATP}+\text{ADP}+\text{AMP}+\text{IMP}+\text{HxR}+\text{Hx})} \times 100$$

K-value of 20% is recommended as the freshness limit and 60% as the rejection point.

In addition, Luong *et al*. (1992) described a new method to evaluate the freshness of fish muscle as hypoxanthine ratio or H value. The usefulness of these freshness indices depends on the fish species being examined.

$$\text{H-value} = \frac{(\text{Hx})}{(\text{IMP}+\text{Hx}+\text{HxR})} \times 100$$

3.3.10: Statistical analysis

All statistical calculations were performed in IBM SPSS Statistics 20.0 Software. Data analysis was performed using analysis of one way analysis of variance (ANOVA) with post-hoc with multiple comparison analysis performed using Duncan

test**.** Correlation analysis between parameters analyzed was done using Pearson Correlation.

3.4: Results

3.4.1: Changes in Rigor index

Rigor index was analyzed in order to get an idea regarding the progress of rigor mortis in whole fish with response to an increase in storage time. The result obtained for the analysis of rigor index of climbing perch, banded snakehead, and stinging catfish stored at ambient temperature for 18 hours is depicted in Table 3.1 and that of iced condition for 18 days in Table 3.2. The obtained values are statistically analyzed using IBM SPSS 20.0 for windows. One-way ANOVA test was employed to compare difference in means of rigor index of selected fish, followed by Duncan-post hoc test to get more details regarding progress of rigor mortis in response to storage time. Statistical analysis was also performed to compare the progress of rigor index among the three selected fish species.

Table. 3.1: Variations in rigor index of climbing perch, banded snakehead and stinging catfish stored at ambient temperature

Storage time (Hours)	Rigor index (%)		
	Climbing Perch	Banded snakehead	Stinging catfish
0	0 ^{a}	0 ^{a}	0 ^{a}
1	5.93±0.71 ^{b}	11.17±1.17 ^{b}	11.95±1.34 ^{b}
2	12.85±3.03 ^{c}	32.24±3.16 ^{d}	52.80±1.68 ^{e}
3	38.78±0.30 ^{d}	53.21±1.12 ^{f}	68.37±0.89 ^{g}
4	65.64±1.91 ^{g}	64.43±2.02 ^{h}	78.37±0.88 ^{i}
5	88.64±1.92 ^{j}	85.93±0.09 ^{j}	78.37±0.88 ^{i}
6	88.64±1.92 ^{j}	85.93±0.09 ^{j}	78.37±0.88 ^{i}
7	88.64±1.92 ^{j}	85.93±0.09 ^{j}	73.66±0.93 ^{h}
8	88.64±1.92 ^{j}	83.71±3.04 ^{j}	64.49±2.11 ^{f}
10	82.27±1.80 ^{i}	77.71±3.24 ^{i}	53.41±2.00 ^{e}
12	71.53±2.08 ^{h}	61.33±2.35 ^{g}	31.13±1.59^{d}
15	58.42±2.23 ^{f}	36.99±0.83 ^{e}	15.06±1.50 ^{c}
18	45.05±2.89 ^{e}	28.92±2.38 ^{c}	10.45±2.05 ^{b}

All values are expressed as mean ± standard deviation, n=3

Different superscripts in the same column indicates significant difference ($p < 0.05$)

Result for one way ANOVA showed that there was significant difference in rigor index in all the three fish stored at ambient temperature in response to the progress of storage time (Appendix 3.1). The results obtained also showed that banded snakehead (85.93%) and climbing perch (88.64%) reached the full rigor condition within 5 hour stored at ambient temperature and it continued till 7th hour and 8th hour respectively. Stinging catfish reached full rigor stage in 4th hour after death (78.37%) and continued until 6th hour, there after noted a gradual decrease in all cases during the period of storage. It was also noticed that there is significant level of difference in progress of rigor mortis among the fish species ($p< 0.05$).

Table 3.2: Variations in rigor index of climbing perch, banded snakehead and stinging catfish stored in ice

Storage time (Days)	Rigor index (%)		
	Climbing Perch	Banded snakehead	Stinging catfish
0	0 ^{a}	0^{a}	0^{a}
1	86.44±3.05^{g}	83.07±1.44^{h}	76.52±2.70^{h}
3	88.51±1.73 ^{g}	83.07±1.44^{h}	75.71±3.24^{h}
5	88.51±1.73 ^{g}	78.64±2.33^{g}	63.66±0.93^{g}
7	82.01±3.21^{f}	70.98±2.02^{f}	58.71±1.81^{f}
9	71.14±2.26^{e}	59.36±0.98^{e}	39.12±2.65^{e}
12	62.19±3.01^{d}	55.21±3.35^{d}	28.66±1.88^{d}
15	54.91±2.92^{c}	35.91±1.29^{c}	19.56±4.85^{c}
18	42.26±4.48 ^{b}	29.51±3.38^{b}	12.95±1.48^{b}

All values are expressed as mean ± standard deviation, n=3

Different superscripts in the same column indicates significant difference ($p < 0.05$)

Table 3.2 and Appendix 3.2 shows the results of changes in values of rigor index in the three fish species stored at iced condition for 18 days. Values of rigor index are expressed in percentage. Data obtained showed that all the fish attained full rigor stage within 1st day of storage in ice. Climbing perch showed highest rigor index (88.51%) and full rigor continued till 5th day. Stinging catfish showed lowest rigor index (76.52%) and it reached post rigor condition within the first day itself. Banded snakehead shown the highest rigor index value of 83.07%, and duration of full rigor was found till third day. Additionally analysis of variance shows that there is a significance difference in progress of rigor among the fish species ($p < 0.05$).

3.4.2: pH changes in post-mortem fish muscle

Data obtained for the analysis of muscle pH of climbing perch, banded snakehead, and stinging catfish stored at ambient temperature (25 ^{O}C ± 2 ^{O}C) for 18 hours and at iced condition for 18 days are given in the Table 3.3 and Table 3.4 respectively. The statistical analysis was done using SPSS. One-way ANOVA test was performed to compare difference in means of pH values obtained during different storage time. Duncan -post hoc analysis was performed to get further information regarding changes in tissue pH value in response to storage time. General linear model test was performed to analyze the changes regarding pH value between three fish species selected for the study.

Table 3.3: Changes in pH of climbing perch, banded snakehead and stinging catfish muscle tissue stored at ambient temperature

Storage time (Hours)	pH		
	Climbing perch	Banded snakehead	Stinging catfish
0	6.94±0.02^{e}	6.90±0.01^{e}	7.07±0.03^{b}
1	6.89±0.02 ^{d}	6.86±0.01^{d}	7.04±0.03^{b}
2	6.85±0.01 $^{b, c, d}$	6.81±0.01$^{b, c}$	6.97±0.00^{a}
3	6.85±0.01$^{b, c,d}$	6.80±0.01$^{b, c}$	6.96±0.01^{a}
4	6.83±0.03$^{a, b, c}$	6.78±0.01$^{a, b}$	6.97±0.02^{a}
5	6.80±0.02^{a}	6.75±0.04^{a}	7.04±0.02^{b}
6	6.82±0.02$^{a, b}$	6.79±0.01$^{b, c}$	7.05±0.03^{b}
7	6.87±0.02 $^{c, d}$	6.82±0.02 ^{c}	7.17±0.05^{c}
8	6.93±0.04 ^{e}	6.87±0.01^{d}	7.24±0.00 ^{d}
10	6.97±0.04 ^{e}	6.96±0.03^{f}	7.26±0.01^{d}
12	7.03±0.02 ^{f}	6.98±0.01^{f}	7.26±0.01^{d}
15	7.06±0.04 ^{f}	7.03±0.03^{g}	7.3±0.01^{e}
18	7.12±0.04^{g}	7.08±0.02^{h}	7.32±0.02^{e}

All values are expressed as mean ± standard deviation, n=3

Different superscripts in the same column indicates significant difference ($p < 0.05$)

The pH in fresh samples of the three species ranged from 6.90 to 7.07. pH value in the samples decreased to minimum level within the 3rd hour in stinging catfish and

5th hour of storage at ambient temperature in banded snakehead and climbing perch. Upon further storage at ambient temperature, its values gradually increased to 7.12, 7.08 and 7.32 in climbing perch, banded snakehead and Asian stinging catfish respectively. Analysis of variance showed that change in pH value was affected by the time at ambient temperature, variation was also observed among the fish species studied ($p< 0.05$) (Appendix 3.3).

Table 3.4: Changes in pH of climbing perch, banded snakehead and stinging catfish fish muscle tissue stored at iced condition

Storage time (Days)	pH		
	Climbing perch	Banded snakehead	Stinging catfish
0	$6.97\pm0.01^{a, b}$	6.94 ± 0.01^{c}	7.00 ± 0.03^{a}
1	6.90 ± 0.04^{a}	6.83 ± 0.02^{a}	6.97 ± 0.01^{a}
3	6.90 ± 0.05^{a}	6.87 ± 0.02^{b}	7.09 ± 0.01^{b}
5	6.95 ± 0.03^{a}	6.82 ± 0.01^{a}	7.10 ± 0.01^{b}
7	$7.02\pm0.03^{b, c}$	6.89 ± 0.02^{b}	7.16 ± 0.01^{c}
9	$7.06\pm0.01^{c, d}$	$6.97\pm0.01^{c, d}$	7.16 ± 0.02^{c}
12	$7.07\pm0.01^{c, d}$	6.98 ± 0.01^{d}	7.22 ± 0.01^{d}
15	$7.08\pm0.02^{c, d}$	7.03 ± 0.02^{e}	7.26 ± 0.01^{e}
18	7.11 ± 0.02^{d}	7.07 ± 0.02^{f}	7.30 ± 0.01^{f}

All values are expressed as mean ± standard deviation, n=3
Different superscripts in the same column indicates significant difference ($p < 0.05$)

Table. 3.4 and Appendix 3. 4 show the post-mortem changes in the pH value of three fish species at ice stored condition. Significant variation in post-mortem muscle tissue pH was observed between the three fish species studied ($p< 0.05$). The pH values of 6.97, 6.94 and 7.00 were shown in the fresh samples of climbing perch, banded snakehead and Asian stinging catfish respectively. Within first day of storage of climbing perch and Asian stinging and third day of storage in banded snakehead, the muscle tissue pH decreased to 6.90, 6.97 and 6.67 respectively. Thereafter an increase in the pH value was noticed with the storage period in all cases. The statistical analysis also showed that the pH value was affected by the storage time in ice ($p< 0.05$).

3.4.3: Changes in expressible water content (EWC), water holding capacity (WHC) and cook loss (CL)

Changes in expressible water content, water holding capacity and cook loss were analyzed to evaluate the quality changes in climbing perch, banded snakehead and Asian stinging catfish stored at ambient temperature and iced condition. ANOVA test

was performed to determine variation in the means of data obtained for expressible water content, water holding capacity and cook loss and among the fish species. Duncan Post- hoc test was also carried out for its further clarification.

Table 3.5: Changes in expressible water content (EWC), water holding capacity (WHC) and cook loss (CL) of climbing perch, banded snakehead and stinging catfish muscle tissue stored at ambient temperature

Time	Climbing perch			Banded snakehead			Stinging catfish		
(Hrs)	EWC(%)	WHC(%)	CL(%)	EWC(%)	WHC(%)	CL(%)	EWC(%)	WHC(%)	CL(%)
0	9.01^{a} ±1.16	$74.54^{d, e,f}$ ±0.43	11.97^{a} ±0.33	9.32^{a} ±1.02	$70.06^{e,f}$ ±0.91	10.48^{a} ±1.20	12.87^{b} ±1.57	$62.42^{d, e}$ ±0.21	$17.77^{a, b}$ ±1.29
1	8.99^{a} ±0.84	$74.93^{d, e,f}$ ±1.92	12.34^{a} ±1.55	10.02^{a} ±0.89	$69.44^{e,f}$ ±0.93	9.82^{a} ±2.02	11.92^{a} ±2.02	63.85^{e} ±0.98	15.19^{a} ±1.85
2	9.02^{a} ±1.03	$75.38^{e,f}$ ±0.67	12.44^{a} ±1.76	9.03^{a} ±1.02	$70.25^{e,f}$ ±0.48	9.73^{a} ±1.92	10.30^{b} ±2.80	$62.32^{c, d, e}$ ±0.58	13.84^{a} ±1.64
3	$9.93^{a, b}$ ±2.87	76.93^{f} ±0.35	10.92^{a} ±1.91	9.02^{a} ±2.04	70.91^{f} ±2.22	10.20^{a} ±1.11	13.73^{b} ±0.74	$62. 04^{d, e}$ ±2.51	$15.43^{a, b}$ ±0.92
4	$10.75^{a, b, c}$ ±2.89	76.99^{f} ±4.5	13.54^{a} ±2.43	9.98^{a} ±1.01	$69.14^{e,f}$ ±4.73	$11.90^{a, b}$ ±3.93	12.36^{b} ±2.77	$63.40^{d, e}$ ±5.33	$16.64^{a, b}$ ±1.93
5	$10.96^{a, b, c}$ ±1.90	$76.58^{e,f}$ ±0.77	$15.54^{b,c}$ ±1.92	10.02^{a} ±2.72	70.50^{f} ±0.27	$13.44^{a, b}$ ±2.44	$14.90^{b, c}$ ±2.97	$61.85^{c, d, e}$ ±0.29	20.02^{b} ±2.97
6	$11.48^{a,b, c, d}$ ±2.71	$72.86^{d, e}$ ±1.77	$18.33^{c,d}$ ±1.00	10.74^{a} ±3.98	$66.67^{e,f}$ ±2.70	$16.44^{b, c}$ ±4.80	12.33^{b} ±4.46	$57.81^{b, c, d}$ ±3.43	25.83^{c} ±3.93
7	$13.69^{b,c,d,e}$ ±2.10	71.86^{d} ±1.64	20.83^{d} ±1.55	$13.55^{a, b}$ ±2.48	$65.57^{d,e}$ ±1.39	19.97^{c} ±0.16	14.24^{b} ±1.19	$58.58^{b, c, d,e}$ ±2.68	27.75^{c} ±2.23
8	$14.79^{c,d,e}$ ±1.92	$73.47^{d, e, f}$ ±1.62	24.33^{e} ±2.56	$15.55^{b, c}$ ±4.50	$61.42^{c,d}$ ±1.50	21.38^{c} ±4.55	$15.96^{b, c}$ ±2.78	$58.75^{b, c, d,e}$ ±1.10	35.82^{d} ±3.08
10	$14.56^{c,d,e}$ ±3.14	67.54^{c} ±0.36	29.23^{f} ±2.01	$17.48^{b, c}$ ±2.94	58.33^{c} ±0.33	27.42^{d} ±4.19	$17.02^{b, c}$ ±1.06	$56.48^{b, c}$ ±1.54	37.79^{d} ±1.21
12	$13.59^{b,c,d,e}$ ±2.19	67.70^{c} ±1.65	$30.06^{f,g}$ ±1.54	19.31^{c} ±2.99	54.04^{b} ±0.80	$32.02^{d,e}$ ±1. 46	19.02^{c} ±3.67	54.03^{b} ±0.90	42.48^{e} ±0.71
15	$15.50^{d,e}$ ±2.69	59.18^{b} ±2.34	$32.91^{g,h}$ ±3.22	20.00^{c} ±2.13	52.16^{b} ±5.32	32.97^{e} ±3.61	24.02^{d} ±3.02	44.97^{a} ±6.94	46.83^{f} ±4.76
18	17.54^{e} ±3.10	51.45^{a} ±3.63	$33. 62^{h}$ ±1.93	25.92^{d} ±2.80	47.15^{a} ±3.21	$35.59^{d,e}$ ±2.78	27.03^{d} ±3.02	44.50^{a} ±1.75	49.00^{f} ±3.43

All values are expressed as mean ± standard deviation, n=3

Different superscripts in the same column indicates significant difference ($p < 0.05$)

The results obtained for EWC, WHC and CL for the three fish species during storage at ambient temperature for 18 hours is given in Table 3.5 and their statistical analysis in Appendix 3. 5. Initial expressible water content ranged between 9.01 and 12.87%, cook loss between 10.48 and 17.77 %. The results show that the expressible

water content and cook loss increased gradually throughout the storage period in all the three fish species ($p < 0.05$). 64.40, 70.55 and 63.35 % of increases in cook loss was noticed during the storage period in climbing perch, banded snakehead and stinging catfish muscle respectively. Water holding capacity of the three fish muscle tissue was found to decrease during the storage period at ambient temperature. A small increase in water holding capacity was noticed at pre-rigor stage and thereafter it decreased. A steep decrease in water holding capacity was observed when the fish entered in to the post-rigor stage, and it is inversely related to the change in expressible water content and cook loss. Analysis of variance shows that there is a significant difference among the fish species in their water holding capacity, expressible water content and cook loss ($p<0.05$).

Table 3.6: Changes in expressible water content (EWC), water holding capacity (WHC) and cook loss (CL) of climbing perch, banded snakehead and stinging catfish muscle tissue stored at iced condition

Time (Days)	Climbing perch			Banded snakehead			Stinging catfish		
	EWC (%)	WHC (%)	CL (%)	EWC (%)	WHC (%)	CL (%)	EWC (%)	WHC (%)	CL (%)
0	12.01 ^{a} ±0.81	70.54 ^{e} ±0.43	15.35 ^{a} ±0.67	8.52 ^{a} ±0.78	73.99^{d,e} ±1.02	16.93 ^{a} ±2.06	10.71 ^{a} ±3.02	60.03 ^{e} ±1.94	17.92 ^{a} ±1.69
1	12.91 ^{a} ±1.76	73.52 ^{f} ±0.13	16.73 ^{a} ±2.26	8.93 ^{a} ±1.97	74.74 ^{e} ±1.08	19.42 ^{a,b} ±1.23	11.63 ^{a} ±2.19	59.48$^{d, e}$ ±1.48	19.75 ^{a} ±0.26
3	12.89 ^{a} ±0.17	72.74 ^{f} ±0.45	21.58 ^{b} ±2.19	11.48 ^{a} ±2.18	74.34 ^{e} ±0.96	23.60$^{b, c}$ ±4.52	15.74 ^{b} ±1.14	60.26 ^{e} ±2.80	25.85 ^{b} ±1.30
5	14.55$^{a, b}$ ±0.71	70.06 ^{e} ±0.82	24.23 ^{c} ±1.27	14.73 ^{b} ±1.39	73.72 $^{d, e}$ ±1.59	24.84 ^{c} ±1.555	17.43 $^{b, c}$ ±2.01	56.89$^{c, d}$ ±0.16	29.07 ^{b} ±1.39
7	16.73 ^{b} ±1.41	65.86 ^{d} ±1.66	25.42 ^{c} ±1.00	16.18 ^{b,c} ±0.65	72.57 $^{d, e}$ ±1.91	27.33 $^{c, d}$ ±1.59	20.88 $^{c, d}$ ±1.60	55.26^{c} ±0.74	34.69 ^{c} ±3.03
9	20.38 ^{c} ±0.71	64.74 ^{d} ±0.32	25.63 ^{c} ±0.48	16.61^{b,c,d} ±1.31	71.15 ^{d} ±1.12	26.23 ^{c} ±4.18	22.02 ^{d} ±0.76	49.70 ^{b} ±1.84	42.86 ^{d} ±2.58
12	23.83 ^{d} ±1.81	61.58 ^{c} ±0.84	31.21 ^{d} ±1.43	20.05^{e} ±2.71	63.59 ^{c} ±1.76	31.02$^{d, e}$ ±1.54	23.48 $^{d, e}$ ±0.73	48.58^{b} ±1.13	45.94 ^{d,e} ±4.10
15	25.36^{d} ±1.85	56.93 ^{b} ±1.53	34.43 ^{e} ±1.27	19.72 ^{de} ±3.12	54.47 ^{b} ±1.92	32.13 ^{e} ±1.22	24.28$^{d, e}$ ±2.42	49.05^{b} ±1.47	46.86 ^{d,e} ±2.60
18	25.68 ^{d} ±1.94	54.90 ^{a} ±0.42	35.27 ^{e} ±1.33	19.36^{c,d,e} ±0.53	47.17 ^{a} ±1.93	37.24 ^{f} ±0.97	26.54 ^{e} ±3.39	43.16 ^{a} ±1.90	48.19 ^{e} ±2.94

All values are expressed as mean ± standard deviation, n=3

Different superscripts in the same column indicates significant difference ($p < 0.05$)

Table 3.6 and Appendix 3.6 show changes in expressible water content, water holding capacity and cook loss in three fish species stored in ice. The initial value of expressible water content in all the three fish muscle ranged between 10.71 and 12.01% and gradually increased ($p < 0.05$). Results of one way ANOVA test shows there was significant difference in expressible water content among the fish species ($p < 0.05$). Initial water holding capacity in fresh samples of climbing perch and banded snakehead and stinging catfish were 70.54%, 73.99 and 60% respectively; showing stinging catfish had low water retention capacity than the two others. A slight increase in water holding capacity was observed in all cases and it increased to peak level on first day (73.52%) in climbing perch and banded snakehead (74.74%) and in third day stinging catfish (60.25 %). With further storage, the water holding capacity showed a decreasing pattern ($p < 0.05$). One way ANOVA result shows that that there is a significant variation between fish species in their water holding capacity ($p < 0.05$). Cook loss also showed a significant level of variation and found to be increased in all cases till the last day of storage in ice ($p < 0.05$). Compared to others, Asian stinging catfish showed high degree of loss during cooking. Results shows that there was a significant level of variation in cooking loss between fish species ($p < 0.05$).

3.4.4: Correlation analysis

On statistical analysis using IBM SPSS 20.0 for Windows, Pearson correlation analysis shows that there is significant level of correlation between muscle pH with WHC, EWC and CL. In other words, change in muscle pH inversely linked to the WHC and directly proportional to the EWC and CL. Correlation between pH and WHC with progress of post-mortem storage of climbing perch, banded snakehead and stinging catfish stored at ambient temperature and iced condition are given in Table 3.7.a and 3.7.b. respectively. The 'r' value obtained from Pearson Correlation analysis between pH and WHC with post-mortem storage time confirmed that changes happen to all the parameters and are greatly dependent on muscle tissue pH.

Table 3.7.a: Correlation between EWC, WHC, CL and pH of perch, snakehead and catfish stored at ambient temperature

			EWC	WHC	CL
Climbing perch	Pearson Correlation	pH	.833**	-.645*	.730*
	Sig. (2-tailed)		.017	.045	.042
Banded snakehead	Pearson Correlation	pH	.837**	-.851**	.801**
	Sig. (2-tailed)		.000	.000	.001
Stinging catfish	Pearson Correlation	pH	.743**	-.832**	.959**
	Sig. (2-tailed)		.004	.000	.000

**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

Table 3.7.b: Correlation between EWC, WHC, CL and pH of perch, snakehead and catfish stored at iced condition for 18 days

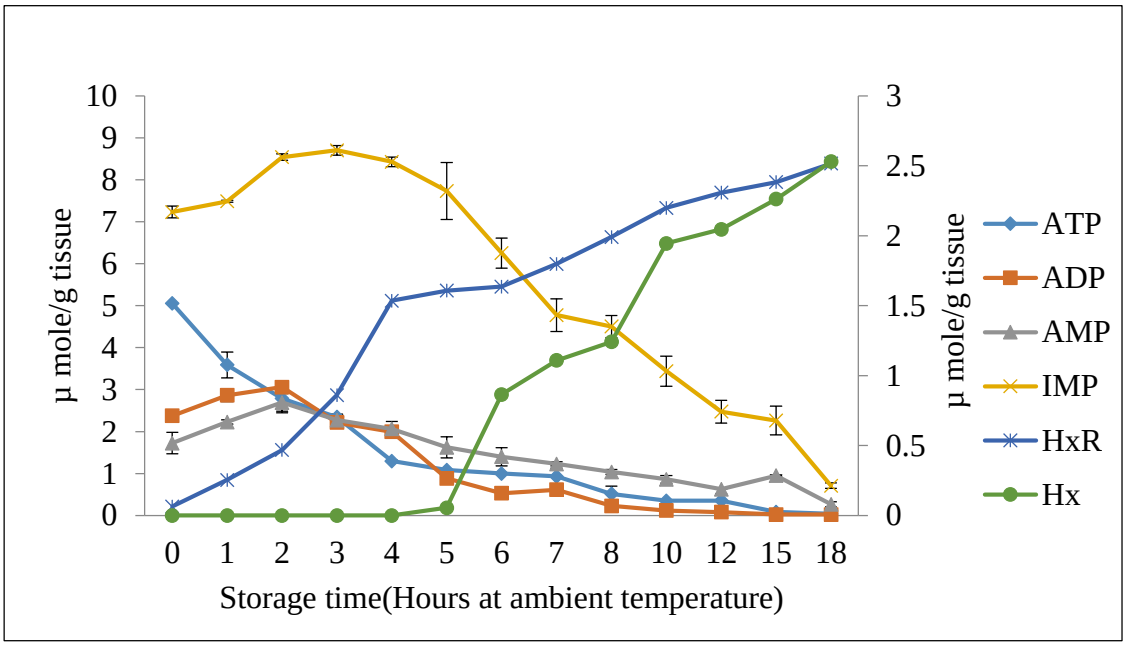
			EWC	WHC	CL
Climbing perch	Pearson Correlation	pH	.933**	-.954**	.852**
	Sig. (2-tailed)		.000	.000	.004
Banded snakehead	Pearson Correlation	pH	.853**	-.904**	.868**
	Sig. (2-tailed)		.003	.001	.002
Stinging catfish	Pearson Correlation	pH	.987**	-.927**	.973**
	Sig. (2-tailed)		.000	.000	.000

**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

3.4.5: Changes in Adenosine nucleotide degradation

Presence and accumulation of nucleotides gave information about the quality of the fish. Hence, an analysis was performed to get data on degradation pattern of ATP and its catabolism products in response to an increase in storage time. The results obtained for the analysis of ATP and its catabolism products such as ADP, AMP, IMP, HxR and Hx of climbing perch, banded snakehead, and stinging catfish stored at ambient temperature for 18 hours are depicted in Fig 3.1 to Fig 3.3. Also the data obtained for the analysis of these three fish stored at iced condition for 18 days are shown in the Fig 3.4 to Fig 3.6. One way ANOVA was used to compare the difference in the means of data obtained followed by analysis of Duncan-post hoc test to determine more details regarding degradation and accumulation patterns of ATP and its catabolism products in response to storage time.

3.4.5. a: Changes in ATP and its degradation compounds in fish muscle tissue stored at ambient temperature



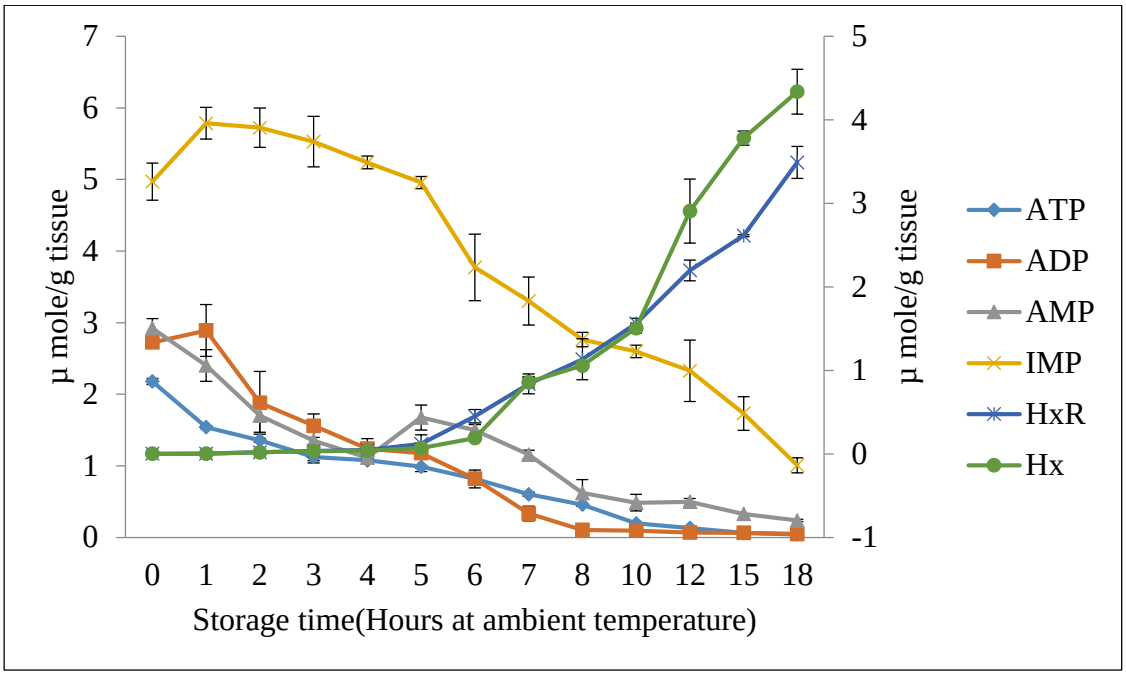
All values are expressed as mean ± standard deviation, n=3

On primary y-axis –ATP, ADP, AMP & IMP

On secondary y-axis –HxR & Hx

Fig 3.1: Changes in the concentration of adenosine triphosphate (ATP) and its degradation compounds in climbing perch muscle tissue stored at ambient temperature

Fig 3.1 shows the results obtained for the analysis of ATP and its catabolism products present in climbing perch fish muscle stored at ambient temperature for 18 hours. The initial concentration of ATP in fish muscle was found to be 5.05 µmoles/g, after 5th hour of incubation its level decreased to below 1.0 µmoles/g. The concentration of ADP, AMP and IMP at initial stage of incubation were noticed as 2.38 and 1.72 and 7.23 µmoles/g respectively and a slight increase in their concentration was observed in the first few hours and it decreased thereafter. Hypoxanthine still remained at a lower level till the 5th hour. After the 5th hour its concentration increased from 0.05 and reached 2.53 µmoles/g by 18th hour of storage. Inosine content was also found to be increased from 0 to 2.52 µmoles/g by the end of storage. Analysis of variance shows that all adenosine nucleotides significantly changed with storage period ($p< 0.05$) (Appendix 3.7).



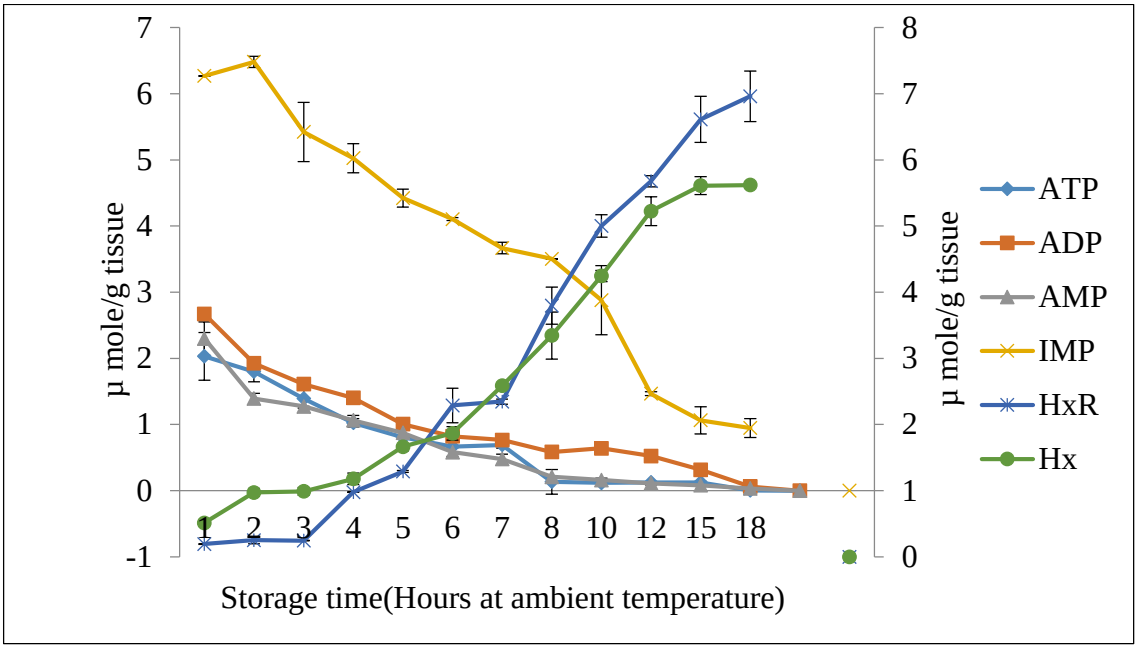
All values are expressed as mean ± standard deviation, n=3

On primary y-axis –ATP, ADP, AMP & IMP

On secondary y-axis –HxR & Hx

Fig 3.2: Changes in the concentration of adenosine triphosphate (ATP) and its degradation compounds in banded snakehead muscle tissue stored at ambient temperature

Fig 3.2 shows concentration of adenosine nucleotides and related compounds in banded snakehead fish muscle during the storage at ambient temperature. Initial concentration of ATP in fish muscle was found to be 2.18 µmoles/g. A steep decrease in concentration was noticed, and it decreased below 1.0 µmoles/g within 5th hour of incubation. Initial concentration of ADP, AMP and IMP noticed as 2.72, 2.92 and 4.97µmoles/g respectively. A noticeable increase in the concentration of ADP and IMP observed in the first few hours of storage and it gradually decreased thereafter. It was found that Inosine and hypoxanthine remained below 0.01 µmoles/g till 5th hour of storage; it increased to 3.49 and 4.34 µmoles/g respectively, showing that the rate of formation of hypoxanthine was higher than inosine content. Analysis of variance shows that concentration of ATP and its degradation products significantly changed with storage period ($p< 0.05$) (Appendix 3.8).



All values are expressed as mean ± standard deviation, n=3

On primary y-axis –ATP, ADP, AMP & IMP

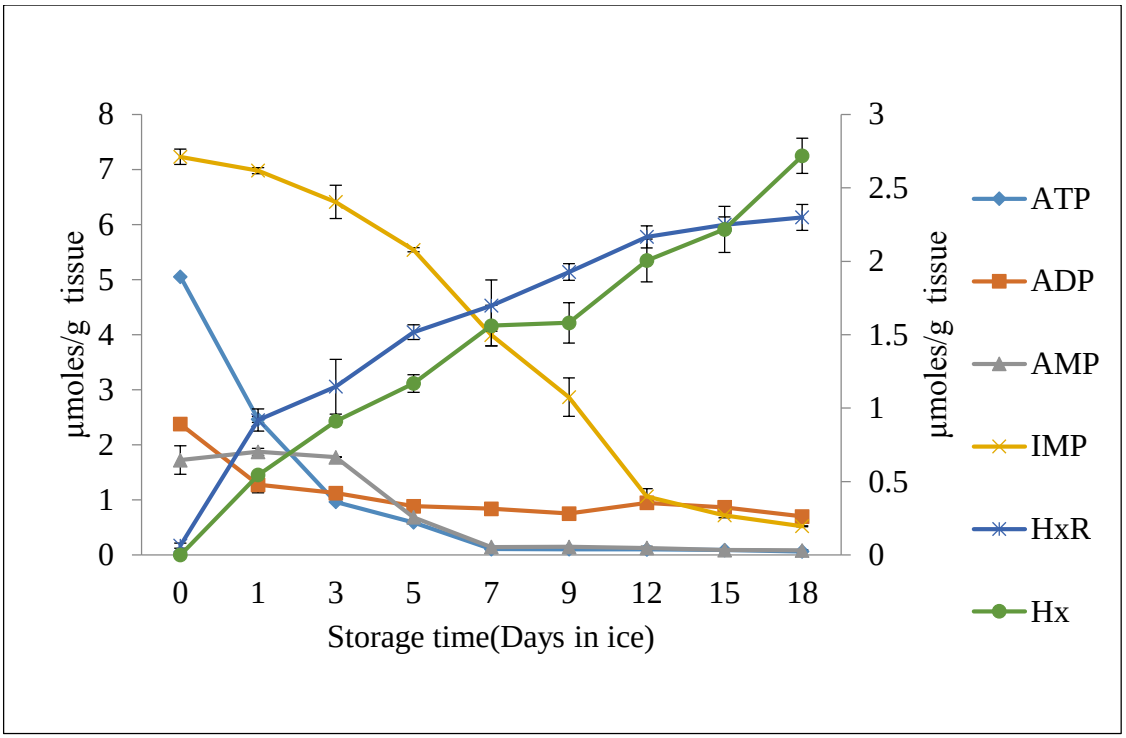
On secondary y-axis –HxR & Hx

Fig 3.3: Changes in the concentration of adenosine triphosphate (ATP) and its degradation compounds in stinging catfish muscle tissue stored at ambient temperature

Data obtained for the analysis of adenosine nucleotides and its metabolites in stinging catfish muscle during the storage at ambient temperature is depicted in Fig 3.3. Data from one way ANOVA shows there were significant changes in the concentration of all nucleotides of fish muscle as storage period increases ($p<0.05$) (Appendix 3.9). Initial concentration of ATP, ADP and AMP in fish muscle found to be 2.03, 2.67 and 2.29 µmoles/g respectively, noticed a decrease in their concentration with increase in the storage time and it decreased below 1.0 µmoles/g after 4th hour of incubation. Initial concentration of IMP was found to be 6.27 µmoles/g and decreased to 0.95 µmoles/g by last hours of storage. A gradual increase in Inosine and hypoxanthine content was observed throughout the study.

Results for one way ANOVA among the fish stored at ambient temperature is given in Appendix 3.10. Results show that changes in the ATP and its degradation compounds degradation pattern vary significantly among the fish species under the study ($p<0.05$).

3.4.5.b : Changes in Adenosine nucleotides and its degradation compounds in fish muscle tissue during iced storage



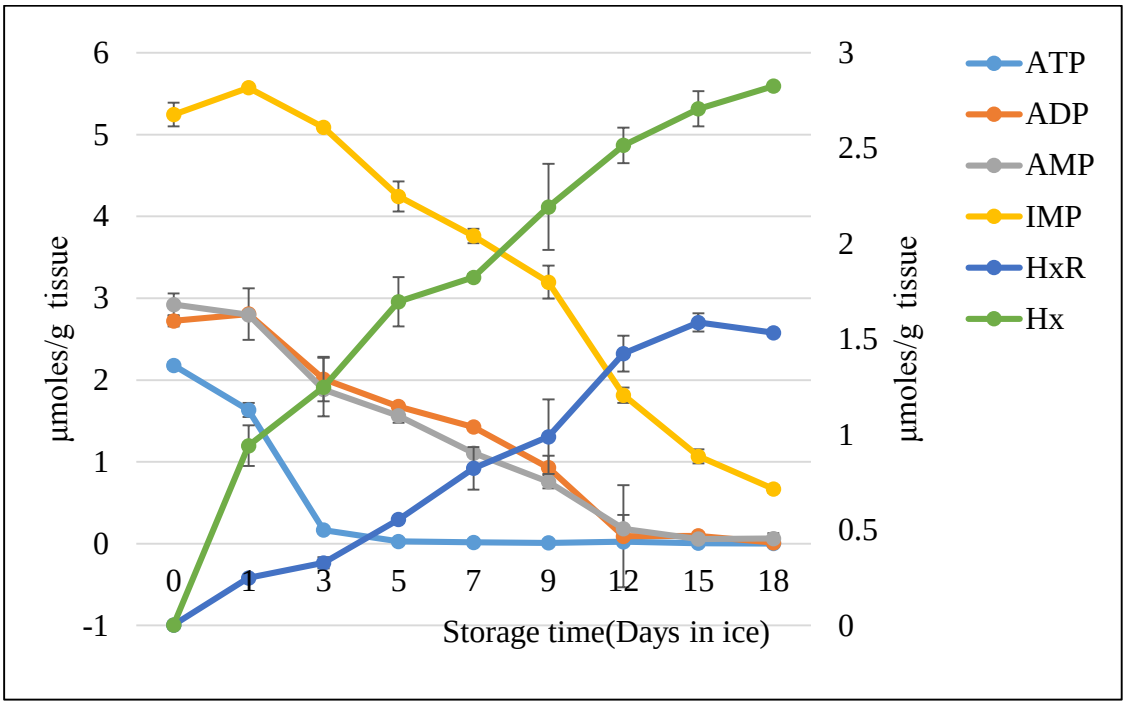
All values are expressed as mean ± standard deviation, n=3

On primary y-axis –ATP, ADP, AMP & IMP

On secondary y-axis –HxR & Hx

Fig 3.4: Changes in the concentration of adenosine triphosphate (ATP) and its degradation compounds in climbing perch muscle tissue during ice storage

Fig 3.4 shows data obtained for the analysis of concentration of adenosine nucleotides and related compounds in climbing perch during storage in ice for 18 days. Analysis of variance shows concentration of ATP degradation products significantly varied with storage in ice ($p<0.05$) (Appendix 3.11). A sharp reduction in the concentration of ATP was noticed during the initial time of storage period, it decreased from 5.05 to 0.96 during first three days of storage in ice. Concentration of ADP, AMP and IMP were also found to be decreasing throughout the storage period, while the content of Inosine and hypoxanthine showed increase with storage days.



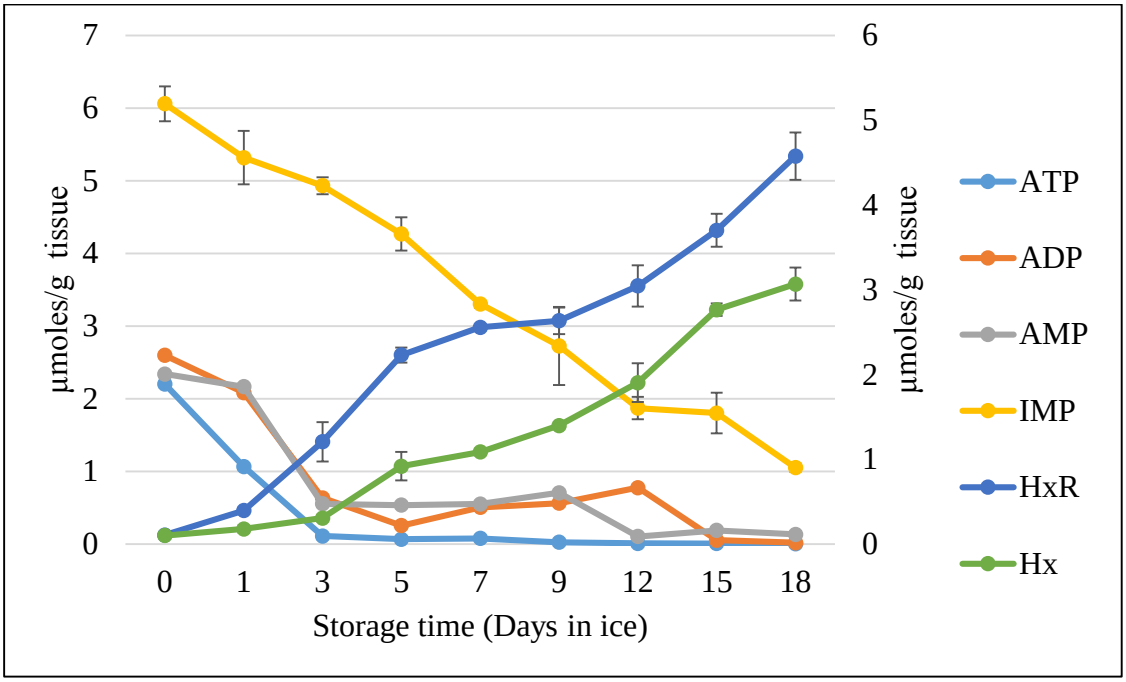
All values are expressed as mean ± standard deviation, n=3

On primary y-axis –ATP, ADP, AMP & IMP

On secondary y-axis –HxR & Hx

Fig 3.5: Changes in the concentration of adenosine triphosphate (ATP) and its degradation compounds in banded snakehead muscle tissue during ice storage

Fig 3.5 shows the variation in concentration of adenosine nucleotides degradation products in Banded snakehead fish muscle stored at iced condition for 18 days. Initial concentration of ATP in fish muscle was found to be 2.18µmoles/g, and it showed a steep decrease reaching below 1.0 µmoles/g after the first day of storage in ice. Initial concentration of ADP, AMP and IMP were 2.72, 2.92 and 5.24 µmoles/g respectively. Their concentrations were almost stable for the 1st day of storage and it then decreased thereafter. Compared to Inosine, concentration of hypoxanthine in fish muscle showed higher value throughout the storage period. Analysis of variance shows that concentration of ATP and its metabolism products significantly varies with storage in ice ($p<0.05$) (Appendix 3.12).



All values are expressed as mean ± standard deviation, n=3

On primary y-axis –ATP, ADP, AMP & IMP

On secondary y-axis –HxR & Hx

Fig 3.6: Changes in the concentration of adenosine triphosphate (ATP) and its degradation products in stinging catfish muscle tissue during ice storage

Data obtained for the examination of the concentration of adenosine nucleotides and related compounds in stinging catfish fish muscle stored in ice for 18 days are given in Fig 3.6. The concentration of ATP, ADP, AMP and IMP decreased during the storage period. Concentration of ATP, ADP and AMP decreased below 1µmoles/g after first day of storage. Concentration of IMP decreased from 6.06 µmoles/g to 1.05 µmoles/g on the last day of storage in ice. Inosine and hypoxanthine content in fish muscle increased from 0.12 and 0.10 to 4.58 and 3.07 respectively towards the last day of storage. Analysis of variance shows that concentration of ATP and its metabolites significantly varied with storage period in ice ($p<0.05$) (Appendix 3.13).

Results for one way ANOVA among the fish stored in ice is given in Appendix 3.14 and confirms significant variation among the fish species under the study($p<0.05$).

3.4.6: K value and H value

Tables 3. 8 and 3. 9 show the K and H value of tissue samples of climbing perch, banded snakehead and stinging catfish subjected to storage at ambient temperature for 18 hours and iced condition for 18 days respectively. K value gives an information regarding the buildup of hypoxanthine and inosine content in the tissue, while H value tells us about accumulation of hypoxanthine and resultant quality loss during the storage time.

Table 3.8: K value and H value of climbing perch, banded snakehead and stinging catfish fish stored at ambient temperature

Time (Hours)	Climbing perch		Banded Snakehead		Stinging catfish	
	K Value	H Value	K Value	H Value	K Value	H Value
0	0.39 ^{a}	0 ^{a}	0.04 ^{a}	0.02 ^{a}	1.56 ^{a}	0.76 ^{a}
1	1.55 ^{a}	0 ^{a}	0.04 ^{a}	0.02 ^{a}	5.05 ^{b}	3.67 ^{b}
2	2.66 ^{a}	0 ^{a}	0.36 ^{a}	0.18 ^{a}	9.56 ^{c}	7.59 ^{c}
3	5.25 ^{b}	0 ^{a}	0.71 ^{a}	0.35 ^{a}	11.27 ^{c}	9.05 ^{c}
4	10.03 ^{c}	0 ^{a}	0.95 ^{a}	0.43 ^{a}	20.20 ^{d}	11.05 ^{d}
5	17.95 ^{d}	0.42 ^{a}	21.32 ^{a}	0.78 ^{a}	29.36 ^{e}	16.54 ^{e}
6	21.40 ^{e}	7.40 ^{b}	24.72 ^{b}	2.51 ^{b}	40.22 ^{f}	18.06 ^{e}
7	27.82 ^{f}	10.61 ^{c}	29.03 ^{c}	12.10 ^{c}	46.82 ^{g}	24.55 ^{f}
8	33.96 ^{g}	13.0 ^{d}	35.70 ^{d}	17.24 ^{d}	61.72 ^{h}	28.92 ^{g}
10	46.49 ^{h}	21.82 ^{e}	47.59 ^{e}	23.35 ^{e}	70.88 ^{i}	32.55 ^{h}
12	55.21 ^{i}	25.94 ^{f}	62.78 ^{f}	35.75 ^{f}	83.07 ^{j}	39.81 ^{i}
15	60.83 ^{j}	28.39 ^{g}	74.49 ^{g}	44.04 ^{g}	88.53 ^{k}	40.64 ^{i}
18	82.92 ^{k}	41.57 ^{h}	85.41 ^{h}	47.32 ^{h}	92.34 ^{l}	41.25 ^{i}

All values are expressed as mean, n=3

Different superscripts in the same column indicates significant difference ($p < 0.01$)

Table 3.8 and Appendix 3.15 show changes in K value and H values in the three fish species during post mortem storage at ambient temperature. Both the values increased in a significant manner ($p<0.01$) during storage. The K value reached above 60 % within 15th hour of storage in climbing perch, 12th hour in banded snakehead, and 8th hour in stinging catfish, which were considered as unacceptable for consumption. It was noted that the H also increased during the storage period at ambient temperature. Results show that banded snakehead has highest content of hypoxanthine with climbing

perch and stinging catfish in decreasing order. Statistical results shows that there is a significant level of variation in K and H values among fish species ($p<0.01$).

Table 3.9: K value and H value of climbing perch, banded snakehead, stinging catfish fish species stored in ice

Time (Days)	Climbing Perch		Banded Snakehead		Stinging catfish	
	K Value	H Value	K Value	H Value	K Value	H Value
0	0.39 [a]	0.00 [a]	0.04 [a]	0.02 [a]	1.53 [a]	0.74 [a]
1	10.41 [b]	4.26 [b]	8.51 [b]	7.45 [b]	5.09 [b]	1.71 [b]
3	16.68 [c]	7.43 [c]	14.70 [c]	11.61 [c]	19.51 [c]	3.94 [c]
5	25.83 [d]	11.23 [d]	23.06 [d]	17.37 [d]	39.052 [d]	11.10 [d]
7	39.06 [e]	18.71 [e]	36.5 [e]	20.36 [e]	45.09 [e]	13.45 [e]
9	47.54 [f]	21.42 [f]	47.3 [f]	27.15 [f]	50.09 [f]	17.35 [f]
12	60.39 [g]	31.27 [g]	65.08 [g]	41.55 [g]	64.21 [g]	24.69 [g]
15	71.66 [h]	35.57 [h]	77.76 [h]	49.01 [h]	75.95 [h]	32.47 [h]
18	78.58 [i]	42.57 [i]	85.36 [i]	55.33 [i]	86.38 [i]	34.66 [h]

All values are expressed as mean n=3

Different superscripts in the same column indicates significant difference ($p <0 .05$)

Table 3.9 and (Appendix 3.16) show changes in K values and H values in the three fish species stored in ice. Result shows an increase of all values in a significant manner ($p<0.05$) as storage time progresses. All the three fish species were with an optimal grade of freshness up to the first 3 days of storage. K value reached above 40 % within 9th day of storage in climbing perch and snakehead and for stinging catfish was on 7th day of storage. All fish reached rejection point of 60% on 12th day of storage. It was noticed that the H values at initial stage of storage also increased as the storage period increased. Statistical analysis shows that there is a significant level of variation in K and H value among fish species ($p<0.05$)

3.4.7: Comparative analysis of progress of rigor with ATP content and K value of fish muscle stored at ambient temperature

Fig 3.7 to 3.9 represent the results obtained on comparative analysis of the progress of rigor mortis with changes in muscular ATP content and K value in the three species stored at ambient temperature for 18 hours. The results for all the three fish

species confirm that the whole fish entered into the full rigor stage when the concentration of ATP decreased to below 1.0µmol/g tissue. The K value increased to 20% and 40% when the fish reached in full rigor stage and post rigor stage respectively.

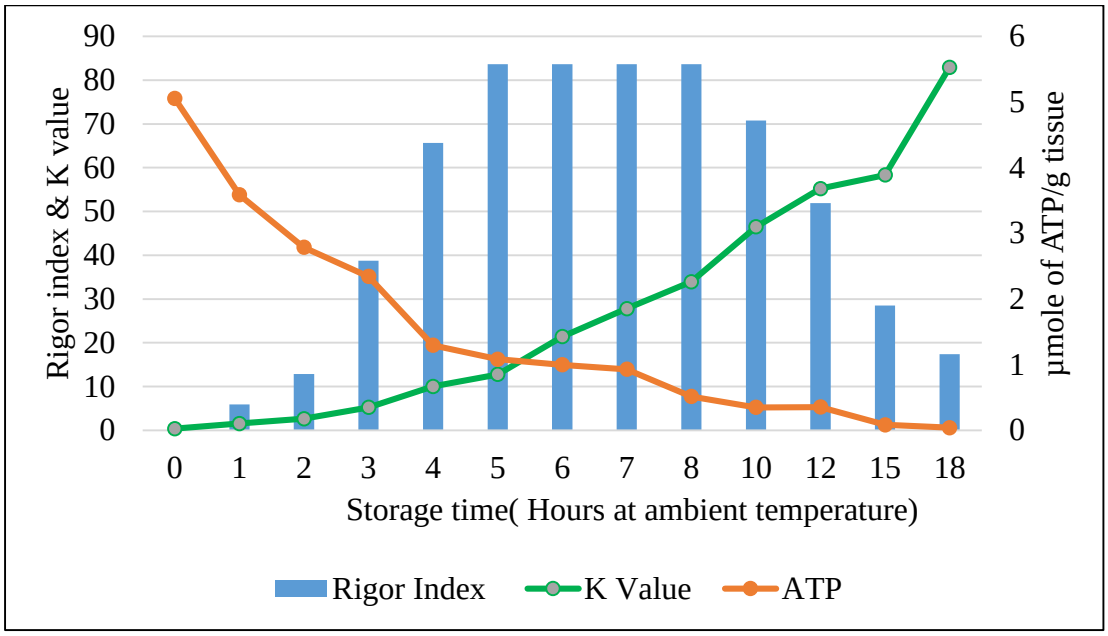


Fig 3.7: Comparative analysis of progress of rigor with ATP content and K value in climbing perch muscle tissue stored at ambient temperature

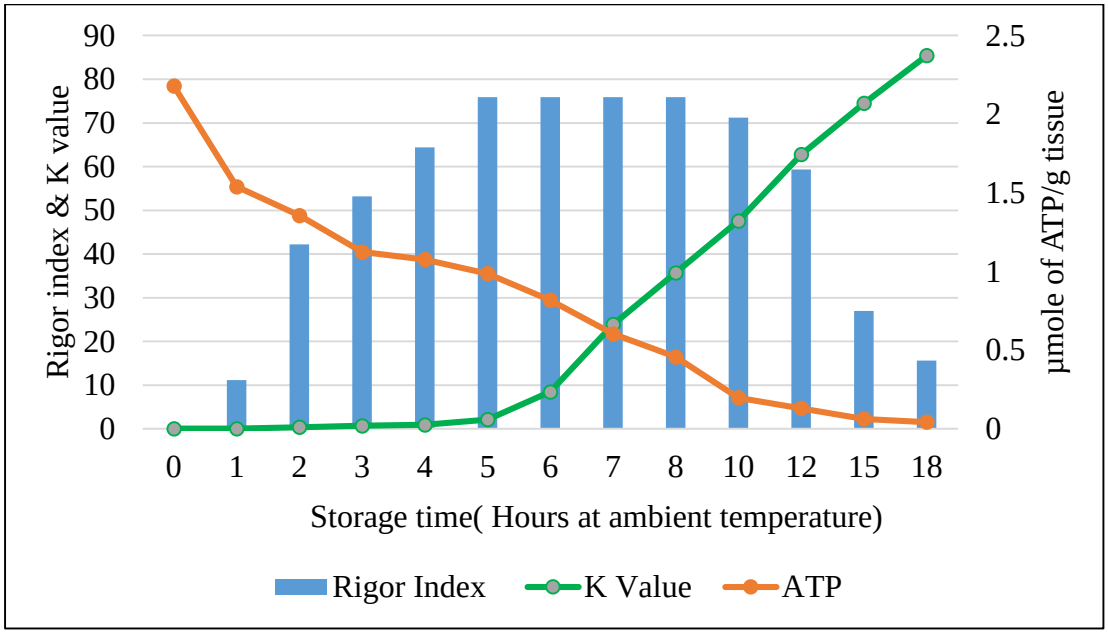
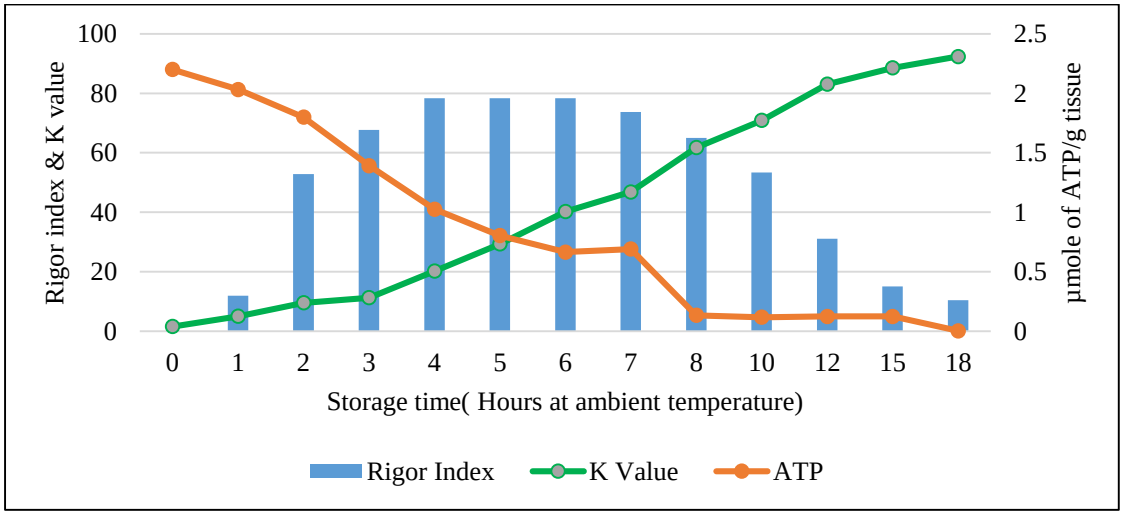


Fig 3.8: Comparative analysis of progress of rigor with ATP content and K value in banded snakehead muscle tissue stored at ambient temperature



3.9: Comparative analysis of progress of rigor with ATP content and K value in stinging catfish muscle tissue stored at ambient temperature

3.4.8: Comparative analysis of progress of rigor with ATP content and K value in ice stored fish muscle

Fig 3.10 to 3.12 shows the results obtained by comparative analysis of rigor index with changes in the K value and muscular ATP content in the fish tissues during ice storage of climbing perch, banded snakehead and stinging catfish respectively for 18 days. Data shows that the whole fish reached in the full rigor condition before concentration of ATP decreased to below 1µmole/g tissue. Additionally, progress of rigor-mortis does not express any direct relation with that of K value in any of the fish selected for the study.

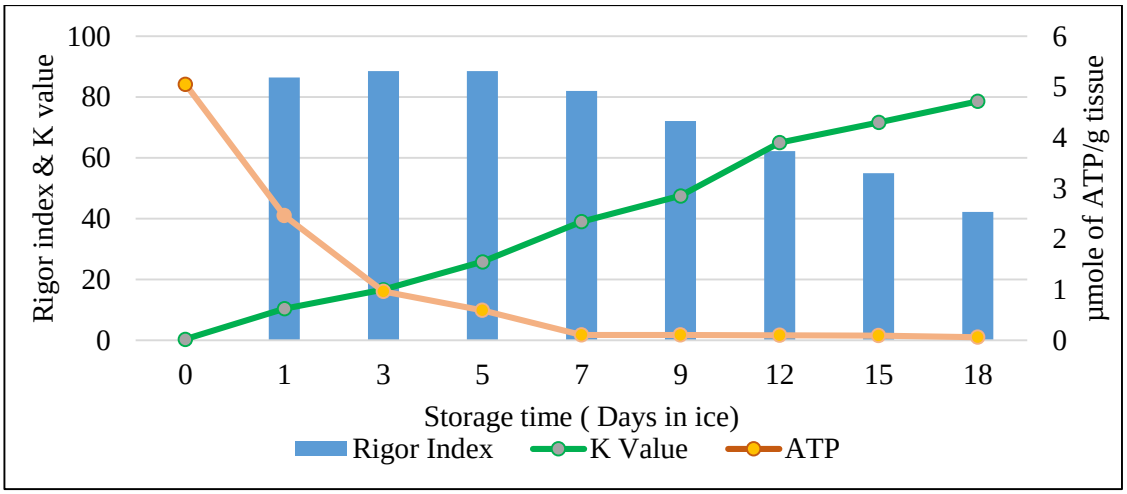


Fig 3.10: Comparative analysis of progress of rigor with ATP content and K value in ice stored climbing perch muscle tissue

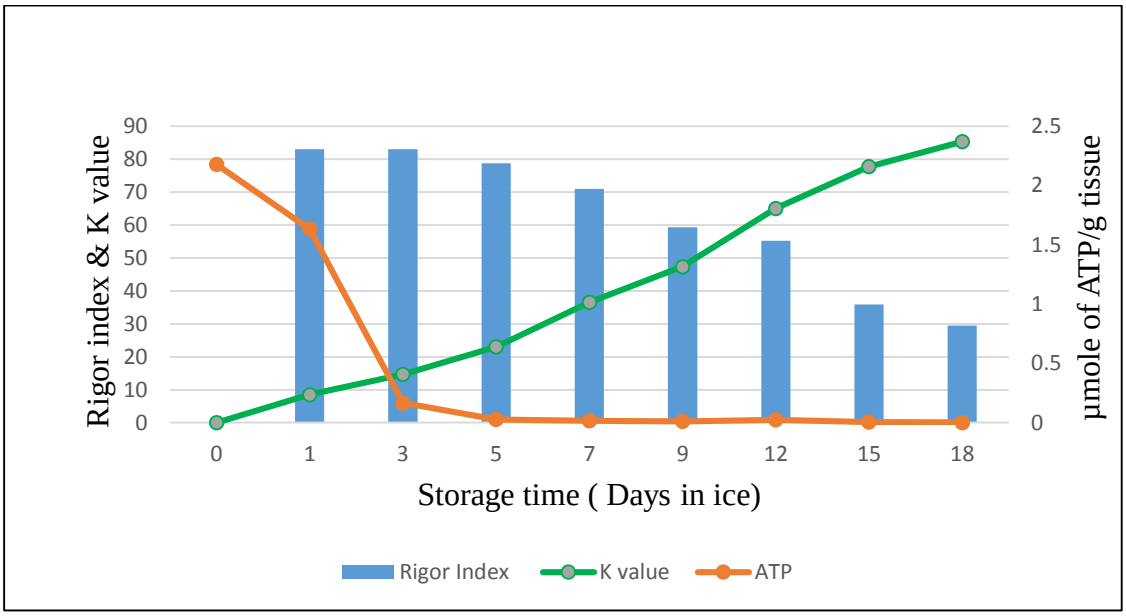


Fig 3.11: Comparative analysis of progress of rigor with ATP content and K value in ice stored banded snakehead muscle tissue

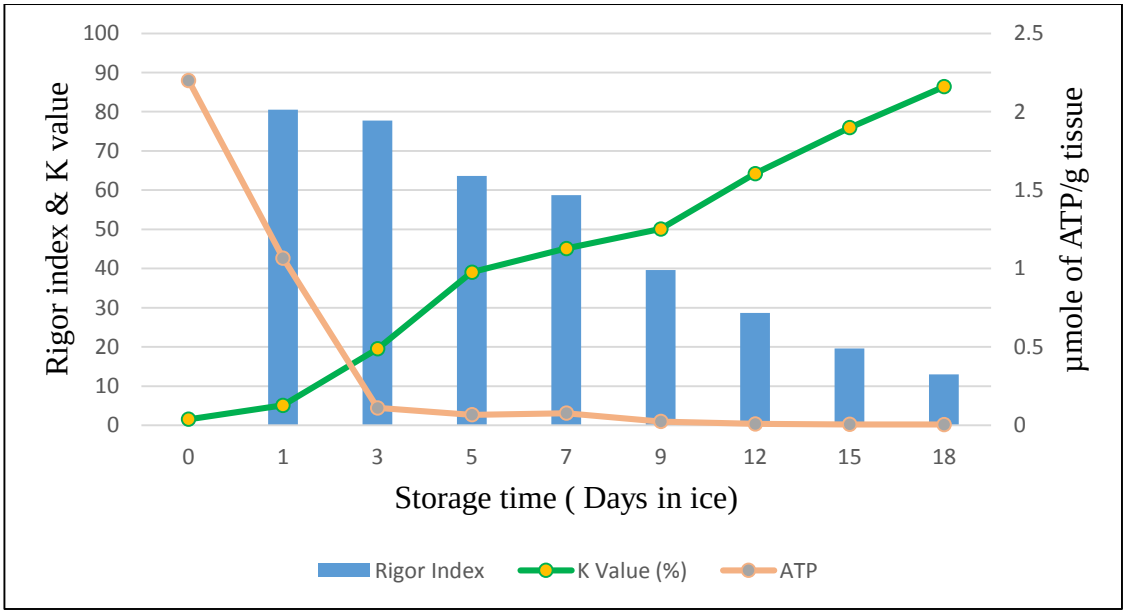


Fig 3.12: Comparative analysis of progress of rigor with ATP content and K value in ice stored stinging catfish muscle tissue

3.5: Discussion

Time and temperature have great impact on quality of fishes during post mortem storage. The stability of quality is influenced by the physico-chemical parameters of the fish muscle tissue. Due to this reason, need for the analysis of physico-chemical changes during post-mortem storage of fish is gaining importance. The results obtained from the analysis of three freshwater fish show that the pH value of fresh fish muscle is close to neutral. Fresh stinging catfish has softer muscle tissue and higher pH value than banded snakehead and climbing perch. It was noticed that upon storage, pH value of fish muscles dropped in a significant manner to a minimum level and which is significantly correlated to the onset of full rigor stage. During this stage the fish muscles were in fully stiffened condition. It is reported that lactic acid accumulation is strongly related to drop in pH in the muscle tissue (Hultin, 1985; Love, 1992; Lougovois & Kyrana, 2005 and Iwamoto *et al*., 1987). Also the rate of change in pH noticed was much accelerated at ambient temperature storage while they were slower at iced storage in all the fish species studied and is in good agreement with the studies of Susanto *et al*. (2011), who stated higher variation in pH value in tropical fish at ambient temperature compared to chilled temperature storage. An increase in pH value was noted in both types of storage conditions in all the species as it proceeded through post-rigor stage.

Diminution of ATP content is also related to the muscular pH value at in-rigor condition due to the accumulation of phosphoric and hydrogen ion liberated by the catabolism of adenosine nucleotides. The results obtained are further validated by observation of different authors (Ruiz-Capillas & Moral 2001; Yongsawatdigul & Park 2002; Hultmann & Rustad 2004; Rammouz *et al*., 2013; England *et al*., 2017). Many studies stated that hike in pH in post-mortem fish muscle is due to the accumulation of basic compound like amines and ammoniacal compounds arising from autolytic and microbial breakdown of complex organic compounds which serve as substrates for spoilage causing bacteria (Pedrosa-Menabrito & Regenstein, 1988; Ruiz-Capillas & Moral, 2001; Susanto *et al.*, 2011).

Reduction in muscular ATP content typically induces the development and progress of rigor-mortis in fish muscle. Fresh muscle tissue of perch has high ATP content than the two others. Present result shows that all the three fish species stored at

ambient temperature reached the full-rigor condition when ATP level in muscle tissue falls down to1µmole/g tissue. This result is in good agreement with Gill (1995), who reported that when the level of ATP in cytosolic fluid reduced to below 1.0 µmole/ g tissue, actomyosin cross-bridge formation trigger the entry of muscle into rigor mortis. From the results obtained, it is observed that progress of rigor mortis was much faster during storage at ambient temperature than iced condition and faster in stinging catfish than the two other species. Unavailability of ATP for actomyosin cross-bridge breakage during muscle contraction is the typical reason for the post-mortem toughness.

Data obtained shows that the progress of rigor mortis in three iced fish is not dependent on the concentration of muscular ATP and is due to cold shortening. Cold shortening happens when the fish muscle becomes chilled prior to the complete use of ATP for muscle contraction (Curran *et al*., 1986 a,b). Chilling will inhibit the Ca^{2+}-pumping and consequently slowdown muscle contraction. Additionally, Lougovois & Kyrana (2005) reports that the body of the fish often assumes a bent form due to the tension developed by antagonistic muscles.

The data obtained in the present study indicated that the magnitude of rigor index is higher in climbing perch than banded snakehead and stinging catfish. Additionally, the post-mortem resolution in stinging catfish happens more quickly than snakehead and perch, which proves that progress of rigor-mortis is typically dependent on the fish species. It was reported that duration of onset of rigor and its resolution vary from species to species and small fish go into full rigor condition faster than medium and large one and duration of rigor of small fish was comparatively less than medium and large fishes (Ali *et al*., 2007).

Based on the results obtained on the progress of rigor mortis, it is observed that upon post-mortem storage, the climbing perch is the most stable freshwater fish while stinging catfish is the least stable one. Compared to a study carried out by Haque *et al*. (1997); Nabi *et al*. (2001), the present study shows that the post-mortem time involved in each stage and the subsequent resolution happens more quickly than marine fishes, indicating that the quality of freshwater fish is less stable than the marine fishes. Duration of rigor mortis in cold blooded animals is shorter than warm

blooded animals (Kato *et al*., 2009). Also temperate freshwater fish is reported to go faster into rigor than tropical fish (Tomlinson *et al.* 1961).

Change in pH value during post-mortem storage act as another causative factor to the loss of water from tissue. Hamm (1966), reported that two third of loss in the water holding capacity of the fish muscle tissue could be due to the breakdown of ATP molecules and one third could be due to the decrease in pH level. Data obtained from analysis of the three fish species shows a significant level of reduction in water holding capacity, increase in the expressible water content and cook loss in both iced storage condition and at ambient temperature. Additionally, the muscle tissue of snakehead has the highest water holding capacity and lowest expressible water content than the two others.

The present study also confirmed that pH variation in the post-mortem fish muscle tissue significantly influenced its water holding capacity and hence expressible water content. This result is strongly supported by Lin *et al*. (2005), who reported that the pH and water holding capacity are interrelated. Amount of water released from the sample is greatly influenced by the water holding capacity and by the magnitude of micro structural damage, which is a function of the textural strength of the sample (Hermansson & Lucisano, 1982; Ofstad *et al*., 1993). This drastic change in water holding capacity, expressible water content and cook loss during post rigor-stage of fish may be due to the denaturation of protein leading to cellular and structural degradation.

The water holding capacity is the property of protein functionality and it gives information on freshness of fish and other processing variables (Douglas- Sehwarz & Lee, 1988). Certain amount of water is essential for the adequate solubility and stability of the tissue protein and their gel formation. A hike in tissue water content than the water binding capacity of the proteins will adversely influence its textural property and is no longer acceptable in market. Hence, on a practical point of view, water-holding capacity is supplementary essential for the optimization and formulation of a fishery product. Sharp & Offer (1992) pointed out that along with fat and water retention, protein gel formation strongly influences the textural and functional properties of a fishery product.

The expressible water content of all the fish muscle increases with storage time in both conditions. Muscle tissue from catfish shows low water holding capacity than banded snakehead and climbing perch. In other words, expressible water content of catfish was found to be higher than two others. This result indicates that water holding capacity is directly linked with affinity of fish muscle to water. Data obtained also confirmed that the fish muscle lose their water holding capacity leading to excessive loss of water in post-rigor stage compared to pre-rigor and in-rigor stage. Increase in expressible water content is due to the denaturation and increased proteolysis, followed by a subsequent loss of structural integrity in the muscle tissue which will thereby affect the ability of proteins for water retention in the muscle tissue. Cook loss increased significantly during storage period both at ambient temperature and iced condition which is directly related with the water holding capacity of the three fish muscle tissue studied. Thermal denaturation during heating also resulted in a severe aggregation of the same. Rammouz *et al*. (2004) also reported a relationship between cook loss and drip loss in Turkey breast muscle. Thus analysis of water holding capacity, expressible water content and cooking loss in the fish muscle tissue selected for the study is of significance to both processing industry and consumers.

A drastic change in the concentration of adenosine nucleotide and its metabolites in the selected fishes were observed throughout the study. Haard (1992) reported that almost complete degradation of ATP happens within the first 24 hours in post-mortem condition. Dephosphorylation of ATP leads to the formation of ADP and AMP and then to IMP by deamination to almost same extent in the muscle tissue of perch, catfish and snakehead stored both at iced and ambient at initial stage. They remain almost constant in a low concentration over the rest of storage. In the meantime, inosine monophosphate gets degraded into inosine and to hypoxanthine and was then accumulated. Depletion of IMP is directly linked with the loss of freshness (Woyewoda *et al*., 1986).

Accumulation of IMP develops a favorable pleasant flavor, while hypoxanthine brought about off-odour in spoiled fish. High value for IMP content was found in the muscle tissue of perch in fresh condition. Buildup of hypoxanthine concentration pointed out initial phase of autolytic deterioration as well as bacterial spoilage (Woyewoda *et al*., 1986). It was reported that the marked accumulation of

hypoxanthine is due to microbial growth (Massa *et al*., 2005). Surette *et al.* (1988) reported that the rate of formation of IMP and its breakdown is found to be same in both sterile and nonsterile samples of Atlantic cod tissue, evidencing the exclusive role of autolytic enzymes for the degradation of ATP to HxR. Upon storage, accumulation of hypoxanthine was almost equal to that of inosine in the muscle tissue of climbing perch. But in banded snakehead, hypoxanthine was found to be higher than inosine, and vice versa in catfish.

Quantitative analysis of freshness and quality by using K value indicates that climbing perch exists in good quality for longer time, with banded snakehead ranked in second position and stinging catfish with the lowest shelf life. Increase in K and H value is typically dependent on the storage time. Decrease in muscular ATP and increase in K value typically influenced the progress of rigor mortis. Data obtained shows that the K value of perch, snakehead and catfish muscle tissue reached above 20% when the muscular ATP level fell below 1.00 µmol/g tissue, both at ambient temperature and iced storage condition. Meanwhile, the fish became rigid when the K value exceeded 20% in the fish stored at ambient temperature in all the three fish species. Additionally, when K value reached above 40%, fish entered in to the post-rigor stage at ambient temperature. These observations were not comparable for ice-stored perch, snakehead and catfish muscle tissue samples. From the data obtained for H value and hypoxanthine content, three fishes are arranged in the order of quality as banded snakehead > climbing perch> stinging catfish. Apart from this, absence of scales on catfish may also favour the loss in freshness compared to the other two freshwater fishes selected for the study.

3.6: Conclusion

Quality loss in fish is due to a series of complex process, including physico-chemical changes followed by spoilage due to microbial contamination. In the present study, various physico-chemical parameters that are related to the post-mortem changes were analyzed during ambient temperature and iced stored condition among three freshwater fish species. Content of ATP, and its catabolism products mainly ADP, AMP, IMP, HxR and Hx, K and H value, pH, water holding capacity , cook loss and expressible water content were estimated and compared with progress of rigor mortis.

Analysis of rigor index revealed that unavailability of ATP for the breakdown of actomyosin cross bridge during muscle contraction induces the whole fish to enter into the full rigor condition within 4 to 5 hours in all the three fish species stored at ambient temperature. But, full rigor in the iced stored fish muscle is not a function of ATP level alone, but is also due to the cold shortening. Stiffness of fish muscle due to the formation of actomyosin cross bridge is also an index of protein functionality. Rate of change in pH value is found to be faster in fish muscle stored at ambient temperature than iced one. The expressible water content of fish muscle increased with storage time. Loss of water holding capacity could be due to the denaturation of protein gel matrix followed by a loss of muscle structural integrity. Cook loss increased significantly with storage period which is directly related to the water holding capacity of protein in muscle tissue. Influence of these parameters in the fish muscle tissue are crucial and will negatively affect processing industry and thereby consumer satisfaction. Drastic reduction of IMP content is directly related to the loss of freshness and flavor. Accumulation of hypoxanthine develops off-odour to the muscle tissue. The study confirmed that banded snakehead is an Hx former species and catfish is an HxR former, while climbing perch produces equal concentration of Hx and HxR. Low temperature storage will slow down the loss of freshness. In addition, properties of muscle proteins and their post-mortem changes is believed to have a crucial role on the final quality. Thus the post-mortem changes of protein fractions in climbing perch, banded snakehead and stinging catfish stored at both ambient temperature and iced conditions are presented in the proceeding chapter.

Chapter 4

Chapter 4

Post-mortem changes in protein fractions

Contents

4.1: Introduction

Proteins act as a major determining factor on quality traits of fish and fishery products. Myofibrillar proteins and connective tissue proteins comprise the fibrous proteins. Collagen act as the major connective tissue protein maintaining the texture of meat by holding myotome bundles together. Disintegration of muscular protein by endogenous protease enzymes is the crucial factor in post-harvest changes of meat. Autolytic as well as microbial action causes an actual loss in protein functionality, nutritive value and finally costumer acceptance. Hence, right information on changes in solubility properties of sarcoplasmic, myofibrillar and connective tissue proteins in fish muscle helps quality enhancement, which further results in customer satisfaction. Extractability of the protein fractions depends on fish species and post-mortem stage of the fish. Therefore, the present study envisages the extent of loss of freshness of the three freshwater fishes selected and changes in the protein fractions with emphasis on changes in collagen fractions and myofibrillar ATPase activity on storage at ambient temperature and iced condition.

4.2: Review of Literature

Based on the solubility properties, proteins in fish muscle tissue are divided into sarcoplasmic, myofibrillar and stroma proteins (Haard, 1992; Foegeding *et al*., 1996). Sarcoplasmic proteins constitute about 20- 35% of the total fish muscle proteins. They are soluble in neutral salt solutions of low ionic strength (<0.15 M). Major part of these proteins are enzymes and heme proteins, where concentration varies depending on the species, breed, muscle fibre type, age of animal and

individual genetics (Damodaran, 2007). Most of sarcoplasmic proteins are of low molecular weight, either rod or globular in shape in nature.

Myofibrillar proteins are the major proteins, which constitute 65-75 % of the total protein in fish white muscles, and are the largest fraction of the muscle tissue (Ashurst & Dennis, 1996). These proteins are soluble in neutral salt solutions of high ionic strength (0.5 M). Myofibrillar proteins are divided into subgroups; myosin, actin, troponin and tropomyosin. Each myosin molecule consists of two heavy and two light chains. The globular head portion of heavy chain contains ATP binding site, actin binding site and light chain binding site. Myosin head region has the ATPase catalytic property (Benjakul *et al*., 2003; Ramachandran *et al*., 2007), whereas the tail region is responsible for the formation of thick filament (McCormick & Schultz, 1994; Foegeding *et al.*, 1996; Lodish *et al*., 2000). Actin constitutes about 22% of total myofibrillar protein, and has a myosin-binding site and during muscle contraction, actin and myosin forms the actomyosin complex (Foegeding *et al*., 1996). Two of F-actin filaments join to form a double helix, a thin filament or I band in muscle tissue. Tropomyosin constitutes about 8-10% of total myofibrillar protein and is the most stable form of myofibrillar protein. Tropomyosin has two polypeptide chains and have double helical structure. Troponin constitute about 8-10% of total myofibrillar protein. Each troponin has three subunits. Troponin-T, interacts with tropomyosin, troponin- I, strongly inhibits the ATPase activity of actomyosin and Troponin-C interact with Ca^{2+} ion and is necessary for the muscle contraction. Tropomyosin and Troponin has a major role in regulating the process of muscle contraction (Foegeding *et al*., 1996).

Stroma protein represents connective tissue protein, constituting approximately 3% of the protein in teleost and about 10 % in elasmobranchs. Stroma proteins cannot be extracted with water, salt, alkaline or weak acidic solution. Collagen is a fibrous glycoprotein with a basic structural unit of tropocollagen and act as the major component of the connective tissue of fish muscle. Each tropocollagen molecule is composed of three polypeptide chains coiled in a helix on a common central axis in a left hand direction and is interrupted at the ends of telopeptides. Tropocollagen chains form an intramolecular (within the tropocollagen units) and/ or intermolecular (between adjacent tropocollagen chains) cross-link giving mechanical

strength to collagen, thus making it insoluble in nature. Cross-link formation does not usually occur in the telopeptides (Suarez *et al*., 2005). Pepsin has been routinely used for solubilizing collagen by cleaving cross link without altering the triple helical structure (Sato 1993; Eckhoff *et al*., 1998; Zhang *et al*., 2011), thereby resulting in disorganization of the collagen molecules.

Acetic acid soluble collagens are telo-collagens, the non-helical telo-peptide regions remains intact while pepsin soluble collagens are atelo-collagen since the pepsin treatment results in cleavage of telo-peptide region (Nalinanon *et al*., 2007; Ali *et al*., 2017). Generally, Type I and Type V collagen has been identified from the fish intramuscular connective tissues and its fraction is very important for the stability and thickness of collagen fibrils. Thinner collagen fibers have high ratio of type V in the fish muscle. Because of high type V collagen fraction in myocommata, degradation of extracellular matrix is likely more easy and rapid (Ando *et al*., 1991; Sato *et al*., 1994). In sardine muscle, type V collagen became solubilized after first day of storage at chilled condition with an associated weakening of pericellular connective tissue and is induced by degradation of thin collagen fibrils with no change in that are located in the interstitial connective tissue (Sato *et al*., 1997). Studies of Sato *et al*. (1991) reports that the type V collagen resides the non-helical region of collagen cross-link.

Post-mortem changes of fish muscle proteins

Degradation of myofibrillar or connective tissue proteins, degradation of bonds and connections that organize and stabilize the structure between the muscle components or both of these mechanisms are responsible for the post-mortem softening of fish muscle (Bremner, 1992). These physico-chemical changes are mainly induced by the influence of endogenous proteases (Sriket *et al*., 2010). Upon muscle tenderization during post-mortem storage condition, the proteolytic enzymes degrade the key muscle tissue proteins of perimysium and endomysium connective tissue, as well as the proteins localized in Z-line and H-zones in the sarcomeres triggering loss of integrity of myofibrillar proteins (Koohmaraie, 1996). These proteolytic enzymes constitute the major fraction of sarcoplasmic proteins. Post-mortem cellular damage results in release of lytic enzyme fractions from its storage site including organelles like endoplasmic reticulum, mitochondria and lysosome

(Kas *et al*., 1983). Therefore releasing of these stored enzymes are crucial in subsequent cellular disintegration and thus the final meat quality (Ladrat *et al*., 2003; Sierra & Olivan, 2013). Although muscle pH and ionic strength significantly influence the solubility of sarcoplasmic proteins, storage temperature and its duration also have a great impact on the same (Rehbein, 1992; Fuente-Betancourt *et al*., 2009; Kaale & Eikevik, 2016; Romotowska *et al*., 2016). It is reported that the sarcoplasmic proteins and actin have poor thermal stability than other proteins (Suzuki, 1981).

Post-mortem storage of fish muscle induces denaturation of myofibrillar protein (Hossain *et al*., 2005b). The aggregate formation of actomyosin and free myosin molecules in fish muscle is more rapid than mammalian species (Shenouda, 1980). Ca^{2+}-ATPase activities in the fish muscle reveal integrity and functionality of myosin molecule in the actomyosin complex (Roura & Crupkin, 1995; Montechia *et al*., 1997). Alteration in ATPase activities induce structural changes in protein due to their denaturation (Sano *et al*., 1994) and therefore measuring ATPase activity provide a direct information about protein denaturation. The alkali soluble protein fraction in fish muscle is from the denatured myofibrillar protein. It is suggested that actomyosin aggregate formation were depolymerized and separated into actin and myosin in high ionic solutions and form insoluble aggregates (Careche *et al*. 2002).

Two forms of collagen viz. type I and type V are present in muscle tissue (Kimura *et al*., 1988; Sato *et al*., 1991; Sato *et al*., 1997). Due to its molecular properties, collagens are considered the most stable proteins in muscle tissue. Collagen surrounded muscle fibers help in connecting the muscle fibers to myocommata and is actually responsible for the stability of the fish muscle. Post-mortem undesirable textural changes and gaping phenomenon is a property of collagen breakdown, contributed by influence of collagenase by disintegrating collagen fibers in myocomata (Ando *et al*., 1995; Bremner & Hallett, 1985; Hernandez-Herrero *et al*., 2003; Sriket *et al*., 2010; Sriket *et al*., 2011a). During chilled storage, the attachment between muscle fibers and myocommata, and the whole sarcolemma, is degraded, and muscle fibers in fish muscle are detached from the myocommatal sheets (Bremner & Hallett, 1985; Hallett & Bremner, 1988). Hydrolysis of myofibrillar and connective tissue protein by endogenous proteolytic enzymes is important in the early deterioration process (Sriket, 2014). The enzymatic

activities are dependent on fish species and its maximum active pH values are close to neutrality or higher. Proteins are degraded into large fragments, and this enhances the susceptibility of the proteins to other proteinases (Ladrat *et al*., 2000).

Several studies demonstrated proteolytic degradation of fishes during postmortem storage such as blue tilapia (Oreochromisaureus) (Korhonen *et al*., 1990), carp (Nakayama *et al*., 1994) Chinook salmon (Jerrett *et al*., 1996), hilsa fish (Tenualosa ilisha Ham.) (Haque *et al*., 1997), shrimp and Prawn (Kamal *et al*., 2000), Carp *(Labeo rohita (Hamilton*) (Jasra *et al*., 2001), Farmed Sea Bream (*Sparus Aurata*) (Suarez *et al*., 2005), farmed Atlantic cod (*Gadus morhua*) (Hultmann & Rustad 2007), Farmed Sea Bream (*Sparus Aurata*) (Suarez *et al*., 2011), Yellow Croaker (*Pseudosciaena crocea*) (Li *et al*., 2014) and Tilapia (*Oreochromis mossambicus*) (Parthiban *et al*.,2015). No detailed study in this regard is available in the selected freshwater fishes selected for the study.

4.3: Materials and methods

4.3.1: Raw material collection and sample preparation

The samples of climbing perch, banded snakehead and stinging catfish were collected and tissue samples were prepared as detailed in 3.3.1.

4.3.2: Extraction of muscle tissue protein

Sarcoplasmic, myofibrillar, denatured protein and stoma protein were extracted and analyzed using the method of Devadasan & Nair (1970) with slight modification. Extraction was done in a cold room at 4 ^{0}C and estimation of each parameter mentioned below were done in triplicate.

Extraction procedure

5 g of homogenized meat was weighed out into a conical flask .Added about 25 ml of KCI-Borate buffer (M-0.05, pH -7.5, 0 ^{O}C) to it. Mixed well and kept overnight at 4 ^{O}C with continuous shaking in a orbital shaker and was centrifuged on the next day at 5000 rpm for 30 minutes at 0°C. Transferred the supernatant to a 100 ml standard flask kept at 0 ^{O}C. The residue was extracted twice with the same buffer and the supernatant collected into the standard flask. The volume was made up to 100ml with the buffer. The residue after extraction of the sarcoplasmic protein was extracted with KCI-Phosphate buffer (KCl-NaH_2P0_4 -Na_2HPO_4 buffer M = 0.6, pH =

7.5 at 0 ^{O}C) in the manner described above. Supernatant collected is myofibrlllar protein and its nitrogen content was determined in an aliquot of the made up solution. The residue after this extraction was similarly extracted with 0.1 N NaOH at room temperature to give the in alkali soluble protein (denatured protein). The residual stroma protein was estimated by direct digestion with sulphuric acid. (Raman & Mathew, 2005). Protein nitrogen content was determined in all the aliquots of protein fractions according to AOAC (2000) and multiplied by the factor 6.25 to get the protein content.

4.3.3: Fractionation of collagen

Extraction of acid soluble, pepsin soluble and insoluble collagen was carried out according to the method of Zhang *et al*. (2011) with slight modification (Raman & Mathew, 2005). All the preparative procedures were performed at 4 ^{O}C. 5 g of homogenized fish meat was treated with 50 ml of 0.1 N NaOH, incubated the sample overnight with continuous shaking, and centrifuged at 5000 rpm for 30 minutes and repeated the procedure twice. Residue obtained was then extracted with 0.5 M acetic acid for 2 days, and were centrifuged at 12,000 rpm for 30 min. Re-extracted the residue with the same solution for 1 day, and these extracts were centrifuged under the same conditions. Combined the supernatants and used as acid soluble collagen. Suspended residues from the acetic acid extraction in 0.5 M acetic acid and digested with 0.5% (w/v) pepsin for 72 h at room temperature. The extraction steps of pepsin soluble collagen (PSC) were the same as the extraction of acid soluble collagen (ASC). Residue obtained after pepsin digestion is considered as insoluble collagen. The nitrogen content was determined according to AOAC (2000).

4.3.4: Preparation of natural actomyosin (NAM)

Natural actomyosin (NAM) was prepared according to the Benjakul *et al*. (1997) with slight modification. 50 g of fish meat was homogenized in 10 volume of pre-chilled 0.6 M KCl, pH 7.5 for 15 minute using a homogenizer and was stirred for 10 minute in ice to allow the complete solubilization. Extracted solution was centrifuged using refrigerated centrifuge (Eppendorf - Centrifuge 5430 R, Hamburg, Germany) for 30 minute at 8000 rpm at 4 ^{O}C. To the supernatant solution, added 10 times volume of chilled deionized water to precipitate the NAM and was collected by

centrifugation at 8000 rpm for 20 minute at 4 ^{O}C. Dissolved the actomyosin pellet obtained in 20 mM Tris-HCl buffer containing 0.6 M KCl (pH 7.5) with a final concentration of 5 mg/ml.

4.3.5: Determination of ATPase activity

ATPase activity was determined according to the procedure developed by Benjakul *et al*. (1997) with slight modification. To 1 ml of actomyosin solution (5 mg/ml), 0.6 ml of 0.5 M tris-maleate buffer (pH 7.5) and 1 ml 10 mM $CaCl_2$ were added and made up to 9.5 ml. To each assay solution, 0.5 ml of 20 mM ATP was added to initiate the reaction. Incubated the solution mixture for 10 minute at 37 ^{0}C, and terminated by the addition of 5ml chilled 15% TCA (w/v). Reaction mixture was then subjected to centrifugation for 5 minutes at 5000 rpm. Inorganic phosphate obtained in the supernatant was estimated by the method of Fiske & Subbarow (1925). Specific activity was expressed as μmoles of inorganic phosphate (Pi) /min/mg protein. A control was performed simultaneously by adding chilled TCA prior to the addition of ATP.

4.3.6: Statistical analysis

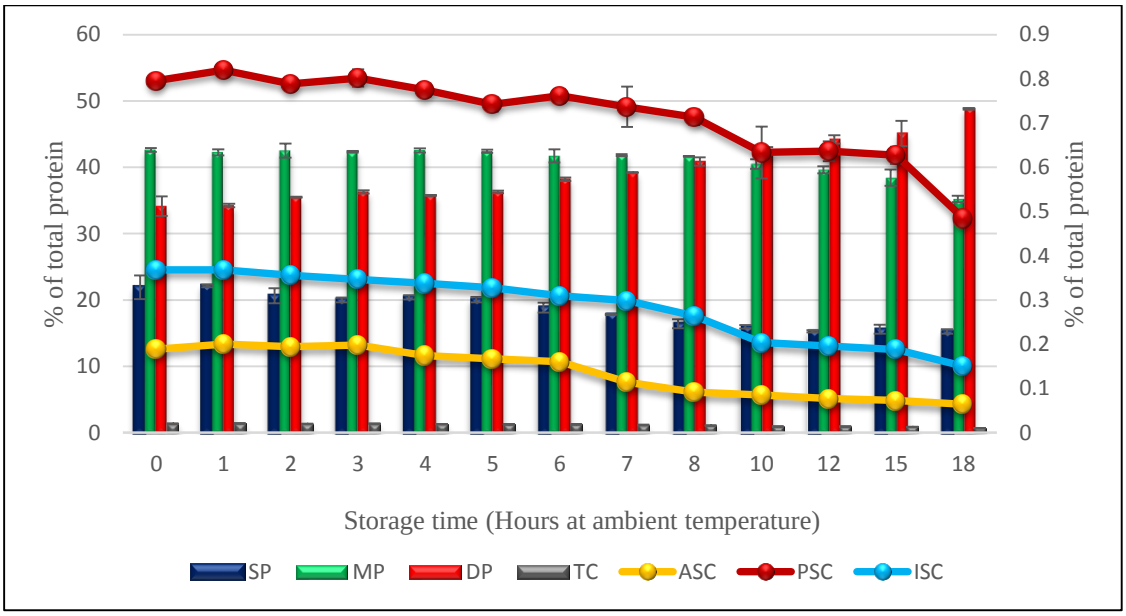
All statistical calculations are performed in IBM SPSS Statistics 20.0 Software. Data analysis was performed using analysis of one way analysis of variance (ANOVA) with post-hoc with multiple comparison analysis performed using Duncan test. Correlation analysis between parameters analyzed was done using Pearson Correlation.

4.4: Results

4.4.1: Solubility changes of the mortem protein fractions

Fig 4.1 - 4.3 show the different protein fractions of tissue samples of climbing perch, banded snakehead and stinging catfish muscle stored at ambient temperature. Table 4.1 - 4.3 show the data for the protein fractions of ice stored fish samples. Expressed the values of the sarcoplasmic protein, myofibrillar protein, alkali soluble protein (denatured protein), total collagens, acid soluble collagen, pepsin soluble collagen and insoluble collagens as percentage of the total protein. With storage both in iced condition and at ambient temperature, the extractability of the protein fractions were significantly altered in all the species under study. One-way ANOVA test was performed to compare differences in means of each protein fractions obtained during

the storage time. Duncan - post hoc analysis was performed to get information regarding changes in muscle tissue protein fractions in response to storage time. Results for one way ANOVA test show that there is a significant difference in protein fraction among the fish species ($p < 0.01$) (Appendix 4.1 and Appendix 4.5).



SP- Sarcoplasmic protein, MP- Myofibrillar protein, DP-Denatured protein, TC- Total collagen, ASC-Acid soluble collagen, PSC- Pepsin soluble collagen, ISC-Insoluble collagen

All values are expressed as mean ± standard deviation, n=3

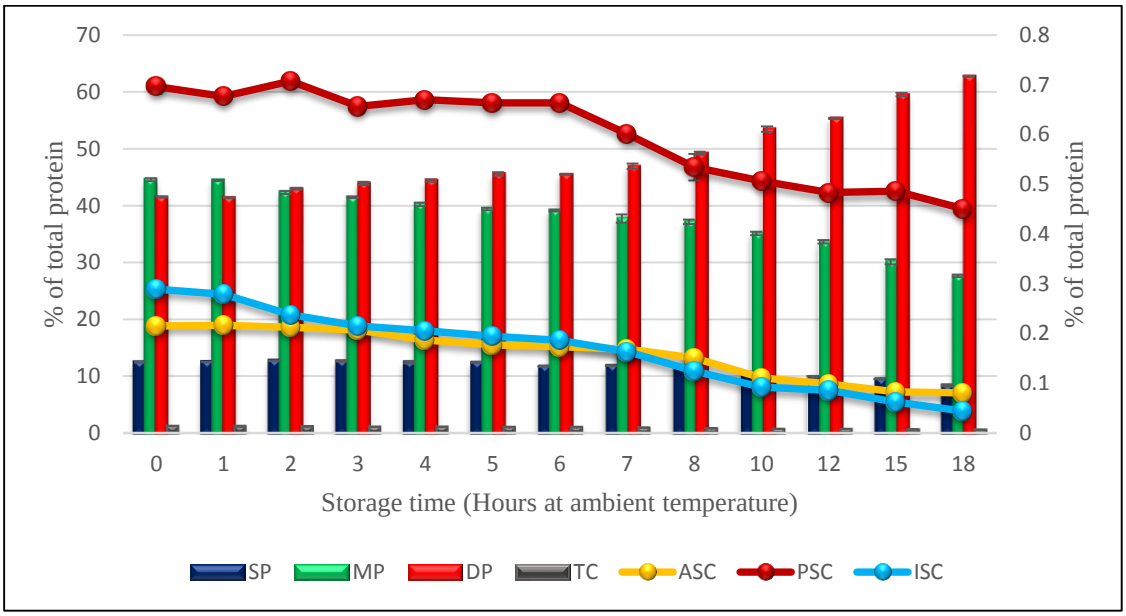
On primary y-axis – SP, MP, DP, TC

On secondary y-axis – ASC, PSC, ISC

Fig 4.1: Protein fractions in the muscle tissue of climbing perch stored at ambient temperature

Fig 4.1 and Appendix 4.2 show the results obtained for the analysis of changes in protein fractions in climbing perch fish muscle stored at ambient temperature for 18 hours. The initial concentration of sarcoplasmic protein in fish muscle (21.90%) decreased to 15.23% at the end of storage period. The concentration of myofibrillar protein and denatured protein (alkali soluble protein) at initial stage of storage was found to be 42.61% and 34.13% respectively. A gradual decrease in the myofibrillar protein and an increase in alkali soluble protein were observed with the progress of post-mortem storage time. Collagen content in the fish muscle was found to be almost stable for the first three hours of storage, which gradually decreased from 1.35% to 0.75%.

The acid soluble, pepsin soluble and insoluble collagen content in the fresh muscle tissue of climbing perch was 0.19%, 0.80% and 0.37%. Acid soluble and insoluble collagen gradually decreased during the study whereas the pepsin soluble collagen remained almost stable till the 6th hour. After the 6 hour the concentration decreased to about 0.52% on storage for 18hours. Analysis of variance shows that all protein fractions significantly changed with storage period ($p < 0.01$).



SP- Sarcoplasmic protein, MP- Myofibrillar protein, DP- Denatured protein, TC- Total collagen, ASC- Acid soluble collagen, PSC- Pepsin soluble collagen, ISC-Insoluble collagen

All values are expressed as mean ± standard deviation, n=3

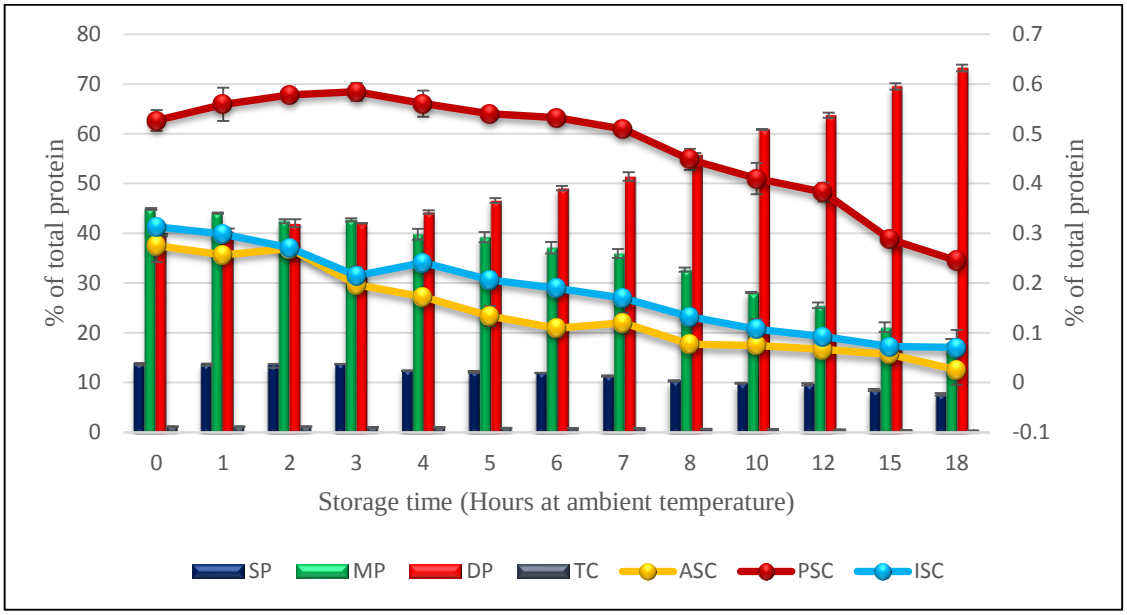
On primary y-axis – SP, MP, DP, TC

On secondary y-axis –ASC, PSC, ISC

Fig 4.2: Protein fractions in the muscle tissue of banded snakehead stored at ambient temperature

Data obtained from the analysis of variation in protein fractions of banded snakehead muscle stored at ambient temperature for 18 hours are given in Fig 4.2 and Appendix 4.3. A decrease from 12.42% to 8.38% in the sarcoplasmic protein was observed at the end of storage period. Myofibrillar protein constitutes the highest protein fraction with a concentration of 44.55% in the fresh flesh, which gradually decreased with increase in storage time. Initial concentration of denatured protein (alkali soluble protein) was found increased to 62.74% on 18 hours of post-mortem storage. 48.33% reduction in total collagen content was observed during the storage time at ambient temperature.

Considering the collagen fractions, pepsin soluble collagen is the highest fraction of total collagen constituting about 0.70% of total protein in the fresh tissue sample. Pepsin soluble collagen was found to be nearly stable for 6 hours of post-mortem storage at ambient temperature, gradually decreased during the study. 0.29% of insoluble collagen and 0.22% acid soluble collagen was observed in the fresh muscle tissue of banded snakehead and a gradual decrease were observed. Analysis of variance shows that all protein fractions significantly changed as storage period advanced ($p < 0.01$).



SP- Sarcoplasmic protein, MP- Myofibrillar protein, DP-Denatured protein, TC- Total collagen, ASC- Acid soluble collagen, PSC- Pepsin soluble collagen, ISC-Insoluble collagen

All values are expressed as mean ± standard deviation, n=3

On primary y-axis – SP, MP, DP, TC

On secondary y-axis –ASC, PSC, ISC

Fig 4.3: Protein fractions in the muscle tissue of stinging catfish stored at ambient temperature

Fig 4.3 and Appendix 4.4 show the results obtained for the analysis of changes in protein fractions present in Stinging catfish muscle stored at ambient temperature for 18 hours. The initial concentration of sarcoplasmic protein in fish muscle was 13.71%, which decreased to 7.62% at the end of storage period. The concentration of myofibrillar protein and denatured protein at the initial stage of storage were 44.87% and 39.65% respectively. A gradual decrease in the myofibrillar protein along with an increase in denatured protein

was observed with the progress of post-mortem storage time. Total collagen content in the fish muscle decreased from 1.12% to 0.36% during the storage period.

The acid soluble, pepsin soluble and insoluble collagen content in the fresh muscle tissue of catfish was 0.38%, 0.41% and 0.33% respectively of total protein content. Acid soluble and insoluble collagen gradually decreased during the study whereas the pepsin soluble collagen remained almost stable till the 4th hour. There after its concentration started decreasing, and reached 0.20 % at 18th hour of storage. Analysis of variance shows that all protein fractions significantly changed as storage period advanced ($p< 0.01$).

Table 4.1: Protein fractions in the muscle tissue of iced stored climbing perch (% of total protein)

Time (Days)	SP	MP	ASP	TC	ASC	PSC	ISC
0	22.90 ^{c} ±1.43	41.97 ^{e} ±0.65	34.57 ^{a} ±2.07	1.30 ^{g} ±0.02	0.20 ^{f} ±0.01	0.74 ^{e} ±0.01	0.35 ^{d} ±0.00
1	23.17 ^{c} ±0.24	40.96 ^{e} ±0.99	34.59 ^{a} ±0.70	1.27 ^{g} ±0.05	0.19 ^{f} ±0.01	0.73 ^{e} ±0.04	0.35 ^{d} ±0.00
3	23.46 ^{c} ±0.43	38.22 $^{c, d}$ ±0.12	37.07 ^{b} ±0.32	1.25 ^{g} ±0.01	0.16 ^{e} ±0.00	0.75 $^{d, e}$ ±0.00	0.33 ^{d} ±0.00
5	23.54 ^{c} ±0.13	36.84 ^{b} ±0.55	38.44 ^{b,c} ±0.45	1.18 ^{f} ±0.02	0.13 ^{d} ±0.01	0.71 $^{d, e}$ ±0.01	0.34 $^{c, d}$ ±0.01
7	23.14 ^{c} ±0.65	37.74 $^{b, c, d}$ ±0.12	37.98 ^{b} ±0.52	1.14 ^{e} ±0.02	0.09 ^{c} ±0.02	0.72 $^{d, e}$ ±0.05	0.32 $^{c, d}$ ±0.01
9	23.43 ^{c} ±0.44	37.60 $^{b, c}$ ±0.01	37.92 ^{b} ±0.36	1.05 ^{d} ±0.06	0.08 ^{c} ±0.01	0.68 ^{d} ±0.00	0.30 ^{c} ±0.05
12	21.20 ^{b} ±0.92	39.28 ±0.24	38.59 ^{b,c} ±1.22	0.92 ^{c} ±0.06	0.04 ^{b} ±0.02	0.60 ^{c} ±0.04	0.28 $^{b, c}$ ±0.03
15	20.28 ^{b} ±2.07	38.93 ^{d} ±0.52	39.97 ^{c} ±1.63	0.82 ^{b} ±0.08	0.03 ^{a,b} ±0.01	0.54 ^{b} ±0.04	0.25 ^{b} ±0.02
18	18.30 ^{a} ±0.65	34.34 ^{a} ±1.33	46.75 ^{d} ±0.72	0.61 ^{a} ±0.05	0.02 ^{a} ±0.00	0.39 ^{a} ±0.03	0.20 ^{a} ±0.01

SP- Sarcoplasmic protein, MP- Myofibrillar protein, ASP-Alkali soluble protein, TC- Total collagen, ASC-Acid soluble collagen, PSC- Pepsin soluble collagen, ISC-Insoluble collagen

All values are expressed as mean ± standard deviation, n=3

Different superscripts in the same column indicates significant difference ($p < 0.01$)

Data obtained for the analysis of changes in protein fractions of ice stored climbing perch muscle tissue is given in Table 4.1 and Appendix 4.6. Concentration of sarcoplasmic fraction was found to be nearly stable for nine days of storage. After that, only a slight decrease (22.90% of total protein to 18.30%) was observed at the end of

storage period. Myofibrillar protein constitutes the highest protein fraction with a concentration of 41.97% in the fresh flesh and a gradual decrease was observed with increase in storage time. Initial concentration of alkali soluble protein was also found to increase during post-mortem storage in ice. 53.08% reduction in total collagen content was observed during the storage period.

Considering the collagen fractions, pepsin soluble collagen is the highest fraction of total collagen constituting about 0.74% in the fresh tissue sample. Pepsin soluble collagen found to be nearly stable for the first three post-mortem days, shows 47% reduction in their concentration on 18th day of storage in ice. 90% reduction in acid soluble collagen was noticed in perch muscle tissue on the last day of storage in ice, whereas insoluble collagen showed only 43% of reduction during the same storage period. Analysis of variance shows that all protein fractions significantly changed as incubation period proceeded ($p< 0.01$).

Table 4.2: Protein fractions in the muscle tissue of ice stored banded snakehead (% of total protein)

Time (Days)	SP	MP	ASP	TC	ASC	PSC	ISC
0	13.15 ^{c} ±0.15	45.09 ^{e} ±0.88	40.64 ^{a} ±1.02	1.12 ^{g} ±0.01	0.20 ^{j} ±0.01	0.68 ^{d} ±0.01	0.24 ^{f} ±0.01
1	13.19 ^{c} ±0.45	44.40 ^{e} ±0.21	41.34 ^{a} ±0.23	1.07 ^{f} ±0.01	0.19 ^{I} ±0.00	0.65 $^{c, d}$ ±0.01	0.23 ^{e,f} ±0.01
3	13.87 ^{c} ±1.02	42.26 ^{d} ±0.66	42.82 ^{b} ±0.34	1.06 ^{f} ±0.02	0.17 ^{h} ±0.00	0.66 ^{c,d} ±0.01	0.22 ^{e} ±0.00
5	13.51 ^{c} ±0.12	41.64 ^{d} ±0.25	43.80 ^{c} ±0.13	1.05 ^{f} ±0.01	0.16 ^{g} ±0.00 ^{f}	0.67 $^{c, d}$ ±0.00	0.22 ^{e} ±0.00
7	13.59 ^{c} ±0.07	39.13 ^{c} ±0.59	46.29 ^{d} ±0. 48	0.99 ^{e} ±0.04	0.14 ^{e} ±0.00	0.65 $^{c, d}$ ±0.02	0.19 ^{d} ±0.01
9	13.21 ^{c} ±0.56	37.26 ^{b} ±0.57	48.60 ^{e} ±0.03	0.94 ^{d} ±0.02	0.13 ^{d} ±0.01	0.64 ^{c} ±0.02	0.17 ^{c} ±0.00
12	13.12 ^{c} ±0.13	37.96 ^{b} ±0.10	48.08 ^{e} ±0.03	0.84 ^{c} ±0.01	0.12 ^{c} ±0.01	0.58 ^{b} ±0.00	0.14 ^{b} ±0.00
15	12.09 ^{b} ±0.06	37.08 ^{b} ±0.65	50.07 ^{f} ±0.57	0.76 ^{b} ±0.01	0.10 ^{b} ±0.01	0.56 $^{a, b}$ ±0.01	0.09 ^{a} ±0.03
18	11.07 ^{a} ±0.35	34.05 ^{a} ±1.07	54.18 ^{g} ±0.68	0.70 ^{a} ±0.04	0.08 ^{a} ±0.00	0.54 ^{a} ±0.04	0.08 ^{a} ±0.00

SP- Sarcoplasmic protein, MP- Myofibrillar protein, ASP-Alkali soluble protein, TC- Total collagen, ASC-Acid soluble collagen, PSC- Pepsin soluble collagen, ISC-Insoluble collagen

All values are expressed as mean ± standard deviation, n=3

Different superscripts in the same column indicates significant difference ($p < 0.01$)

Table 4.2 and Appendix 4.7 show the results of the analysis of changes in protein fractions present in ice stored banded snakehead muscle tissue during 18 days. The sarcoplasmic protein was stable during initial stage of storage in ice, 15.82% reduction in its concentration was observed at the end of storage period. The myofibrillar protein and denatured protein was also unstable, observed a regular decrease in the myofibrillar protein and an increase in denatured protein with progress of post-mortem storage time. Collagen content in the fish muscle was found to be almost stable till 5th day of storage, and a reduction in its concentration was noticed thereafter. About 60%, 20% and 67% reductions in the acid soluble, pepsin soluble and insoluble collagen content were noticed during the study in the muscle tissue of iced stored banded snakehead. Analysis of variance shows that all protein fractions significantly changed with storage period ($p< 0.01$).

Table 4.3: Protein fractions in the muscle tissue of iced stored stinging catfish (% of total protein)

Time (Days)	SP	MP	ASP	TC	ASC	PSC	ISC
0	12.87 ^{d} ±0.65	46.65 ^{e} ±0.47	39.52 ^{a} ±1.11	0.96 ^{g} ±0.01	0.24 ^{g} ±0.01	0.45 ^{d} ±0.00	0.26 ^{f} ±0.01
1	13.29 ^{d} ±0.28	45.54 ^{d} ±0.08	40.24 ^{a} ±0.36	0.93 ^{g} ±0.00	0.23 ^{g} ±0.00	0.44 ^{d} ±0.01	0.26 ^{f} ±0.00
3	12.93 ^{d} ±0.79	43.88 ^{c} ±0.12	42.34 ^{b} ±0.95	0.84 ^{f} ±0.04	0.18 ^{f} ±0.03	0.45 ^{d} ±0.00	0.20 ^{e} ±0.00
5	13.04 ^{d} ±0.11	42.37 ^{c} ±0.92	43.84 ^{c,d} ±0.78	0.76 ^{e} ±0.02	0.15 ^{e} ±0.01	0.42 ^{d} ±0.03	0.19 ^{d} ±0.00
7	13.09 ^{d} ±0.53	43.58 ^{b} ±0.55	42.66 ^{b,c} ±1.05	0.67 ^{d} ±0.03	0.12 ^{d} ±0.00	0.38 ^{c} ±0.03	0.18 ^{c} ±0.00
9	12.62 ^{d} ±0.09	42.29 ^{b} ±0.12	44.48 ^{d} ±0.07	0.61 ^{c} ±0.04	0.09 ^{c} ±0.01	0.35 ^{c} ±0.02	0.16 ^{b} ±0.01
12	10.98 ^{c} ±0.33	41.47 ^{b} ±0.97	47.09 ^{e} ±0.70	0.46 ^{b} ±0.05	0.07 ^{b} ±0.01	0.24 ^{b} ±0.05	0.15 ^{a} ±0.00
15	10.22 ^{b} ±0.34	37.87 ^{a} ±0.43	51.54 ^{f} ±0.10	0.37 ^{a} ±0.01	0.06 ^{a} ±0.01	0.19 ^{a} ±0.01	0.12 ^{a} ±0.00
18	8.50 ^{a} ±0.41	37.08 ^{a} ±0.06	54.05 ^{g} ±0.49	0.37 ^{a} ±0.02	0.06 ^{a} ±0.01	0.18 ^{a} ±0.00	0.13 ^{a} ±0.00

SP- Sarcoplasmic protein, MP- Myofibrillar protein, ASP-Alkali soluble protein, TC- Total collagen, ASC-Acid soluble collagen, PSC- Pepsin soluble collagen, ISC-Insoluble collagen

All values are expressed as mean ± standard deviation, n=3

Different superscripts in the same column indicates significant difference ($p <0.01$)

Table 4.3 and Appendix 4.8 show the results for the analysis of changes in protein fractions in ice stored catfish muscle during 18 days. The sarcoplasmic protein was stable during initial stages of storage in ice and 33.95% reduction in its concentration was observed at the end of storage period. The myofibrillar protein and denatured protein were also unstable, a regular decrease in the myofibrillar protein and an increase in denatured protein were observed with the progress of post-mortem storage time. Observed a gradual reduction in the total collagen content of 75%, 60% and 50% reduction in the acid soluble, pepsin soluble and insoluble collagen content in the muscle tissue of iced stored snakehead muscle tissue. Analysis of variance shows that all protein fractions significantly changed with storage ($p < 0.01$).

4.4.2: Correlation analysis

Correlation of myofibrillar protein and collagen with WHC of three fish stored at ambient temperature and at iced condition was carried out using Pearson analysis in SPSS statistic software. The correlation analysis using SPSS shows that these two proteins have a great impact on holding of water in the fish muscle tissue (Table 4.4 & Table 4.5).

Table 4.4: Correlation study of myofibrillar protein and total collagen with WHC of three fishes stored at ambient temperature

Correlation analysis			Myofibrillar protein	Total collagen
Climbing perch	Pearson Correlation	WHC	.983**	.900**
	Sig. (2-tailed)		.000	.000
Banded snakehead	Pearson Correlation		.980**	.959**
	Sig. (2-tailed)		.000	.000
Stinging catfish	Pearson Correlation		.943**	.914**
	Sig. (2-tailed)		.000	.000

**. Correlation is significant at the 0.01 level (2-tailed).

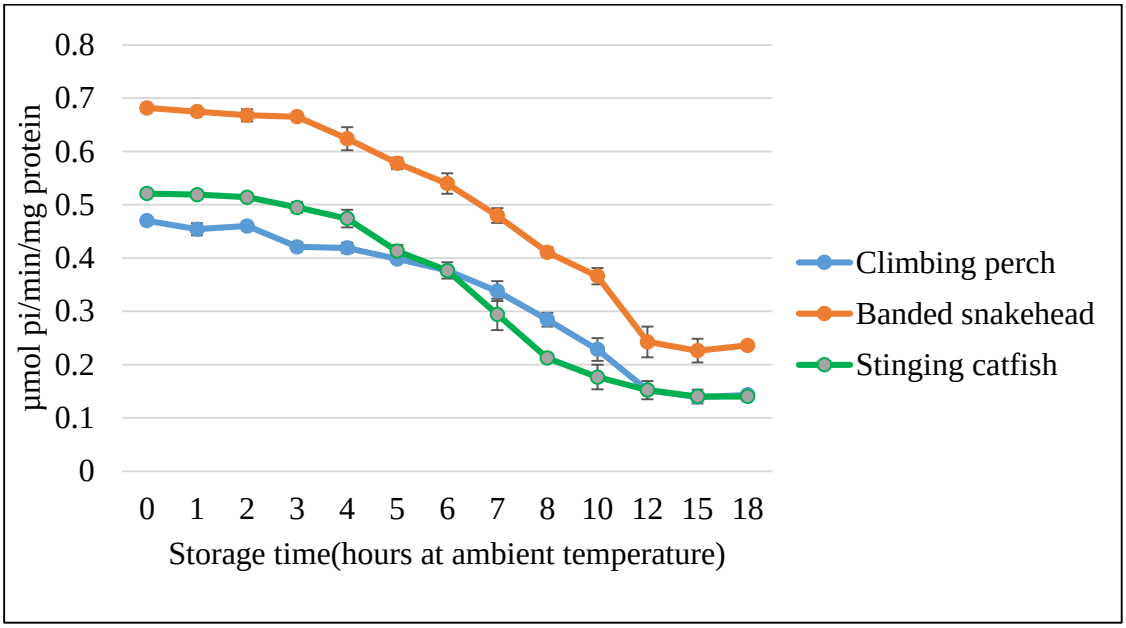
Table 4.5: Correlation study of myofibrillar protein and total collagen with WHC of three fishes stored at iced condition

Correlation analysis			Myofibrillar protein	Total collagen
Climbing perch	Pearson Correlation	WHC	.641*	.969**
	Sig. (2-tailed)		.032	.000
Banded snakehead	Pearson Correlation		.813**	.952**
	Sig. (2-tailed)		.008	.000
Stinging catfish	Pearson Correlation		.884**	.944**
	Sig. (2-tailed)		.002	.000

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

4.4.3: Myofibrillar ATPase activity

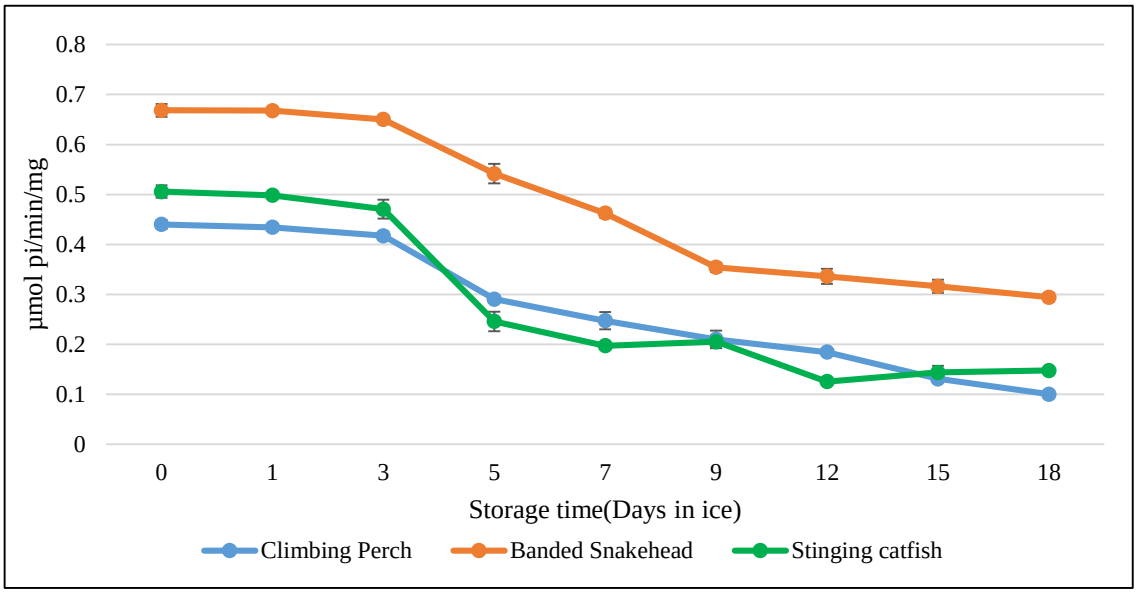


All values are expressed as mean ± standard deviation, n=3

Fig 4.4: Myofibrillar ATPase specific activity of climbing perch, banded snakehead and stinging catfish stored at ambient temperature

Fig 4.4 and Appendix 4.9 show myofibrillar ATPase activity in muscle tissue fraction of climbing perch, banded snakehead and stinging catfish stored at ambient temperature for 18 hours. Myofibrillar ATPase activity at 0 hour of storage of three fishes is in the range 0.47 to 0.68 µmol pi/min/mg protein, banded snakehead showed

higher specific activity and perch showed lower activity. The enzyme activity seemed to be almost constant during the initial period followed by a steep decrease on post-mortem storage. 68%, 65%, 71% reduction in ATPase activity was noticed in perch, snakehead and catfish respectively by 12th hour. ANOVA indicated that post mortem storage of fish at ambient temperature significantly influenced the myofibrillar ATPase activity in all cases and significantly varied between the species ($p< 0.01$).



All values are expressed as mean ± standard deviation, n=3

Fig 4.5: Changes in myofibrillar ATPase specific activity in muscle tissue of iced perch, snakehead and catfish

Fig 4.5 and Appendix 4.10 show data obtained for the myofibrillar ATPase activity in three fresh water fish stored at iced condition for 18 days. The ATPase activities of myofibrillar fraction were 0.44, 0.67, and 0.51 µM Pi/mg protein/min respectively in the fresh climbing perch, banded snakehead and stinging catfish. ATPase activity was found to be almost stable during the initial stage of storage (3 days), followed by a sharp decrease and almost stable towards the end with a very low value. ANOVA result indicated that iced stored condition significantly influenced the myofibrillar ATPase activity in all cases and it significantly varied between and within the species ($p< 0.01$).

4.5: Discussion

4.5.1: Changes in protein fractions during post-mortem storage of fish

Structural level conformational changes in the fish muscle proteins affect their functional properties too. Post-mortem storage temperature induces the fish muscle protein denaturation by altering the secondary, tertiary and quaternary level structural organization. In fresh fish samples, the total sarcoplasmic protein content was high in climbing perch than snakehead and catfish. Compared to other fractions, only a slight decrease of sarcoplasmic protein was noticed in muscle tissue fractions of all the three fish species at both conditions of storage. The results obtained proved that the sarcoplasmic protein content displayed a certain degree of stability during storage. Additionally, in iced stored samples, a slight increase in sarcoplasmic protein fraction was observed during the initial time of storage and decreased by the end. Peculiarity of sarcoplasmic protein is the presence of highly active autolytic enzymes. Additionally, leaching reduces the total sarcoplasmic protein in the muscle tissue extract considerably during the later period at both iced and ambient condition. Further, high temperature abuse at ambient temperature storage induces protein aggregation and the resultant accumulation of these proteins in the inter-fibrillar network. The result is in good agreement with Devadasan & Nair (1970), who studied the sarcoplasmic protein changes in sardine, prawn and mackerel stored at iced condition. Kamal *et al*., (2000) also reported that at the end of 10 days in ice, the sarcoplasmic protein content slightly decreased in shrimp and prawn muscle. The present study suggests that an increase in the sarcoplasmic protein during the initial period of post-mortem storage might be due to the releasing of membrane-stored enzymes or due to the formation of new water-soluble low molecular weight proteins. Further, decrease during the later stage of storage is mainly due to leaching out.

According to myofibrillar protein content in the fresh samples, the fish species selected under the study are arranged in the order as climbing perch< banded snakehead< stinging catfish. Solubility of myofibrillar protein decreased throughout the storage period. Highest myofibrillar protein content was found in stinging catfish muscle tissue. Compared to snakehead and catfish, the extent of post-mortem degradation of myofibrillar protein is least in perch. Presence of alkali soluble protein is higher in snakehead muscle tissue than perch and catfish. It increased with storage

probably due to the action of proteolytic degradation of muscular protein. Pacheco-Aguilar *et al*. (2000) reported that in iced stored sardine, the myofibrillar protein fraction was unstable; sarcoplasmic, myofibrillar and alkali soluble protein of the whole muscles of Monterey sardine sampled during spring decreased. Hossain *et al*. (2005a) reported that there was a gradual decrease in myofibrillar protein solubility in Thai Pangas muscle during the storage period of 25 days in ice.

Loss in myofibrillar protein solubility in fish muscle during storage period is due to the formation of protein aggregate by hydrogen, hydrophobic and disulfide bonds (Jiang *et al*., 1988). Hossain *et al*. (2005b) reported that the myofibrillar protein solubility decreased in queen fish when fish was stored in iced condition for 20 days. Sarma *et al*. (1999) stated that unfolding of myofibrillar protein exposing the hydrophobic groups to the exterior could decrease the extractability of protein. Sano *et al*. (1994) and Sankar & Ramachandran (2005) reported relation between protein insolubility and hydrophobicity. Formation of cross-linking between myosin and actin in the post-rigor muscle could also contribute to the insolubility (Offer & Trinick, 1983). Alteration in myofibrillar protein solubility may also lead to its proteolytic degradation to low molecular weight proteins that are more soluble in water than the native one (Lin & Park, 1996). A significant level of decrease in Ca^{2+} ATPase activity from all the three fish stored at iced and ambient condition indicated the post-mortem denaturation of the myofibrillar proteins. The myofibrillar ATPase activities have been widely used as a measure of actomyosin integrity. The speed of shortening of muscles is related to the activity of the myofibrillar ATPase and can be used to monitor post mortem changes.

The total collagen content in fresh samples was low in stinging catfish compared to climbing perch and banded snakehead. Total collagen content in these fishes decreased during the post-mortem storage conditions. The decrease was gradual at the first half of the storage followed by a rapid decrease in all cases. The rate of post-mortem collagen proteolysis was greater in catfish than other two fishes. The present results indicate that the collagen content in catfish is less stable and could be easily solubilized. Presence of comparatively high amount of total collagen may be the one reason for rigidity of climbing perch than two others. Collagen offers an increased mechanical strength from their cross-links to the muscle tissue.

Kamal *et al*. (2000) reported that the stroma protein slightly decreased in prawn and shrimp during the iced storage period. Suarez *et al*. (2005) stated that collagen content in farmed sea bream muscle decreased slightly over the storage period in ice. The structural change in collagen fibrillar network, separation of collagen fibrils and fibers from endomysium and perimysium resulted in the postmortem tenderization (Liu *et al*., 1994; Ando *et al*., 1995; Nishimura *et al*., 1996).

The water holding capacity is strongly influenced by changes in fish muscle protein structure. Sotelo (1995) supported that change in water holding capacity along with decrease in myofibrillar protein solubility is due to cross-linking between adjacent polypeptide chains by formaldehyde derived from trimethyl amine oxides. Results from the statistical analysis point toward the degradation of connective tissue collagen and myofibrillar protein, which significantly influences loss of water holding capacity of the fish muscle resulting in increase of expressible water content in all the cases. The obtained results also prove that collagen has significant role in holding the moisture content along with myofibrillar protein, further clarifying their role in structural integrity and maintenance of physico-chemical properties in fish muscle. The increase in expressible water content also confirms this phenomenon (Chapter 3). The present study also articulates that changes in water holding capacity, expressible water content and protein denaturation correlate well to the post-mortem storage period in all the three freshwater fish species.

Variation in pH values away from neutral also altered the state of ionization of various charged amino acids in proteins leading to alteration in the electrostatic state and a subsequent conformation change in protein structure. Additionally, pH induced protein aggregate formation reduces its total surface area and could be the reason for partial denaturation followed by a decline in water holding capacity to some extent in the three fish muscle tissues. The minimum repulsion between proteins observed at pH around 5-5.1 increases the water holding capacity (Wismer- Pederson, 1978).

Changes in total collagen and its fractions were found to be highly related to changes in pH in all cases (Chapter 3), proving that structural properties of collagen in post-mortem fish muscle could change in association with variation in pH thus making them more susceptible to attack by endogenous collagenolytic enzymes. The pH of flesh has great influence on the tensile strength of the connective tissue. Additionally,

denaturation of muscular proteins depends on the concentration of autolytic enzymes and other compounds present (Johnston *et al*. 1994; Gartwaite, 1997; Rahman, 1999).

4.5.2: Change in collagen fractions

The muscle tissue from perch, snakehead and catfish contains a high proportion of pepsin soluble collagen followed by insoluble collagen and acid soluble collagen. Upon post-mortem storage, a gradual reduction in acid soluble collagen was observed with progression of storage time both in ice and at ambient temperature. Pepsin soluble collagen was stable during initial storage and decreased thereafter in all cases. The result confirmed that the pepsin soluble collagens from the selected freshwater fish muscle tissues are less susceptible to post-mortem proteolysis than acid soluble collagen and insoluble collagen. Furthermore, pepsin soluble collagen is crucially responsible for post-mortem softening in all the three freshwater fish muscle tissues selected for the study.

Slow degradation rate for the pepsin soluble collagen leads to an increase in the non-helical region than helical ones in muscle tissue collagen, which can be interpreted as the result of action of collagenase enzymes on the helicoid regions. Furthermore, reduction in acid soluble collagen content occurred in pre-rigor stage in all the fish species stored in ice and at ambient temperature. The obtained results are in good agreement with Suarez *et al*. (2005), who investigated that a decline was detected in acid soluble collagen in farmed sea bream in the first post-mortem period. Modzelewska-Kapitula *et al*., (2015) reported a significant level of increase in acid soluble collagen and total collagen along with a reduction in insoluble collagen content in bovine *M. infraspinatus* during aging between 5^{th} to 10^{th} day in vacuum at 3 ^{0}C. High percentage of PSC fraction (57% in perch and 58% in snakehead and 47% in catfish) indicates a high initial proportion of collagen with cross-links and hence of great firmness. Similar values are reported for other fish species, such as trout (35.60%, Sato *et al*., 1991); sardine and tiger puffer (37.30% and 75.00% respectively, Sato *et al*., 1997); salmon (70.50%, Adios *et al*., 1999) and bream (41.00%, Suarez *et al*., 2005).

High insoluble collagen content contributes their peculiar firm texture to the perch muscle than snakehead and catfish. Observed decline in Insoluble collagen content with increasing post-mortem storage time both in iced condition and at ambient

temperature. The result is in good agreement with Eckhoff *et al*. (1998), who reported that in Atlantic salmon, the insoluble collagen content was decreased at 15^{th} day of storage in contrast with day zero. Studies also reports that ISC are primarily allied with the cross linking of collagen molecules, which have an influence on the textural integrity (Montero & Borderias, 1990).

Reduction in collagen is strongly associated with an increased autolysis caused by both native and microbial proteolytic enzymes. (Kubota *et al*., 2003; Sriket, 2014**)**. Sato *et al*. (1987) reported that proteases in fish muscle hydrolyze preferably the non-helical region of collagen, leading to more soluble collagen. The present study established a close relationship between breakage of pepsin soluble collagen and softening of post-mortem fish muscle. From the study, it can be inferred that composition, localization and proportion of different collagen fractions in fish muscle tissue could have a central role in texture determination. The high amount of insoluble collagen than the acid soluble collagen in climbing perch might be the reason for great firmness of the muscle tissue.

4.6: Conclusion

Post-mortem changes in muscular protein content unfavorably affect the total quality and thus the acceptance of fish. The present chapter concludes that, upon post-mortem storage of fish at iced and ambient temperature, the myofibrillar protein underwent a high degree of degradation, accompanied by an increase in total denatured protein (alkali soluble protein). The concentration of sarcoplasmic protein shows the release of stored autolytic enzymes from membrane bound organelles into the cytosolic fluid. Degradation of total collagen is directly related to the post mortem quality loss of fish. Rigidity of the fish muscle is due to the presence of the high insoluble collagen content. In-rigor post mortem fish muscle faces acid soluble collagen degradation, while post-rigor softening is typically due to the degeneration of pepsin soluble collagen.

Chapter 5

Chapter 5

Textural and histochemical changes in fish muscle during post-mortem storage

Contents

5.1: Introduction

Fish and fishery products are considered as "fresh" when its physico-chemical properties are close to those of living condition. Sensory evaluation is a primary assessment method for the consumer to determine the quality of fish and fishery products. Quality decision by consumer is carried out mainly through the visual and non- oral practices. Histochemical and textural analysis is the critical attributes for evaluation of fish and fishery products quality, which is dependent on many external and internal factors. The textural property of food describes the physical properties related to buccal perception during the process of mastication and its swallowing. Whereas, the sensory evaluation analyzes flavors and aroma attributes present in the food system. Normally the texture of fish muscle in full-rigor condition is quite tough and then gradually tenderize as the fish enters in post-rigor stage. The post-mortem fish muscle textural profile is directly or indirectly dependent on myofibrillar proteins and extracellular connective tissue proteins. Evaluation of histochemical changes in post-mortem storage of fish muscle gives cellular and subcellular level information. The histochemical characterization of fish muscle gives an insight on their textural profile and physio-chemical properties. The present chapter conducted studies on changes in textural properties of fish muscle in terms of instrumental textural profile analysis (TPA), histochemical studies and sensory analysis of selected freshwater fishes- climbing perch, banded snakehead and stinging catfish, during post-mortem storage.

5.2: Review of Literature

Fish is a highly delicate and feeble food product, compared to others and its quality and safety are guaranteed based on parameters related to its freshness. According to Cardenas *et al*. (2007), sensory evaluation is considered as the principal method to evaluate the freshness of seafood. Valuation of sensory parameters gives rapid, direct, easy and accurate unique information about the food (Hyldig & Green-Petersen, 2004). Sensory analysis is defined as the scientific method that is used to evoke, analyze, measure and interpret the response that are perceived by human being based on characteristics of food through the senses: sight, taste, smell and touch (Lawless & Heymann, 2010). Loss of freshness of fish is the result of post-mortem physicochemical, biochemical and microbial action and can be detected and evaluated by sight, touch, smell and taste (Huidobro *et al*., 2000). The quality index method (QIM) is a freshness grading system based on significant sensory parameters for a raw fish with a scoring system from zero to three demerit scores (Bremner, 1985; Huidobro *et al*., 2000; Sveinsdottir *et al*., 2002). Enzymatic degradation of protein alters the fish quality by producing peculiar sensory components like nitrogenous bases and volatile amines generated by making a direct change in the structure of proteins, which then change the tissue properties like water holding capacity, firmness, juiciness and gel forming ability (Pacheco-Aguilar *et al*., 2003).

Textural properties generally highlights the sensory perception of fish freshness and physical characters, while the structure mainly focus on subtle changes in internal characteristics and offers additional information on textural changes induced by external factors. Due to this fact, there exist a direct relation between texture and structure (Cheng *et al*., 2014). The structure and texture of fish muscle influences several internal features such as hardness, cohesiveness, springiness and stiffness. According to Coppes *et al*. (2002), Morkore & Einen (2003), textural measurement in fish and fishery products shows a direct correlation with that of sensory and instrumental measurements.

Textural profile analysis (TPA) is defined as the sensory analysis of the texture of a food item in terms of its mechanical, geometrical and compositional properties, the degree of each trait present, and the order in which they appear from the first bite through complex mastication (Brandt *et al*., 1963). It is a parameter that human being

perceives, describes, quantifies, and imitates the satisfactory action of jaw. The texture profile analysis is carried out by manipulating the mechanical properties from a force-time curve generated by compressing the test material at least twice. Textural characteristics of fish muscle tissue are significantly correlated to the time-temperature profile during the storage. Textures are the most delicate factor influencing sensory acceptance by consumers, as they generally prefer firm and elastic meat and is also important for mechanical processing of fish fillet in food industry (Dunajski, 1979; Haard, 1992).

Textural properties of fish muscle is closely associated with two structural proteins namely myofibrillar and connective tissue proteins, which give the tissue its mechanical properties (Fuentes *et al*., 2012). The fish muscle tenderness depends on availability of ATP for breakage of actomyosin cross bridge during muscle contraction. Hultmann & Rustad (2002) said that the salt-soluble protein fraction determines the textural properties of salmon and cod fillet during iced storage. Furthermore, the collagen also influences the fish muscle tissue firmness (Hatae *et al*., 1986; Sato *et al*., 1986, Venugopal & Shahidi, 1996; Suarez *et al*., 2005; Lepetit, 2007; Cheng *et al*., 2014). Haard (1992) found that the fish muscle have about $1/10^{th}$ the collagen content and cross-links compared to muscle tissues of terrestrial animals, and because of this, the texture of fish muscle is more softer than that of terrestrial animal. Anderson *et al*., (1997) reported that textural features of farmed rainbow trout fish have a direct relationship with their fat content. Torgersen *et al*. (2014) stated that there was a significant level of correlation with soft flesh and massive intracellular glycogen accumulation with that of myocyte detachment and altered extracellular matrix protein distribution in Atlantic salmon. Jacobsen *et al.* (2017) reported that the post-mortem muscle tissue gaping and its quality loss closely correlated with post-slaughter cleaning of the abdominal cavity of fillet and firmness in farmed salmon (*Salmo salar L.*)

Histochemical analysis is one of the most effective methods used to evaluate the quality change in fish muscle, which gives direct quality information at cellular and subcellular level. The nutritive value and texture of fish muscle is greatly influenced by structure and composition of muscular proteins (Kiessling *et al*., 2006). Deterioration of muscle is due to the proteolytic degradation of minor components connecting the structural unit together (Olafdottir *et al*., 1997). Disintegration of cytoskeleton and

connective tissue cross-links that are formed along the extracellular components are the crucial factor in the post mortem fillet tenderization (Bahuaud *et al*., 2008). Both low post mortem pH and stress develops an increased tension in connective tissue proteins, triggering the shrinkage of myofibrillar proteins and overall textural deprivation (Bahuaud *et al*. 2010). Previous studies reported that degradation of myofibrillar proteins was the reason for softening of fish muscle during storage in ice (Caballero *et al*., 2009; Ayala *et al*. 2010). Weakening of connective tissue and Z-line are equally responsible for reduction in textural properties in the fish muscle after storage (Sato *et al*. 1997; Ando 1999; Lakshmanan & Piggott 2003). Loss of water content in fish muscle had a negative impact on texture of the fish leading to muscle toughness (Chen *et al*., 1990). Information on the textural parameters of these freshwater species under study is lacking and hence analysis of each of these textural parameters and histochemical changes of structural proteins along with sensory evaluation has been undertaken in this chapter.

5.3: Materials and Methods

5.3.1: Sample Collection

The samples of climbing perch, banded snakehead and stinging catfish were collected and tissue samples were prepared as detailed in 3.3.1.

5.3.2: Sensory Evaluation

Sensory evaluation was carried out using quality index method (QIM) developed by Bremner (1985) with some modifications. Five trained panelists carried out each assessment. Six fish from each storage period were analyzed based on general appearance, muscle stiffness, consistency of belly, shape and colour of eyes, mucus and odour of gills, integrity and brightness of peritoneum and adherence to vertebral column (Appendix A 5.a). Assessed each parameter based on minima of demerit score from zero to a maximum of three, where zero indicated very fresh with optimum quality, while the higher score denoted the degree of spoilage or progress of quality loss. The sum of the total demerit score evaluated for separate characteristics gave an overall sensory demerit score (SDS). The maximum overall demerit score was 32. The total demerit score-point up to five is considered as excellent, 10 as good, 20 as acceptable and above 20 as bad or rejected.

5.3.3: Textural Profile Analysis

Textural profile analysis was carried out using Texture analyzer (Lloyd Instrument, UK, Model LXR PLUS) with NEXYGEN software. Flat-faced cylindrical probe of 50 mm diameter equipped with a load cell of 50 N and a test speed of 12 mm/ min compressed with a bite uniform size of fish piece 5mm thickness twice in a reciprocating motion, imitating the mouth action. Samples were subjected to double compression of 40%. From the force- time plot, hardness 1- after first compression,(Kgf), hardness 2- after second compression (Kgf), cohesiveness, springiness(mm) and stiffness (Kgf/ mm) were evaluated (Fig.5.a).

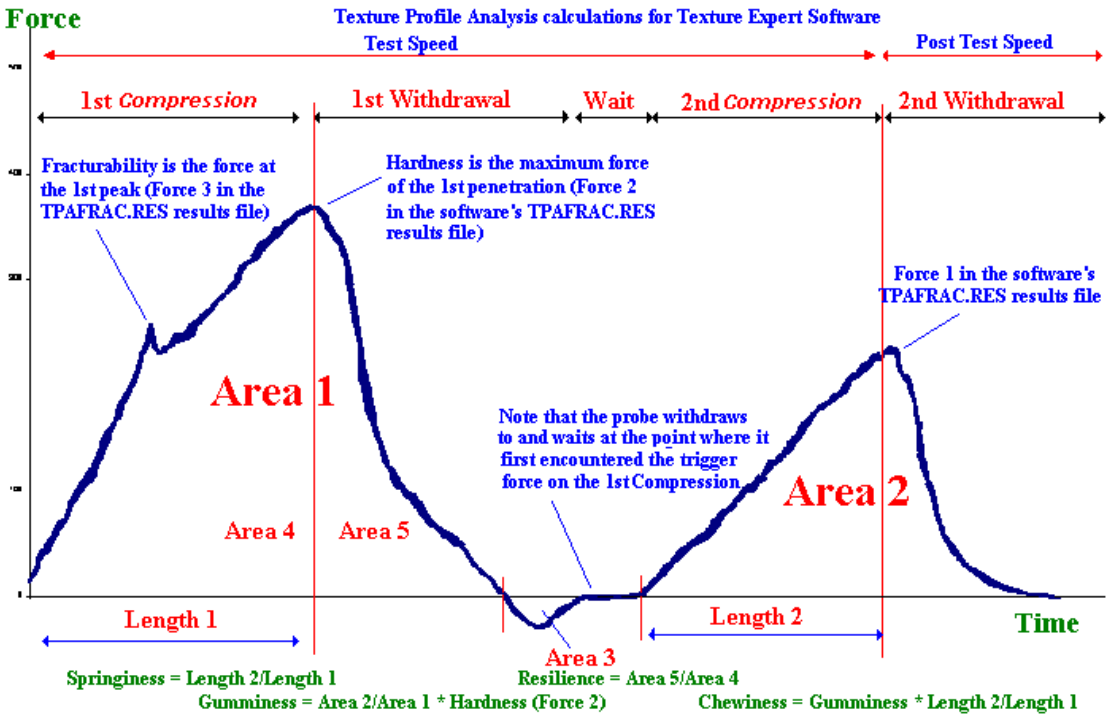


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Fig 5.a: Force –time profile of textual profile analysis

5.3.4: Histochemical evaluation of muscular pattern

Samples of fish muscle were cut into small piece of blocks and fixed overnight using formalin fixative, followed by dehydration in serially diluted alcohol (70-96%). Then the dehydrated samples were treated with xylene to remove alcohol content and embedded in paraffin wax (melting point 60 ^{O}C). Paraffin embedded samples were cut into sections of 5 micron using rotary microtome (Leica RM2155, Germany). Double

staining was performed using Haematoxylin stain and Eosin stain. The myofibrils and collagen were strained yellowish orange and blue respectively, which was then observed under compound light microscope and photographed. (Leica microsystems, CH-9435, Heerbrugg, Switzerland)

5.3.5: Statistical analysis

All statistical calculations are performed in IBM SPSS Statistics 20.0 Software. Data analysis was using one-way analysis of variance (ANOVA) with post-hoc for multiple comparison analysis performed using Duncan test.

5.4: Results

5.4.1: Sensory Evaluation

The evaluation of quality of whole fish was assessed by analyzing the sensory parameters of three fish species selected under the study. The result obtained for the assessment of sensory demerit score of the fish stored at ambient temperature is given in Fig 5.1 and Appendix 5.1. Quality demerit score for fish stored in iced condition is depicted in Fig 5.2 and Appendix .5.2. The variation in the means of data for quality demerit score was determined using ANOVA test. For further clarification, Duncan Post- hoc test was performed.

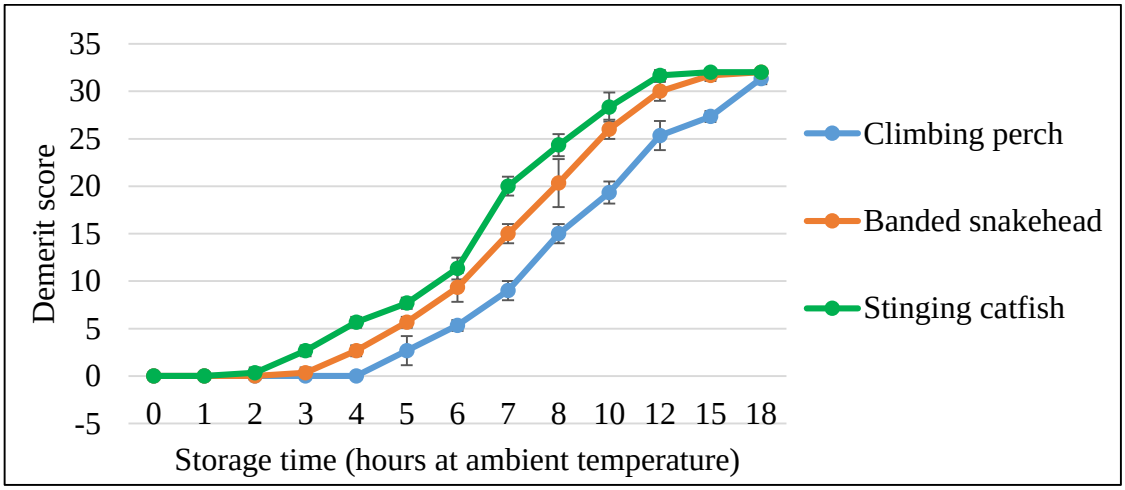


Fig 5.1: Quality demerit score of climbing perch, banded snakehead and stinging catfish stored at ambient temperature

Results showed that climbing perch and banded snakehead were excellent for consumption for the first five hours. Climbing perch was found to be worthy for 7

hours and unacceptable after 10 hours while banded snakehead found to be good for 6 hours and unacceptable after 8 hours. Climbing perch showed highest demerit score of 31.33 on 18 hours of storage. Regarding catfish, the quality demerit score reached above five within 4 hour of storage, indicating that its quality would be excellent only for first three hours at ambient temperature, as good for five hours and unacceptable after 7 hours of storage. Results of one way ANOVA and Duncan post hoc test explained that the sensory demerit score significantly increased with progress of storage time and significantly varied among the species ($p< 0.01$).

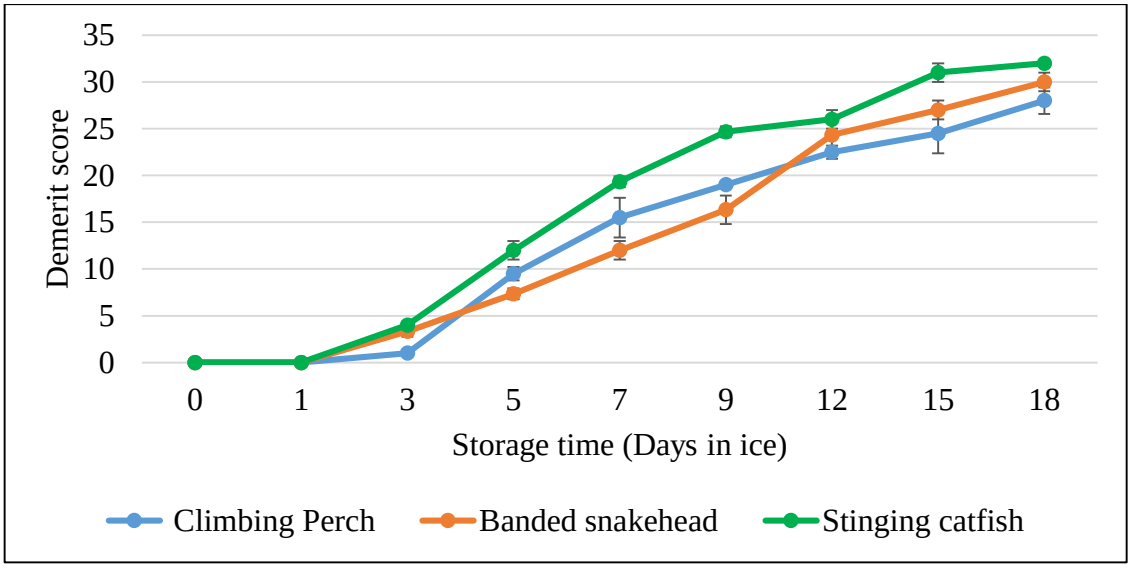


Fig 5.2: Quality demerit score of perch, snakehead and catfish during iced condition

Results showed that the demerit score gradually increased in iced storage also. The sensory demerit score reached above five after day 3 in all three cases, and considered as excellent for consumption. Meanwhile, demerit score reached above 10 within 7 days of storage for perch and snakehead, and suggested that the fish was moderately good for consumption. Stinging catfish attained unacceptable stage after 7 days with maximum demerit score of 32 on 18th day of storage in ice. Banded snakehead and climbing perch showed maximum demerit score of 30 and 28 respectively on the last day of storage in ice and were in acceptable condition until 9th day of storage.

5.4.2: Textural Profile Analysis

Instrumental quality evaluation was carried out by analyzing textural parameters of the three fish species selected for the study. The results obtained regarding texture profile analysis with respect to hardness 1, hardness 2, cohesiveness, chewiness, springiness and stiffness are given in Tables 5.1 to 5.4 and Appendix 5.3 to 5.6. Statistical analysis using ANOVA test determined variation in the means of data obtained for textural profile analysis. Duncan Post- hoc test gave its further clarification.

Table 5.1.a: Hardness 1 and hardness 2 of climbing perch, banded snakehead and stinging catfish muscle tissue stored at ambient temperature

Storage time (hours)	Hardness 1 (kgf)			Hardness 2 (kgf)		
	Perch	Snakehead	Catfish	Perch	Snakehead	Catfish
0	1.49^{f} ±0.10	$0.53^{f,g,h}$ ±0.01	$0.43^{e,f}$ ±0.00	$0.28^{c,d}$ ±0.00	0.22^{e} ±0.01	0.18^{g} ±0.01
1	1.58^{f} ±0.02	0.61^{j} ±0.05	$0.47^{c,d,e}$ ±0.03	$0.38^{d,e,f,g}$ ±0.01	0.18^{d} ±0.02	0.14^{f} ±0.01
2	1.48^{f} ±0.02	$0.61^{i,j}$ ±0.01	0.46^{f} ±0.06	$0.35^{c,d,e,f}$ ±0.01	0.18^{d} ±0.03	$0.11^{e,f}$ ±0.00
3	1.54^{f} ±0.01	$0.54^{f,g,h}$ ±0.01	$0.45^{e,f}$ ±0.03	$0.45^{c,d,g}$ ±0.00	0.19^{d} ±0.03	0.14^{f} ±0.00
4	1.43^{f} ±0.07	$0.52^{f,g}$ ±0.02	$0.48^{e,f}$ ±0.06	$0.38^{e,f,g}$ ±0.09	0.18^{d} ±0.02	$0.07^{c,d}$ ±0.04
5	1.23^{e} ±0.01	$0.57^{h,I}$ ±0.02	0.48^{f} ±0.02	$0.40^{f,g}$ ±0.00	0.19^{d} ±0.01	$0.11^{e,f}$ ±0.01
6	$1.12^{d,e}$ ±0.04	$0.55^{f,g}$ ±0.08	$0.41^{d,e,f}$ ±0.01	$0.29^{c,d,e}$ ±0.02	0.18^{d} ±0.02	$0.09^{d,e}$ ±0.01
7	$1.06^{d,e}$ ±0.05	0.50^{f} ±0.02	$0.34^{c,d}$ ±0.00	$0.22^{c,d,e}$ ±0.02	0.14^{c} ±0.01	$0.09^{d,e}$ ±0.00
8	$0.82^{c,d}$ ±0.13	0.46^{e} ±0.01	0.30^{c} ±0.00	0.20^{c} ±0.01	0.15^{c} ±0.01	$0.09^{d,e}$ ±0.01
10	0.69^{c} ±0.04	0.40^{d} ±0.02	0.21^{b} ±0.06	$0.29^{c,d,e}$ ±0.01	0.10^{b} ±0.01	$0.04^{b,c}$ ±0.01
12	0.67^{c} ±0.02	0.32^{c} ±0.02	$0.20^{a,b}$ ±0.00	$0.29^{c,d,e}$ ±0.08	0.08^{b} ±0.00	$0.04^{a,b}$ ±0.00
15	0.40^{b} ±0.02	0.26^{b} ±0.02	0.13^{a} ±0.01	0.21^{b} ±0.08	0.01^{a} ±0.01	0.02^{a} ±0.00
18	0.14^{a} ±0.03	0.20^{a} ±0.01	$0.10^{a,b}$ ±0.04	0.04^{a} ±0.02	0.01^{a} ±0.00	0.01^{a} ±0.00

All values are expressed as mean ± standard deviation, n=3

Different superscripts in the same column indicates significant difference ($p < 0.01$)

Table 5.1.b. Hardness 1 and Hardness 2 of ice stored muscle tissue samples of perch, snakehead and catfish

Storage time (days)	Hardness1 (kgf)			Hardness2 (kgf)		
	Perch	Snakehead	Catfish	Perch	Snakehead	Catfish
0	1.53 ^{e} ±0.05	0.57 ^{f} ±0.02	0.38 ^{f} ±0.02	0.56 ^{e,f} ±0.01	0.20 ^{e} ±0.00	0.13 ^{e} ±0.00
1	1.56 ^{e} ±0.03	0.63 ^{g} ±0.04	0.37 ^{f} ±0.00	0.58 ^{f} ±0.02	0.20 e ±0.00	0.12 ^{d} ±0.01
3	1.57 ^{e} ±0.02	0.62 ^{g} ±0.00	0.27 ^{e} ±0.01	0.56 ^{e,f} ±0.00	0.22 ^{f} ±0.00	0.11 ^{d} ±0.00
5	1.45 ^{e} ±0.17	0.60 ^{f,g} ±0.01	0.27 ^{d} ±0.00	0.55 ^{e} ±0.03	0.17 ^{d} ±0.01	0.08 ^{c} ±0.02
7	1.31 ^{d} ±0.01	0.49 ^{e} ±0.01	0.19 ^{c} ±0.01	0.37 ^{d} ±0.01	0.13 ^{c} ±0.00	0.08 ^{c} ±0.00
9	1.16 ^{c} ±0.08	0.43 ^{d} ±0.01	0.18 ^{c} ±0.01	0.34 ^{c} ±0.01	0.13 ^{c} ±0.01	0.07 ^{b,c} ±0.00
12	0.98 ^{b} ±0.02	0.37 ^{c} ±0.01	0.16 ^{b} ±0.01	0.31 ^{c} ±0.02	0.10 ^{b} ±0.01	0.06 ^{a,b} ±0.00
15	0.66 ^{a} ±0.01	0.24 ^{b} ±0.02	0.15 ^{b} ±0.01	0.21 ^{a} ±0.01	0.12 ^{c} ±0.0	0.05 ^{a} ±0.00
18	0.56 ^{a} ±0.00	0.19 ^{a} ±0.00	0.08 ^{a} ±0.00	0.25 ^{b} ±0.01	0.07 ^{a} ±0.0	0.06 ^{a,b} ±0.00

All values are expressed as mean ± standard deviation, n=3

Different superscripts in the same column indicates significant difference ($p < 0.01$)

Results of muscle tissue hardness 1 and hardness 2 of perch, snakehead and catfish during post- mortem storage at ambient temperature is depicted in Table 5.1.a. and for ice stored samples in Table 5.1.b. Their statistical results are given in Appendix 5.3 and Appendix 5.4 respectively. Statistical results show that there is significant level of variation in hardness 1 and 2 between fish species ($p< 0.01$). Hardness characterize the peak force requisite to compress the tissue, indicates resistant power of the fish muscle tissue to deform by an external pressure. With post-mortem storage, all the samples showed a significant variation in hardness 1 and hardness 2 ($p< 0.01$). Perch muscle tissue has highest toughness in fresh condition. There is a slight increase in the values for hardness 1 and 2 in the early post-mortem storage period in all cases at both storage conditions.

Table 5.2.a. Springiness, Stiffness and Cohesiveness of perch, snakehead and catfish muscle tissue stored at ambient temperature

Storage time (hours)	Springiness(mm)			Stiffness (kgf/mm)			Cohesiveness		
	Perch	Snakehead	Catfish	Perch	Snakehead	Catfish	Perch	Snakehead	Catfish
0	0.98 ^{c} ±0.00	1.20 ^{e,f} ±0.03	0.89 ^{d} ±0.01	1.65 ^{d,e,f} ±0.10	0.54 ^{b,c} ±0.10	0.59 ^{b,c} ±0.15	0.20 ^{b,c} ±0.01	0.11 ^{d,e,f} ±0.01	0.08 ^{e} ±0.00
1	0.93 ^{c} ±0.03	1.26 ^{f} ±0.06	0.68 ^{c} ±0.01	1.91 ^{e,f} ±0.10	0.56 ^{c,d} ±0.00	0.64 ^{c,d} ±0.05	0.20 ^{b,c} ±0.03	0.12 ^{g} ±0.01	0.07 ^{e} ±0.00
2	0.88 ^{c} ±0.03	1.25 ^{d,e} ±0.00	0.64 ^{c} ±0.01	1.91 ^{e,f} ±0.05	0.56 ^{d,e} ±0.16	0.79 ^{d,e} ±0.00	0.21 ^{c} ±0.01	0.12 ^{e,f,g} ±0.01	0.14 ^{g} ±0.02
3	0.86 ^{c} ±0.01	1.15 ^{c} ±0.04	0.61 ^{c} ±0.02	2.05 ^{f} ±0.04	0.55 ^{e} ±0.16	0.83 ^{e} ±0.00	0.20 ^{b,c} ±0.00	0.12 ^{f,g} ±0.00	0.12 ^{f} ±0.01
4	0.85 ^{c} ±0.04	1.20 ^{c} ±0.09	0.40 ^{a,b} ±0.10	2.02 ^{d,e,f} ±0.21	0.51 ^{g} ±0.38	0.87 ^{g} ±0.05	0.19 ^{a,b} ±0.00	0.13 ^{f,g} ±0.00	0.13 ^{f,g} ±0.02
5	0.68 ^{c} ±0.05	1.20 ^{d} ±0.05	0.58 ^{a,b} ±0.03	2.11 ^{c,d,e,f} ±0.17	0.56 ^{h} ±0.33	0.94 ^{h} ±0.05	0.20 ^{a,b,c} ±0.01	0.10 ^{c,d,e,f} ±0.00	0.04 ^{a} ±0.00
6	0.61 ^{b,c} ±0.06	1.07 ^{c} ±0.06	0.54 ^{b} ±0.03	2.08 ^{c,d,e,f} ±0.30	0.60 ^{f} ±0.15	0.85 ^{f} ±0.00	0.19 ^{a,b} ±0.01	0.09 ^{c,d} ±0.00	0.06 ^{d,e} ±0.01
7	0.55 ^{a,b,c} ±0.03	1.03 ^{c} ±0.03	0.45 ^{b} ±0.04	2.13 ^{c,d,e,f} ±0.24	0.56 ^{e} ±0.17	0.84 ^{e} ±0.17	0.18 ^{a,b} ±0.01	0.09 ^{c,d,e} ±0.00	0.06 ^{d,e} ±0.01
8	0.45 ^{a,b,c} ±0.03	0.96 ^{c} ±0.07	0.46 ^{b} ±0.07	2.0 ^{c,d,e} ±0.44	0.58 ^{d,e} ±0.04	0.77 ^{d,e} ±0.03	0.17 ^{a,b} ±0.01	0.08 ^{b,c,d} ±0.00	0.04 ^{c,d} ±0.01
10	0.43 ^{a,b,c} ±0.02	0.75 ^{b} ±0.09	0.34 ^{a,b} ±0.02	1.99 ^{b,c,d} ±0.20	0.57 ^{c,d} ±0.05	0.65 ^{c,d} ±0.15	0.17 ^{a,b} ±0.02	0.08 ^{b,c} ±0.03	0.04 ^{b,c} ±0.00
12	0.43 ^{a,b,c} ±0.01	0.65 ^{a,b} ±0.11	0.34 ^{a,b} ±0.01	1.87 ^{b,c} ±0.05	0.49 ^{c,d} ±0.02	0.65 ^{c,d} ±0.01	0.13 ^{a} ±0.02	0.05 ^{a,b} ±0.01	0.04 ^{a,b,c} ±0.00
15	0.30 ^{a,b} ±0.01	0.56 ^{a} ±0.13	0.29 ^{a} ±0.02	1.69 ^{b} ±0.05	0.48 ^{a,b} ±0.05	0.49 ^{a,b} ±0.12	0.12 ^{a} ±0.02	0.04 ^{a} ±0.03	0.02 ^{a,b} ±0.00
18	0.17 ^{a} ±0.02	0.53 ^{a} ±0.03	0.25 ^{a,b} ±0.01	0.90 ^{a} ±0.14	0.37 ^{a} ±0.08	0.59 ^{a} ±0.15	0.09 ^{c} ±0.02	0.04 ^{a} ±0.04	0.01 ^{a,b} ±0.00

All values are expressed as mean ± standard deviation, n=3

Different superscripts in the same column indicates significant difference ($p < 0.01$)

Table 5.2.b: Springiness, Stiffness and Cohesiveness of ice stored samples of perch, snakehead and catfish

Storage time (days)	Springiness (mm)			Stiffness (kgf/mm)			Cohesiveness		
	Perch	Snakehead	Catfish	Perch	Snakehead	Catfish	Perch	Snakehead	Catfish
0	0.73 ^{h} ±0.00	0.77 ^{d} ±0.03	0.70 ^{d} ±0.03	2.62 ^{c,d} ±0.07	0.87 ^{c} ±0.05	0.62 ^{c} ±0.00	0.05 ^{d} ±0.01	0.05 ^{d} ±0.00	0.03 ^{d} ±0.00
1	0.67 ^{g} ±0.00	0.76 ^{d} ±0.02	0.68 ^{d} ±0.02	2.75 ^{d} ±0.02	0.96 ^{c} ±0.05	0.64 ^{d} ±0.01	0.05 ^{d} ±0.00	0.05 ^{d} ±0.00	0.02 ^{d} ±0.00
3	0.67 ^{g} ±0.00	0.76 ^{c} ±0.02	0.56 ^{c} ±0.05	2.75 ^{d} ±0.02	0.96 ^{c} ±0.03	0.59 ^{c,d} ±0.03	0.05 ^{d} ±0.00	0.03 ^{d} ±0.00	0.03 ^{d} ±0.01
5	0.65 ^{f} ±0.01	0.75 ^{c} ±0.02	0.55 ^{c} ±0.03	2.65 ^{c,d} ±0.33	0.92 ^{c} ±0.02	0.55 ^{c} ±0.01	0.04 ^{c,d} ±0.01	0.05 ^{c,d} ±0.00	0.02 ^{c,d} ±0.00
7	0.59 ^{e} ±0.01	0.62 ^{b,c} ±0.03	0.53 ^{b,c} ±0.16	2.54 ^{b,c,d} ±0.04	0.89 ^{c} ±0.06	0.46 ^{b} ±0.12	0.04 ^{b,c,d} ±0.00	0.04 ^{b,c,d} ±0.00	0.02 ^{b,c,d} ±0.00
9	0.53 ^{d} ±0.01	0.56 ^{b,c} ±0.04	0.50 ^{b,c} ±0.02	2.52 ^{b,c} ±0.12	0.89 ^{c} ±0.06	0.43 ^{b} ±0.03	0.04 ^{b,c} ±0.00	0.04 ^{b,c} ±0.00	0.02 ^{b,c} ±0.00
12	0.48 ^{c} ±0.01	0.48 ^{b,c} ±0.02	0.46 ^{b,c} ±0.03	2.37 ^{b} ±0.06	0.88 ^{c} ±0.04	0.41 ^{b} ±0.01	0.03 ^{b} ±0.00	0.04 ^{b} ±0.00	0.02 ^{b} ±0.00
15	0.44 ^{b} ±0.01	0.47 ^{a,b} ±0.00	0.44 ^{a,b} ±0.02	1.74 ^{a} ±0.06	0.63 ^{b} ±0.04	0.40 ^{a,b} ±0.01	0.03 ^{b} ±0.00	0.03 ^{b} ±0.00	0.02 ^{b} ±0.00
18	0.43 ^{a} ±0.01	0.45 ^{a} ±0.02	0.35 ^{a} ±0.00	1.61 ^{a} ±0.01	0.51 ^{a} ±0.03	0.33 ^{a} ±0.00	0.02 ^{a} ±0.01	0.01 ^{a} ±0.00	0.01 ^{a} ±0.00

All values are expressed as mean ± standard deviation, n=3

Different superscripts in the same column indicates significant difference ($p < 0.01$)

Results for muscle tissue springiness, stiffness and cohesiveness of perch, snakehead and catfish during post- mortem storage at ambient temperature is shown in Table 5.2.a, and Appendix 5.5 and that of ice stored samples given in Table 5.2.b and Appendix 5.6**.** In general, a decreasing trend was noticed in springiness over the storage period ($p<0.01$). Observed slight increase in stiffness of climbing perch, banded snakehead fish muscle in pre-rigor condition, and decreased thereafter. The stiffness of

catfish muscle decreased throughout the study. The results of One-Way ANOVA confirmed that there is a significant variation in stiffness within the species ($p< 0.01$). Cohesiveness of climbing perch and banded snakehead fish muscle was almost stable in pre-rigor condition and it decreased thereafter. A slight increase observed in the cohesiveness of catfish muscle until the fourth hour of storage and decreased thereafter.

One-way ANOVA results show that there is a significant level variation in springiness, stiffness and cohesiveness among the fish species ($p<0.01$).

5.4.3: Correlation analysis between muscle tissue protein and textural profile parameters

In order to study the influence of biochemical composition of muscle tissue protein (Chapter 4) on the characteristic textural profile of the perch, snakehead and catfish, correlation analysis was conducted using Pearson's Correlation Index (r) in SPSS with a significance level of $p < 0.01$ or $p < 0.05$.

Table 5.3.a: Correlation between muscle tissue protein and textural profile parameters stored at ambient temperature

		Hardness1			Hardness2			Cohesiveness			Springiness			Stiffness		
		Perch	Snakehead	Catfish	Perch	Snakehead	Catfish	Perch	Snakehead	Catfish	Perch	Snakehead	Catfish	Perch	Snakehead	Catfish
MP	Pearson Correlation	.954**	.947**	.938**	.761**	.949**	.913**	.932**	.917**	.715**	.882**	.953**	.930**	.738**	.688**	.633*
	Sig. (2-tailed)	.00	.00	.00	.003	.00	.00	.000	.000	.006	.000	.000	.000	.004	.009	.020
TC	Pearson Correlation	.943**	.940**	.907**	.764**	.926**	.886**	.961**	.927**	.769**	.961**	.972**	.937**	.553*	.582*	.546
	Sig. (2-tailed)	.00	.00	.00	.002	.00	.00	.000	.000	.006	.000	.000	.000	.050	.037	.053
**. Correlation is significant at the 0.01 level (2-tailed). *Correlation is significant at the 0.05 level (2-tailed).								MP: Myofibrillar protein TC: Total collagen.								

Table 5.3.b: Correlation between muscle tissue protein and textural profile parameters in iced condition

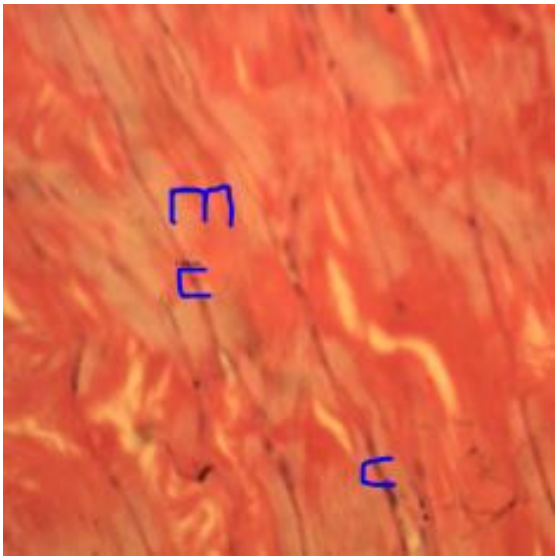
		Hardness1			Hardness2			Cohesiveness			Springiness			Stiffness		
		Perch	Snakehead	Catfish	Perch	Snakehead	Catfish	Perch	Snakehead	Catfish	Perch	Snakehead	Catfish	Perch	Snakehead	Catfish
MP	Pearson Correlation	.534	.903**	.897**	.467	.925**	.894**	.653	.702*	.822**	.518	.934**	.939**	.514	.738*	.888**
	Sig. (2-tailed)	.138	.001	.001	.205	.000	.001	.056	.035	.007	.153	.000	.000	.157	.023	.001
TC	Pearson Correlation	.983**	.971**	.953**	.891**	.903**	.954**	.960**	.743*	.868**	.955**	.955**	.942**	.946**	.860**	.969**
	Sig. (2-tailed)	.000	.000	.000	.001	.001	.000	.000	.022	.002	.000	.000	.000	.000	.003	.000
**. Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed).								MP: Myofibrillar protein TC: Total collagen								

Results obtained for correlation between myofibrillar protein and collagen with textural profile parameters are depicted in Table 5.3.a and Table 5.3.b and the results show that all the textural profile parameters are positively correlated to that of total collagen and myofibrillar protein. The higher 'r' value for fish muscle springiness and cohesiveness of all the three fish point out that springiness and cohesiveness are more influenced by collagen than myofibrillar protein. Perch showed the highest and catfish showed the lowest 'r' value with collagen.

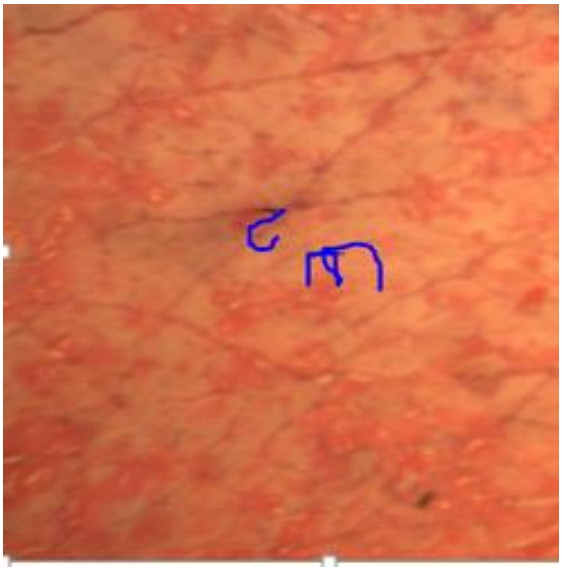
The 'r' value also showed that both myofibrillar proteins and collagen have influence on stiffness and total hardness of fish muscle stored at ambient temperature. Furthermore, compared to collagen, myofibrillar proteins have greater influence on both total hardness and stiffness and in this respect, highest 'r' value was shown by perch and lowest by catfish. In ice stored fish muscle, collagen showed higher correlation with both stiffens and hardness than that of myofibrillar protein. Besides, myofibrillar protein from perch has no significant relation with that of stiffness and hardness ($p > 0.01$), suggesting that along with some other factors, these two structural proteins have a major role in determining the firmness during post-mortem storage of fish muscle.

5.4.4: Histochemical studies

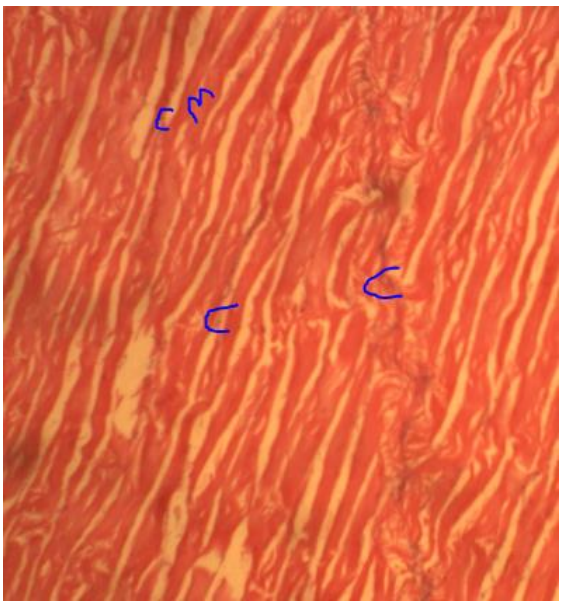
Plates 5.1 to 5.3 depict the photographs of the histochemical changes in the muscle tissue samples of ice-stored perch, snakehead and catfish. Even though the obtained micrographs cover only a very small area of section, it revealed the overall post-mortem histochemical changes in tissues. In all the three fish muscle tissues (Plate 5.1, 5.2, 5.3), the musculature were observed good with regular and well closely packed myofibrillar bundles. The cellular structural design was compact and observed few extra cellular spaces. Collagen present in connective tissue formed myocommata sheath, which cover and hold each myotome bundle together. A slight shrinkage in the extracellular space in all the three fish species was noted when the fish muscle reached full rigor condition (Day 3). As post-mortem storage time advanced to post- rigor stage, large void spaces appeared in all the three samples and increased with storage time. Additionally, partial depletion of both myofibrillar protein and collagen content were also observed on 18^{th}day of storage in all the fish species. Large number of fragmented fibers along with a precipitated material is also seen.



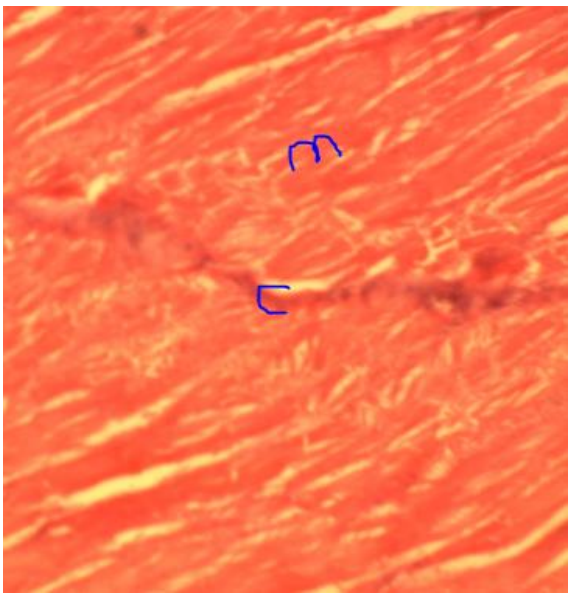
Day 0



Day 3



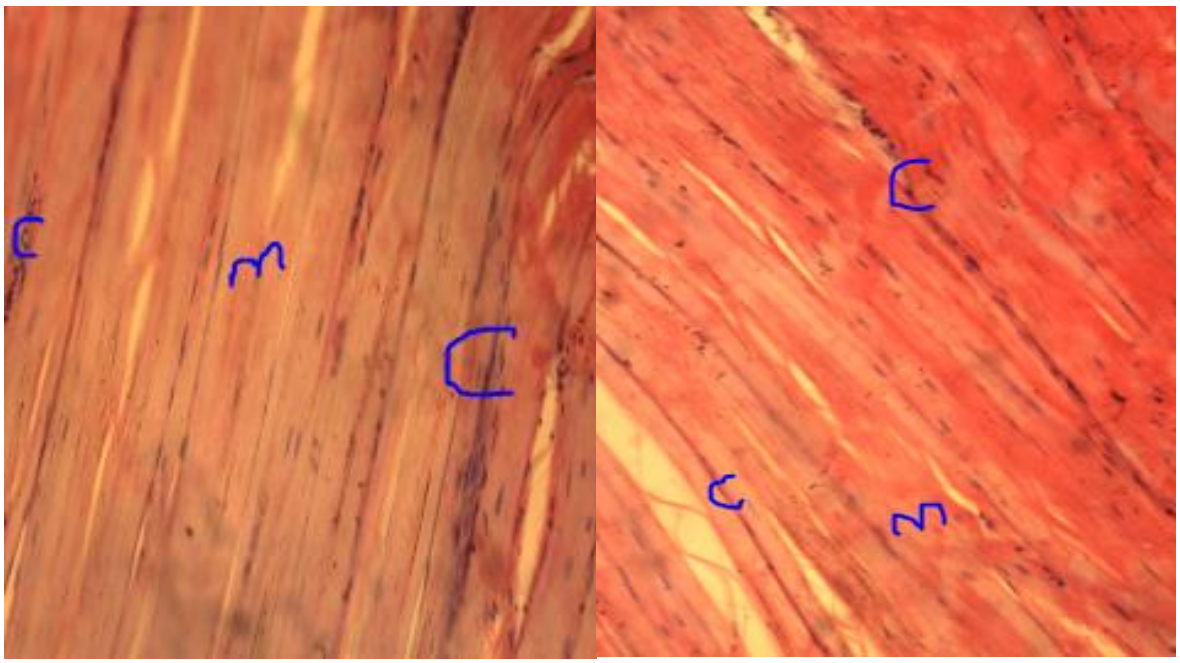
Day 12



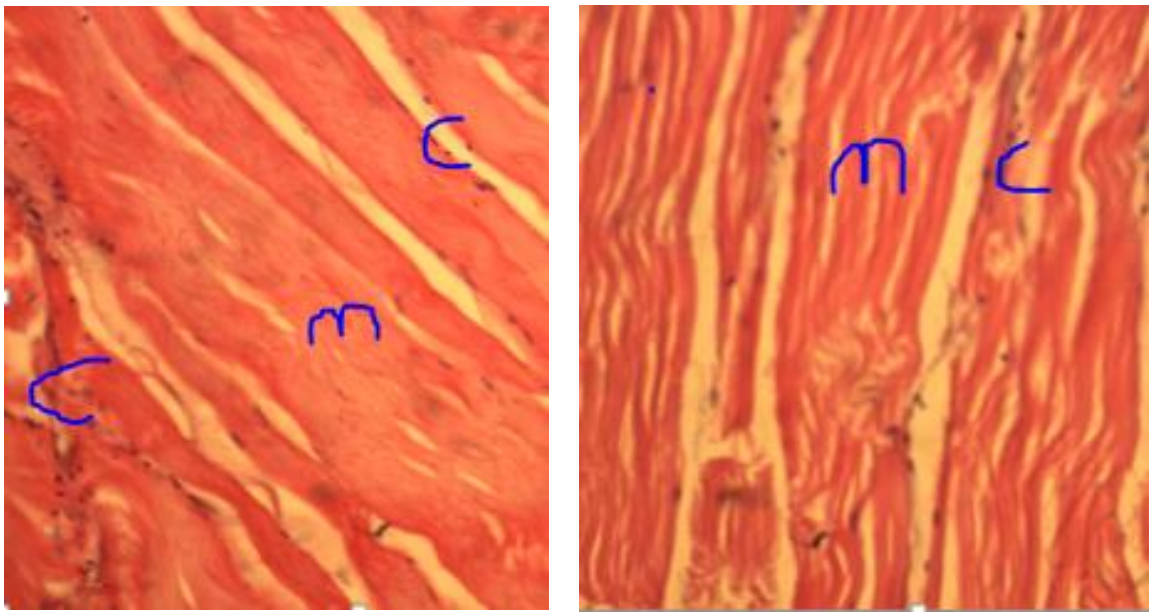
Day 18

m- myofibrillar protein, c- collagen

Plate 5.1: Histochemical changes in muscle tissue of iced climbing perch



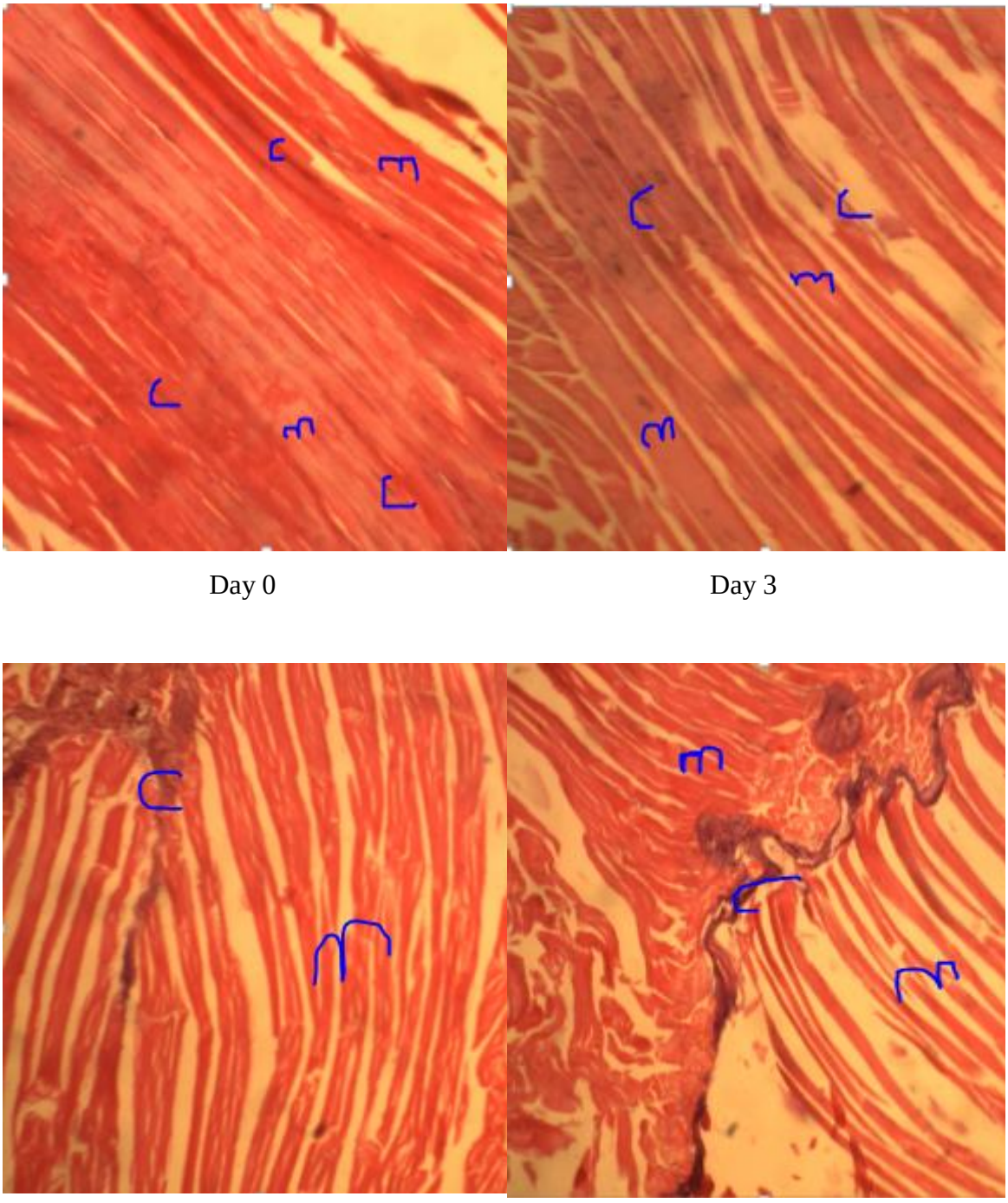
Day 0 Day 3



Day 12 Day 18

m- myofibrillar protein, c- collagen

Plate 5.2: Histochemical changes in muscle tissue of iced banded snakehead



Day 0 Day 3

Day 12 Day 18

m- myofibrillar protein, c- collagen

Plate 5.3: Histochemical changes in muscle tissue of iced stinging catfish

5.5: Discussion

Measurement and evaluation of structural and textural parameters are important for determining the quality of fish. The present chapter discusses the sensory evaluation and instrumentation textural profile analysis of the selected fish species kept at ambient temperature and iced storage. The musculature quality of the iced fish species was evaluated by histochemical analysis. The spoilage of these three fishes were strongly associated with the development of strong fishy, rancid and putrid odour, and resulted in rejection. The fresh fish had quality index mark of zero, and the value increased as deteriorated. High value for demerit score indicated the higher rate of spoilage with storage. Thus, the results obtained for quality index in terms of demerit score provided valid information on storage time and total remaining shelf life of the fish.

Four-phase analysis viz 0 to 5, 6 to 10, 11 to 20 and 21 to 32, based on the sensory demerit score at ambient temperature and ice-stored condition were conducted. Only slight or relatively low level change for sensory score occurred in phase 1 without any loss of natural physical appearance, odor and flavor. A slight reduction in quality without any visible signs of spoilage and/or off-flavor was observed in phase 2. Studies conducted in Chapter 3 showed that the increase of K value and H value was typically due to the buildup of hypoxanthine and inosine in comparison with other metabolites of ATP in the post-mortem fish muscle tissue. This was a clear sign of early spoilage with a slight off-flavor, where texture began to soften showing signs of spoilage and fish enters into the phase 3. Furthermore, the fish muscle turns softer when it entered in post-rigor condition. Eventually in phase 4, the fish began to have putrid odor and its texture began to soften with signs of spoilage and sour odor. The results for K and H values discussed in Chapter 3(section 3.4.5) seems to be directly related to the sensory demerit score in all the three fish species. Together with K and H value, sensory analysis revealed that the perch has longer shelf life than snakehead and catfish has the least shelf life.

Textural profile also provided good information on post-mortem tissue deterioration that significantly correlated to the storage period. Total hardness of the fish species reduced by more than 50% during the storage period at both iced and ambient temperature. Formation of three dimensional protein network could be a reason for the increase in hardness and stiffness together with actomyosin cross bridge

formation during pre-rigor ambient temperature and iced condition. Detachment of myofibers from sarcolemma-endomysium and myocommata along with degradation of collagen and myofibrillar protein could be the reason for reduction in post-rigor muscle tissue hardness.

Results from the correlation analysis show that at ambient temperature, myofibrillar proteins have greater influence on total hardness and stiffness than collagen in all the three fishes. The result also shows that the actomyosin cross-bridge formation is responsible for the characteristic stiffness and hardness. In ice stored fish muscle, collagen shows the maximum correlation with regard to both stiffness and total hardness than that of myofibrillar protein. On the other hand, overlapping of thick and thin myofilaments by cold shortening in ice stored fish muscle can interfere with the hardness and stiffness induced by the actomyosin cross-bridge formation. In this condition, collagen present in the muscle tissue can positively attribute to the texture, thus in ice stored fish muscle, stiffness, and hardness could be a functionality of collagen than myofibrillar protein.

The present study shows that the muscle tissue of perch has highest and catfish has the lowest value for hardness and stiffness. Besides, myofibrillar protein from iced perch has no significant relation with that of stiffness and hardness. Additionally, high total lipid content (Chapter 2) in the muscle tissue of climbing perch would influence the high total hardness and stiffness. The present study suggests that upon cold storage, low temperature induced solidification of fat interferes with the hardness and stiffness induced in the muscle tissue of perch. This result was supported by the studies of Wood *et al*. (2003) who reported that in pork, beef and lamb the melting point of lipid and the firmness/hardness of carcass fat is closely related to the concentration of stearic acid. In addition Stejskal *et al*. (2011) reported that, high value for hardness and stiffness of farmed Eurasian perch muscle tissue is due to the presence of high amount of saturated fatty acids. Values of cohesiveness and springiness also decreased as storage time proceeded, which is influenced by both collagen and myofibrillar protein in the muscle tissue in all the three freshwater fishes and are more dependent on total collagen and myofibrillar protein. Results obtained is supported by other studies (Periago *et al*., 2005; Cheng *et al*., 2014).

Studies of histochemical changes also gave a clear insight on how the myofibrillar and connective tissue proteins are involved in structural changes. It is observed that during post-mortem storage, connection between muscle fibers and myocomata, and whole myocomatta is degraded. Thus both connective tissue collagen and myofibrillar protein content have significant role in textural integrity and toughness of the fish muscle. Ayala *et al.* (2005) observed that rapid onset and change in ultra-structure of the fish muscle tissue is related to the final pH and its rate of decrease. Slight level shrinkage of extracellular space was noticed in all species and could be the functionality of acidic pH induced protein denaturation. Studies of Periago *et al.* (2005) correlated the muscle fiber density with the collagen and hydroxyproline content, and with textural parameters. Collapse of collagen fibers results in the development of post-mortem gaping phenomenon in fish muscle (Anderson *et al*., 1997). Post-mortem gaping in fish muscle is also correlated with that of insoluble collagen content (Espe *et al*., 2004). Furthermore, high value of firmness could be due to higher reducible immature collagen content and hydroxyproline cross link (Johnsen *et al*., 2011).

5.6: Conclusion

The current chapter evaluated the freshness quality of fish with respect to the organoleptic properties. It can be concluded that loss of textural property of the three fish species analyzed were strongly associated with changes in the connective tissue protein and myofibrillar protein. Additionally, it was also found that climbing perch has comparatively longer shelf life while stinging catfish has the least shelf life. Reduction in hardness and stiffness of post-mortem fish muscle gave an information on resolution of rigor-mortis. The results confirm that the actomyosin cross-bridge formation induces the total hardness and stiffness in all the species studied. In ice stored fish muscle, stiffness and hardness are typically a functionality of collagen than myofibrillar protein. The muscle tissue of perch has highest and catfish has the lowest value for hardness and stiffness. High total hardness and stiffness of the muscle tissues of climbing perch can be also attributed to the high total lipid content compared to the other two species. The present study also suggests that upon cold storage, low temperature induced solidification of fat could interfere with the hardness and stiffness brought about by myofibrillar protein. Based on the present study, it is inferred that collagen has high influence than myofibrillar protein on cohesiveness and is high in perch muscle tissue

than two others. Muscle tissue of snakehead showed highest elastic recovering property (springiness) than perch while catfish has minimum springiness. Results obtained from correlation studies show that both collagen and myofibrillar protein has influence on springiness in all the three fish species and is more dependent on total collagen content than myofibrillar protein.

Histochemical studies also confirmed that muscle tissue from catfish has high level of post-mortem quality loss. Additionally, perch fish muscle contain high insoluble collagen content with low gaping score while the catfish muscle tissue has less insoluble collagen and high gaping score. In this point of view, evaluation of texture and structural properties of fish are of great importance in freshness quality assurance and for product development in the food industry.

Chapter 6

Chapter 6

Role of Autolytic enzymes in post-mortem fish muscle softening

Contents

6.1: Introduction

The major factors that determine the post-mortem deterioration include the temperature during storage, rate and extent of pH changes and proteolytic degradation of connective tissue and myofibrillar proteins (Delbarre-Ladrat *et al*., 2006; Hultmann & Rustad, 2007; Ahmed *et al*., 2015). Macromolecules present in the fish muscle undergo a number of biochemical changes after cessation of life due to the activity of both endogenous and exogenous enzymes. Autolytic enzymes reduce the total textural quality at initial phase of rigor mortis in fish muscle without imparting any characteristic off-odors or off-flavors (Hansen *et al*., 1996).

Immediately after death, pH value in fish muscle tissue decreases from neutral to acidic range. Peptides and free amino acids produced by the action of autolytic enzymes on fish muscle proteins induce growth and multiplication of spoilage causing microorganism and its outcome is the production of biogenic amines followed by a rise in pH value (Fraser & Sumar, 1998). Major protease enzymes responsible for post-mortem muscular changes are matrix metallo proteases, calpains and cathepsins. Autolytic activity of enzymes in living organism is regulated by several mechanisms, including compartmentalization, synthesis as inactive zymogen form and action of endogenous inhibitors etc. (Hibbett, *et al*., 1999; Blankenvoorde *et al*., 2000).

Autolytic enzymatic activity with microbial spoilage prevent the long time storage of fish as a food item using normal preservation techniques such as refrigeration or icing. In order to extend the shelf life of these products and thereby enrich the economic value, some alternative method could be used. Marination of fish is considered as one of the most effective processing and preservation techniques. This

is used to improve flavor, aroma, juiciness and tenderness, and to extend shelf life. If these additives are enzyme activity modulators, physico-chemical properties of the food product could be improved and thereby the total shelf life could be enhanced.

In the previous chapter (Chapter 4 & 5), it was found that collagen and myofibrillar proteins undergo post-mortem degradation in a significant manner. Hence, studies on autolytic enzymes that degrade connective tissue collagen and myofibrillar protein are very important. From the findings of Chapter 4, it is clear that the connective tissue collagen in the fish muscle has a major role in the overall textural property. Additionally, presence of activators and inhibitors can alter the storage stability of food. Very little information is available in the literature on the effect of natural herbs and spices on autolytic enzymes mainly collagenase and lysosomal acid phosphatase activities in post-mortem fish muscle. Based on this, study was carried out on the following aspects on the three species of freshwater fishes selected viz. climbing perch, banded snakehead and stinging catfish stored at ambient temperature and chilled condition.

- Assay of total, free and bound lysosomal acid phosphatase enzyme activity
- Assay of Cathepsin D activity
- Determination of collagenase enzyme activity variations at post-mortem storage conditions
- Partial characterization of collagenase by identifying temperature optimum and pH optimum.
- Study on the effect of natural herbs on modulation of collagenase and lysosomal acid phosphatase enzyme activity

6.2: Review of Literature

6.2.1: Endogenous autolytic enzymes and post-mortem fish muscle degradation

Fish muscle tissue undergoes a number of biochemical and enzymatic changes which leads to muscular disintegration. Endogenous proteolytic enzymes are mainly located in sarcoplasm and extracellular fluid. In living condition, they induce protein turnover while after death, biological regulation property is lost which results in hydrolysis of the muscle proteins causing resolution of rigor mortis (Foegeding *et al*., 1996).Compared to muscle activities, visceral and hepatic fractions of enzymes show greater autolytic activity.

Proteolytic enzymes are classified based on their mode of action as endopeptidases and exopeptidases. The endopeptidases hydrolyze specific peptide bond deep inside the peptide chain to cleave them into smaller peptides, while exopeptidase hydrolyze only terminal peptide bond. Based on the principle residues or groups at their active sites, proteolytic enzymes are typically classified as serine, thiol, zinc, metallo and acid proteases. (Palmer & Bonner, 2007; Das, 2010). Along with the endogenous activators or inhibitors, the proteolytic activity of enzymes are greatly influenced by physico-chemical factors like pH and temperature, life cycle of fish and location within the body part. Studies reported that heat activated proteolytic enzymes are actively involved in the softening of fish muscle (Lin & Lanier, 1980). Benjakul *et al*. (2011) suggested that the heat activated metalloproteinases are involved in the autolysis in the kuruma prawn muscle and the maximum activity shown is recorded at 60 ^{0}C.

The rates of enzymatic reactions have a great impact on the quality traits of the fish meat and thus final quality (Haard, 1992). Proteolysis proceeds extensively when the autolytic rate is very high (Yamashita & Konagaya, 1990a). Autolytic activities of enzymes are greatly dependent on the fish species and its maximum activity was exhibited at neutral and/or higher pH (Kolodziejska & Sikorshi, 1996). Rapid decrease in post-mortem pH could contribute to releasing of lysosomal acidic proteinases from its storage site, and thus in turn will act on muscle proteins. It has been also reported that most of the autolytic activity at pH 6.5 is attributable to the lysosomal cysteine protease (Yamashita & Konagaya, 1990a).

Cathepsins, one of cysteine proteases located in lysosome are synthesized as zymogen form and were released at sites of injury or upon cold storage of post mortem muscle (Ashie & Simpson 1997; Nielsen & Nielsen, 2006). Upon post mortem muscle storage, the lysosomes breakdown and cathepsins enzymes are released from the lysosome into the cytoplasmic fluid and intracellular spaces (Bechet *et al*., 2005). Large group of cathepsins have been identified in post-mortem hydrolysis of myofibrillar proteins in fish muscle (Jiang, 2000). Out of this, cathepsin B, D, L, and H are the most significant one within the fish muscle. (Yamashita and Konagaya, 1990b; Cheret *et al*., 2007). Because of wide pH range in activity, cathepsin L and D have an important role in autolytic action, whereas other cathepsins are active at pH values too low of no post-mortem physiological significance. Yamashita & Konagaya (1991) reports the direct

participation of cathepsins L in drastic proteolytic degradation of fine structure of fish muscle proteins. Cathepsin D constitute the major proteolytic activity in the fish muscle at low pH range (Sriket, 2014). Fidalgo *et al*. (2014) reported the activities of Cathepsin B and D in muscle tissues of mackerel and horse mackerel.

Endogenous proteolytic enzymes are mainly responsible for quality loss of fish and shellfish freshness at the initial stages of storage. Thereafter, bacterial metabolism predominates and leads to final spoilage (Pacheco-Aguilar *et al*., 2000). Microbial action on free amino acids results in increase in pH value followed by activation of alkaline proteases enzymes. Kolodziejska & Sikorski (1996) reported that the neutral and alkaline proteases had more impact on the post mortem deterioration in fish muscles than the cathepsins that are active at acidic pH. Hydrolytic activity of cathepsin results in degradation and weakening of many of low molecular weight myofibrillar proteins during late post mortem storage of muscle and subsequent increase in the release of polypeptide fragments and oligopeptides (Ahmed *et al*., 2015). In addition to cathepsin, calpains (calcium activated neutral proteases) also have a role in post mortem degradation of fish muscle, by cleaving myofibrillar proteins (Kolodziejska & Sikorski, 1996; Geesink, *et al*., 1999; Ahmed *et al*., 2015). Calpains degrade many of the myofibrillar proteins, and cleaves peptide bond on proteins at specific sites, but calpains cause only a limited proteolysis. The proteins are degraded into small fragments, and this enhances the susceptibility of the proteins to other proteinases (Ladrat *et al*., 2000).

It is reported that matrix metallo proteinase (MMP) family of proteases acted on neutral pH (Visse & Nagase, 2003). Collagenases are the matrix metallo proteinases that are found in the skeleton muscle (Kolodziejska & Sikorski, 1996). Matrix metallo proteinase breakdown type I, II and III collagen chains after the amino acid glycine in a specific sequence (Gln/Leu)–Gly---(Ile/Leu)– (Ala/Leu) (---the bond breakdown point) (Daboor *et al*., 2010). Breakdown of connective tissue in the fish muscle tissue by endogenous collagenases trigger the development of undesirable textural changes and gaping (Bremner & Hallett, 1985; Ando *et al*., 1995; Ashie *et al*., 1996). During chilled storage, the attachment between muscle fibers and myocommata, and the whole sarcolemma, was degraded, and muscle fibers in fish muscle were detached from the myocommatal sheets (Bremner & Hallett, 1985; Hallett & Bremner, 1988). It was reported that autolytic enzyme mainly responsible for the disintegration of collagen 1 and V, results

in the softening of fish muscle and gapping, presumably due to the action of autolytic collagenolytic enzymes (Bremner & Hallett, 1985; Ando *et al*., 1995; Ashie *et al*., 1996; Kubota *et al*., 2003; Yoshida *et al*., 2009; Sriket, 2014).

The collagenases enzymes are synthesized as inactive zymogen forms and are believed to activate a wide variety of proteolytic enzymes. The actions of activated collagenases in normal cell are controlled by Tissue Inhibitors of Metallo Proteinases (TIMP), which are small cationic glycoproteins (Bremner, 1992). Maximum activity of collagenase enzyme was at pH value close to neutral or higher (7-8) and its activities were strongly dependent on fish species (Kristjansson *et al*., 1995; Teruel and Simpson, 1995; Sivakumar, *et al*., 1999; Kim *et al*., 2002; Park *et al*., 2002). Collagenase enzymes were mainly activated and stabilized by metal ions (Ca^{2+}) and other activators (including stress, injury, infection or heat) and were inhibited by calcium chelators (Bremner, 1992; Bracho & Haard, 1995). The short shelf life of iced chilled fish due to softening of tissue was probably due to the presence of collagenolytic enzymes (Hernandez-Herrero *et al*, 2003; Sriket *et al*., 2011b; Sriket *et al*., 2012).

Post-mortem biochemical and enzymatic reactions are responsible for the initial quality loss, which will further favor the activity of microorganism (Arashisar *et al*., 2004). Collagen present in the connective tissue is mainly responsible for the final integrity of the fillets. Presence of wide range of naturally present autolytic enzymes along with high pH makes the fish a highly perishable food product (Cakli *et al*., 2007).

6.2.2: Partial purification and characterization of collagenolytic enzyme from muscle tissue

Collagenase activity was found in fish muscle tissues including rainbow trout (Saito *et al*., 2000), mackerel (*Scomber japonicas)* (Park *et al*., 2002), Japanese flounder (*Paralichthys olivaceus)* (Kubota *et al*., 2003) and common carp (Wu *et al*., 2008). Two collagenolytic proteinase enzymes known to hydrolyze fish muscle collagen include serine protease and matrix metalloprotease (MMP) (Kubota *et al*., 2003). Presence of serine proteinases have been reported in catfish (Yoshinaka *et al*., 1986), shrimp (Lu *et al*., 1990; Chen *et al*., 1991; Van-Wormhoudt *et al*., 1992), Kamchatka crab (Klimova *et al*., 1990; Sakharov and Litvin, 1992), filefish (Kim *et al*., 2002), Atlantic cod (Kristjansson *et al*., 1995), green-shore crab (Roy *et al*., 1996) and red sea bream (*Pagrus major*) (Yoshida *et*

al., 2009). The collagenolytic metallo-proteinases are one of the principal extra-cellular enzymes, involved in degradation of collagen in the extracellular fluids (Carmeli *et al*., 2004). They contain zinc in its active site and require calcium for its stability (Stricklin *et al*., 1977).

6.2.3: Natural herbs and spices as enzyme activity modulators

Although current technologies are available for processing and preservation of fish and fishery products, it seems impossible to totally remove the risk of spoilage. Marinades are solutions used to improve flavor, aroma, juiciness and tenderness, and to extend shelf life of raw materials (Gokoglu *et al*. 2004; Ibrahim Sallam *et al*., 2007; Cadun *et al*., 2008; Duman *et al*., 2012). Great global trend of restriction in the use of synthetic compounds as food preservatives provides a wide gateway for marinating the fish and fishery product using natural herbs, which could be healthier than synthetic one. Shelf life extension through marinating the aquatic products are reported by many authors. Results of *Rahimabadi et al. (2015)* reported the positive effects of washing with tamarind (*Tamarindus indica L*.) water solution on shelf life extension of silver carp fillet during refrigerator storage. Effect of dip treatment of natural extracts of pomegranate peel extracts (*Punica granatum*) (PPE) and green tea (*Camelia sinensis*) to preserve the fatty fish and extend shelf life of Indian mackerel during ice storage was reported by Shinde *et al*. (2015). Frank *et al*. (2014) studied the protective effects of garlic (*Allium sativum*) and ginger (*Zingiber officinale*) on physicochemical and microbial attributes of liquid smoked silver carp.

Rahman *et al*. (2017) conducted a study to evaluate the impact of spices (turmeric, chili and their combination) treatment on the quality extension of dried Taki (*Channa punctatus*) during a storage period of 2 months. Yildiz (2016) investigated the effect of essential oils from thyme and rosemary on the quality of marinated rainbow trout during storage at $4^{O}C$. The dip treatment of grape seed extract increased the shelf life of mackerel up to 15 days, and papaya seed extract by 12 and 9 days for Indian mackerel during ice storage (Sofi *et al*., 2016). Nurul (2013) studied positive effects of chilies marinade on the microbiological quality of Indian mackerel at chilled storage. Serdaroglu & Felekoglu (2005) studied the effect of using rosemary extract and onıon juice on oxidative stability of sardine (*Sardina pilchardus*). Utami *et al*. (2016), studied preservation effect of Japanese

turmeric and red ginger essential oils on coated frozen patin fillets. Research was carried out by El-Sherif & El-Ghafour (2016) to evaluate the antioxidant activity and antimicrobial effects of individually (1.0%) garlic, ginger and rosemary essential oils (EOs) as natural preservatives to extend the shelf-life of raw Bayad (*Bagrus bayad*) fish sausage. Results of Kumolu-Johnson & Ndimele (2011) showed that samples treated with ginger paste extended the shelf-life of hot-smoked *Clarias gariepinus*. Studies of Das *et al*. (2011) and Biswas *et al*. (2012) show that curry leaf extract is a very effective inhibitor of primary and secondary oxidation products in raw ground and cooked meat and was potential as a natural antioxidant in raw and cooked meat systems. Quality analysis of turmeric treated three different Bangladeshi smoke-dried lean fishes stored at refrigeration temperature (4 ^{O}C) was studied by Latifa *et al*. (2014). Effect of lactic, acetic and citric acids on quality changes of refrigerated green mussel was studied by Masniyom & Benjama (2007).

Even though marination has a crucial role in shelf life extension of aquatic products, their action on the autolytic enzymes are still not clear. With this point of view, the present study investigated the role of some selected natural herbs and spices which are commonly used in kitchen for cooking purpose in collagenase and lysosomal acid phosphatase enzyme activity modulation and are discussed in the present chapter.

6.3: Materials and methods

6.3.1: Raw material collection and sample preparation

The samples of climbing perch, banded snakehead and stinging catfish were collected and tissue samples were prepared as detailed in 3.3.1.

6.3.2: Determination of lysosomal Acid phosphatase enzyme (EC NO. 3.1.3.2) activity:

The lysosomal enzymes activity in terms of acid phosphatase was determined by the method of Warrier, *et al*. (1972). Free, total and membrane bound lysosomal enzyme activity was determined in terms of acid phosphatase using *p*- nitro phenyl phosphate as substrate.

6.3.2.1. Preparation of tissue homogenate

Total activity: The sample tissue was finely minced with scissors and 10% (w/v) tissue suspension was prepared in 0.25 M sucrose containing 1 mM EDTA and 0.1% Triton X-100 using a homogenizer at a speed of 5000 rpm for 20 sec. The homogenate was

centrifuged at 12000 rpm for 30 minutes at 4 ^{O}C. The procedure was repeated twice, pooled and the supernatant were used as enzyme solution.

Free activity: The free activity was determined from 10% w/v tissue suspension in cold 0.25 M sucrose solution that contained 1 mM EDTA.

Bound activity (latent activity): Bound activity was determined by subtracting the free activity from total activity.

$$\textbf{\% bound activity} = \frac{\text{Total activity} - \text{Free activity}}{\text{Total activity}} \times 100$$

6.3.2.2: Assay of acid phosphatase

In a test tube, 0.5 ml of buffer, 0.5 ml of substrate solution and 0.5ml of enzyme extract were taken and incubated the test tube at 37 ^{O}C for 30 minutes. The enzyme activity was stopped by adding 4 ml of 0.1N NaOH. To the control, enzyme extract was added after the addition of NaOH. The absorbance was measured at 405 nm along with blank and standards. Protein in the extract was estimated by the method of Lowry *et al*. (1951).

The lysosomal activity was expressed in terms of acid phosphatase as µmole of *p*-nitro phenol liberated/ min/g tissue (EU).

6.3.3: Determination of cathepsin D (EC 3.4.23.5) activity

The Cathepsin D activity was determined by the method of Bonete *et al*. (1984) with slight modifications.

6.3.3.1: Extraction of Cathepsin D enzyme

1g tissue sample was homogenated in 10 ml extraction buffer (1% KCl containing 1 mM of EDTA) using a homogenizer by setting a minimum speed for 20 seconds. The homogenate was centrifuged for 30 minutes at 12,000 rpm, keeping temperature of 4 ^{O}C. The residue was washed with same buffer and collected the supernatant, pooled the supernatant and was used as enzyme solution.

6.3.3.2: Assay of cathepsin D

Cathepsin D activity was assayed by the method of Leblanc & Gill (1981). 1 ml of enzume extract was added to 2 ml of 0. 2 M glycin-HCl buffer (pH- 3.0) buffer containing 2% hemoglobin and incubated for 2 hours at 37 ^{o}C. The activity was terminated by adding 2 ml of freshly prepared 10% TCA. Control was prepared by adding 10% to the buffered

hemoglobin substrate. Then 1ml of enzyme axtract was added and incubated for 2 hours at 37 ^{O}C. The enzyme activity terminated mixture then filtered throuht Whatman No.4 filterpaper. Liberated TCA soluble peptided were determined by the method of Lowry *et al*. (1951). The enzyme activity was expressed in micromoles of tyrosine per g tissue per min.

6.3.4: Collagenase enzyme (EC 3.4.21.32) activity determination

6.3.4.1: Extraction of collagenase enzyme

1g of muscle tissue was homogenized at a speed of 12,000 rpm for 2 minutes with 10 ml of 50 mM Tris-HCl buffer (pH 7.5) that contained 5 mM $CaCl_2$. The homogenate was centrifuged at 12,000 rpm for 30 minutes at 4^{O}C. This procedure was repeated twice. The supernatants were pooled and was used as enzyme solution.

6.3.4.2: Determination of Collagenase Enzyme activity

Collagenolytic activity was measured by the method of Moore & Stein (1954) with slight modifications. The reaction mixture of 1ml of 50 mM Tris- HCl buffer (pH- 7.5) containing 5mg collagen and 5 mM $CaCl_2$, and 0.1 ml of enzyme solution was incubated at 37 ^{O}C for 1 hour. The reaction was stopped by adding 0.2 ml of 50% Trichloro acetic acid. After 10 minutes of incubation at room temperature, the solution was centrifuged at 3000 rpm for 10 minutes. 0.2 ml of the supernatant was mixed with 3.8 ml of distilled water and 1ml of ninhydrin reagent, and incubated for 15 minutes in a boiling water bath, then cooled to room temperature. The mixture was diluted with 1ml 50% 1- propanol. The absorbance was read at 540 nm. 50 mM Tris-HCl buffer (pH- 7.5) that contained 5 mM $CaCl_2$ was used as reference. A standard curve was prepared using a solution of L- leucine amino acid. One unit of enzyme activity is defined as the micromoles of leucine/minute/g tissue.

6.3.5: Characterization of collagenase enzyme from the muscle tissue

6.3.5.1: Extraction of Collagenase Enzyme

Collagenase enzyme was extracted from the muscle tissue according to the method discussed in 6.3.4.1

6.3.5.2: Fractionation of Collagenase Enzyme

Fractionation of the crude extract of collagenase enzyme was done using ammonium sulfate as described by Teruel & Simpson (1995). Added solid ammonium sulfate to the enzyme extract solution to a concentration of 40% and

incubated for 1 hour at 4 ^{O}C. After incubation, concentration of ammonium sulfate was increased to 80% and incubated for one hour at 4 ^{O}C. Then centrifuged the solution for 30 minutes at 7000 rpm and 4 ^{O}C. Pellet obtained was re-suspended in 2 ml of 50 mM Tris-HCl buffer (containing 5 mM $CaCl_2$, pH of 7.4). The solution was then desalted using 25 mm cellulose membrane dialysis tube (Cat No. D9777, Sigma Aldrich, Oakville, Canada) for three times. Centrifuged the ammonium sulfate free solution at 7000 rpm and 4 ^{O}C for 30 minutes and the supernatant collected and stored at –20 ^{O}C.

6.3.5.3: Partial purification of Collagenase Enzyme

Partial purification of collagenase was done by using Sephadex G-100 column (2 cm × 80 cm) according to the method described by Indra *et al*. (2005). Column was first washed with five volumes of 50 mM Tris-HCl buffer (containing 5 mM CaCl2, pH of 7.4) and then with ten volumes of 50 mM Tris-HCl buffer (containing 5 mM CaCl2 and 0.15 M NaCl , pH of 7.4) at the flow rate of 1ml/min. The enzyme extract was then loaded into the Sephadex G-100 column at 4^{O}C and was eluted with ten volumes of 50 mM Tris-HCl buffer (containing 5 mM $CaCl_2$ and 0.15 M NaCl with a pH of 7.4) with a flow rate 1 ml/min. Collagenase enzyme fractions collected were pooled and stored at –20^{O}C.

6.3.5.4: Determination of pH optimum of the collagenase enzyme

The optimum pH of the collagenase enzyme was determined by incubating the reaction mixture at 37 ^{O}C for one hour as described in Section 6.3.4.2 and the enzyme activity was measured according to the method of Moore & Stein (1954). The buffers used were 50 mM tris buffer for pH 7.0, 7.2, 7.4, 7.6, 7.8, and 8.0 containing 5 mM $CaCl_2$.

6.3.5.5: Determination of temperature optimum of the collagenase enzyme

Determination of temperature optima was carried out by incubating the reaction mixture using 50 mM Tris-HCl buffer (pH 7.5) that contained 5 mM $CaCl_2$ at various temperature (15 – 50^{O}C) for one hour. The activity of the enzyme was assayed as described above (6.3.4.2).

6.3.5.6: Effect of various inhibitors and metal ions

Effect of some metal ions and inhibitors on partially purified collagenolytic enzyme was determined by adding 1 mM each of Ca^{2+}, Mn^{2+}, Zn^{2+}, PMSF and EDTA. Activity was then compared with control.

6.3.6: Effect of natural enzyme activity modulators on Collagenase and Lysosomal acid phosphatase

Determined the effect of some natural herbs and spices by incubating 0.2 ml of 10% water extract of each *Capsicum annuum* (Chilli), *Capsicum annuum* (Bird's Eye chilli), *Piper nigrum* (pepper seed), *Allium sativum* (garlic), *Curcuma longa* (Common Turmeric), *Zingiber officinale* (Ginger), *Murraya koenigii* (curry leaves), *Citrus limon* fruit juice (lemon), *Tamarindus indica* (Tamarind), *Indian garcinia* (Malabar tamarind), *Pimenta dioica* (All spice) and *Camellia sinensis* (Tea leaf)) to the reaction mixture, followed by one hour incubation at 37^{O}C and pH 7.5. The activity of the enzyme was assessed as described above (6.3.4.2).All the sampling and assay were done in triplicate.

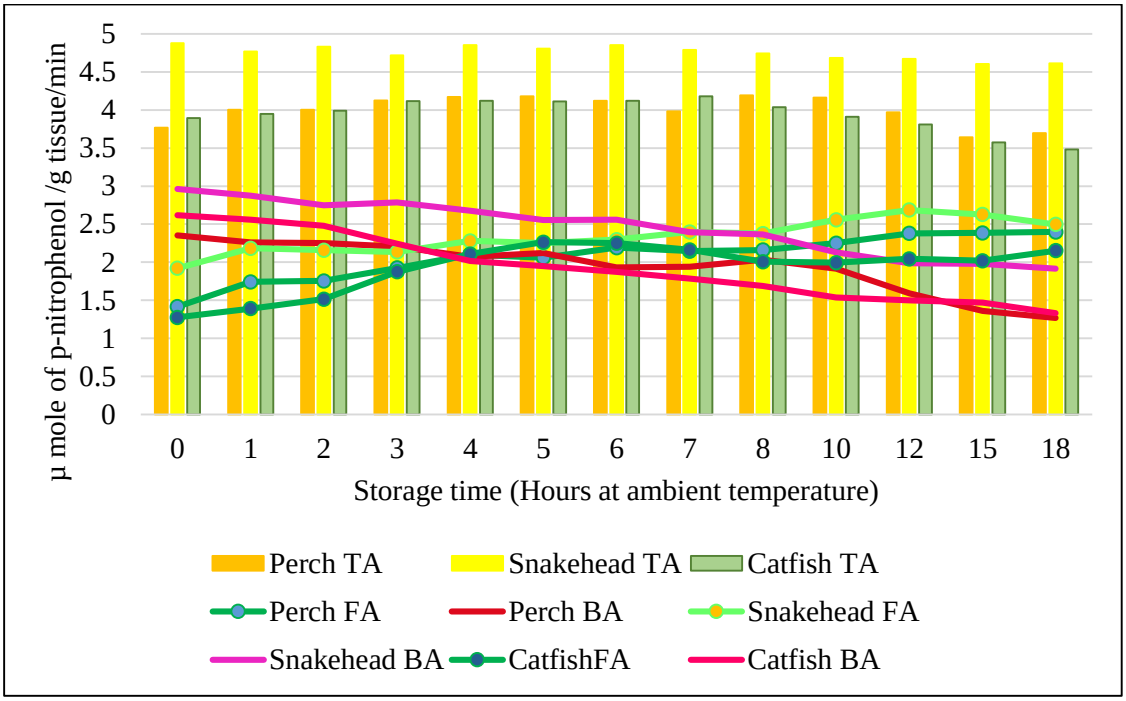
6.3.7: Statistical analysis

All statistical calculations were performed in IBM SPSS Statistics 20.0 Software. Data analysis was performed by one way analysis of variance (ANOVA) with post-hoc with multiple comparison analysis performed using Duncan test.

6.4. Results

6.4.1. Lysosomal acid phosphatase enzyme activity

The lysosomal enzyme activity from muscle tissue fraction of climbing perch, banded snakehead and stinging catfish upon post-mortem storage at ambient temperature and at iced condition are shown in Fig.6.1 and Fig 6.2 respectively. The enzyme activity was determined in terms of acid phosphatase free activity, bound activity and total activity. The value obtained was expressed on micro mole of *p*-nitrophenol /g tissue/min. Statistical test using IBM SPSS 20.0 was performed to determine the variation in the mean value of data obtained for the lysosomal enzyme activity and between the fish species. Duncan Post-hoc test gave a clear information regarding variation in their activity.



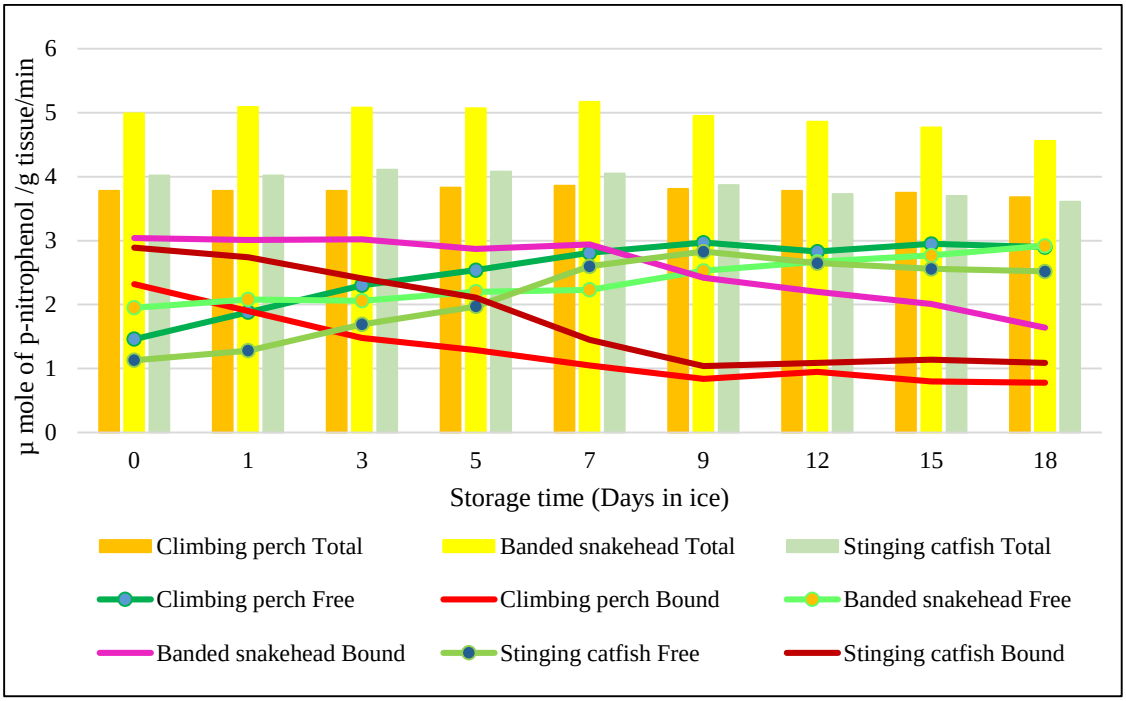
TA- Total activity, FA- Free activity, BA- Bound activity

All values are expressed as mean ± standard deviation, n=3

Fig 6.1: Lysosomal acid phosphatase enzyme activity in the muscle tissue of climbing perch, banded snakehead and stinging catfish stored at ambient temperature

The results obtained for lysosomal enzyme activity in terms of acid phosphatase of perch, snakehead and catfish stored at ambient temperature for 18 hours given in Fig 6.1 and Appendix 6.1. Total lysosomal acid phosphatase enzyme activity in fresh sample ranged between 3.77 and 4.87 µmoles *p*-nitro phenol/g tissue/min. Banded snakehead showed the highest activity, while climbing perch showed the lowest enzyme activity. ANOVA results says that there is no significant level variation in the total acid phosphatase activity in perch and snakehead, but catfish showed a significant level variation upon storage and was decreased with increase in storage time at ambient temperature ($p < 0.01$). Free acid phosphatase activity in the tissue extract increased with increase in storage period ($p< 0.01$). A slight diminution in their activity was noticed by the end of the storage period in post-rigor muscle tissue fraction in all the three fishes. The bound enzyme activity varied in all the three fishes with increase during storage period. Compared to the bound enzyme activity on the zeroth hour of storage, 46%, 36% and 49% reduction in the activity was noticed in perch, snakehead and catfish respectively ($p < 0.01$) by 18th hour of storage. Results obtained from the

statistical analysis shows that there was a significant level variation in lysosomal enzyme activity among the three fish species selected for study ($p < 0.01$).



TA- Total activity, FA- Free activity, BA- Bound activity

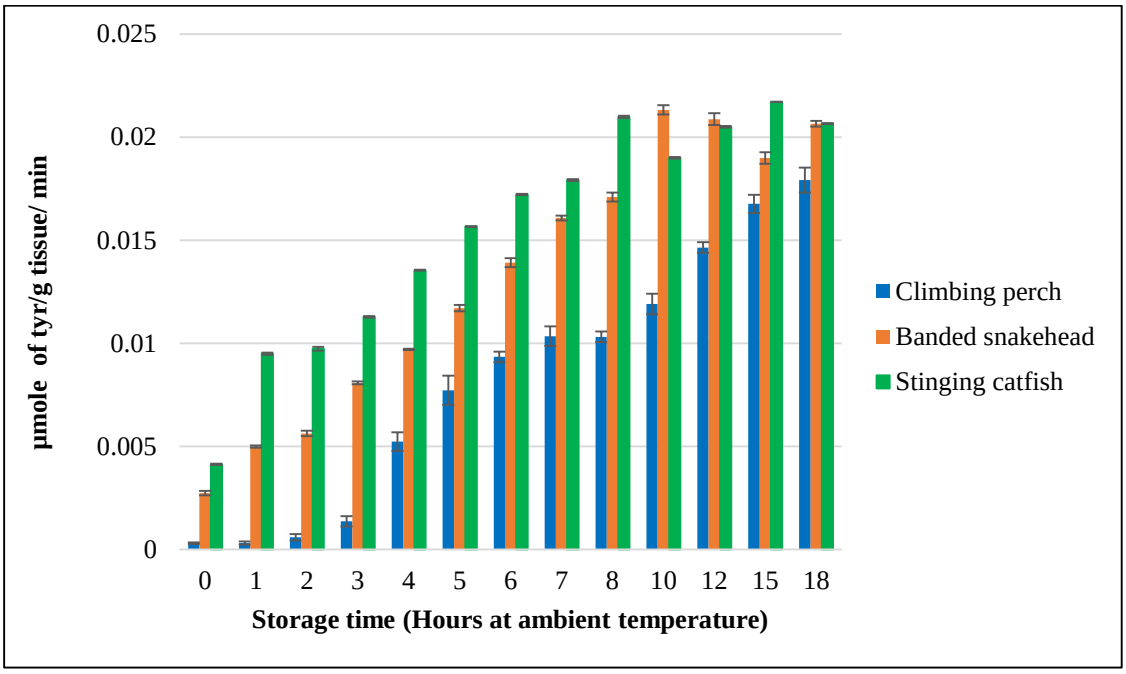
All values are expressed as mean ± standard deviation, n=3

Fig 6.2: Lysosomal acid phosphatase enzyme activity in the muscle tissue of climbing perch, banded snakehead and stinging catfish stored in iced condition

Fig 6.2 and Appendix 6.2 illustrated the data obtained for the lysosomal enzyme activity in terms of acid phosphatase of perch, snakehead and catfish stored at ambient temperature for 18 days. There is no significant level variation in total acid phosphatase activity in perch and snakehead muscle tissue extract at the end of the storage period ($p > 0.01$) the total enzyme activity significantly varied in all the three fish species ($p< 0.01$). Free acid phosphatase activity inreased with an increase in storage period of all the three fish species ($p< 0.01$). The bound enzyme activity vary in all the three fish with increase in storage period. Compared to the bound enzyme activity on the zeroth day of storage, 58%, 46% and 62 % reduction in the activity in perch, snakehead and catfish was noticed by the end of storage in ice. Result obtained from the statistical analysis shows that there was a significant level variation in lysosomal enzyme activity among the three fish species ($p< 0.01$).

6.4.2: Cathepsin D enzyme activity

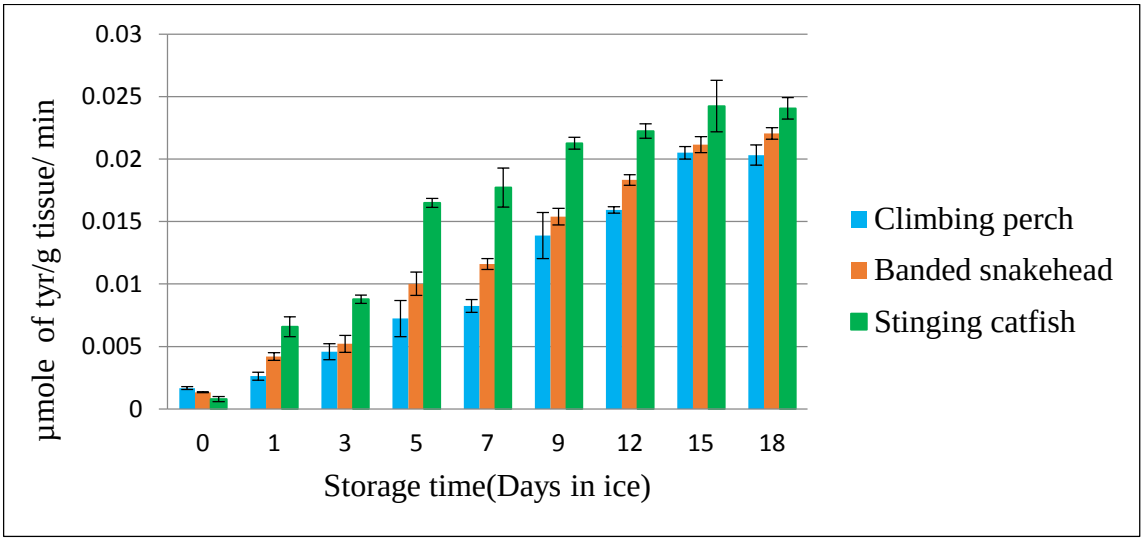
The Cathepsin D activity from muscle tissue fraction of climbing perch, banded snakehead and stinging catfish upon post-mortem storage at ambient temperature and iced condition are illustrated in Fig 6.3 and 6.4 respectively. The unit enzyme activity was determined using denatured hemoglobin as substrate. The value obtained was expressed as micromole of tyr/g tissue/ min. Statistical test using IBM SPSS 20.0 was performed to determine the variation in the mean value of data obtained for the Cathepsin D enzyme activity and between the fish species. Duncan Post-hoc test was carried out to study regarding variation in their activity.



All values are expressed as mean ± standard deviation, n=3

Fig 6.3: Cathepsin D activity in muscle tissue of climbing perch, banded snakehead and stinging catfish stored at ambient temperature

Fig 6.3 and Appendix 6.3 shows the results obtained from the assay of cathepsin D unit activity in muscle fraction of perch, snakehead and catfish stored at ambient temperature for 18 hours. The enzyme activity increased with increase in storage time. The Cathepsin D activity was significantly different across the fish species and with varying time period tested ($p < 0.01$).

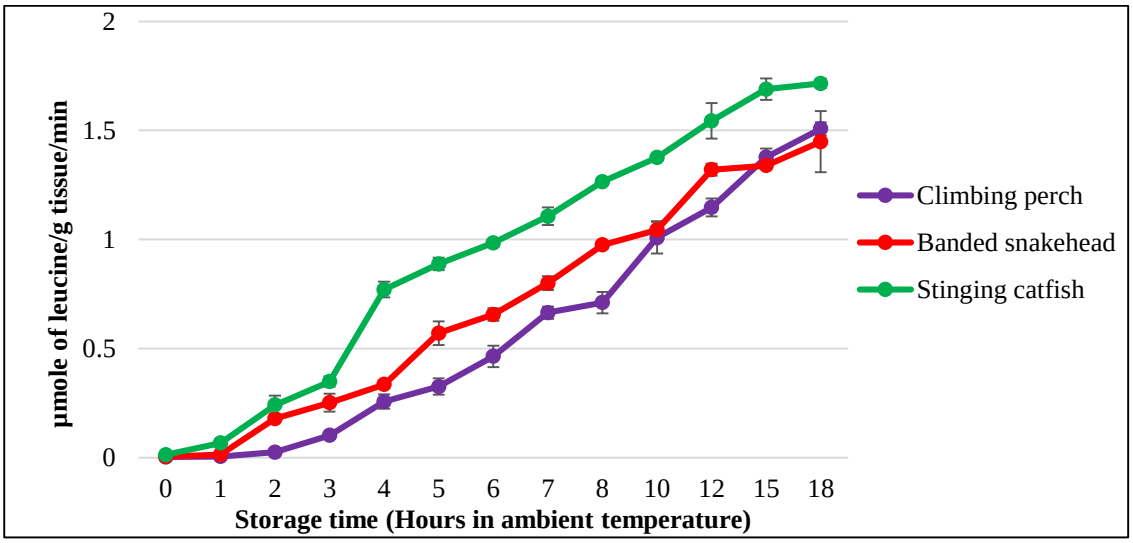


All values are expressed as mean ± standard deviation, n=3

Fig 6.4: Cathepsin D activity in muscle tissue of ice stored climbing perch, banded snakehead and stinging catfish

Fig 6.4 and Appendix 6.4 show the results from the determination of Cathepsin D activity in muscle fraction of perch, snakehead and catfish stored in ice for 18 days. The activity of Cathepsin D activity increased with increase in storage time. ANOVA results shows shat there is a significant level difference in Cathepsin D activity among the fish species analyzed and between days of storage within the fish ($p< 0.01$).

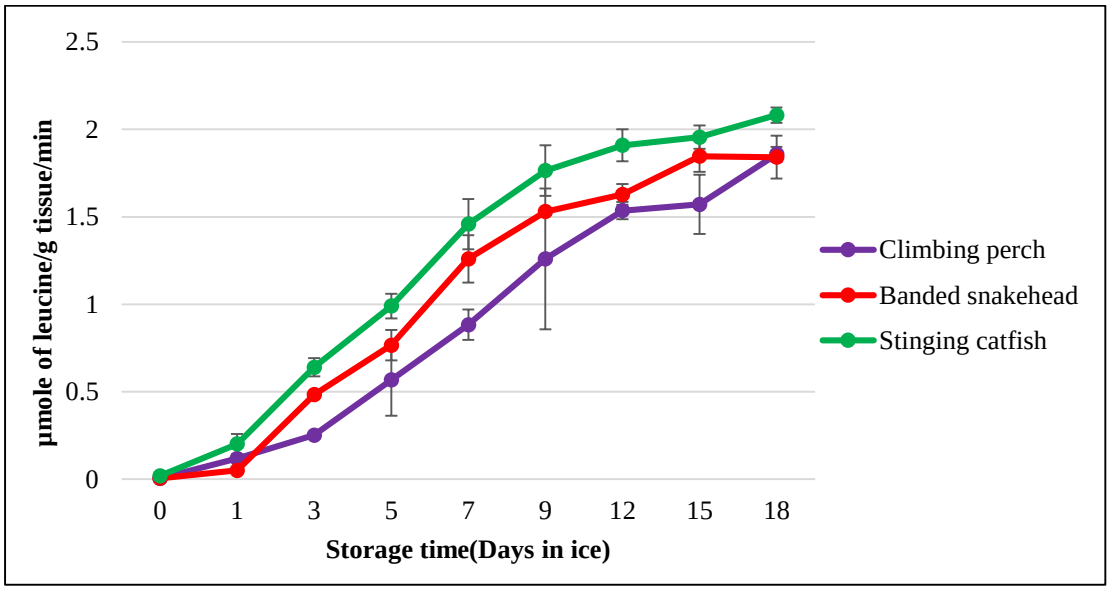
6.4.3: Collagenase enzyme activity



All values are expressed as mean ± standard deviation, n=3

Fig 6.5: Collagenolytic enzyme activity in muscle tissue of climbing perch, banded snakehead and stinging catfish stored at ambient temperature

Data obtained for determination of collagenolytic enzyme activity in muscle tissue fraction of climbing perch, banded snakehead and stinging catfish kept at ambient temperature for 18 hours are given in Fig 6. 5 and Appendix 6.5. The enzyme activity was found to be almost stable during the early post-mortem storage time followed by a steep increase with storage period. There was a significant variation in enzyme activity within the species and between fish species ($p< 0.01$).



All values are expressed as mean ± standard deviation, n=3

Fig 6.6: Collagenolytic enzyme activity in muscle tissue of perch, snakehead and catfish stored at chilled stored condition

Fig 6.6 and Appendix 6.6 showed the collagenase activity at iced storage condition in three fish species. All the species showed a high degree of proteolytic activity. However, catfish showed a higher activity than snakehead and perch during the storage period. A steep increase in the collagenase enzyme activity was noticed after first day of storage. The collagenase enzyme activity showed a significant variation across three fish species and within the species as well ($p< 0.01$).

6.4.4: Determination of pH optimum and temperature optimum for collagenase enzyme activity

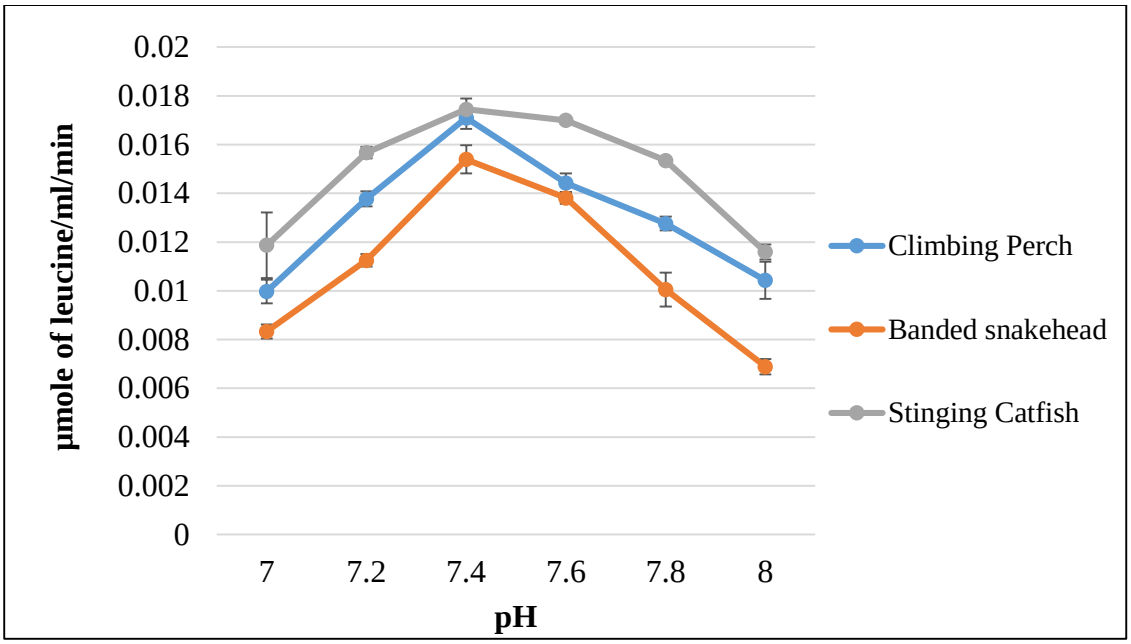


Fig 6.7: pH optimum for collagenase enzyme activity

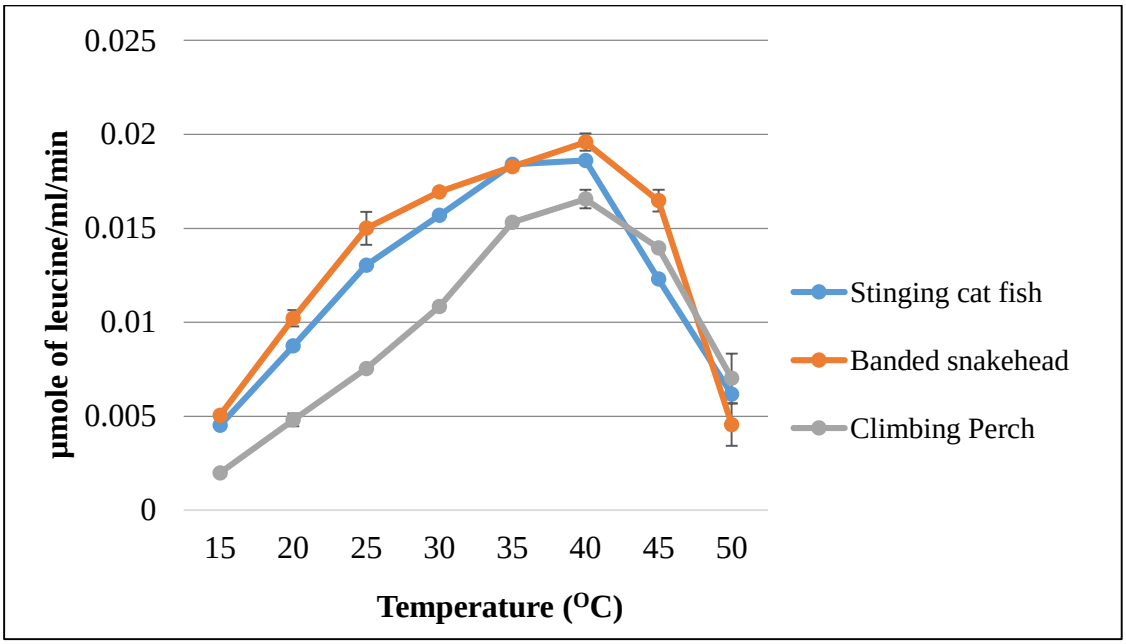


Fig 6.8: Temperature optimum for collagenase enzyme activity

Fig 6.7 and 6.8 show the data for pH optima and temperature optima of collagenase enzyme isolated from the muscle tissue of climbing perch, banded snakehead and stinging catfish. The optimum pH of the enzyme from the three freshwater fish was 7.4 and temperature optima was 40 ^{O}C.

6.4.5: Determination of effect of synthetic activity modulators for collagenase enzyme activity

Table 6.1: Effect of synthetic activity modulators for collagenase enzyme activity (µmole of leucine/ml/min)

Modulators	Climbing perch	Banded snakehead	Stinging catfish
	Activity (%)	Activity (%)	Activity (%)
Control	100	100	100
PMSF	52.03	50.18	38.88
EDTA	92.67	88.17	87.01
Ca^{2+}	115.4	119.5	103.89
Zn^{2+}	19.00	9.08	7.53
Mn^{2+}	33.09	26.87	17.01

Table 6.1 shows the data obtained for the modification effect of some metal ions and enzyme inhibitors on the partially purified collagenase enzyme from the muscle tissue of climbing perch, banded snakehead and stinging catfish. Collagenase enzyme was slightly inhibited by EDTA, and a 50% inhibition was noticed with PMSF. Considering the effect of metal ions, the enzyme activity was enhanced by the addition of Ca^{2+}, were strongly inhibited by Zn^{2+} and Mn^{2+}.

6.4.6: Determination of modulatory effect of natural herbs on enzyme activity

Table 6.2: Effect of natural herbs on collagenase and lysosomal acid phosphatase enzyme

Treatments	Collagenase (µmole of leucine/ml/min)			Acid phosphatase (µmole of *p*-nitro phenol/ml/min)		
	Climbing Perch	Banded Snakehead	Stinging Catfish	Climbing Perch	Banded Snakehead	Stinging Catfish
Control	22.85 ^{a}	16.95 ^{a}	18.66 ^{a}	576.46 ^{g}	614.00 ^{e}	441.80 ^{d}
	±0.32	±1.04	±0.22 ^{h}	±10.42	±11.31	±14.85
Capsicum annuum (Chilli)	28.93 ^{g}	27.48 ^{h}	25.71	4.25 ^{a}	26.44 ^{a,b,c}	13.73 ^{a}
	±0.58	±2.36	±0.80	±0.78	±4.28	±2.68
Capsicum annuum (Bird's Eye chilli)	26.81 ^{h}	31.94 $^{d, e}$	19.73 ^{f}	25.91 ^{b}	14.59 $^{a.b}$	12.48 ^{a}
	±0.27	±0.49	±0.07	±7.19	±0.74	±3.88
Piper nigrum (Pepper seed)	57.32 ^{h}	28.93 ^{g}	39.61 ^{i}	32.52 ^{b,c}	4.29 ^{a}	6.05 ^{a}
	±1.68	±2.02	±0.08	±9.56	±0.74	±1.45
Allium sativum (Garlic)	40.38 ^{i}	35.7 ^{i}	42.14 ^{j}	2.27 ^{a}	3.86 ^{a}	12.61 ^{a}
	±2.76	±2.42	±3.80	±1.76	±1.29	±3.58
Curcuma longa (Common Turmeric)	23.66 ^{f}	21.68 ^{d}	23.76 ^{f}	2.27 ^{a}	3.14 ^{a}	1.80 ^{a}
	±1.12	±0.35	±0.71	±0.88	±0.84	±0.84
Zingiber officinale (Ginger)	32.16 ^{g}	27.58 ^{f,g}	26.79 ^{g}	2.10 ^{a}	1.46 ^{a}	8.41 ^{a}
	±0.80	±1.46	±0.04	±0.71	±0.20	±1.93
Murraya koenigii (Curry leaves)	24.38 ^{f}	25.53 ^{e,f}	27.27 ^{g}	27.89 ^{a}	48.35 ^{c}	6.86 ^{a}
	±0.10	±0.61	±0.18	±1.97	±7.32	±2.97
Citrus × limon fruit juice (lemon)	12.63 ^{e}	16.83 ^{c}	17.63 ^{c}	585.47 ^{g}	690.28 ^{f}	528.11 ^{g}
	±0.46	±1.41	±0.46	±12.01	±4.52	±15.35
Tamarindus indica (Tamarind)	15.70 ^{d}	10.01 ^{b}	13.90 ^{d,e}	530.04 ^{e}	666.60 ^{f}	504.89 ^{f}
	±0.46	±0.61	±0.06	±13.50	±41.28	±12.15
Indian garcinia (Malabar tamarind)	13.82 ^{c}	13.17 ^{c}	7.79 ^{b}	555.57 ^{f}	839.15 ^{g}	480.62 ^{e}
	±0.16	±0.45	±0.57	±15.44	±12.68	±5.38
Pimenta dioica (All spice)	14.06 ^{b}	16.23 ^{c}	18.12 ^{e}	51.22 ^{c,d}	36.59 ^{b,c}	59.89 ^{c}
	±0.17	±1.62	±0.63	±3.79	±2.56	±8.41
Camellia sinensis (Tea leaf)	10.03 ^{a}	14.42 ^{b,c}	14.33 ^{c,d}	54.14 ^{d}	28.44 ^{d}	32.64 ^{b}
	±1.21	±1.01	±1.63	±1.42	±28.38	±2.67

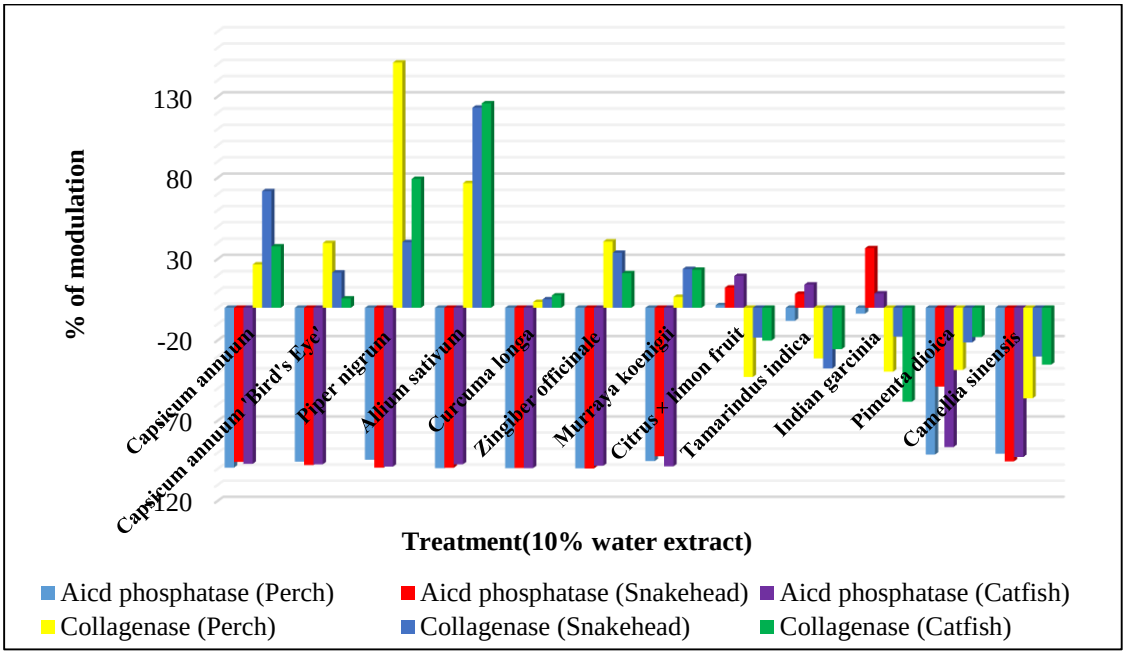


Fig. 6.9: Percentage of modulation effect of collagenase and acid phosphatase activity

Table 6.2 shows the results for the modulatory effect of selected herbs and spices on collagenase and acid phosphatase activity and Fig 6.9 shows the graphical representation of percent of modulatory effect on collagenase enzyme and acid phosphatase enzyme activity. Out of twelve treatments, the water extracts of lemon juice, Tamarind, Malabar tamarind, All spice and Tea leaf showed the inhibitory effect on collagenase enzyme activity; curry leaves have almost no effect where as other treatment stimulate the enzyme activity in all the three fish species. On the other hand, the juice extracts from lemon, Tamarind and Malabar tamarind stimulate the acid phosphatase activity while the rest of the treatments strongly inhibit the activity. Statistical results show that there is a significant level variation in collagenase enzyme and acid phosphatase activity between treatments in all the three freshwater fishes under the study ($p < 0.05$) (Appendix 6.7).

6.5: Discussion

6.5.1: Autolytic enzymes on post-mortem fish muscle

The lysosomal enzymes mainly cathepsins play significant role in postmortem proteolysis and meat tenderization because they degrade many of the same proteins that are degraded in post-mortem muscle. Many lysosomal enzymes also exhibit significant

activity at pH values that are close to the tissue pH values in pre-rigor meat. It was reported that the autolytic activity in mantle of *Ommastrephus sloani pacificus* and *Loiigo peale*iwas is maximum at lower pH with activity dropping considerably in the neutral and alkaline range (Sakai & Matsumoto, 1981). Jiang *et al*. (1992) reported that the proteolysis caused by lysosomal enzymes was highest at pH 6.5 and correlated with the muscular pH value. This suggested the role of lysosomal enzymes in the fragmentation of muscle proteins.

Determination of free, bound and total lysosomal enzyme activity in terms of acid phosphatase in climbing perch, banded snakehead and stinging catfish muscle tissue during storage at ambient temperature and chilled condition gives information regarding release of membrane bound enzymes in fish muscle tissue and their role in post-mortem softening. It was reported that 40-60% of acid phosphatase enzymes were bound to lysosomal membranes and with low pressures was found to cause disruption of lysosomes and leakage of the enzyme (Ohmori *et al*., 1992 ; Teixeira *et al*., 2013; Fidalgo *et al*., 2015). According to Ohmori *et al.* (1992), pressures of about 200-300 MPa caused an increase of acid phosphatase activity in the cytosolic fraction and a decrease in the lysosomal fraction. In this study, reduction in bound enzyme activity was observed in the tissue fraction of perch, snakehead and catfish during storage at ambient temperature and iced condition. The results obtained point out to the instability of lysosomal membrane followed by the release of stored lytic enzymes into the cytoplasm. This result was strongly supported by Etherington (1984), who reported that the fall in pH during post-mortem glycolysis weakens the walls of organelles such as lysosomes and consequently promote the release of lysosomal proteases. Results obtained also confirmed that stinging catfish has less lysosomal membrane stability and hence poor quality than two others.

Additionally, leaching out of water soluble proteins from the muscle tissue resulted in a reduction in free and total enzyme activity during the later stage of post-mortem storage of perch, snakehead and catfish at ambient temperature and iced condition.

Cathepsin D activity in three freshwater fish species increased with increase in the post-mortem storage time both in iced and at ambient temperature and is due to the

rupture of lysosomal membranes. Results obtained also confirmed that the lysosomal membrane of stinging catfish has less membrane stability and hence highest activity for cathepsin D than perch and snakehead. By comparing with other autolytic protease enzymes studied, the activity of cathepsin D determined in both condition in three fish species was extremely low, indicating its less participation in tissue protein degradation during post mortem condition. Porter *et al.* (1995) also found extremely low activity of cathepsin D in four species; Pacific whiting, arrowtooth flounder, Alaska pollack and Pacific cod. Aoki *et al.* (2000) detected cathepsin D activity in red or white muscle among 24 species, and no difference was seen between red- and white-flesh fish, or freshwater. However, Ladrat *et al*. (2003) reports that cathepsin D has a role in the post-mortem proteolysis by the release of α-actinin. Lund and Nielsen (2001) reported that cathepsins are involved in the degradation of sarcoplasmic and myofibillar proteins in salmon fish muscle. Jiang *et al*. (1992) also investigated the ability of cathepsin D to degrade myofibrillar proteins in tilapia muscle tissue, where the highest activity was shown at pH 5.5.

Post-mortem degradation of collagen results in the 'gaping phenomenon' along with unusual softening of the muscle tissue and is discussed in the previous chapters. The present study also discussed the role of collagenase enzyme in the post-mortem fish muscle softening and is supported by the studies of Suarez *et al*. (2005) who reported that the post-mortem fish muscle softening is a by-product of action of specific collagenase on insoluble fraction of the collagen. Collagenases are capable of degrading the polypeptide backbone in collagen (Sikorski *et al*., 1984). The obtained result showed that the collagenase enzyme has a significant role on breakdown of collagen. Extracellular matrix collagenases regarded as initiators of breakdown are active against collagen type I, IV and V (Bremner, 1992).

Upon storage at ambient temperature and ice stored condition, collagenolytic enzymes showed highest level of activity in post-rigor stage than the pre-rigor stage. Additionally, from the result, it is also observed that the pepsin soluble collagen is the major target for the collagenase enzyme activity. Furthermore, initial attack on the collagen triple helix is by specific collagenases. It was reported that once the initial cleavage has been achieved, other non-specific proteases can pursue the attack (Kristjansson, *et al*., 1995; Sriket *et al.* 2010). The Cathepsin L and serine protease are

capable of hydrolyzing major muscle structure proteins, such as telopeptide collagen (Yamashita & Konagaya, 1991). Most of telopeptide collagens are acid soluble in nature. Asghar & Henrickson (1982) reported that the mammalian native collagen was not very susceptible to attack by proteolytic enzymes, but lysosomal enzymes could degrade collagen denatured with lactic acid. Studies reported that collagenase enzymes are more easily released and activated before other lysosomal proteinases are released. In addition, cathepsin B released from the lysosome because of their disruption could also activate the collagenase enzyme. (Tschesche *et al*., 1989).

Changes in concentration of insoluble collagen and pepsin soluble collagen during storage confirm the role of collagenase enzyme in collagen degradation followed by muscle texture softening (Chapter III & Chapter V). The mushiness development in fish muscle during ice storage was perhaps by the diffusion of digestive proteolytic enzymes from autolyzed digestive tract due to gaping or breakdown of myotome (Sriket *et al*., 2011b). Additionally, the activity of collagenase against collagen was very high, which indicate that matrix metalloproteinases could participate in degradation of collagen and other extracellular matrix proteins and thereby have a role in the loss of integrity of the muscle. Furthermore, degradation of collagen type I and V is crucial for the softening of shellfish muscle, which is due to the autolytic action of collagenase enzymes (Ezquerra *et al*., 2003; Sriket *et al*., 2011b, Sriket *et al*., 2012).

6.5.2: Optimization of temperature and pH of collagenase enzyme

The temperature dependence of collagenase enzyme was investigated and the results indicate that the isolated enzyme from the muscle tissue of three freshwater fishes have the temperature optima of 40 ^{0}C, which is the same temperature optima that was reported for the skeletal muscles of winter flounder (*Pseudopleuronectes americanus*) (Teruel & Simpson,1995). This temperature is lower than that of 60 ^{0}C of the tissue of file fish (*Novoden modestrus*) (Kim *et al*., 2002), 55 ^{0}C for the internal organs of filefish, and mackerel (*Comber japonicas*) (Park *et al*., 2002), intestines of Atlantic cod (between 45 and 50°C) (Kristjánsson *et al*., 1995). This temperature was higher than that of the 30^{o}C optimum temperature reported for Atlantic cod (*Cadus morhua*) (Kristjansson *et al*., 1995) and greenshore crab (*Carcinus maenas*) (Roy *et al*.,

1996). In addition, pH dependence of the partially purified collagenase showed an optima of 7.4. Teruel & Simpson (1995) reported the optimum pH for collagenolytic enzyme activity from the skeletal muscle of winter flounder as 7.5. Investigations report that the pH values ranging from 7.0 to 8.0 be the optimum for the activity of collagenase enzyme (Sivakumar, *et al*., 1999; Kim *et al*., 2002; Park *et al*., 2002).

6.5.3: Partial characterization of collagenase enzyme

In general, the metallo-collagenolytic enzyme specifically requires Zn^{2+} in its active site for optimum activity and stability. PMSF and EDTA act as an inhibitor for serine proteases and EDTA for metalloproteases. From the results obtained, the partially purified enzyme is inhibited by Zn^{2+} and Mn^{2+} and activated by Ca^{2+}. Additionally, more than 85% activity was detected with the addition of EDTA, however a 50% inhibition was noticed with PMSF. Therefore, the results obtained suggest that the isolated collagenasec enzymes are serine proteases.

6.5.4: Modulatory effect of selected natural herbs and spices on collagenase enzyme activity

Spices and herbs have displayed many of health benefits in treating and preventing a wide variety of inflammatory, cardiovascular, neurologic and metabolic diseases. The activity modulation of collagenolytic enzyme and lysosomal enzyme activity by the water extracts of some herbs and spices commonly used in kitchen for cooking was investigated. The data obtained showed that the extract of garlic, ginger, green chili, Bird's Eye chili, black pepper and curry leaves enhanced the collagenolytic activity. While, lemon juice and extract of *Pimenta dioica* leaves, *Camellia sinensis*, *Tamarindus indica* and *Indian garcinia* inhibited its activity of three fish species. The present data also shows that the curcumin extract has no effect on the activity of collagenase enzyme in fish muscle. It is supported by the studies of Shinde *et al*. (2015) who reported decrease in the muscle tissue pH in green tea dip treated Indian mackerel (*Rastriliger kanagurta*) during its storage period. They also reported that the green tea extract has a partial inhibitory effect on the endogenous enzyme activity in the first stage of storage. Thring *et al*. (2009) reported that the plant extracts, with activity against collagenase enzyme, had high phenolic content. From the results obtained for the modulatory effect on lysosomal acid phosphatase, addition of lemon juice, tamarind

and Malabar tamarind stimulate the acid phosphatase activity while rest of the treatment strongly inhibit the activity. Thus the present findings confirm the use of *Pimenta dioica* leaves and *Camellia sinensis* to extend the shelf life of the selected fish by inhibiting the activity of both collagenase enzyme and lysosomal acid phosphatase.

6.6: Conclusion

Post-mortem fish muscle underwent a number of quality deterioration if not processed and preserved properly. The autolytic enzymes act on tissue constituents contributing to post-mortem degradation in fish muscle and fishery products during storage and processing. The study confirmed the correlation between the degradation of collagen fractions with collagenase enzyme activity at post rigor stage. The study also substantiated the previous results about disintegration of collagen during early post-mortem stage attributed by the action of lysosomal proteolytic enzymes. Furthermore, determination of free, bound and total lysosomal enzyme activity in terms of acid phosphatase in three freshwater fish species provided a detailed picture on post-mortem instability of lysosomes followed by release of membrane bound enzymes and degradation of muscular proteins. The effect of cathepsin D in post-mortem muscle protein disintegration at various stages of storage was also confirmed. Lysosomal membrane instability showed more in stinging catfish than perch and snakehead. Moreover, a high cathepsin D activity was found in catfish muscle tissue fraction. Compared to other proteolytic enzymes studied, involvement of cathepsin D on post-mortem tissue degradation was very low in the selected fish species.

The partial purification and characterization of collagenolytic enzyme in muscle tissue confirms that the collagenase enzyme present in the muscle tissue of freshwater fishes selected are serine proteases with the pH optima of 7.4 and temperature optima of 40 ^{0}C.

From the modulation effect of herbs and spices on the enzymes, it is recommended that leaf extracts of All spices (*Pimenta dioica*) and Tea (*Camellia sinensis)* could be used to extend their shelf life by inhibiting the activity of both collagenase and lysosomal acid phosphatase enzymes. Thus, use of all-spices and tea extracts could improve the consumer satisfaction, as they are natural and more healthy than those of synthetic food additives.

Chapter 7

Chapter 7

Summary and Conclusion

The quality attributes of the fish muscle is mainly influenced by its organoleptic and physico-chemical properties which is a functionality of muscle tissue proteins. Post-mortem degradation of fish muscle is influenced by both intrinsic and extrinsic factors. Action of autolytic enzymes on post-mortem fish muscle induces a number of unfavorable biochemical changes. In this respect, a coordinated study on biochemical, histochemical and textural changes in relation to onset and resolution of rigor have been done in three freshwater species of fish namely, climbing perch, banded snakehead and stinging catfish stored at ambient temperature for 18 hours and iced condition for 18 days. In the General Introduction (Chapter1) the significance of the study with a brief description of the freshwater fish species selected and objectives of the work are given.

A preliminary investigation was conducted to study the influence of different seasons on the proximate composition of the freshwater fishes selected viz; climbing perch, banded snakehead and stinging catfish (Chapter 2). The proximate composition in fish muscle varies significantly during various months of the year, the reasons being physiological, environmental and nutritional. High protein and lipid content were detected during non-spawning period and minimum during spawning months, and inversely co-related to the moisture content. Banded snakehead (17.99%) had highest average protein content in their muscle tissue and climbing perch (15.46%) had the least protein content. The muscle tissue from climbing perch had higher lipid content (12.87%) than banded snakehead (4.65%) and stinging catfish (2.42%). The average moisture content was found to be highest in stinging catfish (78.72%) and least in climbing perch (68.86%).

Post-mortem fish muscle quality is quantitatively determined by analyzing the changes in major physical and chemical parameters including rigor index, pH, water holding capacity, expressible water content and cook loss (Chapter 3). Muscle tissue of stinging catfish showed high pH value than banded snakehead and climbing perch

immediately after their death. Magnitude of rigor index was higher in climbing perch than banded snakehead and stinging catfish. Among these fishes, stinging catfish recorded more rapid progress of rigor mortis and lowest rigor index value. The banded snakehead (85.93%) and climbing perch (88.64%) reached the full rigor condition within 5 hours on storage at ambient temperature and retained the rigor till the 7th hour and 8th hour respectively. Stinging catfish reached full rigor stage at 4th hour after death (78.37%) and continued until 6th hour. All the three fishes stored in ice entered into full rigor condition within first day of storage. The perch showed the highest rigor index value with maximum value of 88.51% and full rigor continued till 5^{th} day. Banded snakehead showed the maximum rigor index value of 83.07%, and duration of full rigor was found till third day. Catfish showed maximum rigor index of 76.52% on first day and reached post rigor condition after the first day itself on iced storage.

All the three fish muscle tissues showed a significant level of reduction in water holding capacity, increase in expressible water content and cook loss, both during iced storage condition and at ambient temperature as they entered into post rigor condition. Initial water holding capacity in fresh samples of climbing perch and banded snakehead and stinging catfish were 70.54%, 73.99 and 60% respectively with stinging catfish having lowest water retention capacity. Catfish had highest expressible water content and cook loss than the other two .WHC of the post-mortem fish muscle was typically dependent on pH.

Presence and accumulation of nucleotides give information about the quality of the fish. Hence, an analysis was performed to get data on degradation pattern of ATP in response to increase in storage time (Chapter 3). Concentration of ATP in fresh muscle tissue sample of perch, snakehead and catfish was 5.05, 2.18 and 2.03 μmoles /g respectively. Decrease in muscle ATP and increase in K value typically influenced the progress of rigor mortis. Data obtained showed that the K value of perch, snakehead and catfish muscle reached above 20% when the ATP level fell below 1.00 μmol/g tissue, both at ambient temperature and iced storage condition. Meanwhile, the fish became rigid when the K value exceeded 20% of all the three species stored at ambient temperature. Additionally, when K value reached above 40%, fish entered into the post-rigor stage at ambient temperature. These observations were not comparable for iced stored fishes. Data obtained shows that the progress of rigor mortis in the three iced fish

is not dependent on the concentration of muscular ATP and is due to cold shortening. Cold shortening happens when the fish muscle becomes chilled prior to the complete use of ATP for muscle contraction and chilling will inhibit the Ca^{2+}-pumping and consequently slowdown muscle contraction.

Detailed quantitative analysis of protein fractions in post-mortem muscle tissue of perch, snakehead and catfish (Chapter 4) revealed that the myofibrillar protein was subjected to a high degree of degradation accompanied by an increase in alkali soluble protein (denatured protein). The myofibrillar protein content was high in muscle tissue of catfish than two others in fresh condition. The extent of post-mortem degradation of myofibrillar protein was comparatively lesser in perch than two others. To establish the instability of myosin, myofibrillar ATPase activity was determined and the enzyme activity was almost constant during the initial period followed by a steep decrease with storage time, reached very low value towards the end of storage period. More than 60% reduction in myofibrillar ATPase activity was observed during the storage period in all the cases. The sarcoplasmic protein content remained rather stable compared to other protein fractions studied. The myofibrillar ATPase activities have been widely used as a measure of actomyosin integrity. The speed of shortening of muscles is related to the activity of the myofibrillar ATPase and can be used to monitor post mortem changes.

Total collagen integrity was found to be inversely proportional to the extent of rigor-mortis in fish muscle. Total tissue collagen content in perch was higher than snakehead and catfish, their concentration ranging between 0.9 to 1.4% of total protein. The rate of post-mortem collagen proteolysis was greater in catfish than other two fishes. The results indicated that the collagen content in catfish is less stable and could be easily solubilized. Presence of comparatively high amount of total collagen may be the one reason for rigidity of climbing perch than two others. Collagen offers an increased mechanical strength from their cross-links to the muscle tissue.

All the fishes had high proportion of pepsin soluble collagen than insoluble and acid soluble collagen. Upon post-mortem storage, gradual reduction in acid soluble collagen was observed with progression of storage time both in ice and at ambient temperature. Pepsin soluble collagen which was stable initially decreased thereafter in all three species. The result confirmed that the pepsin soluble collagens from the

selected freshwater fish muscle tissues are less susceptible to post-mortem proteolysis than acid soluble collagen and insoluble collagen, indicated by high percentage of initial content of PSC fraction, 57% in perch, 58% in snakehead and 47% in catfish.

A study was also conducted in Chapter 5 on changes in organoleptic properties, histochemical studies, and instrumental textural profile analysis. Four-phase sensory analysis based on the sensory demerit score at ambient temperature and ice-stored condition showed signs of early spoilage in phase 3,where all the three fishes entered into post-rigor condition. Together with K and H value, sensory analysis revealed that perch had longer shelf life than snakehead and catfish had the least shelf life.

TPA parameters also varied differently in all the three species during storage. Total hardness was reduced by more than 50% during the storage period at both iced and ambient temperature, with perch showing the highest and catfish showing the lowest value for hardness and stiffness. At ambient temperature, myofibrillar protein had greater influence on total hardness and stiffness than collagen in all the three fishes. In ice stored fish muscle, collagen showed the maximum correlation with regard to both stiffness and total hardness than that of myofibrillar protein. Additionally, high total lipid content along with high collagen content in the tissue of climbing perch would influence the high total hardness and stiffness. The present study also suggested that upon cold storage, low temperature induced solidification of fat interferes with the hardness and stiffness. Values of cohesiveness and springiness also decreased with storage, influenced by both total collagen and myofibrillar protein. Studies of histochemical changes also gave a clear insight on how the myofibrillar and connective tissue proteins are involved in post mortem structural changes attributing textural integrity and toughness to fish muscle tissue.

Influence of autolytic enzymes mainly lysosomal acid phosphatase, Cathepsin D and collagenase in post-mortem muscle tenderization is discussed in Chapter 6. Determination of free, bound and total lysosomal enzyme activity in terms of the indicator enzyme, acid phosphatase in climbing perch, banded snakehead and stinging catfish muscle tissue during storage at ambient temperature and chilled condition showed release of membrane bound enzymes into cytoplasm resulting in post-mortem softening. On comparison among the three freshwater fishes, lysosomal membrane of

stinging catfish had least stability and hence of poor quality than two others. Results obtained for the assay of cathepsin D activity in muscle tissue fraction of the three fish species also confirmed this. On comparison with other autolytic protease enzymes, the activity of cathepsin D assayed in both storage conditions in three fish species was extremely low, indicating the less participation of the same in tissue protein degradation during post mortem condition.

The role of collagenase enzyme in the post-mortem fish muscle softening was studied in detail. The obtained result showed that the collagenase enzyme has a significant role on breakdown of collagen. On storage of the fish at ambient temperature and ice stored condition, collagenolytic enzymes showed highest level of activity in post-rigor stage than in pre-rigor stage. The present study confirmed a close relationship between breakage of pepsin soluble collagen and softening of fish muscle during post rigor stage. Thus composition, localization and proportion of different collagen fractions in fish muscle tissue in the species studied are important factors contributing to the muscle textural qualities along with myofibrillar fraction.

Partial purification and characterization of collagenolytic enzyme suggest that the enzyme present in the muscle tissue of freshwater fish under study belong to serine proteases with a pH optimum of 7.4 and temperature optimum of 40^{o}C. A study on the effect of natural herbs and spices on modulation of collagenase and lysosomal acid phosphatase enzyme was conducted The study strongly suggested that out of many, the water extract of leaves of All spices (*Pimenta dioica*) and Tea (*Camellia sinensis*) could be used to extend the shelf life of the fish species by inhibiting the activity of both collagenase and lysosomal acid phosphatase enzymes. A systematic study in this respect is recommended so as to draw fruitful information on the effect of herbs and spices from nature in altering the basic biochemical and autolytic changes of fish muscle tissue on post mortem so as to improve the consumer satisfaction, as they are natural and healthier than those of synthetic food additives.

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Appendices

Appendix A

Appendix 5. a: Sensory characteristics and scores used in the panel score sheet

Appearance	Skin (both dark and white side)	Bright, iridescent pigmentation	0
		Bright but not lustrous	1
		Dull, becoming discolored	2
		Dull and discolored pigmentation	3
	Mucus	Clear, not clotted	0
		Slightly milky	1
		Milky, opaque	2
		Yellowish gray and clotted	3
Consistency	Texture (black side)	Rigid (This demerit point is given only to fish still in rigor)	0
		Firm and elastic	1
		Slightly soft, less elastic	2
		Very soft	3
	Belly	Firm elastic	0
		Soft	1
		Burst	2
Eyes	Color	Transparent cornea, black shining pupil	0
		Slightly opalescent cornea, black pupil	1
		Mat cornea, opaque pupil	2
		Milky cornea, gray pupil	3
	Shape	Convex	0
		Convex but slightly sunken	1
		Flat	2
		Sunken	3
Gills	Color	Bright light rose-red	0
		Pale rose-red	1
		Slightly discolored, especially at the end of gill filaments	2
		Brown, yellowish, gray	3
Odor	Fresh	marine and seaweed-like	0
		Neutral	1
		Slight of fish, acid, some unpleasant smell	2
		Very unpleasant smell, rotten	3
	Mucus	Not mucus	0
		Slight traces of milk mucus	1
		Yellowish, brown, slightly clotted	2
Fillets	Color of flesh	Clear color, mother-of-pearl	0
		Waxy, milky	1
		Opaque, discolored, yellow, brown	2
	Adherence to the vertebral column	Breaks instead of coming	0
		Sticks	1
		Sticks slightly	2
		Does not stick	3
	Peritoneum	Glossy brilliant white, difficult to tear from flesh	0
		Slightly dull, intact, difficult to tear from flesh	1
		Complete, fairly easy to tear from flesh	2
Total			0–32

Appendix B

Appendix 2.1: Seasonal variation in proximate composition in the muscle tissue of Perch

Appendix 2.1.1: ANOVA result- Seasonal variation in proximate composition of Perch

Source	Sum of Squares	df	Mean Square	F	Sig.
Protein	61.993	11	5.636	35.963	.000
Lipid	24.250	11	2.205	23.691	.000
Moisture	134.658	11	12.242	56.461	.000
Ash	2.328	11	.212	81.448	.000

Appendix 2.1.2: Duncan post hoc test- Seasonal variation in proximate composition in the muscle tissue of Perch

Appendix 2.1.2.a: Duncan post hoc test- Seasonal variation in protein content in the muscle tissue of Perch

Months	N	Subset for alpha = 0.01				
		a	b	c	d	e
July	3	13.0183				
August	3	13.5959	13.5959			
September	3		14.1104			
June	3			15.0850		
January	3			15.2100		
October	3			15.4645	15.4645	
December	3				16.3667	16.3667
February	3					16.5273
May	3					16.5273
November	3					16.6335
April	3					17.0083
March	3					17.0153
Sig.		.087	.125	.279	.010	.088

Appendix 2.1.2.b: Duncan post hoc test- Seasonal variation in lipid content in the muscle tissue of Perch

Months	N	Subset for alpha = 0.01				
		a	b	c	d	e
July	3	11.0816				
August	3	11.6617				
December	3		12.5305			
September	3		12.7107			
January	3		12.7742			
October	3		12.8530			
March	3		12.9333			
May	3		13.0699	13.0699		
November	3		13.2466	13.2466	13.2466	
February	3			13.7163	13.7163	13.7163
June	3				13.8441	13.8441
April	3					14.0634
Sig.		.029	.018	.021	.031	.200

Appendix 2.1.2.c: Duncan post hoc test- Seasonal variation in moisture content in the muscle tissue of Perch

Months	N	Subset for alpha = 0.01							
		a	b	c	d	e	f	g	h
April	3	66.7090							
March	3	66.9057							
Nov	3	67.4052	67.4052						
Jan	3	67.8277	67.8277	67.8277					
Dec	3		68.1040	68.1040	68.1040				
Feb	3		68.3557	68.3557	68.3557	68.3557			
May	3			68.7851	68.7851	68.7851			
June	3				69.0908	69.0908			
Oct	3					69.3695	69.3695		
Sept	3						70.4079		
Aug	3							71.9778	
July	3								73.3684
Sig.		.011	.029	.028	.024	.021	.012	1.000	1.000

Appendix 2.1.2.d: Duncan post hoc test- Seasonal variation in ash content in the muscle tissue of Perch

Months	N	Subset for alpha = 0.01								
		a	b	c	d	e	f	g	h	i
June	3	.9056								
February	3	.9499	.9499							
May	3		1.0327	1.0327						
August	3		1.0660	1.0660	1.0660					
December	3			1.0891	1.0891					
January	3				1.1809	1.1809				
July	3					1.2223				
September	3					1.2946	1.2946			
November	3						1.3974	1.3974		
October	3							1.4808		
April	3								1.6031	
March	3									1.7533
Sig.		.298	.013	.212	.014	.015	.021	.057	1.000	1.000

Appendix 2.1.3: Pearson Correlation results

		Protein	Lipid	Moisture	Ash
Protein	Pearson Correlation	1	.749**	-.929**	.326
	Sig. (2-tailed)		.005	.000	.302
	N	12	12	12	12
Lipid	Pearson Correlation	.749**	1	-.798**	.079
	Sig. (2-tailed)	.005		.002	.807
	N	12	12	12	12
Moisture	Pearson Correlation	-.929**	-.798**	1	-.370
	Sig. (2-tailed)	.000	.002		.236
	N	12	12	12	12
Ash	Pearson Correlation	.326	.079	-.370	1
	Sig. (2-tailed)	.302	.807	.236	
	N	12	12	12	12

**. Correlation is significant at the 0.01 level (2-tailed).

Appendix 2.2: Seasonal variation in proximate composition in the muscle tissue of snakehead

Appendix 2.2.1: ANOVA result- Seasonal variation in proximate composition in the muscle tissue of snakehead

Source	Sum of Squares	df	Mean Square	F	Sig.
Protein	51.885	11	4.717	44.890	.000
Lipid	20.574	11	1.870	10.413	.000
Moisture	143.047	11	13.004	21.643	.000
Ash	1.823	11	.166	3.454	.005

Appendix 2.2.2: Duncan post hoc test- Seasonal variation in proximate composition in the muscle tissue of snakehead

Appendix 2.2.2.a: Duncan post hoc test- Seasonal variation in protein content in the muscle tissue of snakehead

Months	N	Subset for alpha = 0.01						
		a	b	c	d	e	f	g
July	3	15.9374						
August	3	16.0915	16.0915					
June	3	16.4386	16.4386					
December	3		16.7905	16.7905				
January	3			17.2667	17.2667			
November	3				17.7437	17.7437		
October	3					18.4042	18.4042	
September	3						18.5742	
March	3						18.9202	18.9202
February	3						18.9320	18.9320
May	3						19.0294	19.0294
April	3							19.4537
Sig.		.085	.019	.085	.084	.020	.043	.076

Appendix 2.2.2.b: Duncan post hoc test- Seasonal variation in lipid content in the muscle tissue of snakehead

Months	N	Subset for alpha = 0.01	
		a	b
June	3	3.4042	
August	3	3.4935	
July	3	3.4981	
December	3	3.6958	
September	3		4.7857
November	3		4.7988
January	3		4.9110
May	3		4.9494
April	3		5.0336
October	3		5.0777
March	3		5.4343
February	3		5.4941
Sig.		.450	.088

Appendix 2.2.2.c: Duncan post hoc test- Seasonal variation in moisture content in the muscle tissue of snakehead

Months	N	Subset for alpha = 0.01		
		a	b	c
May	3	72.8211		
November	3	72.8361		
March	3	72.8448		
April	3	73.0491		
January	3	73.0708		
February	3	73.1949		
September	3	73.4313		
October	3	73.7694		
December	3		75.9881	
June	3		76.8425	76.8425
August	3		77.7434	77.7434
July	3			78.1893
Sig.		.206	.014	.054

Appendix 2.2.2.d: Duncan post hoc test- Seasonal variation in ash content in the muscle tissue of snakehead

Months	N	Subset for alpha = 0.01	
		a	b
June	3	1.2971	
October	3	1.3341	1.3341
September	3	1.3724	1.3724
April	3	1.3893	1.3893
August	3	1.4009	1.4009
July	3	1.4602	1.4602
March	3	1.4994	1.4994
May	3	1.7687	1.7687
December	3	1.8349	1.8349
November	3	1.8407	1.8407
January	3	1.8428	1.8428
February	3		1.8920
Sig.		.015	.013

Appendix 2.2.3: Pearson Correlation for proximate composition-Snakehead

		Protein	Lipid	Moisture	Ash
Protein	Pearson Correlation	1	.903**	-.881**	.115
	Sig. (2-tailed)		.000	.000	.722
	N	12	12	12	12
Lipid	Pearson Correlation	.903**	1	-.934**	.298
	Sig. (2-tailed)	.000		.000	.346
	N	12	12	12	12
Moisture	Pearson Correlation	-.881**	-.934**	1	-.377
	Sig. (2-tailed)	.000	.000		.227
	N	12	12	12	12
Ash	Pearson Correlation	.115	.298	-.377	1
	Sig. (2-tailed)	.722	.346	.227	
	N	12	12	12	12

**. Correlation is significant at the 0.01 level (2-tailed).

Appendix 2.3: Seasonal variation in proximate composition in the muscle tissue of catfish

Appendix 2.3.1: ANOVA result- Seasonal variation in proximate composition in the muscle tissue of catfish

Source	Sum of Squares	df	Mean Square	F	Sig.
Protein	106.975	11	9.725	75.441	.000
Lipid	6.104	11	.555	11.431	.000
Moisture	172.948	11	15.723	17.148	.000
Ash	9.114	11	.829	9.728	.000

Appendix 2.3.2: Duncan post hoc test- Seasonal variation in proximate composition in the muscle tissue of catfish

Appendix 2.3.2.a: Duncan post hoc test- Seasonal variation in protein content in the muscle tissue of catfish

Months	N	Subset for alpha = 0.01					
		a	b	c	d	e	f
Aug	3	13.0145					
July	3		13.8730				
June	3		14.3000				
Sept	3		14.4827	14.4827			
Oct	3			15.2187	15.2187		
Jan	3				15.9692	15.9692	
Feb	3				16.0517	16.0517	
Dec	3					16.2767	
Nov	3						17.4634
May	3						17.9741
April	3						18.2273
March	3						18.3228
Sig.		1.000	.059	.019	.012	.332	.012

Appendix 2.3.2.b: Duncan post hoc test- Seasonal variation in lipid content in the muscle tissue of catfish

Months	N	Subset for alpha = 0.01		
		a	b	c
June	3	1.8703		
Feb	3	2.0063		
Sept	3	2.0748		
Oct	3	2.1342		
Aug	3	2.2162		
July	3	2.2706	2.2706	
Jan	3		2.7568	2.7568
Dec	3			2.8027
March	3			2.8568
Nov	3			2.8601
May	3			2.8940
April	3			3.1153
Sig.		.059	.012	.090

Appendix 2.3.2.c: Duncan post hoc test- Seasonal variation in moisture content in the muscle tissue of catfish

Months	N	Subset for alpha = 0.01				
		a	b	c	d	e
April	3	75.0067				
May	3	75.0652				
March	3	77.0144	77.0144			
Nov	3	77.0643	77.0643			
Dec	3		77.6478			
Oct	3		78.7848	78.7848		
Feb	3		79.0442	79.0442		
Sept	3		79.3624	79.3624	79.3624	
Jan	3		79.4675	79.4675	79.4675	
Aug	3			80.5570	80.5570	80.5570
July	3				81.6710	81.6710
June	3					82.0144
Sig.		.022	.010	.051	.011	.089

Appendix 2.3.2.d: Duncan post hoc test- Seasonal variation in ash content in the muscle tissue of catfish

Months	N	Subset for alpha = 0.01		
		a	b	c
April	3	1.1664		
July	3	1.1807		
Oct	3	1.2040		
Jan	3	1.2222		
March	3	1.2736		
Feb	3	1.3174		
June	3	1.3534		
Aug	3	1.6280	1.6280	
Nov	3	1.8861	1.8861	
Sept	3	1.8953	1.8953	
May	3		2.1018	
Dec	3			2.9062
Sig.		.014	.080	1.000

Appendix 2.3.3: Pearson Correlation studies in proximate composition of catfish

Catfish		Protein	Lipid	Moisture	Ash
Protein	Pearson Correlation	1	.808**	-.898**	.108
	Sig. (2-tailed)		.001	.000	.737
	N	12	12	12	12
Lipid	Pearson Correlation	.808**	1	-.814**	.248
	Sig. (2-tailed)	.001		.001	.436
	N	12	12	12	12
Moisture	Pearson Correlation	-.898**	-.814**	1	-.284
	Sig. (2-tailed)	.000	.001		.371
	N	12	12	12	12
Ash	Pearson Correlation	.108	.248	-.284	1
	Sig. (2-tailed)	.737	.436	.371	
	N	12	12	12	12

**. Correlation is significant at the 0.01 level (2-tailed).

Appendix 2.4: seasonal variation in proximate composition among the three fish species

Source	Sum of Squares	df	Mean Square	F	Sig.
Protein	34.818	2	17.409	32.689	.000
Lipid	725.688	2	362.844	1061.285	.000
Moisture	549.046	2	274.523	214.754	.000
Ash	.917	2	.458	3.351	.054

Appendix 3.1: Variation in rigor index of climbing perch, banded snakehead and stinging catfish muscle tissue stored at ambient temperature

Appendix 3.1.1: ANOVA results- Variation in rigor index of climbing perch, banded snakehead and stinging catfish stored at ambient temperature

Source	Sum of Squares	df	Mean Square	F	Sig.
Climbing perch	39628.248	12	3302.354	1747.795	.000
Banded Snakehead	32491.336	12	2707.611	787.880	.000
Stinging catfish	31630.106	12	2635.842	2616.173	.000
Among fish	1597.892	2	898.946	16.80	.021

Appendix 3.1.2: Duncan post hoc test Variation in rigor index of climbing perch, banded snakehead and stinging catfish stored at ambient temperature

Appendix 3.1.2.a: Duncan post hoc test - Rigor index of climbing perch at ambient temperature

Hours	N	Subset for alpha = 0.05									
		a	b	c	d	e	f	g	h	i	j
0	3	.00									
1	3		5.93								
2	3			12.85							
3	3				38.78						
18	3					45.05					
15	3						58.42				
4	3							65.64			
12	3								71.53		
10	3									82.27	
5	3										88.64
6	3										88.64
7	3										88.64
8	3										88.64
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Appendix 3.1.2.b: Duncan post hoc test -Rigor index of Banded Snakehead at ambient temperature

Hour	N	Subset for alpha = 0.05								
		a	b	c	d	e	f	g	h	i
0	3	0.00								
1	3		11.17							
18	3			28.92						
2	3				32.24					
15	3					36.98				
3	3						53.21			
12	3							62.89		
4	3							64.42		
10	3								77.71	
8	3									83.71
5	3									85.93
6	3									85.93
7	3									85.93
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	.321	1.000	.191

Appendix 3.1.2.c: Duncan post hoc test -Rigor index of Stinging catfish at ambient temperature

Hours		Subset for alpha = 0.05								
	N	a	b	c	d	e	f	g	h	i
0	3	.00								
18	3		10.45							
1	3		11.95							
15	3			15.06						
12	3				31.13					
2	3					52.81				
10	3					53.42				
8	3						64.49			
3	3							68.37		
7	3								73.66	
4	3									78.37
5	3									78.37
6	3									78.37
Sig.		1.000	.079	1.000	1.000	.461	1.000	1.000	1.000	1.000

Appendix 3.2: Variation in rigor index of iced perch, snakehead and catfish

Appendix 3.2.1: ANOVA results- Variation in rigor index of iced perch, snakehead and catfish

Source	Sum of Squares	df	Mean Square	F	Sig.
Climbing perch	20207.415	8	2525.927	663.460	.000
Banded Snakehead	19357.493	8	2419.687	1104.066	.000
Stinging catfish	19118.633	8	2389.829	737.426	.000
Among fish	2277.208	2	1138.604	28.053	.000

Appendix 3.2.2: Duncan post hoc test -Variation in rigor index of climbing perch, banded snakehead and stinging catfish stored at ambient temperature

Appendix 3.2.2.a: Duncan post hoc test - Rigor index of iced Perch

Days	N	Subset for alpha = 0.05						
		a	b	c	d	e	f	g
0	3	0.00						
18	3		42.26					
15	3			54.91				
12	3				62.19			
9	3					71.14		
7	3						82.01	
1	3							86.45
3	3							88.51
5	3							88.51
Sig.		1.00	1.00	1.00	1.00	1.00	1.00	.235

Appendix 3.2.2.b: Duncan post hoc test -Rigor index of iced Snakehead

Days	N	Subset for alpha = 0.05							
		a	b	c	d	e	f	g	h
0	3	.00							
18	3		29.52						
15	3			35.91					
12	3				55.21				
9	3					59.36			
7	3						70.98		
5	3							78.64	
1	3								83.06
3	3								83.16
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000	.935

Appendix 3.2.2.c: Duncan post hoc test -Rigor index of iced Catfish

Days	N	Subset for alpha = 0.05							
		a	b	c	d	e	f	g	h
0	3	.00							
18	3		12.95						
15	3			19.56					
12	3				28.66				
9	3					39.13			
7	3						58.71		
5	3							63.66	
3	3								75.71
1	3								76.52
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000	.587

Appendix 3.3: Variation in pH of perch, snakehead and catfish muscle tissue stored at ambient temperature

Appendix 3.3.1: ANOVA results- Variation in pH of perch, snakehead and catfish muscle tissue stored at ambient temperature

Source	Sum of Squares	df	Mean Square	F	Sig.
Climbing perch	.367	12	.031	53.659	.000
Banded Snakehead	.387	12	.032	106.083	.000
Stinging catfish	.682	12	.057	147.708	.000
Among fish	.452	2	.226	123.316	.000

Appendix 3.3.2: Duncan post hoc test - Variation in pH of perch, snakehead and catfish muscle tissue stored at ambient temperature

Appendix 3.3.2.a: Duncan post hoc test - pH of Perch at ambient temperature

Hours	N	Subset for alpha = 0.05						
		a	b	c	d	e	f	g
	3	6.80						
6	3	6.81	6.81					
4	3	6.82	6.82	6.82				
3	3		6.84	6.84	6.84			
2	3		6.85	6.85	6.85			
7	3			6.86	6.86			
1	3				6.88			
8	3					6.92		
0	3					6.93		
10	3					6.96		
12	3						7.02	
15	3						7.06	
18	3							7.12
Sig.		.207	.111	.082	.070	.073	.071	1.000

Appendix 3.3.2.b: Duncan post hoc test - pH of Snakehead at ambient temperature

Hours	N	Subset for alpha = 0.05							
		a	b	c	d	e	f	g	h
5	3	6.75							
4	3	6.77	6.77						
6	3		6.79	6.79					
3	3		6.80	6.80					
2	3		6.80	6.80					
7	3			6.81					
1	3				6.85				
8	3				6.87				
0	3					6.90			
10	3						6.97		
12	3						6.98		
15	3							7.02	
18	3								7.07
Sig.		.072	.063	.119	.302	1.000	.113	1.000	1.000

Appendix 3.3.2.c: Duncan post hoc test - pH of Catfish at ambient temperature

Hours	N	Subset for alpha = 0.05				
		a	b	c	d	e
3	3	6.94				
4	3	6.96				
2	3	6.97				
5	3		7.03			
1	3		7.03			
6	3		7.04			
0	3		7.06			
7	3			7.16		
8	3				7.24	
10	3				7.26	
12	3				7.26	
15	3					7.3
18	3					7.32
Sig.		.087	.080	1.000	.249	.358

Appendix 3.4: Variation in pH of iced perch, snakehead and catfish muscle tissue

Appendix3.4.1: ANOVA results- Variation in pH of iced perch, snakehead and catfish muscle tissue

Source	Sum of Squares	df	Mean Square	F	Sig.
Climbing perch	.151	8	.019	24.300	.000
Banded Snakehead	.183	8	.023	85.781	.000
Stinging catfish	.299	8	.037	168.412	.000
Among fish	.198	2	.099	62.897	.000

Appendix 3.4.2: Duncan post hoc test - Variation in pH of iced perch, snakehead and catfish

Appendix 3.4.2.a: Duncan post hoc test - pH of iced Perch

Days	N	Subset for alpha = 0.05			
		a	b	c	d
3	3	6.90			
1	3	6.90			
5	3	6.95	6.95		
0	3		6.97		
7	3			7.02	
9	3			7.06	7.06
12	3			7.07	7.07
15	3				7.08
18	3				7.11
Sig.		.051	.391	.051	.057

Appendix 3.4.2.b: Duncan post hoc test - pH of iced Snakehead

Days	N	Subset for alpha = 0.05					
		a	b	c	d	e	f
5	3	6.82					
1	3	6.83					
3	3		6.87				
7	3		6.89				
0	3			6.94			
9	3				6.97		
12	3				6.98		
15	3					7.03	
18	3						7.07
Sig.		.463	.151	1.000	.463	1.000	1.000

Appendix 3.4.2.c: Duncan post hoc test - pH of iced Catfish

Days	N	Subset for alpha = 0.05						
		a	b	c	d	e	f	g
1	3	6.97						
0	3		7.00					
3	3			7.09				
5	3			7.10				
7	3				7.16			
9	3				7.16			
12	3					7.22		
15	3						7.26	
18	3							7.30
Sig.		1.000	1.000	.422	1.000	1.000	1.000	1.000

Appendix 3.5: Changes in expressible water content (EWC), water holding capacity (WHC) and cook loss (CL) of Climbing perch, Banded snakehead and Stinging catfish muscle tissue stored at ambient temperature

Appendix 3.5.1: ANOVA results- Changes in expressible water content (EWC), water holding capacity (WHC) and cook loss (CL) of Climbing perch, Banded snakehead and Stinging catfish muscle tissue stored at ambient temperature

Source		Sum of Squares	df	Mean Square	F	Sig.
Climbing perch	EWC	287.126	12	23.927	4.308	.001
	WHC	2146.432	12	178.869	42.162	.000
	CL	2701.194	12	225.100	55.114	.000
Banded Snakehead	EWC	1062.329	12	88.527	12.925	.000
	WHC	2363.999	12	197.000	31.449	.000
	CL	2995.732	12	249.644	28.481	.000
Stinging catfish	EWC	1323.666	12	110.305	16.750	.000
	WHC	1504.238	12	125.353	14.428	.000
	CL	5854.364	12	487.864	74.590	.000
Among fish	EWC	49.787	2	24.893	4.083	.030
	WHC	1095.535	2	547.768	148.738	.000
	CL	581.043	2	290.521	48.618	.000

Appendix 3.5.2: Duncan post hoc test - Changes in EWC, WHC and CL of Climbing perch, Banded snakehead and Stinging catfish muscle tissue stored at ambient temperature

Appendix 3.5.2.1: Duncan post hoc test - Changes in EWC of Climbing perch, Banded snakehead and Stinging catfish muscle tissue stored at ambient temperature

Appendix 3.5.2.1.a: Duncan post hoc test - Changes in EWC of perch at ambient temperature

Hours	N	Subset for alpha = 0.05				
		a	b	c	d	e
1	3	8.9865				
0	3	9.0072				
2	3	9.0145				
3	3	9.9329	9.9329			
4	3	10.7542	10.7542	10.7542		
5	3	10.9632	10.9632	10.9632		
6	3	11.4801	11.4801	11.4801	11.4801	
12	3		13.5910	13.5910	13.5910	13.5910
7	3		13.6907	13.6907	13.6907	13.6907
10	3			14.5643	14.5643	14.5643
8	3			14.7904	14.7904	14.7904
15	3				15.5019	15.5019
18	3					17.5401
Sig.		.269	.096	.077	.075	.080

Appendix 3.5.2.1.b: Duncan post hoc test - Changes in EWC of Snakehead at ambient temperature

Hours	N	Subset for alpha = 0.05			
		a	b	c	d
2	3	8.6858			
3	3	9.0191			
0	3	9.3201			
4	3	9.9829			
1	3	10.0159			
5	3	10.0182			
6	3	10.7353			
7	3	13.5485	13.5485		
8	3		15.5483	15.5483	
10	3		17.4805	17.4805	
12	3			19.3086	
15	3			20.0010	
18	3				25.9237
Sig.		.058	.093	.066	1.000

Appendix 3.5.2.1.c: Duncan post hoc test - Changes in EWC of Catfish at ambient temperature

Hours	N	Subset for alpha = 0.05			
		a	b	c	d
1	3	10.9210			
6	3		12.3256		
4	3		12.3584		
0	3		12.8739		
2	3		13.2708		
3	3		13.7292		
7	3		14.2415		
5	3		14.8981	14.8981	
8	3		15.9570	15.9570	
10	3		17.0219	17.0219	
12	3			19.0219	
15	3				24.0218
18	3				27.0327
Sig.		1.000	.064	.082	.163

Appendix 3.5.2.2: Duncan post hoc test - Changes in WHC of Climbing perch, Banded snakehead and Stinging catfish muscle tissue stored at ambient temperature

Appendix 3.5.2.2.a: Duncan post hoc test - Changes in WHC of Perch at ambient temperature

Hours	N	Subset for alpha = 0.05					
		a	b	c	d	e	f
18	3	51.4472					
15	3		59.1780				
10	3			67.5432			
12	3			67.6963			
7	3				71.8576		
6	3				72.8560	72.8560	
8	3				73.4717	73.4717	73.4717
0	3				74.5401	74.5401	74.5401
1	3				74.9328	74.9328	74.9328
2	3					76.5203	76.5203
5	3					76.5810	76.5810
3	3						76.9335
4	3						76.9906
Sig.		1.000	1.000	.928	.112	.060	.078

Appendix 3.5.2.2.b: Duncan post hoc test - Changes in WHC of Snakehead at ambient temperature

Hours	N	Subset for alpha = 0.05					
		a	b	c	d	e	f
18	3	47.1510					
15	3		52.1550				
12	3		54.0396				
10	3			58.3322			
8	3			61.4179	61.4179		
7	3				65.5680	65.5680	
6	3					66.6698	66.6698
4	3					69.1438	69.1438
1	3					69.4436	69.4436
0	3					70.0550	70.0550
2	3					70.1095	70.1095
5	3						70.4560
3	3						70.9107
Sig.		1.000	.365	.143	.053	.059	.080

Appendix 3.5.2.2.c: Duncan post hoc test - Changes in WHC of Catfish at ambient temperature

Hours	N	Subset for alpha = 0.05				
		a	b	c	d	e
18	3	44.4950				
15	3	44.9660				
12	3		54.0349			
10	3		56.4825	56.4825		
6	3		57.8081	57.8081	57.8081	
7	3		58.5781	58.5781	58.5781	58.5781
8	3		58.7492	58.7492	58.7492	58.7492
2	3			61.7272	61.7272	61.7272
5	3			61.8539	61.8539	61.8539
3	3				62.3994	62.3994
0	3				62.4243	62.4243
4	3				63.3952	63.3952
1	3					63.8489
Sig.		.846	.090	.058	.054	.068

Appendix 3.5.2.3: Duncan post hoc test - Changes in CL of Climbing perch, Banded snakehead and Stinging catfish muscle tissue stored at ambient temperature

Appendix 3.5.2.3.a: Duncan post hoc test - Changes in CL of Perch at ambient temperature

Hours	N	Subset for alpha = 0.05							
		a	b	c	d	e	f	g	h
3	3	10.9210							
2	3	11.3750							
0	3	11.9675	11.9675						
1	3	12.3440	12.3440						
4	3	13.5439	13.5439						
5	3		15.5430	15.5430					
6	3			18.3299	18.3299				
7	3				20.8306				
8	3					24.3258			
10	3						29.2341		
12	3						30.0601	30.0601	
15	3							32.9054	32.9054
18	3								33.6238
Sig.		.166	.056	.103	.142	1.000	.621	.097	.667

Appendix 3.5.2.3.b: Duncan post hoc test - Changes in CL of Snakehead at ambient temperature

Hours	N	Subset for alpha = 0.05				
		a	b	c	d	e
1	3	9.8210				
3	3	10.1962				
2	3	10.3111				
0	3	10.4821				
4	3	11.9019	11.9019			
5	3	13.4390	13.4390			
6	3		16.4395	16.4395		
7	3			19.9731		
8	3			21.3797		
10	3				27.4231	
18	3				31.5946	31.5946
12	3				32.0206	32.0206
15	3					32.9733
Sig.		.199	.086	.063	.083	.596

Appendix 3.5.2.3.c: Duncan post hoc test - Changes in CL of Catfish at ambient temperature

Hours	N	Subset for alpha = 0.05					
		a	b	c	d	e	f
2	3	15.1423					
1	3	15.1851					
3	3	15.4329	15.4329				
4	3	16.6436	16.6436				
0	3	17.7732	17.7732				
5	3		20.0217				
6	3			25.8327			
7	3			27.7504			
8	3				35.8175		
10	3				37.7855		
12	3					42.4790	
15	3						46.8321
18	3						49.0018
Sig.		.271	.053	.367	.355	1.000	.308

Appendix 3.6: Changes in expressible water content (EWC), water holding capacity (WHC) and cook loss (CL) of Climbing perch, Banded snakehead and Stinging catfish muscle tissue stored at iced condition

Appendix 3.6.1: ANOVA results- Changes in expressible water content (EWC), water holding capacity (WHC) and cook loss (CL) of Climbing perch, Banded snakehead and Stinging catfish muscle tissue stored at iced condition

Source		Sum of Squares	df	Mean Square	F	Sig.
Climbing perch	EWC	760.799	8	95.100	49.727	.000
	WHC	485.909	8	60.739	17.789	.000
	CL	759.854	8	94.982	21.231	.000
Banded Snakehead	EWC	1094.048	8	136.756	172.383	.000
	WHC	2450.775	8	306.347	130.625	.000
	CL	911.179	8	113.897	41.315	.000
Stinging catfish	EWC	1213.891	8	151.736	73.247	.000
	WHC	962.992	8	120.374	20.291	.000
	CL	3412.896	8	426.612	70.351	.000
Among fish	EWC	84.371	2	42.185	21.920	.000
	WHC	1007.359	2	503.680	54.023	.000
	CL	441.760	2	220.880	17.820	.000

Appendix 3.6.2: Duncan post hoc test - Changes in EWC, WHC and CL of Climbing perch, Banded snakehead and Stinging catfish muscle tissue stored at iced condition

Appendix 3.6.2.1: Duncan post hoc test - Changes in EWC of Climbing perch, Banded snakehead and Stinging catfish muscle tissue stored at iced condition

Appendix 3.6.2.1.a: Duncan post hoc test - Changes in EWC of iced perch

Days	N	Subset for alpha = 0.05			
		a	b	c	d
0	3	12.0072			
3	3	12.8889			
1	3	12.9145			
5	3	14.5527	14.5527		
7	3		16.7292		
9	3			20.3774	
12	3				23.8291
15	3				25.3620
18	3				25.6799
Sig.		.051	.070	1.000	.137

Appendix 3.6.2.1.b: Duncan post hoc test - Changes in EWC of iced snakehead

Days	N	Subset for alpha = 0.05				
		a	b	c	d	e
0	3	8.5179				
1	3	8.9307				
3	3	11.4767				
5	3		14.7262			
7	3		16.1818	16.1818		
9	3		16.6104	16.6104	16.6104	
18	3			19.3568	19.3568	19.3568
15	3				19.7152	19.7152
12	3					20.0455
Sig.		.078	.252	.060	.066	.672

Appendix 3.6.2.1.c: Duncan post hoc test - Changes in EWC of iced catfish

Days	N	Subset for alpha = 0.05				
		a	b	c	d	e
0	3	10.7122				
1	3	11.6288				
3	3		15.7357			
5	3		17.4257	17.4257		
7	3			20.8817	20.8817	
9	3				22.0211	
12	3				23.4837	23.4837
15	3				24.2823	24.2823
18	3					26.5351
Sig.		.602	.341	.061	.086	.111

Appendix 3.6.2.2: Duncan post hoc test - Changes in WHC of Climbing perch, Banded snakehead and Stinging catfish muscle tissue stored at iced condition

Appendix 3.6.2.2.a: Duncan post hoc test - Changes in WHC of iced perch

Days	N	Subset for alpha = 0.05					
		a	b	c	d	e	f
18	3	54.9004					
15	3		56.9266				
12	3			61.5763			
9	3				64.7428		
7	3				65.8615		
5	3					70.0607	
0	3					70.5401	
3	3						72.7366
1	3						73.5228
Sig.		1.000	1.000	1.000	.141	.518	.294

Appendix 3.6.2.2.b: Duncan post hoc test - Changes in WHC of iced snakehead

Days	N	Subset for alpha = 0.05				
		a	b	c	d	e
18	3	47.1741				
15	3		54.4702			
12	3			63.5873		
9	3				71.1524	
7	3				72.5672	72.5672
5	3				73.7213	73.7213
0	3				73.9881	73.9881
3	3					74.3421
1	3					74.7395
Sig.		1.000	1.000	1.000	.050	.134

Appendix 3.6.2.2.c: Duncan post hoc test - Changes in WHC of iced catfish

Days	N	Subset for alpha = 0.05				
		a	b	c	d	e
18	3	43.1642				
12	3		48.5807			
15	3		49.0480			
9	3		49.7019			
7	3			55.2611		
5	3			56.8861	56.8861	
1	3				59.4761	59.4761
0	3					60.0291
3	3					60.2552
Sig.		1.000	.445	.246	.072	.594

Appendix 3.6.2.3: Duncan post hoc test - Changes in CL of Climbing perch, Banded snakehead and Stinging catfish muscle tissue stored at iced condition

Appendix 3.6.2.3.a: Duncan post hoc test - Changes in CL of iced perch

Days	N	Subset for alpha = 0.05				
		a	b	c	d	e
0	3	15.3518				
1	3	16.7307				
3	3		21.5821			
5	3			24.2271		
7	3			25.4194		
9	3			25.6321		
12	3				31.2076	
15	3					34.4275
18	3					35.2717
Sig.		.256	1.000	.273	1.000	.482

Appendix 3.6.2.3.b: Duncan post hoc test - Changes in CL of iced snakehead

Days	N	Subset for alpha = 0.05					
		a	b	c	d	e	f
0	3	16.9323					
1	3	19.4218	19.4218				
3	3		23.5956	23.5956			
5	3			24.8376			
9	3			26.2341			
7	3			27.3258	27.3258		
12	3				31.0206	31.0206	
15	3					32.1264	
18	3						37.2386
Sig.		.227	.050	.101	.080	.585	1.000

Appendix 3.6.2.3.c: Duncan post hoc test - Changes in CL of iced catfish

Days	N	Subset for alpha = 0.05				
		a	b	c	d	e
0	3	17.9227				
1	3	19.7474				
3	3		25.8511			
5	3		29.0744			
7	3			34.6881		
9	3				42.8559	
12	3				45.9440	45.9440
15	3				46.8573	46.8573
18	3					48.1918
Sig.		.376	.126	1.000	.074	.304

Appendix 3.7: Changes in the ATP and related compound degradation pattern in perch muscle tissue stored at ambient temperature

Appendix 3.7.1: ANOVA results- Changes in the ATP and related compound degradation pattern in perch stored at ambient temperature

	Sum of Squares	df	Mean Square	F	Sig.
ATP	55.763	12	4.647	192.487	.000
ADP	32.537	12	2.711	426.907	.000
AMP	12.410	12	1.034	45.295	.000
IMP	177.754	12	14.813	162.350	.000
HxR	16.810	12	1.401	95.561	.000
Hx	23.665	12	1.972	205.667	.000

Appendix 3.7.2: Duncan post hoc test- Changes in the ATP and related compound degradation pattern in perch stored at ambient temperature

Appendix 3.7.2.a: Duncan post hoc test- Changes in the ATP in perch stored at ambient temperature

Hours	N	Subset for alpha = 0.05							
		a	b	c	d	e	f	g	h
18	3	.04							
15	3	.08							
10	3	.35	.35						
12	3	.35	.35						
8	3		.51						
7	3			.93					
6	3			.99	.99				
5	3			1.08	1.08				
4	3				1.29				
3	3					2.34			
2	3						2.78		
1	3							3.58	
0	3								5.05
Sig.		.083	.330	.363	.091	1.000	1.000	1.000	1.000

Appendix 3.7.2.b: Duncan post hoc test- Changes in the ADP in perch stored at ambient temperature

Hours	N	Subset for alpha = 0.05							
		a	b	c	d	e	f	g	h
18	3	.0229							
15	3	.0238							
12	3	.0803	.0803						
10	3	.1187	.1187						
8	3		.2299						
6	3			.5294					
7	3			.6126					
5	3				.8854				
4	3					1.9965			
3	3						2.2143		
0	3						2.3768		
1	3							2.8642	
2	3								3.0576
Sig.		.286	.097	.316	1.000	1.000	.062	1.000	1.000

Appendix 3.7.2.c: Duncan post hoc test- Changes in the AMP in perch stored at ambient temperature

Hours	N	Subset for alpha = 0.05							
		a	b	c	d	e	f	g	h
18	3	.2664							
12	3		.6251						
10	3		.8622	.8622					
15	3		.9510	.9510	.9510				
8	3			1.0382	1.0382				
7	3				1.2264	1.2264			
6	3					1.3989	1.3989		
5	3						1.6250		
0	3						1.7243		
4	3							2.0626	
1	3							2.2288	
3	3							2.2714	
2	3								2.6933
Sig.		1.000	.060	.289	.106	.274	.061	.212	1.000

Appendix 3.7.2.d: Duncan post hoc test- Changes in the IMP in perch stored at ambient temperature

Hours	N	Subset for alpha = 0.05						
		a	b	c	d	e	f	g
18	3	.7107						
15	3		2.2614					
12	3		2.4734					
10	3			3.4393				
8	3				4.5031			
7	3				4.7750			
6	3					6.2561		
0	3						7.2311	
1	3						7.4875	
5	3						7.7362	
4	3							8.4292
2	3							8.5412
3	3							8.7033
Sig.		1.000	.495	1.000	.384	1.000	.135	.404

Appendix 3.7.2.e: Duncan post hoc test- Changes in the HxR in perch stored at ambient temperature

Hours	N	Subset for alpha = 0.05							
		a	b	c	d	e	f	g	h
0	3	.0642							
1	3	.2544	.2544						
2	3		.4675						
3	3			.8601					
4	3				1.5359				
5	3				1.6078				
6	3				1.6358				
7	3				1.7991	1.7991			
8	3					1.9913	1.9913		
10	3						2.1991	2.1991	
12	3							2.3082	2.3082
15	3							2.3838	2.3838
18	3								2.5159
Sig.		.140	.102	1.000	.065	.136	.110	.170	.126

Appendix 3.7.2.f: Duncan post hoc test- Changes in the Hx in perch stored at ambient temperature

Hours	N	Subset for alpha = 0.05					
		a	b	c	d	e	f
0	3	.0000					
1	3	.0000					
2	3	.0000					
3	3	.0000					
4	3	.0000					
5	3	.0547					
6	3		.8648				
7	3			1.1087			
8	3			1.2412			
10	3				1.9451		
12	3				2.0462		
15	3					2.2620	
18	3						2.5294
Sig.		.621	1.000	.199	.321	1.000	1.000

Appendix 3.8: Changes in the ATP and related compound degradation pattern in snakehead muscle tissue stored at ambient temperature

Appendix 3.8.1: ANOVA results- Changes in the ATP and related compound degradation pattern in snakehead stored at ambient temperature

	Sum of Squares	df	Mean Square	F	Sig.
ATP	10.484	12	.874	384.500	.000
ADP	28.578	12	2.382	68.213	.000
AMP	17.035	12	1.420	62.405	.000
IMP	65.881	12	5.490	77.036	.000
HxR	32.957	12	2.746	259.440	.000
Hx	57.805	12	4.817	264.451	.000

Appendix 3.8.2: Duncan post hoc test- Changes in the ATP and related compound degradation pattern in snakehead stored at ambient temperature

Appendix 3.8.2.a: Duncan post hoc test- Changes in the ATP degradation pattern in snakehead stored at ambient temperature

Hours	N	Subset for alpha = 0.05								
		a	b	c	d	e	f	g	h	i
18	3	.04301								
15	3	.06366								
12	3	.13073	.13073							
10	3		.19750							
8	3			.45710						
7	3				.60347					
6	3					.81824				
5	3						.98723			
4	3						1.07638	1.07638		
3	3							1.12452		
2	3								1.50829	
1	3								1.53932	
0	3									2.17931
Sig.	3	.103	.185	1.000	1.000	1.000	.084	.331	.526	1.000

Appendix 3.8.2.b: Duncan post hoc test- Changes in the ADP degradation pattern in snakehead stored at ambient temperature

Hours	N	Subset for alpha = 0.05			
		a	b	c	d
18	3	.0483			
15	3	.0650			
12	3	.0687			
10	3	.0962			
8	3	.1059			
7	3	.3349			
6	3		.8194		
5	3		1.1777	1.1777	
4	3		1.2401	1.2401	
3	3			1.5599	
2	3				2.6264
0	3				2.7241
1	3				2.8906
Sig.		.190	.051	.073	.202

Appendix 3.8.2.c: Duncan post hoc test- Changes in the AMP degradation pattern in snakehead stored at ambient temperature

Hours	N	Subset for alpha = 0.05						
		a	b	c	d	e	f	g
18	3	.2370						
15	3	.3292	.3292					
10	3	.4855	.4855					
12	3	.4981	.4981					
8	3		.6208					
4	3			1.1164				
7	3			1.1585	1.1585			
3	3			1.3529	1.3529	1.3529		
6	3				1.4995	1.4995		
5	3					1.6765		
2	3						2.0815	
1	3						2.4030	
0	3							2.9226
Sig.		.133	.096	.160	.050	.061	.053	1.000

Appendix 3.8.2.d: Duncan post hoc test- Changes in the IMP degradation pattern in snakehead stored at ambient temperature

Hours	N	Subset for alpha = 0.05							
		a	b	c	d	e	f	g	h
18	3	1.0087							
15	3		1.7326						
12	3			2.3291					
10	3			2.5994					
8	3			2.7646	2.7646				
7	3				3.3039	3.3039			
6	3					3.7723			
5	3						4.9577		
0	3						4.9713		
4	3						5.2383	5.2383	
3	3						5.5276	5.5276	5.5276
1	3							5.7857	5.7857
2	3								5.8636
Sig.		1.000	1.000	.144	.064	.103	.069	.072	.253

Appendix 3.8.2.e: Duncan post hoc test- Changes in the HxR degradation pattern in snakehead stored at ambient temperature

Hours	N	Subset for alpha = 0.05							
		a	b	c	d	e	f	g	h
0	3	.0027							
1	3	.0032							
2	3	.0075							
3	3	.0351							
4	3	.0452							
5	3	.1214							
6	3		.4498						
7	3			.8373					
8	3				1.1330				
10	3					1.5626			
12	3						2.1986		
15	3							2.6140	
18	3								3.4910
Sig.		.316	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Appendix 3.8.2.f: Duncan post hoc test- Changes in the Hx degradation pattern in snakehead stored at ambient temperature

Hours	N	Subset for alpha = 0.05					
		a	b	c	d	e	f
1	3	.0022					
0	3	.0027					
2	3	.0053					
3	3	.0336					
4	3	.0375					
5	3	.0703					
6	3	.1897					
7	3		.8591				
8	3		1.0587				
10	3			1.5052			
12	3				2.9069		
15	3					3.7813	
18	3						4.3373
Sig.		.234	.163	1.000	1.000	1.000	1.000

Appendix 3.9: Changes in the ATP and related compound degradation pattern in catfish muscle tissue stored at ambient temperature

Appendix 3.9.1: ANOVA results- Changes in the ATP and related compound degradation pattern in catfish stored at ambient temperature

	Sum of Squares	df	Mean Square	F	Sig.
ATP	14.649	12	1.221	61.418	.000
ADP	15.193	12	1.266	14.496	.000
AMP	15.482	12	1.290	129.544	.000
IMP	89.570	12	7.464	156.404	.000
HxR	158.188	12	13.182	317.606	.000
Hx	95.253	12	7.938	444.383	.000

Appendix 3.9.2: Duncan post hoc test- Changes in the ATP and related compound degradation pattern in catfish stored at ambient temperature

Appendix 3.9.2.a: Duncan post hoc test- Changes in the ATP degradation pattern in catfish stored at ambient temperature

Hours	N	Subset for alpha = 0.05					
		a	b	c	d	e	f
18	3	.00358					
10	3	.11823					
12	3	.12390					
15	3	.12394					
8	3	.13326					
6	3		.66570				
7	3		.69041				
5	3		.80355	.80355			
4	3			1.02553			
3	3				1.39186		
2	3					1.79842	
1	3					2.03204	2.03204
0	3						2.19988
Sig.		.417	.370	.139	1.000	.121	.255

Appendix 3.9.2.b: Duncan post hoc test- Changes in the ADP degradation pattern in catfish stored at ambient temperature

Hours	N	Subset for alpha = 0.05						
		a	b	c	d	e	f	g
18	3	.0615						
15	3	.3170	.3170					
12	3	.5227	.5227					
8	3	.5834	.5834					
10	3	.6395	.6395					
7	3	.7652	.7652	.7652				
6	3		.8223	.8223				
5	3		1.0060	1.0060	1.0060			
4	3			1.4028	1.4028	1.4028		
3	3				1.6109	1.6109		
2	3					1.9248	1.9248	
0	3						2.2856	2.2856
1	3							2.6696
Sig.		.051	.058	.066	.073	.116	.244	.216

Appendix 3.9.2.c: Duncan post hoc test- Changes in the AMP degradation pattern in catfish stored at ambient temperature

Hours	N	Subset for alpha = 0.05					
		a	b	c	d	e	f
18	3	.0317					
15	3	.0812					
12	3	.1095					
10	3	.1606					
8	3	.2085					
7	3		.4786				
6	3		.5827				
5	3			.8755			
4	3			1.0635	1.0635		
3	3				1.2736	1.2736	
2	3					1.3915	
1	3						2.2990
0	3						2.3398
Sig.		.131	.316	.082	.055	.259	.689

Appendix 3.9.2.d: Duncan post hoc test- Changes in the IMP degradation pattern in catfish stored at ambient temperature

Hours	N	Subset for alpha = 0.05							
		a	b	c	d	f	g	h	8
18	3	.9465							
15	3	1.0623	1.0623						
12	3		1.4662						
10	3			2.8795					
8	3				3.5037				
7	3				3.6676	3.6676			
6	3					4.1057	4.1057		
5	3						4.4234		
4	3							5.0257	
3	3							5.4229	
0	3								6.0633
1	3								6.2692
2	3								6.4819
Sig.		.605	.087	1.000	.466	.066	.170	.092	.091

Appendix 3.9.2.e: Duncan post hoc test- Changes in the HxR degradation pattern in catfish stored at ambient temperature

Hours	N	Subset for alpha = 0.05						
		a	b	c	d	e	f	g
0	3	.1057						
1	3	.1923						
3	3	.2431						
2	3	.2524						
4	3		.9772					
5	3		1.2900					
6	3			2.2893				
7	3			2.3461				
8	3				3.7952			
10	3					4.9999		
12	3						5.6787	
15	3							6.6121
18	3							6.9619
Sig.		.517	.149	.785	1.000	1.000	1.000	.110

Appendix 3.9.2.f: Duncan post hoc test- Changes in the Hx degradation pattern in catfish stored at ambient temperature

Hours	N	Subset for alpha = 0.05								
		a	b	c	d	e	f	g	h	i
0	3	.0992								
1	3		.5134							
2	3			.9735						
3	3			.9891						
4	3			1.1790						
5	3				1.6640					
6	3				1.8664					
7	3					2.5855				
8	3						3.3454			
10	3							4.2454		
12	3								5.2254	
15	3									5.6110
18	3									5.6204
Sig.		1.000	1.000	.167	.154	1.000	1.000	1.000	1.000	.945

Appendix 3.10: ANOVA Results among the fishes- Changes in the ATP and related compound degradation pattern in three fishes stored at ambient temperature

	Sum of Squares	df	Mean Square	F	Sig.
ATP	3.786	2	1.893	7.321	.003
ADP	1.172	2	.86	3.973	.029
AMP	2.568	2	1.284	11.175	.000
IMP	24.990	2	12.495	25.061	.000
HxR	21.796	2	10.898	10.714	.000
Hx	21.821	2	10.910	29.757	.000

Appendix 3.11: Changes in the ATP and related compound degradation pattern in iced perch muscle tissue

Appendix 3.11.1: Changes in the ATP and related compound degradation pattern in iced perch muscle tissue

	Sum of Squares	df	Mean Square	F	Sig.
ATP	45.620	8	5.702	12519.429	.000
ADP	4.273	8	.534	131.011	.000
AMP	10.526	8	1.316	147.794	.000
IMP	120.416	8	15.052	452.508	.000
HxR	8.728	8	1.091	105.317	.000
Hx	11.627	8	1.453	127.799	.000

Appendix 3.11.2: Duncan post hoc test- Changes in the ATP and related compound degradation pattern in iced perch

Appendix 3.11.2.a: Duncan post hoc test- Changes in the ATP degradation pattern in iced perch

Days	N	Subset for alpha = 0.05				
		a	b	c	d	e
18	3	.0630				
15	3	.0900				
12	3	.1018				
9	3	.1057				
7	3	.1080				
5	3		.5932			
3	3			.9645		
1	3				2.4629	
0	3					5.0531
Sig.		.084	1.000	1.000	1.000	1.000

Appendix 3.11.2.b: Duncan post hoc test- Changes in the ADP degradation pattern in iced perch

Days	N	Subset for alpha = 0.05					
		a	b	c	d	e	f
18	3	.6996					
9	3	.7524	.7524				
7	3	.8385	.8385	.8385			
15	3		.8656	.8656			
5	3		.8856	.8856			
12	3			.9471			
3	3				1.1242		
1	3					1.2740	
0	3						2.3781
Sig.		.067	.083	.146	1.000	1.000	1.000

Appendix 3.11.2.c: Duncan post hoc test- Changes in the AMP degradation pattern in iced perch

Days	N	Subset for alpha = 0.05		
		a	b	c
18	3	.0827		
15	3	.0900		
12	3	.1287		
7	3	.1444		
9	3	.1479		
5	3		.6868	
0	3			1.7243
3	3			1.7763
1	3			1.8731
Sig.		.536	1.000	.166

Appendix 3.11.2.d: Duncan post hoc test- Changes in the IMP degradation pattern in iced perch

Days	N	Subset for alpha = 0.05						
		a	b	c	d	e	f	g
18	3	.5219						
15	3	.7212	.7212					
12	3		1.0645					
9	3			2.8658				
7	3				3.9950			
5	3					5.5423		
3	3						6.4105	
1	3							6.9795
0	3							7.2311
Sig.		.303	.092	1.000	1.000	1.000	1.000	.201

Appendix 3.11.2.e: Duncan post hoc test- Changes in the HxR degradation pattern in iced perch

Days	N	Subset for alpha = 0.05				
		a	b	c	d	e
0	3	.0642				
1	3		.9186			
3	3		1.1459			
5	3			1.5173		
7	3			1.6981	1.6981	
9	3				1.9271	
12	3					2.1658
15	3					2.2496
18	3					2.2988
Sig.		1.000	.052	.109	.051	.243

Appendix 3.11.2.f: Duncan post hoc test- Changes in the Hx degradation pattern in iced perch

Days	N	Subset for alpha = 0.05						
		a	b	c	d	e	f	g
0	3	.0000						
1	3		.5444					
3	3			.9108				
5	3				1.1676			
7	3					1.5618		
9	3					1.5811		
12	3						2.0054	
15	3						2.2170	
18	3							2.7177
Sig.		1.000	1.000	1.000	1.000	.860	.078	1.000

Appendix 3.12: Changes in the ATP and related compound degradation pattern in iced snakehead muscle tissue

Appendix 3.12.1: Changes in the ATP and related compound degradation pattern in iced snakehead muscle tissue

	Sum of Squares	df	Mean Square	F	Sig.
ATP	11.219	8	1.402	1352.964	.000
ADP	17.541	8	2.193	33.564	.000
AMP	19.364	8	2.421	330.955	.000
IMP	53.073	8	6.634	355.122	.000
HxR	5.444	8	.680	97.448	.000
Hx	13.443	8	1.680	126.799	.000

Appendix 3.12.2: Duncan post hoc test- Changes in the ATP and related compound degradation pattern in iced snakehead

Appendix 3.12.2.a: Duncan post hoc test- Changes in the ATP degradation pattern in iced snakehead

Days	N	Subset for alpha = 0.05			
		a	b	c	d
18	3	.0024			
15	3	.0060			
9	3	.0109			
7	3	.0168			
12	3	.0250			
5	3	.0275			
3	3		.1670		
1	3			1.6341	
0	3				2.1793
Sig.		.486	1.000	1.000	1.000

Appendix 3.12.2.b: Duncan post hoc test- Changes in the ADP degradation pattern in iced snakehead

Days	N	Subset for alpha = 0.05				
		a	b	c	d	e
18	3	.0120				
15	3	.0963				
12	3	.5318	.5318			
9	3		.9289	.9289		
7	3			1.4263	1.4263	
5	3				1.6762	
3	3				2.0116	
0	3					2.7241
1	3					2.8074
Sig.		.083	.155	.083	.056	.752

Appendix 3.12.2.c: Duncan post hoc test- Changes in the AMP degradation pattern in iced snakehead

Days	N	Subset for alpha = 0.05						
		a	b	c	d	e	f	g
15	3	.0574						
18	3	.0629						
12	3		.3020					
9	3			.7579				
7	3				1.1069			
5	3					1.5630		
3	3						1.8836	
1	3							2.7982
0	3							2.9226
Sig.		.950	1.000	1.000	1.000	1.000	1.000	.180

Appendix 3.12.2.d: Duncan post hoc test- Changes in the IMP degradation pattern in iced snakehead

Days	N	Subset for alpha = 0.05							
		a	b	c	d	e	f	g	h
18	3	.6703							
15	3		1.0685						
12	3			1.7482					
9	3				3.1975				
7	3					3.7621			
5	3						4.2449		
0	3							4.9713	
3	3							5.0880	
1	3								5.5736
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	.415	1.000

Appendix 3.12.2.e: Duncan post hoc test- Changes in the HxR degradation pattern in iced snakehead

Days	N	Subset for alpha = 0.05					
		a	b	c	d	e	f
0	3	.0027					
1	3		.2496				
3	3		.3282				
5	3			.5550			
7	3				.8235		
9	3				.9890		
12	3					1.3582	
18	3					1.5336	1.5336
15	3						1.5875
Sig.		1.000	.372	1.000	.079	.065	.535

Appendix 3.12.2.f: Duncan post hoc test- Changes in the Hx degradation pattern in iced snakehead

Days	N	Subset for alpha = 0.05						
		a	b	c	d	e	f	g
0	3	.0027						
1	3		.9422					
3	3			1.2490				
5	3				1.6963			
7	3				1.8239			
9	3					2.1929		
12	3					2.4497	2.4497	
15	3						2.7067	2.7067
18	3							2.8260
Sig.		1.000	1.000	1.000	.296	.053	.053	.327

Appendix 3.13: Changes in the ATP and related compound degradation pattern in iced catfish muscle tissue

Appendix 3.13.1: Changes in the ATP and related compound degradation pattern in iced catfish muscle tissue

	Sum of Squares	df	Mean Square	F	Sig.
ATP	9.177	8	1.147	77.956	.000
ADP	10.988	8	1.373	78.832	.000
AMP	11.500	8	1.438	112.209	.000
IMP	49.370	8	6.171	92.444	.000
HxR	35.050	8	4.381	151.234	.000
Hx	19.049	8	2.381	168.549	.000

Appendix 3.13.2: Duncan post hoc test- Changes in the ATP and related compound degradation pattern in iced catfish

Appendix 3.13.2.a: Duncan post hoc test- Changes in the ATP degradation pattern in iced catfish

Days	N	Subset for alpha = 0.05		
		a	b	c
18	3	.0047		
15	3	.0056		
12	3	.0091		
9	3	.0239		
5	3	.0664		
7	3	.0768		
3	3	.1107		
1	3		1.0659	
0	3			2.1999
Sig.		.437	1.000	1.000

Appendix 3.13.2.b: Duncan post hoc test- Changes in the ADP degradation pattern in iced catfish

Days	N	Subset for alpha = 0.05			
		a	b	c	d
18	3	.0180			
15	3	.0526			
5	3	.2538	.2538		
7	3		.5032	.5032	
9	3		.5618	.5618	
3	3			.6342	
12	3			.7761	
1	3				2.0828
0	3				2.2856
Sig.		.121	.052	.085	.159

Appendix 3.13.2.c: Duncan post hoc test- Changes in the AMP degradation pattern in iced catfish

Days	N	Subset for alpha = 0.05		
		a	b	c
12	3	.1023		
18	3	.1312		
15	3	.1868		
5	3		.5350	
7	3		.5505	
3	3		.5523	
9	3		.7038	
1	3			2.1662
0	3			2.3398
Sig.		.493	.196	.159

Appendix 3.13.2.d: Duncan post hoc test- Changes in the IMP degradation pattern in iced catfish

Days	N	Subset for alpha = 0.05					
		a	b	c	d	e	f
18	3	1.0512					
15	3		1.8019				
12	3		1.8705				
9	3			2.7272			
7	3			3.3040			
5	3				4.2680		
3	3					4.9314	
1	3					5.3187	
0	3						6.0633
Sig.		1.000	.797	.052	1.000	.168	1.000

Appendix 3.13.2.e: Duncan post hoc test- Changes in the HxR degradation pattern in iced catfish

Days	N	Subset for alpha = 0.05						
		a	b	c	d	e	f	g
0	3	.1057						
1	3	.3936						
3	3		1.2055					
5	3			2.2292				
7	3			2.5560	2.5560			
9	3				2.6340			
12	3					3.0458		
15	3						3.7011	
18	3							4.5767
Sig.		.125	1.000	.087	.657	1.000	1.000	1.000

Appendix 3.13.2.f: Duncan post hoc test- Changes in the Hx degradation pattern in iced catfish

Days	N	Subset for alpha = 0.05					
		a	b	c	d	e	f
0	3	.0992					
1	3	.1768					
3	3	.3047					
5	3		.9177				
7	3		1.0861				
9	3			1.3966			
12	3				1.9025		
15	3					2.7638	
18	3						3.0673
Sig.		.132	.190	1.000	1.000	1.000	1.000

Appendix 3.14: ANOVA Results among the fishes- Changes in the ATP and related compound degradation pattern in iced fishes

	Sum of Squares	df	Mean Square	F	Sig.
ATP	2.443	2	1.222	4.170	.035
ADP	1.26	2	.513	3.134	.045
AMP	1.438	2	.719	5.359	.017
IMP	1.415	2	.707	5.847	.019
HxR	9.322	2	4.661	17.134	.000
Hx	1.093	2	.547	8.193	.004

Appendix 3.15: Change in K value and H values in three fish species stored at ambient temperature

Appendix 3.15.1: ANOVA results-Change in K value and H values in three fish species stored at ambient temperature

		Sum of Squares	df	Mean Square	F	Sig.
Climbing perch	K Value	25334.498	12	2111.208	1674.790	.000
	H value	6964.712	12	580.393	1467.836	.000
Banded snakehead	K Value	35873.163	12	2989.430	4025.388	.000
	H value	11541.435	12	961.786	2740.278	.000
Stinging catfish	K Value	39745.821	12	3312.152	2872.712	.000
	H value	7554.220	12	629.518	1130.303	.000
Among fish	K Value	2245.342	2	1122.671	28.327	.000
	H value	642.523	2	321.261	18.747	.000

Appendix 3.15.2: Duncan post hoc test-Change in K value and H values in three fish species stored at ambient temperature

Appendix 3.15.2.1: Duncan post hoc test -Change in K value in three fish species stored at ambient temperature

Appendix 3.15.2.1.a: Duncan post hoc test -K value of Perch at ambient temperature

Hours	N	Subset for alpha = 0.01										
		a	b	c	d	e	f	g	h	i	j	k
0	3	.38										
1	3	1.54										
2	3	2.66										
3	3		5.24									
4	3			10.03								
5	3				12.79							
6	3					21.41						
7	3						27.81					
8	3							33.97				
10	3								46.51			
12	3									55.37		
15	3										60.89	
18	3											82.92
Sig.		.026	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Appendix 3.15.2.1.b: Duncan post hoc test -K value of Snakehead at ambient temperature

Hours	N	Subset for alpha = 0.01							
		a	b	c	d	e	f	g	h
0	3	.0417							
1	3	.0442							
2	3	.19							
3	3	.71							
4	3	.94							
5	3	2.12							
6	3		8.46						
7	3			23.92					
8	3				35.66				
10	3					47.59			
12	3						62.79		
15	3							74.50	
18	3								85.42
Sig.		.013	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Appendix 3.15.2.1.c: Duncan post hoc test -K value of Catfish at ambient temperature

Hours		Subset for alpha = 0.01											
	N	a	b	c	d	e	f	g	h	i	j	k	l
0	3	1.56											
1	3		5.04										
2	3			9.58									
3	3			11.26									
4	3				20.21								
5	3					29.32							
6	3						40.22						
7	3							46.82					
8	3								61.74				
10	3									70.87			
12	3										83.05		
15	3											88.52	
18	3												92.33
Sig.		1.000	1.000	.066	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Appendix 3.15.2.2: Duncan post hoc test -H value of three fishes stored at ambient temperature

Appendix 3.15.2.2.a: Duncan post hoc test -H value of Perch at ambient temperature

Hours	N	Subset for alpha = 0.01							
		a	b	c	d	e	f	g	h
0	3	.00							
1	3	.00							
2	3	.00							
3	3	.00							
4	3	.00							
5	3	.42							
6	3		7.40						
7	3			10.59					
8	3				13.00				
10	3					21.79			
12	3						26.16		
15	3							28.59	
18	3								41.57
Sig.		.479	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Appendix 3.15.2.2.b: Duncan post hoc test -H value of Snakehead at ambient temperature

Hours	N	Subset for alpha = 0.01							
		a	b	c	d	e	f	g	h
1	3	.01							
0	3	.02							
2	3	.08							
3	3	.34							
4	3	.42							
5	3	.77							
6	3		2.51						
7	3			12.09					
8	3				17.24				
10	3					23.34			
12	3						35.71		
15	3							44.05	
18	3								47.31
Sig.		.178	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Appendix 3.15.2.2.c: Duncan post hoc test -H value of Catfish at ambient temperature

Hours	N	Subset for alpha = 0.01								
		a	b	c	d	e	f	g	h	i
0	3	.75								
1	3		3.67							
2	3			7.61						
3	3			9.04						
4	3				11.05					
5	3					16.53				
6	3					18.06				
7	3						24.52			
8	3							28.92		
10	3								32.54	
12	3									39.80
15	3									40.65
18	3									41.25
Sig.		1.000	1.000	.027	1.000	.018	1.000	1.000	1.000	.032

Appendix 3.16: Change in K value and H values in three fish species stored at iced condition

Appendix 3.16.1: ANOVA results-Change in K value and H values in three fish species stored at iced condition

		Sum of Squares	df	Mean Square	F	Sig.
Climbing perch	K Value	19084.676	8	2385.585	1411.847	.000
	H value	5313.036	8	664.129	788.765	.000
Banded snakehead	K Value	22577.944	8	2822.243	538.062	.000
	H value	8775.808	8	1096.976	391.021	.000
Stinging catfish	K Value	21622.355	8	2702.794	1526.756	.000
	H value	3936.889	8	492.111	833.674	.000
Among fish	K Value	306.811	2	153.406	5.955	.041
	H value	453.581	2	226.791	17.552	.000

Appendix 3.16.2: Duncan post hoc test-Change in K value and H values in three fish species stored at iced condition

Appendix 3.16.2.1: Duncan post hoc test -Change in K value in three fish species stored at iced condition

Appendix 3.16.2.1.a: Duncan post hoc test -Change in K value in iced perch

Days	N	Subset for alpha = 0.05								
		a	b	c	d	e	f	g	h	i
0	3	.38								
1	3		10.41							
3	3			16.68						
5	3				25.83					
7	3					39.03				
9	3						47.57			
12	3							65.03		
15	3								71.65	
18	3									78.57
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Appendix 3.16.2.1.b: Duncan post hoc test -Change in K value in iced snakehead

Days	N	Subset for alpha = 0.05								
		a	b	c	d	e	f	g	h	i
0	3	.0414								
1	3		8.5172							
3	3			14.7048						
5	3				23.0594					
7	3					29.5429				
9	3						39.4326			
12	3							61.4717		
15	3								77.7694	
18	3									85.3750
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Appendix 3.16.2.1.c: Duncan post hoc test -Change in K value in iced catfish

Days	N	Subset for alpha = 0.05								
		a	b	c	d	e	f	g	h	i
0	3	1.55								
1	3		5.09							
3	3			19.48						
5	3				38.03					
7	3					45.09				
9	3						50.23			
12	3							64.16		
15	3								75.99	
18	3									86.37
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Appendix 3.16.2.2: Duncan post hoc test -Change in H value in three fish species stored at iced condition

Appendix 3.16.2.2.a: Duncan post hoc test -Change in H value in iced perch

Days	N	Subset for alpha = 0.05								
		a	b	c	d	e	f	g	h	i
0	3	.0000								
1	3		3.8742							
3	3			7.3850						
5	3				11.2360					
7	3					18.7025				
9	3						21.4472			
12	3							31.2656		
15	3								35.5570	
18	3									42.5690
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Appendix 3.16.2.2.b: Duncan post hoc test -Change in H value in iced snakehead

Days	N	Subset for alpha = 0.05								
		a	b	c	d	e	f	g	h	i
0	3	.02								
1	3		6.73							
3	3			11.64						
5	3				17.37					
7	3					20.35				
9	3						27.21			
12	3							39.42		
15	3								49.01	
18	3									55.34
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Appendix 3.16.2.2.c: Duncan post hoc test -Change in H value in iced catfish

Days	N	Subset for alpha = 0.05							
		a	b	c	d	e	f	g	h
0	3	.7517							
1	3	1.5795							
3	3		3.9403						
5	3			11.0836					
7	3				13.4467				
9	3					17.4288			
12	3						24.6584		
15	3							32.4994	
18	3								34.6602
Sig.		.203	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Appendix 4.1: ANOVA Results among the fishes- Changes in protein fractions of three freshwater fishes stored at ambient temperature

	Sum of Squares	df	Mean Square	F	Sig.
SP	432.867	2	216.434	335.310	.000
MP	253.172	2	126.586	9.944	.001
ASP	1136.171	2	568.085	45.159	.000
TC	.769	2	.384	238.091	.000
ASC	.075	2	.002	2.891	.04
PSC	.383	2	.191	251.457	.000
ISC	.099	2	.050	9.633	.001

Appendix 4.2: Changes in protein fractions in Climbing perch fish muscle stored at ambient temperature

Appendix 4.2.1: ANOVA results- Changes in protein fractions in Climbing perch fish muscle stored at ambient temperature

	Sum of Squares	df	Mean Square	F	Sig.
SP	235.150	12	19.596	77.289	.000
MP	168.717	12	14.060	71.783	.000
ASP	781.162	12	65.097	240.363	.000
TC	1.777	12	.148	327.286	.000
ASC	.169	12	.014	1.845	.093
PSC	.157	12	.013	.116	1.000
ISC	.285	12	.024	.380	.959

Appendix 4.2.2: Duncan post hoc test- Changes in protein fractions in Climbing perch fish muscle stored at ambient temperature

Appendix 4.2.2.a: Duncan post hoc test- Changes in sarcoplasmic protein fractions in Climbing perch fish muscle stored at ambient temperature

hrs	N	Subset for alpha = 0.01			
		a	b	c	d
18	3	15.233933			
12	3	15.282650			
15	3	15.573173			
10	3	15.952910			
8	3	16.424360			
7	3		17.815687		
6	3		18.858220		
3	3			20.022223	
5	3			20.052790	
4	3			20.403923	
2	3			20.628943	
0	3				21.901903
1	3				22.098910
Sig.		.014	.018	.189	.636

Appendix 4.2.2.b: Duncan post hoc test- Changes in myofibrillar protein fractions in Climbing perch fish muscle stored at ambient temperature

hrs	N	Subset for alpha = 0.01			
		a	b	c	d
18	3	35.244157			
15	3		38.429150		
12	3			39.617710	
10	3			40.496087	
8	3				41.645150
6	3				41.702587
7	3				41.830580
1	3				42.261740
3	3				42.345780
5	3				42.420803
2	3				42.531643
4	3				42.550590
0	3				42.612483
Sig.		1.000	1.000	.022	.028

Appendix 4.2.2.c: Duncan post hoc test- Changes in alkali soluble protein in Climbing perch fish muscle stored at ambient temperature

Hrs	N	Subset for alpha = 0.01						
		a	b	c	d	e	f	g
0	3	34.132890						
1	3	34.250790						
2	3		35.500393					
4	3		35.758670					
3	3		36.285600					
5	3		36.289610					
6	3			38.207873				
7	3			39.204440				
8	3				40.861723			
10	3					42.628760		
12	3						44.189997	
15	3						45.109790	
18	3							48.823053
Sig.		.784	.100	.027	1.000	1.000	.040	1.000

Appendix 4.2.2.d: Duncan post hoc test- Changes in total collagen in Climbing perch fish muscle stored at ambient temperature

hrs	N	Subset for alpha = 0.01						
		a	b	c	d	e	f	g
8	3	.698858						
15	3		.887885					
12	3		.909643					
10	3		.922241					
8	3			1.068766				
7	3				1.149287			
6	3					1.231318		
5	3					1.236804		
4	3						1.286814	
2	3							1.339019
3	3							1.346394
0	3							1.352720
1	3							1.388558
Sig.		1.000	.071	1.000	1.000	.755	1.000	.013

Appendix 4.3: Changes in protein fractions in snakehead muscle stored at ambient temperature

Appendix 4.3.1: ANOVA results- Changes in protein fractions in banded snakehead muscle tissue stored at ambient temperature

	Sum of Squares	df	Mean Square	F	Sig.
SP	71.382	12	5.949	712.587	.000
MP	959.601	12	79.967	716.623	.000
ASP	1750.894	12	145.908	2058.730	.000
TC	1.770	12	.148	716.983	.000
ASC	.093	12	.008	1584.982	.000
PSC	.314	12	.026	196.924	.000
ISC	.233	12	.019	421.254	.000

Appendix 4.3.2: Duncan post hoc test- Changes in protein fractions in snakehead muscle tissue stored at ambient temperature

Appendix4.3.2.a: Duncan post hoc test- Changes in sarcoplasmic protein fractions in snakehead muscle tissue stored at ambient temperature

hrs	N	Subset for alpha = 0.01						
		a	b	c	d	e	f	g
18	3	8.38						
15	3		9.50					
12	3			9.85				
10	3				10.60			
8	3					11.70		
6	3					11.73		
7	3					11.84		
5	3						12.41	
0	3						12.42	
4	3						12.43	
1	3						12.53	12.53
3	3						12.61	12.61
2	3							12.74
Sig.		1.000	1.000	1.000	1.000	.082	.020	.010

Appendix 4.3.2.b: Duncan post hoc test- Changes in myofibrillar protein fractions in snakehead muscle tissue stored at ambient temperature

hrs	N	Subset for alpha = 0.01									
		a	b	c	d	e	f	g	h	i	J
18	3	27.65									
15	3		30.12								
12	3			33.64							
10	3				35.09						
8	3					37.15					
7	3					37.72					
6	3						39.13				
5	3						39.43				
4	3							40.21			
3	3								41.42		
2	3									42.31	
1	3										44.42
0	3										44.55
Sig.		1.000	1.000	1.000	1.000	.048	.282	1.000	1.000	1.000	.621

Appendix 4.3.2.c: Duncan post hoc test- Changes in alkali soluble protein in snakehead muscle tissue stored at ambient temperature

hrs	N	Subset for alpha = 0.01									
		a	b	c	d	e	f	g	h	i	j
1	3	41.37									
0	3	41.51									
2	3		42.98								
3	3			43.92							
4	3			44.43							
6	3				45.49						
5	3				45.59						
7	3					46.92					
8	3						49.23				
10	3							53.47			
12	3								55.34		
15	3									59.53	
18	3										62.74
Sig.		.541	1.000	.028	.638	1.000	1.000	1.000	1.000	1.000	1.000

Appendix 4.3.2.d: Duncan post hoc test- Changes in total collagen in snakehead muscle tissue stored at ambient temperature

hrs	N	Subset for alpha = 0.01										
		a	b	c	d	e	f	g	h	i	j	k
18	3	.57										
15	3		.63									
12	3			.66								
10	3				.71							
8	3					.80						
7	3						.93					
6	3							1.02				
5	3							1.03	1.04			
4	3								1.06	1.06		
3	3									1.08		
2	3										1.157	
1	3										1.17	1.17
0	3											1.20
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	.329	.029	.188	.222	.023

Appendix 4.3.2.e: Duncan post hoc test- Acid soluble collagen content in snakehead muscle tissue stored at ambient temperature

hrs	N	Subset for alpha = 0.01								
		a	b	c	d	e	f	g	h	i
18	3	.08								
15	3	.08								
12	3		.09							
10	3			.11						
8	3				.15					
7	3					.169				
6	3					.17	.17			
5	3						.17			
4	3							.186		
3	3								.21	
2	3									.21
0	3									.21
1	3									.21
Sig.		.235	1.000	1.000	1.000	.018	.064	1.000	1.000	.068

Appendix 4.3.2.f: Duncan post hoc test- Pepsin soluble collagen in snakehead muscle tissue stored at ambient temperature

hrs	N	Subset for alpha = 0.01						
		a	b	c	d	e	g	H
18	3	.4506						
12	3		.4833					
15	3		.485					
10	3		.507					
8	3			.5342				
7	3				.6015			
3	3					.6565		
6	3					.6634		
5	3					.6634		
4	3					.6700	.670	
1	3					.6767	.676	
0	3						.696	.696
2	3							.707
Sig.		1.000	.024	1.000	1.000	.064	.012	.266

Appendix 4.3.2.g: Duncan post hoc test- Insoluble collagen in snakehead muscle tissue stored at ambient temperature

hrs	N	Subset for alpha = 0.01									
		a	b	c	d	e	f	g	h	i	j
18	3	.04									
15	3		.06								
12	3			.08							
10	3			.09							
8	3				.12						
7	3					.16					
6	3						.16				
5	3						.19	.19			
4	3							.21	.21		
3	3								.22		
2	3									.24	
1	3										.28
0	3										.29
Sig.		1.000	1.000	.222	1.000	1.000	.156	.060	.100	1.000	.085

Appendix 4.4: Changes in protein fractions in catfish muscle stored at ambient temperature

Appendix 4.4.1: ANOVA results- Changes in protein fractions in catfish muscle tissue stored at ambient temperature

	Sum of Squares	df	Mean Square	F	Sig.
SP	151.061	12	12.588	638.562	.000
MP	2891.322	12	240.943	850.210	.000
ASP	4722.867	12	393.572	2158.287	.000
TC	2.586	12	.215	815.063	.000
ASC	.263	12	.022	155.546	.000
PSC	.448	12	.037	201.940	.000
ISC	.233	12	.019	182.927	.000

Appendix 4.4.2: Duncan post hoc test- Changes in protein fractions in catfish muscle tissue stored at ambient temperature

Appendix 4.4.2.a: Duncan post hoc test- Changes in sarcoplasmic protein in catfish muscle tissue stored at ambient temperature

hrs	N	Subset for alpha = 0.01								
		a	b	c	d	e	f	g	h	i
18	3	7.62								
15	3		8.47							
12	3			9.65						
10	3			9.83						
8	3				10.32					
7	3					11.31				
6	3						11.92			
5	3						12.11	12.11		
4	3							12.37		
2	3								13.35	
1	3								13.57	13.57
3	3									13.70
0	3									13.71
Sig.		1.000	1.000	.123	1.000	1.000	.105	.033	.062	.267

Appendix 4.4.2.b: Duncan post hoc test- Changes in myofibrillar protein in catfish muscle tissue stored at ambient temperature

hrs	N	Subset for alpha = 0.01									
		a	b	c	d	e	f	g	h	i	j
18	3	18.03									
15	3		21.14								
12	3			25.51							
10	3				28.07						
8	3					32.68					
7	3						35.91				
6	3							37.43			
5	3								39.24		
4	3								39.78		
2	3									42.41	
3	3									42.68	
1	3										44.06
0	3										44.87
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000	.222	.544	.076

Appendix 4.4.2.c: Duncan post hoc test- Changes in alkali soluble protein in catfish muscle tissue stored at ambient temperature

hrs	N	Subset for alpha = 0.01										
		a	b	c	d	e	f	g	h	i	j	k
0	3	39.64										
1	3	40.42										
3	3		41.99									
2	3		42.01									
4	3			44.27								
5	3				46.59							
6	3					48.65						
7	3						51.41					
8	3							55.79				
10	3								60.89			
12	3									63.74		
15	3										69.52	
18	3											73.20
Sig.		.035	.977	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Appendix 4.4.2.d: Duncan post hoc test- Changes in total collagen in catfish muscle tissue stored at ambient temperature

hrs	N	Subset for alpha = 0.01									
		a	b	c	d	e	f	g	h	i	j
18	3	.34									
15	3		.42								
12	3			.54							
10	3				.59						
8	3					.65					
7	3						.79				
6	3							.83			
5	3								.87		
4	3									.97	
3	3									.99	
1	3										1.11
0	3										1.11
2	3										1.12
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.115	.771

Appendix 4.4.2.e: Duncan post hoc test- Changes in acid soluble collagen in catfish muscle tissue stored at ambient temperature

hrs	N	Subset for alpha = 0.01				
		a	b	c	d	e
18	3	.025774				
15	3		.056600			
12	3		.066585			
10	3		.074580			
8	3		.077042			
6	3			.113384		
7	3			.120068		
5	3			.133632		
4	3				.172425	
3	3				.196908	
1	3					.256297
2	3					.269090
0	3					.275187
Sig.		1.000	.063	.057	.018	.075

Appendix 4.4.2.f: Duncan post hoc test- Changes in pepsin soluble collagen in catfish muscle tissue stored at ambient temperature

hrs	N	Subset for alpha = 0.01							
		a	b	c	d	e	f	g	h
8	3	.245090							
15	3		.288229						
12	3			.382486					
10	3			.410006					
8	3				.448435				
7	3					.509057			
0	3					.526460	.526460		
6	3					.532138	.532138	.532138	
5	3					.540212	.540212	.540212	
1	3						.558970	.558970	.558970
4	3							.560665	.560665
2	3								.577874
3	3								.584245
Sig.		1.000	1.000	.020	1.000	.015	.011	.025	.045

Appendix 4.4.2.g: Duncan post hoc test- Changes in insoluble collagen in catfish muscle tissue stored at ambient temperature

hrs	N	Subset for alpha = 0.01								
		a	b	c	d	e	f	g	h	i
8	3	.132165								
7	3		.169360							
6	3		.191367	.191367						
5	3			.206150						
3	3			.214340						
4	3				.240725					
18	3				.245090					
2	3					.270910				
15	3					.288229	.288229			
1	3						.298454	.298454		
0	3							.312765		
12	3								.382486	
10	3									.410006
Sig.		1.000	.015	.015	.608	.049	.235	.100	1.000	1.000

Appendix 4.5: ANOVA Results among the fishes- Changes in protein fractions of three freshwater fishes stored in ice (p=.05)

	Sum of Squares	df	Mean Square	F	Sig.
SP	568.556	2	284.278	806.122	.000
MP	70.317	2	35.159	14.952	.000
ASP	320.876	2	160.438	82.745	.000
TC	.752	2	.376	101.097	.000
ASC	.027	2	.014	49.570	.000
PSC	.517	2	.259	126.919	.000
ISC	.140	2	.070	145.824	.000

Appendix 4.6: Changes in protein fractions in ice stored climbing perch muscle

Appendix 4.6.1: ANOVA results- Changes in protein fractions in ice stored Climbing perch

	Sum of Squares	df	Mean Square	F	Sig.
SP	81.188	8	10.149	27.689	.000
MP	113.384	8	14.173	52.425	.000
ASP	318.604	8	39.826	85.255	.000
TC	1.288	8	.161	149.861	.000
ASC	.114	8	.014	252.209	.000
PSC	.346	8	.043	78.829	.000
ISC	.062	8	.008	26.973	.000

Appendix 4.6.2: Duncan post hoc test- Changes in protein fractions in ice stored perch

Appendix 4.6.2.a: Duncan post hoc test- Changes in sarcoplasmic protein in ice stored perch

Days	N	Subset for alpha = 0.01		
		a	b	c
18	3	18.302333		
15	3		20.277770	
12	3		21.204690	
0	3			22.897917
7	3			23.139063
1	3			23.173280
9	3			23.425957
3	3			23.459103
5	3			23.540440
Sig.		1.000	.077	.262

Appendix 4.6.2.b: Duncan post hoc test- Changes in myofibrillar protein in ice stored perch

Days	N	Subset for alpha = 0.01					
		a	b	c	d	e	f
18	3	34.337277					
5	3		36.839690				
9	3		37.598780	37.598780			
7	3		37.744547	37.744547	37.744547		
3	3			38.216790	38.216790	38.216790	
15	3				38.931400	38.931400	
12	3					39.284450	
1	3						40.961343
0	3						41.582010
Sig.		1.000	.057	.184	.015	.027	.161

Appendix 4.6.2.c: Duncan post hoc test- Changes in alkali soluble protein in ice stored perch

Days	N	Subset for alpha = 0.01			
		a	b	c	d
0	3	34.235290			
1	3	34.593970			
3	3		37.073730		
9	3		37.920857		
7	3		37.976720		
5	3		38.439700	38.439700	
12	3		38.588007	38.588007	
15	3			39.970010	
18	3				46.748740
Sig.		.528	.024	.017	1.000

Appendix 4.6.2.d: Duncan post hoc test- Changes in total collagen in ice stored perch

Days	N	Subset for alpha = 0.01						
		a	b	c	d	e	f	g
18	3	.611649						
15	3		.820819					
12	3			.922852				
9	3				1.054406			
7	3					1.139671		
5	3					1.180170	1.180170	
3	3						1.250381	1.250381
1	3							1.271407
0	3							1.284784
Sig.		1.000	1.000	1.000	1.000	.148	.017	.239

Appendix 4.6.2.e: Duncan post hoc test- Changes in acid soluble collagen in ice stored perch

Days	N	Subset for alpha = 0.01					
		a	b	c	d	e	f
18	3	.019538					
15	3	.029772	.029772				
12	3		.043932				
9	3			.078609			
7	3			.094811			
5	3				.129851		
3	3					.162784	
1	3						.191687
0	3						.200365
Sig.		.113	.033	.017	1.000	1.000	.175

Appendix 4.6.2.f: Duncan post hoc test- Changes in pepsin soluble collagen in ice stored perch

Days	N	Subset for alpha = 0.01				
		a	b	c	d	e
18	3	.391084				
15	3		.539167			
12	3			.603095		
9	3				.677230	
5	3				.706920	.706920
7	3				.722871	.722871
1	3				.732751	.732751
0	3					.740371
3	3					.748017
Sig.		1.000	1.000	1.000	.015	.067

Appendix 4.6.2.g: Duncan post hoc test- Changes in insoluble collagen in ice stored perch

Days	N	Subset for alpha = 0.01				
		a	b	c	d	e
18	3	.201027				
15	3		.251880			
12	3		.275825	.275825		
9	3			.298567	.298567	
7	3				.321988	.321988
3	3				.339580	.339580
5	3					.343400
0	3					.344048
1	3					.346969
Sig.		1.000	.101	.118	.011	.121

Appendix 4.7: Changes in protein fractions in ice stored banded snakehead muscle

Appendix 4.7.1: ANOVA results- Changes in protein fractions in ice stored snakehead

	Sum of Squares	df	Mean Square	F	Sig.
SP	18.051	8	2.256	23.234	.000
MP	328.073	8	41.009	206.451	.000
ASP	479.490	8	59.936	474.533	.000
TC	.538	8	.067	280.485	.000
ASC	.040	8	.005	433.968	.000
PSC	.058	8	.007	47.384	.000
ISC	.091	8	.011	178.644	.000

Appendix 4.7.2: Duncan post hoc test- Changes in protein fractions in ice-stored snakehead

Appendix 4.7.2.a: Duncan post hoc test- Changes in sarcoplasmic protein fractions in ice stored snakehead

Days	N	Subset for alpha = 0.01		
		a	b	c
18	3	11.070458		
15	3		12.094653	
12	3			13.123967
0	3			13.150586
1	3			13.193132
9	3			13.205585
5	3			13.508931
7	3			13.586832
3	3			13.870349
Sig.		1.000	1.000	.018

Appendix 4.7.2. b: Duncan post hoc test- Changes in myofibrillar protein in ice stored snakehead

Days	N	Subset for alpha = 0.01				
			b	c	d	e
18	3	34.045938				
15	3		37.075248			
9	3		37.260318			
12	3		37.959766			
7	3			39.130867		
5	3				41.644143	
3	3				42.256076	
1	3					44.399176
0	3					45.091805
Sig.		1.000	.032	1.000	.110	.073

Appendix 4.7.2.c: Duncan post hoc test- Changes in alkali soluble protein fractions in ice stored snakehead

Days	N	Subset for alpha = 0.01						
		a	b	c	d	e	f	g
0	3	40.635295						
1	3	41.340336						
3	3		42.817765					
5	3			43.797174				
7	3				46.292770			
12	3					48.079565		
9	3					48.595195		
15	3						50.073728	
18	3							54.184387
Sig.		.026	1.000	1.000	1.000	.092	1.000	1.000

Appendix 4.7.2.d: Duncan post hoc test- Changes in total collagen in ice stored snakehead

Days	N	Subset for alpha = 0.01						
		a	b	c	d	e	f	g
18	3	.699217						
15	3		.756371					
12	3			.836702				
9	3				.938902			
7	3					.989531		
5	3						1.049752	
3	3						1.055810	
1	3						1.067355	
0	3							1.122314
Sig.		1.000	1.000	1.000	1.000	1.000	.204	1.000

Appendix 4.7.2.e: Duncan post hoc test- Changes in acid soluble collagen in ice stored snakehead

Days	N	Subset for alpha = 0.01								
		a	b	c	d	e	f	g	h	i
18	3	.079966								
15	3		.098813							
12	3			.117889						
9	3				.133120					
7	3					.143014				
5	3						.163239			
3	3							.172480		
1	3								.187175	
0	3									.203215
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Appendix 4.7.2.f: Duncan post hoc test- Changes in pepsin soluble collagen in ice stored snakehead

Days	N	Subset for alpha = 0.01			
		a	b	c	d
18	3	.543714			
15	3	.563603	.563603		
12	3		.582894		
9	3			.637690	
1	3			.647181	.647181
7	3			.654818	.654818
3	3			.660614	.660614
5	3			.669009	.669009
0	3				.675912
Sig.		.065	.073	.011	.019

Appendix 4.7.2.g: Duncan post hoc test- Changes in insoluble collagen in ice stored snakehead

Days	N	Subset for alpha = 0.01					
		a	b	c	d	e	f
18	3	.075537					
15	3	.093955					
12	3		.135919				
9	3			.168093			
7	3				.191700		
5	3					.217504	
3	3					.222716	
1	3					.232999	.232999
0	3						.243187
Sig.		.011	1.000	1.000	1.000	.036	.136

Appendix 4.8: Changes in protein fractions in ice stored stinging catfish muscle

Appendix 4.8.1: ANOVA results- Changes in protein fractions in ice stored catfish

	Sum of Squares	df	Mean Square	F	Sig.
SP	67.057	8	8.382	83.273	.000
MP	243.287	8	30.411	216.157	.000
ASP	587.543	8	73.443	280.322	.000
TC	1.262	8	.158	380.230	.000
ASC	.066	8	.008	516.135	.000
PSC	.307	8	.038	148.409	.000
ISC	.122	8	.015	199.659	.000

Appendix 4.8.2: Duncan post hoc test- Changes in sarcoplasmic protein in ice stored catfish

Appendix 4.8.2.a: Duncan post hoc test- Changes in protein fractions in ice stored catfish

Days	N	Subset for alpha = 0.01			
		a	b	c	d
18	3	8.504364			
15	3		10.219168		
12	3			10.983140	
9	3				12.617371
0	3				12.874402
3	3				12.932822
5	3				13.037394
7	3				13.092220
1	3				13.290276
Sig.		1.000	1.000	1.000	.032

Appendix 4.8.2.b: Duncan post hoc test- Changes in myofibrillar protein in ice stored catfish

Days	N	Subset for alpha = 0.01				
		a	b	c	d	e
18	3	37.077734				
15	3	37.873128				
12	3		41.470425			
9	3		42.292412			
5	3		42.370024			
7	3			43.580785		
3	3			43.883571		
1	3				45.535312	
0	3					46.649491
Sig.		.018	.011	.336	1.000	1.000

Appendix 4.8.2.c: Duncan post hoc test- Changes in alkali soluble protein in ice stored catfish

Days	N	Subset for alpha = 0.01						
		a	b	c	d	e	f	g
0	3	39.516545						
1	3	40.239635						
3	3		42.343211					
7	3		42.656114	42.656114				
5	3			43.835895	43.835895			
9	3				44.482919			
12	3					47.090046		
15	3						51.537036	
18	3							54.050150
Sig.		.101	.464	.011	.139	1.000	1.000	1.000

Appendix 4.8.2.d: Duncan post hoc test- Changes in total collagen in ice stored catfish

Days	N	Subset for alpha = 0.01						
		1a	b	c	d	e	f	g
18	3	.367752						
15	3	.370668						
12	3		.456388					
9	3			.607297				
7	3				.670881			
5	3					.756687		
3	3						.840397	
1	3							.934776
0	3							.959562
Sig.		.863	1.000	1.000	1.000	1.000	1.000	.153

Appendix 4.8.2.e: Duncan post hoc test- Changes in acid soluble collagen in ice stored catfish

Days	N	Subset for alpha = 0.01						
		a	b	c	d	e	f	g
15	3	.121492						
18	3	.126713						
12	3		.148865					
9	3			.161909				
7	3				.176409			
5	3					.188877		
3	3						.204198	
1	3							.262579
0	3							.263748
Sig.		.127	1.000	1.000	1.000	1.000	1.000	.724

Appendix4.8.2.f: Duncan post hoc test- Changes in pepsin soluble collagen in ice stored catfish

Days	N	Subset for alpha = 0.01			
		a	b	c	d
18	3	.179968			
15	3	.190975			
12	3		.239165		
9	3			.354687	
7	3			.377413	
5	3				.418758
1	3				.443972
0	3				.454072
3	3				.454499
Sig.		.413	1.000	.101	.021

Appendix4.8.2.g: Duncan post hoc test- Changes in insoluble collagen in ice stored catfish

Days	N	Subset for alpha = 0.01					
		1a	b	c	d	e	f
15	3	.058201					
18	3	.061071					
12	3	.068358					
9	3		.090702				
7	3			.117059			
5	3				.149052		
3	3					.181699	
1	3						.228225
0	3						.241742
Sig.		.193	1.000	1.000	1.000	1.000	.074

Appendix 4.9: ATPase activity in three fish species stored at ambient temperature

Appendix 4.9.1: ANOVA results- ATPase activity in three fishes stored at ambient temperature

Source	Sum of Squares	df	Mean Square	F	Sig.
Climbing perch	.570	12	.048	233.147	.000
Banded Snakehead	1.134	12	.095	402.128	.000
Stinging catfish	.911	12	.076	354.840	.000
Among fish	.213	2	.107	98.321	.000

Appendix 4.9.2: Duncan post-hoc test- ATPase activity in three fishes stored at ambient temperature

Appendix 4.9.2.a: Duncan post-hoc test- ATPase activity in perch stored at ambient temperature

days	N	Subset for alpha = 0.01						
		a	b	c	d	e	f	g
15	3	.139267						
18	3	.143333						
12	3	.152333						
10	3		.228667					
8	3			.284333				
7	3				.338000			
6	3					.376667		
5	3					.398333	.398333	
4	3						.419000	
3	3						.421000	
1	3							.454000
2	3							.460000
0	3							.470000
Sig.		.300	1.000	1.000	1.000	.074	.076	.206

Appendix 4.9.2.b: Duncan post-hoc test- ATPase activity in snakehead stored at ambient temperature

days	N	Subset for alpha = 0.01							
		a	b	c	d	e	f	f	h
15	3	.226333							
18	3	.236000							
12	3	.242667							
10	3		.366000						
8	3			.410833					
7	3				.479667				
6	3					.539733			
5	3						.577667		
4	3							.624000	
3	3								.665000
2	3								.667667
1	3								.674667
0	3								.681667
Sig.		.229	1.000	1.000	1.000	1.000	1.000	1.000	.235

Appendix 4.9.2.c: Duncan post-hoc test- ATPase activity in catfish stored at ambient temperature

days	N	Subset for alpha = 0.01							
		a	b	c	d	f	g	h	i
15	3	.140310							
18	3	.140333							
12	3	.152200	.152200						
10	3		.176800						
8	3			.212333					
7	3				.294240				
6	3					.376667			
5	3						.412933		
4	3							.474143	
3	3							.494963	.494963
2	3								.514000
1	3								.519000
0	3								.521000
Sig.		.357	.050	1.000	1.000	1.000	1.000	.093	.055

Appendix 4.10: ATPase activity in three fishes stored at iced condition

Appendix 4.10.1: ANOVA results- ATPase activity in three fishes stored at iced condition

Source	Sum of Squares	df	Mean Square	F	Sig.
Climbing perch	.412	8	.052	502.872	.000
Banded Snakehead	.604	8	.076	548.479	.000
Stinging catfish	.626	8	.078	549.572	.000
Among fish	.239	2	.119	105.059	.000

Appendix 4.10.2: Duncan post-hoc test- ATPase activity in three fishes stored at iced condition

Appendix 4.10.2.a: Duncan post-hoc test- ATPase activity in iced perch

days	N	Subset for alpha = 0.01						
		a	b	c	d	e	f	g
18	3	.100324						
15	3		.131000					
12	3			.184667				
9	3				.210000			
7	3					.247333		
5	3						.290500	
3	3							.417333
1	3							.434333
0	3							.439967
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	.017

Appendix 4.10.2.b: Duncan post-hoc test- ATPase activity in iced snakehead

days	N	Subset for alpha = 0.01					
		a	b	c	d	e	f
18	3	.294433					
15	3	.316167	.316167				
12	3		.336333	.336333			
9	3			.354300			
7	3				.462667		
5	3					.541667	
3	3						.650233
1	3						.667667
0	3						.668400
Sig.		.036	.050	.077	1.000	1.000	.088

Appendix 4.10.2.c: Duncan post-hoc test- ATPase activity in iced catfish

days	N	Subset for alpha = 0.01				
		1a	b	c	d	e
12	3	.125650				
15	3	.143950				
18	3	.147567				
7	3		.197238			
9	3		.205300			
5	3			.245983		
3	3				.470667	
1	3				.498500	.498500
0	3					.505833
Sig.		.046	.419	1.000	.010	.461

Appendix 5.1: Sensory evaluation of three freshwater fishes at ambient temperature

Appendix 5.1.1: ANOVA result for sensory evaluation of Climbing perch, Banded snakehead and Stinging catfish at ambient temperature

Source	Sum of Squares	df	Mean Square	F	Sig.
Climbing perch	4808.308	12	400.692	459.618	.000
Banded Snakehead	6196.308	12	516.359	516.359	.000
Stinging catfish	6351.436	12	529.286	897.486	.000
Among fish	144.313	2	72.157	19.042	.000

Appendix 5.1.2 : Duncan Post hoc test- SDS of three fishes stored at ambient temperature

Appendix 5.1.2.a: Duncan Post hoc test- SDS of Climbing perch at ambient temperature

Hours	N	Subset for alpha = 0.01								
		a	b	c	d	e	f	g	h	i
0	3	.00								
1	3	.00								
2	3	.00								
3	3	.00								
4	3	.00								
5	3		2.67							
6	3			5.33						
7	3				9.00					
8	3					13.33				
10	3						16.67			
12	3							24.67		
15	3								27.33	
18	3									31.33
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Appendix 5.1.2.b: Duncan Post hoc test- SDS of Banded snakehead at ambient temperature

Hours	N	Subset for alpha = 0.01							
		1a	b	c	d	e	f	g	h
0	3	.00							
1	3	.00							
2	3	.00							
3	3	.33							
4	3		2.67						
5	3			5.67					
6	3				9.33				
7	3					15.00			
8	3						20.33		
10	3							26.00	
12	3								30.00
15	3								31.67
18	3								32.00
Sig.		.714	1.000	1.000	1.000	1.000	1.000	1.000	.027

Appendix 5.1.2.c: Duncan Post hoc test- SDS of Stinging catfish at ambient temperature

Hours	N	Subset for alpha = 0.01								
		a	b	c	d	e	f	g	h	i
0	3	.00								
1	3	.00								
2	3	.33								
3	3		2.67							
4	3			5.67						
5	3				7.67					
6	3					11.33				
7	3						20.00			
8	3							24.33		
10	3								28.33	
12	3									31.67
15	3									32.00
18	3									32.00
Sig.		.621	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.621

Appendix 5.2: Sensory evaluation of three freshwater fishes at iced condition

Appendix 5.2.1: ANOVA for Sensory evaluation of Climbing perch, Banded snakehead and Stinging catfish at iced condition

Source	Sum of Squares	df	Mean Square	F	Sig.
Climbing perch	2949.000	8	368.625	552.938	.000
Banded Snakehead	3263.630	8	407.954	440.590	.000
Stinging catfish	4009.333	8	501.167	1230.136	.000
Among fish	61.588	2	30.794	9.289	.002

Appendix 5.2.2: Duncan Post hoc test- SDS of three fishes stored at iced condition

Appendix 5.2.2.a: Duncan Post hoc test- SDS of iced Climbing perch

Days in ice	N	Subset for alpha = 0.01						
		a	b	c	d	e	f	g
0	3	.00						
1	3	.00						
3	3	1.00						
5	3		9.00					
7	3			15.50				
9	3				19.00			
12	3					22.50		
15	3						24.50	
18	3							28.00
Sig.		.172	1.000	1.000	1.000	1.000	1.000	1.000

Appendix 5.2.2.b: Duncan Post hoc test- SDS of iced Banded snakehead

Days in ice	N	Subset for alpha = 0.01							
		1a	b	c	d	f	g	h	i
0	3	.00							
1	3	.00							
3	3		3.33						
5	3			7.33					
7	3				12.00				
9	3					16.33			
12	3						24.33		
15	3							27.00	
18	3								30.00
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Appendix 5.2.2.c: Duncan Post hoc test- SDS of iced Stinging catfish

Days in ice	N	Subset for alpha = 0.01					
		a	b	c	d	e	f
0	3	.00					
1	3	.00					
3	3		4.00				
5	3			12.00			
7	3				19.33		
9	3					24.67	
12	3					26.00	
15	3						31.00
18	3						32.00
Sig.		1.000	1.000	1.000	1.000	.020	.071

Appendix 5.3: Hardness 1 and Hardness 3 of perch, snakehead and catfish stored at ambient temperature

Appendix 5.3.1: ANOVA results for Hardness 1 and Hardness 2 of perch, snakehead and catfish stored at ambient temperature

Source		Sum of Squares	df	Mean Square	F	Sig.
Climbing perch	Hardness 1	7.449	12	.621	80.830	.000
	Hardness 2	.438	12	.037	22.948	.000
Banded Snakehead	Hardness 1	.711	12	.059	182.550	.000
	Hardness 2	.192	12	.016	166.949	.000
Stinging catfish	Hardness 1	.625	12	.052	48.048	.000
	Hardness 2	.092	12	.008	62.698	.000
Among fish	Hardness 1	3.759	2	1.879	45.745	.000
	Hardness 2	.293	2	.147	57.371	.000

Appendix 5.3.2: Duncan Post hoc test- Hardness 1(kgf) of three fishes at ambient temperature

Appendix 5.3.2.1: Duncan Post hoc test- Hardness 1(kgf) of three fishes at ambient temperature

Appendix 5.3.2.1.a: Duncan Post hoc test- Hardness1 (kgf) of perch at ambient temperature

Storage time	N	Subset for alpha = 0.01					
		a	b	c	d	e	f
18	3	.17					
15	3		.45				
12	3			.78			
10	3			.82			
8	3			.92	.92		
7	3				1.06	1.06	
6	3				1.12	1.12	
5	3					1.23	
4	3						1.43
2	3						1.48
0	3						1.49
3	3						1.54
1	3						1.58
Sig.		1.000	1.000	.080	.011	.037	.061

Appendix 5.3.2.1.b: Duncan Post hoc test- Hardness 1(kgf) of snakehead at ambient temperature

Storage time	N	Subset for alpha = 0.01									
		a	b	c	d	e	f	g	h	i	j
18	3	.1950									
15	3		.2504								
12	3			.3201							
10	3				.3952						
8	3					.4563					
7	3						.5032				
4	3						.5206	.5206			
0	3						.5327	.5327	.5327		
3	3						.5413	.5413	.5413		
6	3							.5534	.5534		
5	3								.5698	.5698	
2	3									.6096	.6096
1	3										.6147
Sig.		1.000	1.000	1.000	1.000	1.000	.022	.047	.025	.011	.732

Appendix 5.3.2.1.c: Duncan Post hoc test- Hardness 1(kgf) of catfish at ambient temperature

Storage time	N	Subset for alpha = 0.01					
		1a	b	c		e	f
15	3	.1270					
18	3	.1342	.1342				
12	3	.2004	.2004				
10	3		.2110				
8	3			.3025			
7	3			.3406	.3406		
1	3			.3689	.3689	.3689	
6	3				.4100	.4100	.4100
0	3					.4339	.4339
4	3					.4399	.4399
3	3					.4451	.4451
2	3						.4536
5	3						.4849
Sig.		.014	.010	.024	.019	.015	.018

Appendix 5.3.2.2: Duncan Post hoc test- Hardness 2 of three fishes at ambient temperature

Appendix 5.3.2.2.a: Duncan Post hoc test- Hardness 2(kgf) of perch at ambient temperature

Storage time	N	Subset for alpha = 0.01						
		1a	b	c	d	e	f	g
18	3	.0331						
15	3		.1651					
8	3			.2657				
0	3			.2773	.2773			
12	3			.2897	.2897	.2897		
6	3			.2913	.2913	.2913		
10	3			.2915	.2915	.2915		
7	3			.2980	.2980	.2980		
2	3			.3531	.3531	.3531	.3531	
1	3				.3763	.3763	.3763	.3763
4	3					.3832	.3832	.3832
5	3						.3977	.3977
3	3							.4544
Sig.		1.000	1.000	.024	.011	.016	.218	.033

Appendix 5.3.2.2.b: Duncan Post hoc test- Hardness 2(kgf) of snakehead at ambient temperature

Storage time	N	Subset for alpha = 0.01				
		a	b	c	d	e
18	3	.0063				
15	3	.0072				
12	3		.0835			
10	3		.0973			
7	3			.1394		
8	3			.1473		
1	3				.1762	
2	3				.1789	
4	3				.1803	
6	3				.1819	
3	3				.1850	
5	3				.1921	
0	3					.2231
Sig.		.919	.094	.326	.087	1.000

Appendix 5.3.2.2.c: Duncan Post hoc test- Hardness 2 (kgf) of catfish at ambient temperature

Storage time	N	Subset for alpha = 0.01						
		a	b	c	d	e	f	g
18	3	.0127						
15	4	.0178						
12	3	.0366	.0366					
10	3		.0570	.0570				
4	3			.0658	.0658			
8	3				.0886	.0886		
7	3				.0905	.0905		
6	3				.0914	.0914		
2	3					.1115	.1115	
5	3					.1145	.1145	
3	3						.1364	
1	3						.1367	
0	3							.1805
Sig.		.017	.032	.333	.013	.014	.014	1.000

Appendix 5.4: Hardness 1 and Hardness 3 of perch, snakehead and catfish stored at iced condition

Appendix 5.4.1: ANOVA results for Hardness 1 and Hardness of ice stored perch, snakehead and catfish

Source		Sum of Squares	df	Mean Square	F	Sig.
Climbing perch	Hardness 1	3.564	8	.445	195.158	.000
	Hardness 2	.523	8	.065	539.963	.000
Banded Snakehead	Hardness 1	.654	8	.082	286.577	.000
	Hardness 2	.059	8	.007	202.282	.000
Stinging catfish	Hardness 1	.251	8	.031	673.493	.000
	Hardness 2	.019	8	.002	58.693	.000
Among fish	Hardness 1	4.611	2	2.306	93.135	.000
	Hardness 2	.002	2	.001	30.528	.000

Appendix 5.4.2: Duncan Post hoc test- Hardness 1 and Hardness 2 of three ice stored fishes

Appendix 5.4.2.1: Duncan Post hoc test- Hardness 1of three ice stored fish

Appendix 5.4.2.1.a: Duncan Post hoc test- Hardness 1of ice stored perch

Days in ice	N	Subset for alpha = 0.01				
		a	b	c	d	e
18	3	.5624				
15	3	.6605				
12	3		.9837			
9	3			1.1647		
7	3				1.3054	
5	3					1.4482
0	3					1.5313
1	3					1.5551
3	3					1.5657
Sig.		.022	1.000	1.000	1.000	.012

Appendix 5.4.2.1.b: Duncan Post hoc test- Hardness 1of ice stored snakehead

Days in ice	N	Subset for alpha = 0.01						
		a	b	c	d	e	f	g
18	3	.1937						
15	3		.2388					
12	3			.3663				
9	3				.4256			
7	3					.4883		
0	3						.5715	
5	3						.6047	.6047
3	3							.6168
1	3							.6312
Sig.		1.000	1.000	1.000	1.000	1.000	.027	.084

Appendix 5.4.2.1.c: Duncan Post hoc test- Hardness 1of ice stored catfish

Days in ice	N	Subset for alpha = 0.01					
		a	b	c	d	e	f
18	3	.0832					
15	3		.1512				
12	3		.1584				
9	3			.1790			
7	3			.1945			
5	3				.2569		
3	3					.2739	
1	3						.3733
0	3						.3842
Sig.		1.000	.214	.012	1.000	1.000	.065

Appendix 5.4.2.2: Duncan Post hoc test- Hardness 2 of three ice stored fish

Appendix 5.4.2.2.a: Duncan Post hoc test- Hardness 2 of ice stored perch

Days in ice	N	Subset for alpha = 0.01					
		a	b	c	d	e	f
15	3	.2075					
18	3		.2515				
12	3			.3124			
9	3			.3377			
7	3				.3658		
5	3					.5453	
0	3					.5554	.5554
3	3					.5613	.5613
1	3						.5812
Sig.		1.000	1.000	.011	1.000	.108	.013

Appendix 5.4.2.2.b: Duncan Post hoc test- Hardness 2 of ice stored snakehead

Days in ice	N	Subset for alpha = 0.01					
		a	b	c	d	e	f
18	3	.0697					
12	3		.1031				
15	3			.1208			
7	3			.1329			
9	3			.1336			
5	3				.1701		
1	3					.1954	
0	3					.1994	
3	3						.2192
Sig.		1.000	1.000	.023	1.000	.429	1.000

Appendix 5.4.2.2.c: Duncan Post hoc test- Hardness 2 of ice stored catfish

Days in ice	N	Subset for alpha = 0.01				
		a	b	c	d	e
15	3	.0487				
12	3	.0604	.0604			
18	3	.0624	.0624			
9	3		.0741	.0741		
5	3			.0815		
7	3			.0819		
3	3				.1066	
1	3				.1179	
0	3					.1336
Sig.		.023	.023	.177	.045	1.000

Appendix 5.5: Cohesiveness, Springiness and Stiffness of perch, snakehead and catfish at ambient temperature

Appendix 5.5.1: ANOVA result- Cohesiveness, Springiness and Stiffness of perch, snakehead and catfish at ambient temperature

Source		Sum of Squares	df	Mean Square	F	Sig.
Climbing perch	Cohesiveness	.366	12	.02	4.147	.000
	Stiffness	16.388	12	1.366	17.738	.000
	Springiness	.364	12	.030	5.195	.000
Banded Snakehead	Cohesiveness	.045	12	.004	23.668	.000
	Stiffness	.171	12	.014	10.432	.000
	Springiness	3.228	12	.269	84.453	.000
Stinging catfish	Cohesiveness	.068	12	.006	59.440	.000
	Stiffness	2.808	12	.234	58.417	.000
	Springiness	1.126	12	.094	28.905	.000
Among fish	Cohesiveness	.088	2	.044	139.776	.000
	Stiffness	13.700	2	6.850	289.754	.000
	Springiness	1.594	2	.797	116.183	.000

Appendix 5.5.2: Duncan Post hoc test - Cohesiveness, Springiness and Stiffness of perch, snakehead and catfish at ambient temperature

Appendix 5.5.2.1: Duncan Post hoc test - Cohesiveness of perch, snakehead and catfish at ambient temperature

Appendix 5.5.2.1: Duncan Post hoc test- Cohesiveness of three fishes at ambient temperature

Appendix 5.5.2.1.a: Duncan Post hoc test- Cohesiveness of perch at ambient temperature

Storage time	N	Subset for alpha = 0.01		
		a	b	c
15	3	.0106		
12	3	.0134		
10	3	.0168	.0168	
8	3	.0174	.0174	
7	3	.0176	.0176	
4	3	.0189	.0189	
6	3	.0191	.0191	
5	3	.0197	.0197	.0197
1	3		.0201	.0201
0	3		.0201	.0201
3	3		.0204	.0204
18	3			.0223
2	3			.0226
Sig.				

Appendix 5.5.2.1.b: Duncan Post hoc test- Cohesiveness of snakehead at ambient temperature

Storage time	N	Subset for alpha = 0.01						
		a	b	c	d	e	f	g
15	3	.04						
18	3	.04						
12	3	.05	.05					
10	3		.08	.08				
8	3		.08	.08	.08			
6	3			.09	.09			
7	3			.09	.09	.09		
5	3			.10	.10	.10	.10	
0	3				.11	.11	.11	
2	3					.12	.12	.12
3	3						.12	.12
4	3						.13	.13
1	3							.15
Sig.		.367	.011	.062	.014	.012	.012	.018

Appendix 5.5.2.1.c: Duncan Post hoc test- Cohesiveness of catfish at ambient temperature

Storage time (Hours)	N	Subset for alpha = 0.01						
		a	b	c	d	e	F	g
5	3	.0130						
15	4	.0146	.0146					
18	3	.0199	.0199					
12	3	.0355	.0355	.0355				
10	3		.0376	.0376				
8	3			.0446	.0446			
7	3				.0614	.0614		
6	3				.0622	.0622		
1	3					.0706		
0	3					.0769		
3	3						.1155	
4	3						.1251	.1251
2	3							.1436
Sig.		.013	.011	.290	.042	.081	.231	.026

Appendix 5.5.2.2: Duncan Post hoc test- springiness of three fishes at ambient temperature

Appendix 5.5.2.2.a: Duncan Post hoc test- Springiness of perch at ambient temperature

Storage time (Hours)	N	Subset for alpha = 0.01		
		a	b	C
18	3	.3031		
15	3	.3324	.3324	
7	3	.4649	.4649	.4649
8	3	.4668	.4668	.4668
10	3	.4693	.4693	.4693
12	3	.4752	.4752	.4752
6	3		.5181	.5181
5	3			.5542
2	3			.5627
3	3			.5652
4	3			.5903
0	3			.5931
1	3			.6151
Sig.		.019	.012	.048

Appendix 5.5.2.2.b: Duncan Post hoc test- Springiness of snakehead at ambient temperature

Storage time (Hours)	N	Subset for alpha = 0.01					
		a	b	c	d	e	f
18	3	.5295					
15	4	.5582					
12	3	.6500	.6500				
10	3		.7514				
8	3			.9631			
3	3			1.0054			
7	3			1.0266			
4	3			1.0634			
6	3			1.0671			
5	3				1.2020		
2	3				1.2510	1.2510	
0	3					1.3633	1.3633
1	3						1.4130
Sig.		.018	.035	.049	.293	.021	.286

Appendix 5.5.2.2.c: Duncan Post hoc test- Springiness of catfish at ambient temperature

Storage time (Hours)	N	Subset for alpha = 0.01			
		a	b	c	d
15	3	.2812			
12	3	.3364	.3364		
10	3	.3512	.3512		
18	3	.3517	.3517		
5	3	.3884	.3884		
4	3	.4011	.4011		
7	3		.4546		
8	3		.4565		
6	3		.4664		
3	3			.6149	
2	3			.6420	
1	3			.6802	
0	3				.8903
Sig.		.028	.020	.191	1.000

Appendix 5.5.2.3: Duncan Post hoc test- Stiffness of three fishes at ambient temperature

Appendix 5.5.2.3.a: Duncan Post hoc test- Stiffness of perch at ambient temperature

Storage time	N	Subset for alpha = 0.01					
		a	b	c	d	e	f
18	3	.75					
15	3		1.48				
12	3		1.96	1.96			
10	3		2.07	2.07	2.07		
8	3			2.38	2.38	2.38	
6	3			2.44	2.44	2.44	2.44
5	3			2.58	2.58	2.58	2.58
7	3			2.62	2.62	2.62	2.62
4	3				2.74	2.74	2.74
0	3				2.75	2.75	2.75
1	3					2.88	2.88
2	3					2.95	2.95
3	3						3.12
Sig.		1.000	.019	.013	.012	.037	.012

Appendix 5.5.2.3.b: Duncan Post hoc test- Stiffness(Kg/mm) of snakehead at ambient temperature

Storage time (Hours)	N	Subset for alpha = 0.01							
		a	b	c	d	e	f	g	h
18	3	.4098							
15	4	.4820	.4820						
0	3		.5888	.5888					
1	3			.6426	.6426				
12	3			.6505	.6505				
10	3			.6693	.6693				
8	3				.7687	.7687			
2	3				.7941	.7941			
3	3					.8346			
7	3					.8492			
6	3						.9929		
4	3							1.1763	
5	3								1.4008
Sig.		.170	.046	.161	.012	.161	1.000	1.000	1.000

Appendix 5.5.2.3.c: Duncan Post hoc test- Stiffness of catfish at ambient temperature

Storage time	N	Subset for alpha = 0.01							
		a	b	c	d	e	f	g	h
18	3	.4098							
15	3	.4820	.4820						
0	3		.5888	.5888					
1	3			.6426	.6426				
12	3			.6505	.6505				
10	3			.6693	.6693				
8	3				.7687	.7687			
2	3				.7941	.7941			
3	3					.8346			
7	3					.8492			
6	3						.9929		
4	3							1.1763	
5	3								1.4008
Sig.		.170	.046	.161	.012	.161	1.000	1.000	1.000

Appendix 5.6: Cohesiveness, Springiness and Stiffness of ice stored perch, snakehead and catfish

Appendix 5.6.1: ANOVA results- Cohesiveness, Springiness and Stiffness of ice stored perch, snakehead and catfish

Source		Sum of Squares	df	Mean Square	F	Sig.
Climbing perch	Cohesiveness	.001	8	.000	18.890	.000
	Stiffness	4.364	8	.545	70.417	.000
	Springiness	.265	8	.033	1511.355	.000
Banded Snakehead	Cohesiveness	.004	8	.000	67.007	.000
	Stiffness	.577	8	.072	35.679	.000
	Springiness	.463	8	.058	87.029	.000
Stinging catfish	Cohesiveness	.001	8	.000	28.220	.000
	Stiffness	.292	8	.037	37.465	.000
	Springiness	.322	8	.040	23.733	.000
Among fish	Cohesiveness	.002	2	.001	30.528	.000
	Stiffness	18.486	2	9.243	272.924	.000
	Springiness	.039	2	.020	13.500	.000

Appendix 5.6.2: Duncan Post hoc test- Cohesiveness, Springiness and Stiffness of ice stored perch, snakehead and catfish

Appendix 5.6.2.1: Duncan Post hoc test- Cohesiveness of ice stored perch, snakehead and catfish

Appendix 5.6.2.1.a: Duncan Post hoc test- Cohesiveness of ice stored perch

Days in ice	N	Subset for alpha = 0.01			
		a	b	c	d
18	3	.0232			
12	3		.0312		
15	3		.0350		
9	3		.0363	.0363	
7	3		.0386	.0386	.0386
5	3			.0430	.0430
0	3				.0454
1	3				.0456
3	3				.0456
Sig.		1.000	.013	.020	.019

Appendix 5.6.2.1.b: Duncan Post hoc test- Cohesiveness of ice stored snakehead

Days in ice	N	Subset for alpha = 0.01			
		a	b	c	d
18	3	.0232			
12	3		.0312		
15	3		.0350		
9	3		.0363	.0363	
7	3		.0386	.0386	.0386
5	3			.0430	.0430
0	3				.0454
1	3				.0456
3	3				.0456
Sig.		1.000	.013	.020	.019

Appendix 5.6.2.1.c: Duncan Post hoc test- Cohesiveness of ice stored catfish

Days in ice	N	Subset for alpha = 0.01			
		a	b	c	d
18	3	.0232			
12	3		.0312		
15	3		.0350		
9	3		.0363	.0363	
7	3		.0386	.0386	.0386
5	3			.0430	.0430
0	3				.0454
1	3				.0456
3	3				.0456
Sig.		1.000	.013	.020	.019

Appendix 5.6.2.2: Duncan Post hoc test- springiness of three ice stored fishes

Appendix 5.6.2.2.a: Duncan Post hoc test- springiness of ice stored perch

Days in ice	N	Subset for alpha = 0.01							
		a	b	c	d	e	f	g	h
18	3	.4279							
15	3		.4401						
12	3			.4804					
9	3				.5298				
7	3					.5864			
5	3						.6505		
1	3							.6707	
3	3							.6719	
0	3								.6904
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	.744	1.000

Appendix 5.6.2.2.b: Duncan Post hoc test- springiness of ice stored snakehead

Days in ice	N	Subset for alpha = 0.01			
		a	b	c	d
18	3	.3478			
15	3	.4403	.4403		
12	3		.4577	.4577	
9	3		.5019	.5019	
7	3		.5305	.5305	
5	3			.5461	
3	3			.5568	
1	3				.6754
0	3				.7250
Sig.		.013	.023	.015	.158

Appendix 5.6.2.2.c: Duncan Post hoc test- springiness of ice stored catfish

Days in ice	N	Subset for alpha = 0.01			
		a	b	c	d
18	3	.3478			
15	3	.4403	.4403		
12	3		.4577	.4577	
9	3		.5019	.5019	
7	3		.5305	.5305	
5	3			.5461	
3	3			.5568	
1	3				.6754
0	3				.7250
Sig.		.013	.023	.015	.158

Appendix 5.6.2.3: Duncan Post hoc test- Stiffness of three ice stored fishes

Appendix 5.6.2.3.a: Duncan Post hoc test- Stiffness of ice stored perch

Days in ice	N	Subset for alpha = 0.01			
		a	b	c	d
18	3	1.6080			
15	3	1.7369			
12	3		2.3732		
9	3		2.5162	2.5162	
7	3		2.5382	2.5382	2.5382
0	3			2.6201	2.6201
5	3			2.6478	2.6478
3	3				2.7477
1	3				2.7520
Sig.		.090	.042	.108	.014

Appendix 5.6.2.3.b: Duncan Post hoc test- Stiffness of ice stored snakehead

Days in ice	N	Subset for alpha = 0.01		
		a	b	c
18	3	.5121		
15	3		.6307	
0	3			.8738
12	3			.8783
9	3			.8870
7	3			.8930
5	3			.9220
1	3			.9562
3	3			.9564
Sig.		1.000	1.000	.062

Appendix 5.6.2.3.c: Duncan Post hoc test- Stiffness of ice stored catfish

Days in ice	N	Subset for alpha = 0.01			
		a	b	c	d
18	3	.3289			
15	3	.3987	.3987		
12	3		.4124		
9	3		.4311		
7	3		.4619		
5	3			.5453	
3	3			.5891	.5891
0	3				.6221
1	3				.6401
Sig.		.014	.034	.103	.073

Appendix 6.1: Assay of lysosomal free, bound and total acid phosphatase unit activity in muscle fraction of perch, snakehead and catfish stored at ambient temperature

Appendix 6.1.1: ANOVA result- Assay of lysosomal free, bound and total acid phosphatase unit activity in muscle fraction of perch, snakehead and catfish stored at ambient temperature

Source		Sum of Squares	df	Mean Square	F	Sig.
Climbing perch	Free activity	3.068	12	.256	70.918	.000
	Bound activity	4.608	12	.384	7.609	.000
	Total bound	1.338	12	.112	2.140	.051
Banded Snakehead	Free activity	1.964	12	.164	8.967	.000
	Bound activity	3.597	12	.300	6.607	.000
	Total bound	.305	12	.025	1.573	.162
Stinging catfish	Free activity	3.831	12	.319	50.181	.000
	Bound activity	5.181	12	.432	36.259	.000
	Total bound	1.679	12	.140	25.207	.000
Among fish	Free activity	1.261	2	.631	34.583	.000
	Bound activity	1.627	2	.813	48.898	.000
	Total bound	5.371	2	2.686	208.840	.000

Appendix 6.1.2: Duncan post hoc test -Assay of Assay of lysosomal free, bound and total acid phosphatase unit activity in muscle fraction of perch stored at ambient temperature

Appendix 6.1.2.1.a: Duncan post hoc test - Assay of lysosomal free acid phosphatase unit activity in muscle fraction of perch stored at ambient temperature

Hours	N	Subset for alpha = 0.01						
		a	b	c	d	e	f	g
0	3	1.4152						
1	3		1.7412					
2	3		1.7533					
3	3			1.9250				
5	3			2.0599	2.0599			
4	3				2.1008			
7	3				2.1448	2.1448		
8	3				2.1609	2.1609		
6	3				2.1922	2.1922		
10	3					2.2509	2.2509	
12	3						2.3792	2.379
15	3						2.3850	2.385
18	3							2.397
Sig.		1.000	.808	.011	.021	.057	.015	.733

Appendix 6.1.2.2.a: Duncan post hoc test - Assay of lysosomal bound acid phosphatase unit activity in muscle fraction of perch stored at ambient temperature

Hours	N	Subset for alpha = 0.01			
		a	b	c	d
15	3	1.2578			
18	3	1.2987			
12	3	1.5903	1.5903		
7	3	1.7386	1.7386	1.7386	
10	3		1.9140	1.9140	1.9140
6	3		1.9306	1.9306	1.9306
8	3		2.0318	2.0318	2.0318
4	3		2.0699	2.0699	2.0699
5	3		2.1225	2.1225	2.1225
3	3			2.2033	2.2033
2	3			2.2509	2.2509
1	3			2.2611	2.2611
0	3				2.3536
Sig.		.022	.016	.020	.048

Appendix 6.1.2.3.a: Duncan post hoc test - Assay of lysosomal total acid phosphatase unit activity in muscle fraction of perch stored at ambient temperature

Hours	N	Subset for alpha = 0.01
		a
15	3	3.6428
18	3	3.6958
0	3	3.7688
7	3	3.8834
12	3	3.9695
1	3	4.0023
2	3	4.0042
6	3	4.1228
3	3	4.1283
10	3	4.1649
4	3	4.1707
5	3	4.1825
8	3	4.1927
Sig.		.018

Appendix 6.1.2.1.b: Duncan post hoc test - Assay of lysosomal free acid phosphatase unit activity in muscle fraction of snakehead stored at ambient temperature

Hours	N	Subset for alpha = 0.01			
		a	b	c	d
0	3	1.9194			
2	3	2.1582	2.1582		
1	3	2.1830	2.1830		
3	3	2.2355	2.2355	2.2355	
5	3	2.2525	2.2525	2.2525	
4	3		2.2800	2.2800	
6	3		2.2978	2.2978	
8	3		2.3770	2.3770	2.3770
7	3		2.3970	2.3970	2.3970
10	3			2.5574	2.5574
15	3				2.6601
12	3				2.6871
18	3				2.6968
Sig.		.010	.071	.015	.015

Appendix 6.1.2.2.b**:** Duncan post hoc test - Assay of lysosomal bound acid phosphatase unit activity in muscle fraction of snakehead stored at ambient temperature

Hours	N	Subset for alpha = 0.01			
		1a	b	c	d
18	3	1.9165			
15	3	1.9452			
12	3	1.9869	1.9869		
10	3	2.1290	2.1290	2.1290	
8	3	2.3668	2.3668	2.3668	
7	3	2.3942	2.3942	2.3942	
3	3		2.4860	2.4860	2.4860
5	3			2.5564	2.5564
6	3			2.5572	2.5572
4	3			2.5761	2.5761
1	3			2.5873	2.5873
2	3			2.6750	2.6750
0	3				2.9616
Sig.		.021	.015	.011	.023

Appendix 6.1.2.3.b**:** Duncan post hoc test - Assay of lysosomal total acid phosphatase unit activity in muscle fraction of snakehead stored at ambient temperature

Hours	N	Subset for alpha = 0.01
		a
15	3	4.6053
18	3	4.6133
12	3	4.6741
10	3	4.6864
3	3	4.7215
8	3	4.7438
1	3	4.7703
7	3	4.7912
5	3	4.8088
2	3	4.8332
6	3	4.8550
4	3	4.8561
0	3	4.8810
Sig.		.033

Appendix 6.1.2.1.c**:** Duncan post hoc test -Assay of lysosomal free acid phosphatase unit activity in muscle fraction of catfish stored at ambient temperature

Hours	N	Subset for alpha = 0.01				
		a	b	c	d	e
0	3	1.2742				
1	3	1.3902	1.3902			
2	3		1.5133			
3	3			1.8733		
10	3			1.9944	1.9944	
8	3			2.0062	2.0062	
15	3			2.0190	2.0190	
12	3			2.0442	2.0442	
4	3				2.1069	2.1069
18	3				2.1509	2.1509
7	3				2.1618	2.1618
6	3					2.2509
5	3					2.2632
Sig.		.087	.070	.025	.032	.039

Appendix 6.1.2.2.c**:** Duncan post hoc test -Assay of lysosomal bound acid phosphatase unit activity in muscle fraction of catfish stored at ambient temperature

Hours	N	Subset for alpha = 0.01					
		1a	b	c	d	e	f
18	3	1.3315					
15	3	1.5569	1.5569				
12	3		1.7675	1.7675			
5	3			1.8506			
6	3			1.8716			
10	3			1.9154			
4	3			2.0174	2.0174		
7	3			2.0179	2.0179		
8	3			2.0299	2.0299		
3	3				2.2451	2.2451	
2	3					2.4794	2.4794
1	3						2.5602
0	3						2.6194
Sig.		.018	.026	.015	.025	.014	.149

Appendix 6.1.2.3.c: Duncan post hoc test -Assay of lysosomal total acid phosphatase unit activity in muscle fraction of catfish stored at ambient temperature

Hours	N	Subset for alpha = 0.01				
		a	b	c	d	e
18	3	3.4824				
15	3	3.5759				
12	3		3.8117			
0	3		3.8936	3.8936		
10	3		3.9098	3.9098		
1	3		3.9504	3.9504	3.9504	
2	3		3.9927	3.9927	3.9927	3.9927
8	3			4.0361	4.0361	4.0361
5	3				4.1138	4.1138
3	3				4.1184	4.1184
6	3				4.1225	4.1225
4	3				4.1243	4.1243
7	3					4.1796
Sig.		.137	.011	.044	.018	.011

Appendix 6.2: Assay of lysosomal free, bound and total acid phosphatase unit activity in muscle fraction of ice stored perch, snakehead and catfish

Appendix 6.2.1: ANOVA result- Assay of lysosomal free, bound and total acid phosphatase unit activity in muscle fraction of ice stored perch, snakehead and catfish

Source		Sum of Squares	df	Mean Square	F	Sig.
Climbing perch	Free activity	6.918	8	.865	255.920	.000
	Bound activity	7.006	8	.876	246.862	.000
	Total bound	.062	8	.008	16.931	.000
Banded Snakehead	Free activity	2.929	8	.366	252.443	.000
	Bound activity	6.562	8	.820	472.731	.000
	Total bound	.868	8	.108	84.830	.000
Stinging catfish	Free activity	9.780	8	1.223	632.019	.000
	Bound activity	14.076	8	1.760	378.808	.000
	Total bound	.832	8	.104	39.228	.000
Among fish	Free activity	.659	2	.330	6.157	.010
	Bound activity	7.807	2	3.903	52.743	.000
	Total bound	7.388	2	3.694	437.039	.000

Appendix 6.2.2.1: Duncan post hoc test - Assay of lysosomal free, bound and total acid phosphatase unit activity in muscle fraction of ice stored perch

Appendix 6.2.2.1.a: Duncan post hoc test - Assay of lysosomal free acid phosphatase unit activity in muscle fraction of ice stored perch

Days in ice	N	Subset for alpha = 0.01					
		a	b	c	d	e	f
0	3	1.4560					
1	3		1.8793				
3	3			2.2997			
5	3				2.5393		
7	3					2.8120	
12	3					2.8340	2.8340
18	3					2.9000	2.9000
15	3					2.9510	2.9510
9	3						2.9680
Sig.		1.000	1.000	1.000	1.000	.014	.017

Appendix 6.2.2.1.b: Duncan post hoc test - Assay of lysosomal bound acid phosphatase unit activity in muscle fraction of ice stored perch

Days in ice	N	Subset for alpha = 0.01						
		1a	b	c	d	e	f	g
18	3	.7779						
15	3	.8007	.8007					
9	3	.8407	.8407					
12	3		.9463	.9463				
7	3			1.0493				
5	3				1.2863			
3	3					1.4777		
1	3						1.8980	
0	3							2.3177
Sig.		.237	.010	.048	1.000	1.000	1.000	1.000

Appendix 6.2.2.1.c: Duncan post hoc test - Assay of lysosomal total acid phosphatase unit activity in muscle fraction of ice stored perch

Days in ice	N	Subset for alpha = 0.01			
		1a	b	c	d
18	3	3.6779			
15	3		3.7517		
0	3		3.7737	3.7737	
1	3		3.7773	3.7773	
3	3		3.7773	3.7773	
12	3		3.7803	3.7803	
9	3			3.8087	
5	3			3.8257	3.8257
7	3				3.8613
Sig.		1.000	.157	.016	.057

Appendix 6.2.2.2: Duncan post hoc test - Assay of lysosomal free, bound and total acid phosphatase unit activity in muscle fraction of ice stored snakehead

Appendix 6.2.2.2.a: Duncan post hoc test - Assay of lysosomal free acid phosphatase unit activity in muscle fraction of ice stored snakehead

Days in ice	N	Subset for alpha = 0.01						
		a	b	c	d	e	f	g
0	3	1.9520						
3	3		2.0600					
1	3		2.0800					
5	3			2.2000				
7	3			2.2287				
9	3				2.5267			
12	3					2.6660		
15	3						2.7637	
18	3							2.9230
Sig.		1.000	.529	.369	1.000	1.000	1.000	1.000

Appendix 6.2.2.2.b: Duncan post hoc test - Assay of lysosomal bound acid phosphatase unit activity in muscle fraction of ice stored snakehead

Days in ice	N	Subset for alpha = 0.01					
		a	b	c	d	e	f
18	3	1.6397					
15	3		2.0104				
12	3			2.1984			
9	3				2.4197		
5	3					2.8674	
7	3					2.9370	2.9370
1	3						3.0140
3	3						3.0247
0	3						3.0400
Sig.		1.000	1.000	1.000	1.000	.056	.011

Appendix 6.2.2.2.c: Duncan post hoc test - Assay of lysosomal total acid phosphatase unit activity in muscle fraction of ice stored snakehead

Days in ice	N	Subset for alpha = 0.01						
		1a	b	c	d	e	f	g
18	3	4.5627						
15	3		4.7740					
12	3			4.8644				
9	3			4.9463	4.9463			
0	3				4.9920	4.9920		
5	3					5.0674	5.0674	
3	3						5.0847	5.0847
1	3						5.0940	5.0940
7	3							5.1657
Sig.		1.000	1.000	.012	.135	.019	.401	.016

Appendix 6.2.2.3: Duncan post hoc test - Assay of lysosomal free, bound and total acid phosphatase unit activity in muscle fraction of ice stored catfish

Appendix 6.2.2.3.a: Duncan post hoc test - Assay of lysosomal free acid phosphatase unit activity in muscle fraction of ice stored catfish

Days in ice	N	Subset for alpha = 0.01						
		a	b	c	d	e	f	g
0	3	1.1333						
1	3		1.2777					
3	3			1.6937				
5	3				1.9657			
18	3					2.5237		
15	3					2.5627	2.5627	
7	3					2.6033	2.6033	
12	3						2.6450	
9	3							2.8313
Sig.		1.000	1.000	1.000	1.000	.049	.042	1.000

Appendix 6.2.2.3.b: Duncan post hoc test - Assay of lysosomal bound acid phosphatase unit activity in muscle fraction of ice stored catfish

Days in ice	N	Subset for alpha = 0.01				
		a	b	c	d	e
9	3	1.0410				
12	3	1.0870				
18	3	1.0870				
15	3	1.1400				
7	3		1.4490			
5	3			2.1103		
3	3				2.4140	
1	3					2.7433
0	3					2.8880
Sig.		.118	1.000	1.000	1.000	.018

Appendix 6.2.2.3.c: Duncan post hoc test - Assay of lysosomal total acid phosphatase unit activity in muscle fraction of ice stored catfish

Days in ice	N	Subset for alpha = 0.01		
		a	b	c
18	3	3.6107		
15	3	3.7027		
12	3	3.7320		
9	3		3.8723	
1	3			4.0210
0	3			4.0213
7	3			4.0523
5	3			4.0760
3	3			4.1077
Sig.		.013	1.000	.078

Appendix 6.3: Assay of cathepsin D unit activity in muscle fraction of perch, snakehead and catfish stored at ambient temperature

Appendix 6.3.1: ANOVA results -Assay of cathepsin D unit activity in muscle fraction of perch, snakehead and catfish stored at ambient temperature

	Sum of Squares	df	Mean Square	F	Sig.
perch	.001	12	.000	755.582	.000
snakehead	.002	12	.000	4161.673	.000
catfish	.001	12	.000	1527.290	.000
Among the fish	.000	2	.000	77.590	.000

Appendix 6.3.2.a: Duncan post hoc test -Assay of cathepsin D unit activity in muscle fraction of perch stored at ambient temperature

hours	N	Subset for alpha = 0.01									
		a	b	c	d	e	f	g	h	i	j
0	3	.000310									
1	3	.000313									
2	3	.000595	.000595								
3	3		.001373								
4	3			.005229							
5	3				.007723						
6	3					.009341					
8	3						.0106				
7	3						.0103				
10	3							.0119			
12	3								.0146		
15	3									.0167	
18	3										.017
Sig.		.413	.023	1.000	1.000	1.000	.922	1.000	1.000	1.000	1.000

Appendix 6.3.2.b: Duncan post hoc test -Assay of cathepsin D unit activity in muscle fraction of perch stored at ambient temperature

hours	N	Subset for alpha = 0.01											
		a	b	c	d	e	f	g	h	i	j	k	l
0	3	.0027											
1	3		.0049										
2	3			.0056									
3	3				.0080								
4	3					.0097							
5	3						.0117						
6	3							.0139					
7	3								.0160				
8	3									.0170			
15	3										.0189		
18	3											.0206	
12	3											.0208	
10	3												.0213
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.132	1.000

Appendix 6.3.2.c: Duncan post hoc test -Assay of cathepsin D unit activity in muscle fraction of perch stored at ambient temperature

hours	N	Subset for alpha = 0.01										
		a	b	c	d	e	f	g	h	i	j	k
0	3	.0042										
2	3		.0091									
1	3			.0098								
3	3				.0110							
4	3					.0136						
5	3						.0157					
6	3							.0173				
7	3								.0181			
10	3									.0189		
12	3										.0202	
18	3										.0207	
8	3											.0213
15	3											.0216
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.011	.117

Appendix 6.4. Assay of cathepsin D unit activity in muscle fraction of ice stored perch, snakehead and catfish

Appendix 6.4.1: ANOVA results -Assay of cathepsin D unit activity in muscle fraction of ice stored perch, snakehead and catfish

	Sum of Squares	df	Mean Square	F	Sig.
perch	.566	8	.071	217.605	.000
snakehead	.454	8	.057	539.190	.000
catfish	.421	8	.053	198.561	.000
Among the fish	.000	2	6.500E-5	22.822	.000

Appendix 6.4.2: Duncan post hoc test -Assay of cathepsin D unit activity in muscle fraction of ice stored perch, snakehead and catfish

Appendix 6.4.2.a: Duncan post hoc test -Assay of cathepsin D unit activity in muscle fraction of ice stored perch

Day	N	Subset for alpha = 0.01				
		a	b	c	d	e
0	3	.014296				
1	3		.119000			
3	3		.159100			
5	3			.298803		
7	3			.321033		
9	3				.385293	
12	3				.402930	.402930
18	3					.435810
15	3					.439003
Sig.		1.000	.014	.148	.246	.031

Appendix 6.4.2.b: Duncan post hoc test -Assay of cathepsin D unit activity in muscle fraction of iced snakehead

day	N	Subset for alpha = 0.01						
		a	b	c	d	e	f	g
0	3	.024180						
1	3		.076049					
3	3		.094379					
5	3			.181533				
7	3				.210257			
9	3					.278667		
12	3						.332100	
15	3							.383200
18	3							.399603
Sig.		1.000	.042	1.000	1.000	1.000	1.000	.066

Appendix 6.4.2.c: Duncan post hoc test -Assay of cathepsin D unit activity in muscle fraction of iced catfish

day	N	Subset for alpha = 0.01				
		a	b	c	d	e
0	3	.030164				
1	3	.047533	.047533			
3	3		.083017			
5	3			.131110		
7	3			.149333		
9	3				.251400	
12	3				.288440	
18	3					.368100
15	3					.371403
Sig.		.208	.016	.187	.012	.807

Appendix 6.5: Assay of collagenase enzyme unit activity in muscle fraction of perch, snakehead and catfish stored at ambient temperature

Appendix 6.5.1: ANOVA results -Assay of collagenase enzyme unit activity in muscle fraction of perch, snakehead and catfish stored at ambient temperature

	Sum of Squares	df	Mean Square	F	Sig.
perch	10.218	12	.852	632.357	.000
snakehead	9.395	12	.783	352.505	.000
catfish	13.020	12	1.085	848.922	.000
Among the fish	.786	2	.393	37.408	.000

Appendix 6.5.2: Duncan post hoc test -Assay of collagenase enzyme unit activity in muscle fraction of perch, snakehead and catfish stored at ambient temperature

Appendix 6.5.2.a: Duncan post hoc test -Assay of collagenase enzyme unit activity in muscle fraction of perch stored at ambient temperature

hours	N	Subset for alpha = 0.01								
		a	b	c	d	e	f	g	h	i
0	3	.0025								
1	3	.0053								
2	3	.0251	.0251							
3	3		.1018							
4	3			.256						
5	3			.3259						
6	3				.4640					
7	3					.6648				
8	3					.7106				
10	3						1.0079			
12	3							1.1469		
15	3								1.3769	
18	3									1.5072
Sig.		.484	.017	.029	1.000	.138	1.000	1.000	1.000	1.000

Appendix 6.5.2.b: Duncan post hoc test -Assay of collagenase enzyme unit activity in muscle fraction of snakehead stored at ambient temperature

hours	N	Subset for alpha = 0.01							
		a	b	c	d	e	f	g	h
0	3	.0050							
1	3	.0137							
2	3		.1792						
3	3		.2512	.2512					
4	3			.3351					
5	3				.5706				
6	3				.6556				
7	3					.7999			
8	3						.9747		
10	3						1.0441		
12	3							1.3201	
15	3							1.338	
18	3								1.4478
Sig.		.824	.073	.038	.036	1.000	.083	.628	1.000

Appendix 6.5.2.c: Duncan post hoc test -Assay of collagenase enzyme unit activity in muscle fraction of catfish stored at ambient temperature

hours	N	Subset for alpha = 0.01										
		a	b	c	d	e	f	g	h	i	j	k
0	3	.0127										
1	3	.0666										
2	3		.2411									
3	3			.3485								
4	3				.7704							
5	3					.8876						
6	3						.98417					
7	3							1.1061				
8	3								1.2650			
10	3									1.3751		
12	3										1.5437	
15	3											1.6888
18	3											1.7154
Sig.		.076	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.371

Appendix 6.6: Assay of collagenase enzyme unit activity in muscle fraction of ice stored perch, snakehead and catfish

Appendix 6.6.1: ANOVA results -Assay of collagenase enzyme unit activity in muscle fraction of ice stored perch, snakehead and catfish

	Sum of Squares	df	Mean Square	F	Sig.
perch	11.966	8	1.496	55.051	.000
snakehead	12.740	8	1.592	270.553	.000
catfish	15.362	8	1.920	258.845	.000
Among the fish	.491	2	.246	22.533	.000

Appendix 6.6.2: Duncan post hoc test -Assay of collagenase enzyme unit activity in muscle fraction of ice stored perch, snakehead and catfish

Appendix 6.6.2.a: Duncan post hoc test -Assay of collagenase enzyme unit activity in muscle fraction of ice stored perch

day	N	Subset for alpha = 0.01					
		a	b	c	d	e	f
0	3	.005103					
1	3	.117977					
3	3	.251845	.251845				
5	3		.567721	.567721			
7	3			.882962	.882962		
9	3				1.259777	1.259777	
12	3					1.535958	1.535958
15	3					1.663695	1.663695
18	3						1.863628
Sig.		.098	.031	.031	.012	.010	.032

Appendix 6.6.2.b: Duncan post hoc test -Assay of collagenase enzyme unit activity in muscle fraction of ice stored snakehead

day	N	Subset for alpha = 0.01						
		a	b	c	d	e	f	g
0	3	.005811						
1	3	.050643						
3	3		.483307					
5	3			.766139				
7	3				1.260173			
9	3					1.529596		
12	3					1.627732	1.627732	
18	3						1.750087	1.750087
15	3							1.861910
Sig.		.483	1.000	1.000	1.000	.135	.067	.091

Appendix 6.6.2.c: Duncan post hoc test -Assay of collagenase enzyme unit activity in muscle fraction of ice stored catfish

day	N	Subset for alpha = 0.01					
		a	b	c	d	e	f
0	3	.019950					
1	3	.201442					
3	3		.639768				
5	3			.989868			
7	3				1.458630		
9	3					1.764358	
12	3					1.909166	
15	3					1.955443	
18	3						2.161293
Sig.		.019	1.000	1.000	1.000	.018	1.000

6.7: Effect of natural modulators on Collagenase enzyme and lysosomal acid phosphatase enzyme activity

6.7.1: ANOVA results- Effect of natural modulators on Collagenase enzyme and lysosomal acid phosphatase enzyme activity

Fish	Enzyme	Sum of Squares	df	Mean Square	F	Sig.
Perch	Collagenase	86905.785	12	7242.149	473.643	.000
	Acid phosphatase	2433029.438	12	202752.453	1417.458	.000
Snakehead	Collagenase	90039.010	12	7503.251	92.310	.000
	Acid phosphatase	3860161.316	12	321680.110	1434.046	.000
Catfish	Collagenase	115680.046	12	9640.004	240.967	.000
	Acid phosphatase	1868638.174	12	155719.848	2658.769	.000

6.7.2: Duncan post hoc test results- Effect of natural modulators on Collagenase enzyme and lysosomal acid phosphatase enzyme activity

6.7.2.1: Duncan post hoc test results- Effect of natural modulators on Collagenase enzyme activity

6.7.2.1.a: Duncan post hoc test results- Effect of natural modulators on Collagenase enzyme activity of perch

Treatment	N	Subset for alpha = 0.05								
		a	b	c	d	e	f	g	h	i
control	3	22.84								
Tea leaf	3	24.37								
All spice	3		34.18							
Malabar tamarind	3			60.48						
Tamarind	3				68.72					
Lemon	3					76.72				
Curcumin	3						106.16			
Curry leaves	3						107.90			
Chilli	3							126.65		
Ginger	3							126.82		
Pepper	3								138.70	
Bird’s Eye chilli	3								139.82	
Garlic	3									176.74
Sig.		.635	1.000	1.000	1.000	1.000	.590	.957	.728	1.000

6.7.2.1.b: Duncan post hoc test results- Effect of natural modulators on Collagenase enzyme activity of snakehead

Treatment	N	Subset for alpha = 0.05								
		a	b	c	d	e	f	g	h	i
control	3	16.95								
Tamarind	3		59.03							
Tea leaf	3		69.95	69.95						
Malabar tamarind	3			77.66						
All spice	3			78.73						
Lemon	3			81.68						
Curcumin	3				105.17					
Bird's Eye Chili	3				114.83	114.83				
Curry leaves	3					123.85	123.85			
Ginger	3						133.84	133.84		
Pepper	3							140.35		
Chilli	3								162.12	
Garlic	3									210.58
Sig.		1.000	.150	.157	.201	.231	.187	.385	1.000	1.000

6.7.2.1.c: Duncan post hoc test results- Effect of natural modulators on Collagenase enzyme activity of catfish

Treatment	N	Subset for alpha = 0.05									
		a	b	c	d	e	f	g	h	i	j
control	3	18.66									
Malabar tamarind	3		41.75								
Lemon	3			57.18							
Tea leaf	3			64.88	64.88						
Tamarind	3				74.49	74.49					
All spice	3					82.01					
Bird's Eye Chili	3						105.72				
Curcumin	3						107.54				
Ginger	3							121.27			
Curry leaves	3							123.42			
Chilli	3								137.75		
Pepper	3									179.29	
Garlic	3										225.81
Sig.		1.000	1.000	.148	.074	.158	.727	.681	1.000	1.000	1.000

6.7.2.2.a: Duncan post hoc test results- Effect of natural modulators on lysosomal acid phosphatase activity of perch

Treatment	N	Subset for alpha = 0.05						
		a	b	c	d	e	f	g
Ginger	3	2.10						
Garlic	3	2.27						
Curcumin	3	2.27						
Chilli	3	4.24						
Bird's Eye Chili	3		25.91					
Curry leaves	3		27.88					
Pepper	3		32.52	32.51				
All spice	3			51.22	51.22			
Tea leaf	3				54.14			
Tamarind	3					530.04		
Malabar tamarind	3						555.57	
control	3							576.46
Lemon	3							585.47
Sig.		.843	.530	.066	.768	1.000	1.000	.365

6.7.2.2.b: Duncan post hoc test results- Effect of natural modulators on lysosomal acid phosphatase activity of snakehead

Treatment	N	Subset for alpha = 0.05						
		a	b	c	d	e	f	g
Ginger	3	1.45						
Curkumin	3	3.14						
Garlic	3	3.86						
Pepper	3	4.29						
Bird's Eye Chili	3	14.58	14.58					
Chilli	3	26.43	26.43	26.43				
All spice	3		36.59	36.59				
Curry leaves	3			48.34				
Tea leaf	3				174.60			
control	3					614.00		
Tamarind	3						666.59	
Lemon	3						690.28	
Malabar tamarind	3							839.14
Sig.		.082	.100	.101	1.000	1.000	.064	1.000

6.7.2.2.c: Duncan post hoc test results- Effect of natural modulators on lysosomal acid phosphatase activity of catfish

Treatment	N	Subset for alpha = 0.05						
		a	b	c	d	e	f	g
Curkumin	3	1.80						
Pepper	3	6.04						
Curry leaves	3	6.86						
Ginger	3	8.40						
Bird's Eye Chili	3	12.48						
Garlic	3	12.61						
Chilli	3	13.72						
Tea leaf	3		32.64					
All spice	3			59.89				
control	3				441.79			
Malabar tamarind	3					480.62		
Tamarind	3						504.89	
Lemon	3							528.11
Sig.		.107	1.000	1.000	1.000	1.000	1.000	1.000

APPENDIX C

List of Publications

- Treesa Varghese & Saleena Mathew (2017). Assessment of the textural variation of iced stored Anabas testudineus (Bloch, 1792) muscle tissue with emphasis on their collagen and myofibrillar protein content. Journal of Food Science and Technology, 54(8),2512-2518

- Treesa Varghese & Saleena Mathew (2016). Postmortem autolytic changes of iced stored banded snakehead (*Channa striata*) (Bloch, 1793). International Journal of Fisheries and Aquatic Studies, 4(4), 262-267

- Treesa Varghese & Saleena Mathew (2016). Seasonal variations in the proximate composition of Asian Stinging Catfish (*Heteropneustes fossilis*) (bloch, 1794) and Banded Snakehead (*Channa striata*) (bloch, 1793) collected from paddy field of Kerala. Trends in Fisheries Research, 5(3), 2319–4758

APPENDIX D

TREESA VARGHESE

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Objective:
To work on any process those synchronously enriches my knowledge, living standard and contribute towards achieving the goals of employer.

Experience:

- *Asst. Professor* – Currently working as Asst. Professor at School of Biosciences, Mar Athanasios College for Advanced Studies Tiruvalla (MACFAST) from November 2007 to till date
- *Part-time research scholar*, School of Industrial Fisheries, CUSAT

Academic Qualifications:

- Pursuing Ph. D: School of Industrial Fisheries, CUSAT, Cochin, Kerala.
  Research topic: Role of autolytic enzymes in muscle and resultant physico-chemical changes during the post-mortem storage of selected freshwater fishes
  Research Guide: Dr. Saleena Mathew, Professor, School of Industrial Fisheries, CUSAT, Cochin
- Master of Science in Biochemistry: Mahatma Gandhi University, Kottayam.
  Result – 1256/1800 (70%), First Class.
- Bachelor of Science in Biochemistry: Calicut University.
  Result- 913/1200 (76%), First Class.

Research:

- **M. Sc. Project Thesis:** "*Preliminary Investigation on the Correlation of Serum Parameter of Iron Metabolism with Iron Status in Neutrophils and Platelets"*
- **Completed Project**: "*Fish Collagen from Underutilized Marine Resources of Kerala*"-Three year Kerala State Council for Science Technology and Environment (KSCSTE), Kerala - SRS Scheme funded project.

Technical Skills:

- On–hand experience with UV-VIS Spectrophotometer and Atomic absorption spectrophotometer.
- Well experienced in electrophoresis techniques.
- Experienced in Chromatographic techniques (HPLC, TLC & Paper) for separating biological compounds.
- Experienced with use of Lyophilizer, Rotary vacuum evaporator
- Experienced in qualitative and quantitative analysis of biomolecules
- MS Office (Word, Excel, Power point),

Publications:

- Treesa Varghese & Saleena Mathew (2017). Assessment of the textural variation of iced stored Anabas testudineus (Bloch, 1792) muscle tissue with emphasis on their collagen and myofibrillar protein content. Journal of Food Science and Technology, 54(8),2512-2518
- Treesa Varghese & Saleena Mathew (2016). Postmortem autolytic changes of iced stored banded snakehead (*Channa striata*) (Bloch, 1793). International Journal of Fisheries and Aquatic Studies, 4(4), 262-267
- Treesa Varghese & Saleena Mathew (2016). Seasonal variations in the proximate composition of Asian Stinging Catfish (*Heteropneustes fossilis*) (bloch, 1794) and Banded Snakehead (*Channa striata*) (bloch, 1793) collected from paddy field of Kerala. Trends in Fisheries Research, 5(3), 2319–4758
- Quality changes in iced stored Banded snakehead, International Conference on Advances in Bioprocess Technology,26-28 November 2015
- Biomedical and Industrial Applications of Collage 1st Kerala Women's Science Congress, held on 10-12 August 2010, at St. Teresa's College, Ernakulum, Kerala.
- Presented a *Poster* on "*Antibodies are the Most Important Milestones*" in south zone Inter Collegiate Meet organized by CMS College Kottayam held on 14th October 2006.

Seminars attended:

- Work shop on research methodology and data analysis at SB College Changanacherry on 7-8 September 2017
- National conference on life style diseases and management organized by Dept. of Biochemistry, Believers Church Medical College, Tiruvalla on 30/6/2017 & 1/7/2017
- Lecture workshop on recent advances in biophysics organized by MACFAST on 15 & 16/ June 2017
- National conference on recent advances in cancer diagnosis and research organized by Pushpagiri institute of medical science and research Centre, Tiruvalla on 8/7/2016
- Two day training programme on Laboratory safety & management organized by Pushpagiri research Centre on 21 & 22/ 3/ 2016
- International symposium *"Computational Biology and Drug Design"* at Mar Athanasios College for Advanced Studies, Tiruvalla held on 10-12 July 2013
- *"Relevance of GM Crops in Food Security"* at Mar Athanasios College for Advanced Studies, Tiruvalla held on 27 February 2013
- Lecture Workshop on *"Phytonutraceuticals, Pharmacovigilance, CADD and Drug Development"* at Pushpagiri College of Pharmacy
- "*Advances in Herbal Science and Technology"* at Mar Athanasios College for Advanced Studies, Tiruvalla held on 25-27 November 2011.
- International symposium on "*Second Green Revolution: Priorities, Programmes, Social and Ethical Issues*" organized by School of Biosciences, Mar Athanasios College for Advanced Studies, Tiruvalla held on 2- 4 July, 2009.
- Two day lecture Work shop on "*Evolution and Natural Selection*" organized by School of Biosciences, Mar Athanasios College for Advanced Studies Tiruvalla, on 29th & 30th January 2009.

- National Level Seminar on "*Current Advances in Chemical Science*" organized by the Dept. of Chemistry, Sacred Heart College, Thevara, Kochi, Kerala on 26 &27 November 2008.
- Lecture Workshop on "*Gene Structure and Function- Concepts to New Developments*" during 31st January &1st February 2008 organized by School of Biosciences, Mar Athanasios College for Advanced Studies, Tiruvalla.
- International symposium on "*Advances in Food Biotechnology and Nutrition*" organized by School of Biosciences, Mar Athanasios College for Advanced Studies, Tiruvalla held on 30th Nov. and 1st Dec. 2007.
- State level seminar on "*Cell Signaling and Apoptosis*" organized by Mar Athanasios College for Advanced Studies, Tiruvalla held on 25th October 2007.
- 11th ADNAT Convention Symposium on "*Advances in Structural Biology and Structure Prediction*" organized by Centre for Cellular and Molecular Biology, Hyderabad, India held on 23rd February- 25th February, 2007.
- State level seminar on "*Relevance of Biological Science in the Present Scenario*" organized by Dept. of Biochemistry, Biotechnology & Microbiology, M.A College Kothamangalam, held on 30th March 2006.
- National level Seminar on "*Frontiers in Chemistry*" conducted by Dept. of Applied Chemistry, Cochin University of Science and Technology, Cochin held on 24 & 25 March 2006.

Personal profile:

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