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CICLO XXX

"INNOVATIVE APPROACHES IN AQUACULTURE FOR CONSERVATION AND MANAGEMENT OF THREATENED FISH SPECIES: THE CASE OF DIFFERENT STRAINS OF BROWN TROUT (*Salmo trutta* and *Salmo ghigii*)"

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Innovative approaches in aquaculture for Conservation and management of threatened fish species: the case of different strains of brown trout (*Salmo trutta* and *Salmo ghigii*)

Abstract

In Salmonid species, direct factors that affect sperm concentration are culture conditions, stripping time, age, feeding and presence of males. It is reported that inside culture conditions, temperature of water can strongly influence quality of fish sperm, especially duration of motility. The aim of first part of this work is evaluate the quantity of fish milt in different Salmonid lineages; Atlantic brown trout (*Salmo trutta*), an albino strain of Mediterranean brown trout (*Salmo ghigii*), a pure Mediterranean lineage (*Salmo cettii*) and marble trout (*Salmo marmoratus*). Brown trout (*Salmo trutta*) have long been a favourite sport-fish among anglers, and have been transported primarily on the basis of recreational fishing. A balanced diet and proper feeding practices are important in aquaculture. It is necessary to have a proper amount of feed with ingredients necessary for fish such as protein for normal tissue function, maintenance and renewal of the fish body, carbohydrates and lipids for energy source, vitamins and minerals for body functions, growth, reproduction and maintenance of fish metabolism. The second part of an experiment conducted at the University of Parma in Italy in the summer 2016, and the aim is to investigate the effects of dietary black carbon on growth performance and survival rate of brown trout (*Salmo trutta*).

Different factors such as Chemical, Physical, and perceived stressors can all evoke non-specific responses in fish, which are considered adaptive to enable the fish to cope with the disturbance and maintain its homeostatic state. Fish faced with stressful stimuli launch an endocrine stress response through activation of the hypothalamic-pituitary-interrenal (HPI) axis to release cortisol in to the blood. Scientifically validated biomarkers to capture systemic cortisol exposure over

longer periods of time are of utmost importance to assess chronic stress in governmental, wild life, aquaculture and scientific settings. Cortisol is the main corticosteroid hormone in teleost secreted in response to stressor exposure and plays a key role in stress adaptation. The aim of last part of study is to check difference in stress resistance between wild type of Atlantic brown trout (*Salmo trutta*) and albino Mediterranean trout (*Salmo ghigii*). An experiment conducted at Monchio fish hatchery of Parma province in Italy during the summer 2017. Considering that, stress parameters effect on behavioral changes, and mentioned changes can increase stress, as well as reducing reproduction and decreasing growth rate and health status, consequently, the ability of stress factor managing and reducing these parameters have an important role for protection and conservation of endangered species.

Approcci innovativi nell'acquacultura per la conservazione e la gestione di specie ittiche minacciate: il caso di differenti linee del complex trota fario (*Salmo trutta* e *Salmo ghigii*)

Abstract

Nelle specie di salmonidi, i diretti fattori che influenzano la concentrazione di sperma sono le condizioni di coltura, il tempo di strippaggio, l'età, l'alimentazione e la presenza di maschi. E' stato riportato che le condizioni di coltura interne, la temperatura dell'acqua può influenzare fortemente la qualità dello sperma dei pesci, specialmente la durata della motilità. Lo scopo della prima parte di questo lavoro è valutare la quantità del liquido seminale di differenti linee di salmonidi; la trota fario atlantica (*Salmo trutta*), una linea albina di trota mediterranea (appenninica-adriatica) del mediterraneo (*Salmo ghigii*), una linea pura di trota mediterranea (*Salmo cettii*) e la trota marmorata (*Salmo marmoratus*). La trota fario atlantica (*Salmo trutta*) è stata a lungo la specie ittica favorito dai pescatori per la pesca sportiva ed è stato introdotto principalmente per le esigenze di ripopolamento della pesca ricreativa. Una dieta bilanciata e pratiche di alimentazione idonee sono importanti nell'acquacultura. E' necessario avere un giusto quantitativo di cibo con ingredienti necessari alla saluta accrescimento corporeo , come le proteine per il normale funzionamento dei tessuti eil mantenimento e il rinnovo del corpo del pesce; carboidrati e lipidi come riserva energetica, vitamine e minerali per le funzioni del corpo, la crescita, la riproduzione e il mantenimento del metabolismo del pesce. La seconda parte dell'esperimento condotto presso l'Università di Parma (Italia) nell'estate del 2016 ha avuto lo scopo di studiare gli effetti del black carbon (biochar) sulla performance di crescita e il tasso di sopravvivenza della trota marrone (*Salmo trutta*).

Diversi fattori come gli stressor percepito chimici e fisisic possono evocare una risposta non specifica nei pesci, che sono considerate risposte adattative con il fine di render capace il pesce di far fronte ai fattori di disturbo e di mantenere l'omeostasi. I pesci che hanno a che fare con stimoli stressanti lanciano una risposta endocrina allo stress attraverso l'attivazione dell'asse HPI (hypothalamic-pituitary-interrenal) per rilasciare cortisolo nel sangue. Biomarker scientificamente convalidati per rilevare il cortisolo sistemico in lunghi periodi di tempo sono di massima importanza per valutare lo stress cronico in ambiente selvatico, in acquacultura e in setting scientifici. Il cortisolo è il principale ormone corticosteroide nella risposta secreta dei teleostei all'esposizione agli stressor e gioca un ruolo chiave nella risposta adattativa allo stress. L'obiettivo dell'ultima parte dello studio è di registrare le differenze nella resistenza allo stress tra le trote wild-type (*Salmo trutta*) e la trota albina (*Salmo ghigii*), in un esperimento condotto presso nell'impianto per allevamento di Monchio nella provincia di Parma durante l'estate del 2017. Considerando che, gli effetti ai parametri di stress relativi ai cambiamenti comportamentali, ai già menzionati cambiamenti che possono aumentare lo stress, così come una riduzione della riproduzione e una decrescita del tasso di crescita e dello status di salute, e conseguentemente, l'abilità nella gestione dei fattori di stress e una riduzione di questi parametri, hanno un ruolo importante nella protezione e nella conservazione delle specie in via di estinzione.

Dedicated to my father, my mother and my brother who have been a source of inspiration and contributed immensely to the success of this thesis

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CHAPTER 1

1.1 Aquaculture: increasing sustainability by defining its shortcomings and problems and possible mitigation strategies

1.1.1 In General

As the total protein coming from aquaculture has increased enormously over the last decade and fisheries meet its limits, providing food in a sustainable manner is one of the global priorities (FAO, 2016). Providing food for more than 9billion people by 2050 and taking into account climate change and increased competition for natural resources poses one of the major challenges in the world in this framework. Unprecedented commitments have been made in September 2015 by the international community as UN member states adopted the 2030 agenda for sustainable development. The 2030 Agenda also sets aims for the contribution and conduct of fisheries and aquaculture towards food security and nutrition in the use of natural resources so as to ensure sustainable development in economic, social and environmental terms (FAO, 2016). Among the fish species, the Salmonid family is one of the most important taxon because of scientific, social and economic importance. They occupy an extended ecological niche, and can be found in marine fresh water habitats (Macqueen, et al 2017). They have been introduced in south America Australia, Africa, the Middle East and also throughout the northern hemisphere (Pennell, in preparation). As many wild populations are decreasing in numbers ,a greater effort is directed towards their conservation and management with respect to anthropogenic-driven change (Waples RS, 2008). Salmonids include at least 70 species (but are sometimes classified as many as > 200) (Davidson, 2010) and hold an array of adaptations and life-history strategies (Hendry AP, 2004). Different characteristics such

as a widely distributed original range, a broad environmental tolerance, high genetic variability, short generation time, rapid growth, and early sexual maturation are general attributes of successful invasive species (Ramussen 1998). All of these characteristics are needed for species kept in aquaculture; therefore the possibility of many aquaculture species to become encroaching is high. In aquaculture, most species grown are basically wild, but some have been selectively bred for priority development, faster growth and other characteristics (Hulata 2001). Some species have been modified by hybridization or polyploidy to produce infertile individuals to the culture (Hulata 2001). Atlantic salmon (*Salmo salar*) do have inherently modified genotypes developed for higher growth rates, but until now none of these have been commercially cultured. Even though domestication rates may be quite rapid in fish, the genetic structure of most species in aquaculture resembles that of the same species in the wild (Duarte CM 2007).

1.1.2 Salmonid: a global overview and Italian endangered taxa

1.1.2.1 Overall characteristics

Salmonids represent an important economic as well as cultural aspect in the life of many people along the coast of the Atlantic and Pacific Oceans (Schindler, 2003). Because of their astounding abilities to perform long migrations and navigate accurately back to the streams where they were born they are biologically remarkable. As anadromous fish, they utilize many habitats in both fresh and marine waters and in evolutionary and ecological terms, must be considered extremely successful animals. The spawning season of most salmonids is in autumn. Herby some species migrate back to their spawning grounds. There, the females dig nests, called redds, in the gravel and lay their eggs. As they do, the

male partner releases milt which fertilizes the eggs. A few sea-run trout return to the ocean and may spawn again, but all Pacific salmon spawn only once, then die. The eggs stay in the gravel until spring, when they hatch. The offspring, called "alevins", remain in the gravel at first, living on nutrients in the yolk sac. After these nutrients have been depleted, the fish now called "fry", autonomously have to catch food. Some species (pink (*O. gorbuscha*) and most chum (*O. keta*)) migrate to sea at once, while others stay in fresh water for three years or more. All spend a period of time in river estuaries while they adapt to salt water. The estuaries are very rich in food and the young salmonids grow quickly during this time. Once they move out into the open ocean some travel thousands of kilometers before they return to spawn. In recent decades, however, many salmonid populations, including genetically distinct groups of fish, have been decimated or severely under threat by over-fishing and habitat damage. In parallel, salmonid farming has become a large international industry producing a greater weight of fish than the wild harvests combined. Salmonids , consist of 3 sub-families(Table: 1.1): (a)Genus *Oncorhynchus:* seven species of Pacific salmon(*O.tshawytscha*, *O.keta*, *O.kisutch*, *O.gorbuscha* and *O.nerka*) , rainbow trout (*O. mykiss*), cutthroat trout (O. *clarkii*), and related species all with native ranges in the North Pacific Ocean or land masses adjacent to the Pacific; (b) Genus *Salmo*, the Atlantic trout, Atlantic salmon (*Salmo salar*), and brown trout (*Salmo trutta*), all with native ranges in the North Atlantic Ocean and surrounding land masses, the list of species belonging to Salmo genus could even be larger, considering the different strains of Mediterranean trouts (i.e. *S. cettii* and *S. ghigii* in Italy) ; and (c) genus *Salvelinus*, the charr (lake charr (*S. namaycush*), brook charr (*S. fontinalis*), Arctic charr (*S. alpinus*), Dolly Varden charr (*S. malma*), and bull trout(*S.*

confluentis)), many of which are either Pacific shore species or circumpolar in their distributions. Naturally, there are a lot of different sub-populations (strains) within each species making it a point of discussion for many taxonomists, suggestion the origin of many other species.

Table1.1: there are three genera and twenty three species of salmonids, worldwide, each genera and the species that comprise it are listed here. Along with their geographic distribution prior to stocking programs, more species could be added for the genus *Salmo* considering the Mediterranean taxa

Salmon		
Pacific	**Common name**	**Original Distribution**
Oncorhynchus tshawytscha	Chinook or king	Pacific Ocean
O. kisutch	Silver or Coho	Pacific Ocean
O. nerka	Sockeye	Pacific Ocean
O. keta	Chum	Pacific Ocean
O. gorbuscha	Pink	Pacific Ocean
O. masou	Cherry	Pacific Ocean
Atlantic		
Salmo salar	Atlantic salmon	Atlantic Ocean
Charr		
Salvelinus fontinalis	Brook trout	North America
S. malma	Dolly varden	North America
S. namaycush	Lake trout	North America
S. alpinus	Artic charr	Palearctic
S. confluentis	Bull trout	North America

S. leucomaenis	Kundsna charr	Asia
S. kronicus	Stone charr	Asia
S. anaktuvukensis	Angayukaksurak charr	Asia
Trout		
Oncorhynchus mykiss	Rainbow trout	Pacific Ocean
O. clarki	Cutthroat Trout	North America
O. aquabonita	Golden trout	North America
O. apache	Apache trout	United State
O. gilae	Gila trout	United state
O. chrysogaster	Mexican Trout	Mexico
O. chrysogaster	Rio Mayo Trout	Mexico
Salmo trutta	Brown trout	Europe, Asia
S. obtusirostris	Soft mounth trout	Turkey
S. marmoratus	Marble trout	Italy and Southern Balkans
S. letnica		Macedonia and Albania
S. ischchan	Sevan trout	Armenia

(Despammier, 2016)

Some strains and even some species are endangered, some are supported entirely by hatchery production, and many strains have become extinct. There are several faces for Aquaculture of Salmonids including food production on salmon and trout farms, ocean ranching (release of hatchery fish into the ocean for later capture), and conservation

hatcheries designed to preserve threatened strains. Some hatcheries produce fish solely for sports. Farmed salmon and trout production has increased dramatically over the last 2 decades and now a day equals worldwide harvest in biomass and with a significantly higher value. Some exhibit extended ocean migrations and spend very little time in fresh water (e.g. chum and pink salmon) while other species spend only a short time in the ocean (e.g. the anadromous cutthroat trout and some Arctic charr which go to sea only for the summer and stay in coastal waters near the home stream). A few species or strains never swim in ocean waters, for example the lake charr (*S. alpinus*) and the resident (fresh water) rainbow trout (*O. mykiss*).

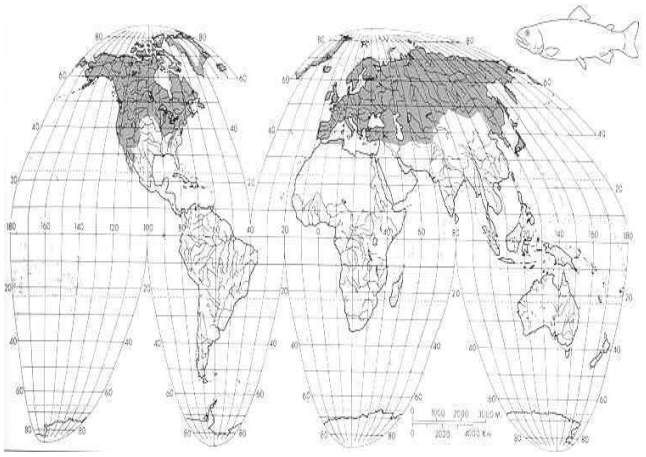


Figure 1.1: Distrubution of salmonid in the world

According to the IUCN Red List there are 2271 species of fishes extinct in the wild or threatened to different extent (IUCN 2015). In Italy, a high number of freshwater fishes are considered autochthonous taxa (Gandolfi et al., 1991; Zerunian, 2002; Kottelat and Freyhof, 2007) with a high number of endemic and sub-endemic species.

In Italy, the nomenclatural confusion and lack of taxonomic updating of Italian endemic or native species, still considered to be of Danubian or transalpine origin, have allowed the legal introduction of central European fish stocks, mainly under the name barbels and chubs, as well as various genera of carps and crucian carps, with all the consequences of introduced alien species. This has reduced the Po basin to a branch of the Danube, and the Tuscany-Latium basin to a dominant complex of the Po and Danube species (Bianco, 1995a; Bianco, 1998). Thus, updating the taxa is of a necessity to determine the conservation status of native species, especially where genetic analyses have revealed unique haplotypes.

Genus Salmo**:** Some 17 taxa were reported by Kottelat and Freyhof (2007) to be in Italy and northern Mediterranean areas, but this was probably an overestimation. In fact, while the taxonomy position of *S. marmoratus* can be clarified, the Mediterranean brown trout complex deals with recent isolations, as shown by molecular genetic studies conducted in Italy and the Mediterranean in an attempt to characterize and clarify their taxonomic position (Giuffra et al., 1994, 1996; Bernatchez, 2001; Caputo, 2003; Lorenzoni et al., 2004; Lucentini et al., 2006). Moreover, the Mediterranean brown trout taxa represent a complex of ecophenotypes recurring in similar environmental situations with variable biology, not unequivocally separable from one another. A further complication is that even the trout relegated to rivers tend to present homing behavior, forming populations that assume habitat-dependent colour patterns, and tending to form reproductively isolated populations with local genetic differentiation (Ryman et al., 1979; Halvorsen and Stabell, 1990). In this context, we give a short description of genus Salmo in Italy.

Salmo cettii Rafinesque Schmaltz, 1810: 32. Type locality: Val Demone and Val di Noto, Sicily. This species, representative of the Tyrrhenian haplotypes lineage of the brown trout complex, is a senior synonym of *S. macrostigma*, considered a possibly endemic trout to the Maghreb area (Kottelat and Freyhof, 2007). The native trout population originally inhabiting the Biferno River basin such as the Adriatic and Tyrrhenian basins in the Molise region (South Italy) belongs to the Mediterranean brown trout species. It is reported as critically threatened and endangered under the taxonomical term Salmo trutta macrostigma by IUCN (1996). Italian populations of Mediterranean brown trout are currently listed in the Italian IUCN Red List (Bianco et al., 2011) as "critically endangered" under the scientific name Salmo cettii.

Salmo marmoratus Cuvier, 1829: Type locality: lakes of Lombardy.

Salmo cenerinus Chiereghin, 1847: Type locality: Chioggia lagoons and connecting rivers.

Salar genivittatus Heckel & Kner, 1858: Type locality: Sala brook, Isonzo river drainage.

Trutta adriatica Kolombatovic, 1890: Type locality: Adriatic Sea at Vranjic, Croatia.

Salmo ghigii Pomini, 1941: Type localities, Sagittario and Gizio rivers (Pescara-Aterno River system), central Italy According to Kottelat and Freyhof (2007), there are no taxa described for Italy that represent the Adriatic lineage of brown trout. Nevertheless, various taxa have been described for the eastern coast of the middle and upper Adriatic Sea. Gridelli (1935) considered the existence of three distinct species of brown trout in Italy under the name *Salmo trutta*. One was the 'Sagittario River trout', very different from the Danubian *Salmo trutta*, especially in the number and conformation of the

vertebrae (Henking and Altnoeder, 1931); it was subsequently described by Pomini (1941) as a separate species, *Salmo ghigii*.

1.1.3 Farming Methods

There are several methods for culturing salmonid species; employing varying levels of infrastructure and manpower (Neil, 2013). Pond and flow through (raceway or tank) systems typically require intermediate levels of infrastructure and Personal. Both methods have been since quite a long time, going back as far as the hatcheries and farms of the 19th century and were typically located in close proximity to a natural water source, either fresh or marine water respectively. Pond farms are more enclosed than flow through systems, the latter relying on diverted water from a waterway, such as a stream, river or well. The water is diverted through manmade channels (earthen or concrete) containing the fish before typically being treated and returned to the source. Flow through systems are utilized by fish farmers in the United States to raise rainbow trout but are heavily regulated and monitored by the government with regard to water quality and pollution (Neil, 2013). Pond systems, which also utilize natural water sources, typically use less water and are better equipped to contain and treat the waste water. Typically, pond/raceway facilities are used as hatcheries in the culture of anadromous salmonids; to produce smolts from fertilized eggs as opposed to raising fish to Technical Report Culturing salmonids in RAS systems 6 marketable size (Anon, 2004). In the case of Atlantic salmon juvenile fish are regarded as smolts when they have undergone a physiological transformation which includes the development of a silvery coloration. This usually takes place in the spring, typically when 12 - 18 months old. At this point in anadromous species the smolts are ready to move to the marine environment

and are transferred to floating sea cages or net pens (Anon, 1980). These are typically located in sheltered coastal waters, e.g. Scottish sea lochs and Norwegian Fjords, can be square or circular and range considerably in volume with the largest housing up to 90,000 fish. In purely freshwater strains of salmonid, i.e. cultured non-anadromous Arctic charr, these pens/cages are positioned in freshwater lakes. The fish are grown in these cage pens, using pelleted feed, until they reach a marketable size, typically after 12 - 24 months. The length of this growth period is dependent on numerous factors including water temperature, stocking density (generally 8-18kg per m3), parasite load and feed conversion rates (FAO, 2013).

1.1.3.1 Raceways systems

Cold water species have been cultured in raceways that take advantage of large water supplies in locations where space is limited. Making the most of a large water resource, water is often serially reused as it flows by gravity from raceway to raceway that are stair-stepped down a hill side. However, water quality deteriorates as it moves from one raceway to the next. Dissolved and particulate wastes accumulate in the water column. Capture of particulate wastes is relatively inefficient in a raceway system due to the short retention time. In addition, managing particulate wastes within raceway systems and their quiescent zones can account for 25% of farm labor (Steven, 2004; IDEQ, 1998). More stringent water pollution control and water use permitting, as well as limited availability of large high-quality water resources, have recently increased interest in alternate fish culture system designs that can economically sustain, or even increase, fish production levels using less water and achieving better waste capture efficiencies(Bostock, 2010; Zohar et al, 2005., Steven,2004).

1.1.3.2 Flow-through systems

Feed is the main source of the direct and in direct production of pollutants in aquaculture process waters. For instance, it is known that salmonids retain only about 30% feed nitrogen (N) and phosphorus (P), if all the feed is consumed (Roger, 2005; Ramseyer and Garling, 1997). The presence and subsequent dissociation of uneaten feed and metabolic byproducts (fish fecal matter) result in both dissolved and suspended waste products which exert an oxygen demand on receiving waters and increase nutrient (nitrogen and phosphorus) loading. Consequently, facilities in which more fish are reared are in need of more feed, and subsequently have higher levels of this product. Current approaches to waste management in flow-through aquaculture systems include the use of sedimentation to produce clarified effluent and to concentrate biosolids (Hinshaw and Fornshell, 2002). Other approaches such as the reduction of dissolved pollutant loading through diet optimization have also been studied (Gatlin and Hardy, 2002; Cho, 1997).

1.1.3.3 Offshore systems

Offshore aquaculture installations have been drawing increasing attention from researchers, industry and policy makers as a promising opportunity for large-scale expansion of the aquaculture industry. This type of production system is based on the management and harvest of marine species in their natural environment, enclosed in specially built structures (cages, rafts). Main species produced are high valued species (rainbow trout (*Oncorhynchus mykiss*), yellowtail (*Seriola lalandi*), gilthead sea bream (*Sparus aurata*), seabass (*Dicentrarchus labrax*), and cobia (*Rachycentron canadum*)) as well as an increasing number of omnivorous species (carps, tilapias, catfish). Currently

there is a wide variety of culture systems used, from artisanal systems used in some Asian countries, to the high tech systems used in Europe and the Americas (Tacon and Halwart, 2007).

1.1.4 Management

The success of fish farming depends on good management, which is related to a comprehensive understanding of the biology of fish cultured and its aquatic environment. Successful fish health management begins with prevention of diseases rather than treatment. The containment of disease outbreaks is accomplished through good water quality management nutrition and sanitation without this foundation it is impossible to prevent outbreak of opportunistic disease with the introduction of new exotic fish species in aquaculture, there is always a risk of pathogen transfer, disease incursion and subsequent outbreaks of disease in the existing aquaculture systems. even though disease diagnosis and control program have generally tended to focus on more intensive aquaculture systems, it is known that small scale rural aquaculture system are also prone to disease. Fish problems have a negative impact on the livelihood of rural fish farmers and their dependents through loss of production, income and assets (Ackerman, 2008).

1.1.5 Feeding strategy

Feed and feeding are among the most important factors influencing growth, feed conversion efficiency and tissue composition of the fish in intensive culture. Thus, much work has been carried out to elucidate nutritional requirements, feeding rates, growth and feed conversion ratios of salmonids (Okumuú, 2002). The feed production such as (foundation of feed raw materials, nutrient content, process ability of the raw material,

sources of supply-continuity, its price, feed formulation and techniques of production etc.) is pivotal for a sustainable growth of the aquaculture industry (Demir. 2011; Yavuzcan et al., 2010). In developing countries most production is realized from semi-intensive practices, pond-based or open-water extensive, improved extensive and using polyculture farming technologies. Fish and other aquatic animals have specific growth, health and reproduction that are primarily dependent upon an adequate supply of nutrients, irrespective of the culture system in which they are kept. The supply of (feeds, fertilizers, etc.) has to be ensured so that the nutrients and energy requirements of the species under cultivation are met and the production goals of the system are achieved. Newly the study in aquaculture highlights the need to optimize feed production and on-farm feed management practices. Firstly its analysis is based on country- and species-specific case studies and regional and specialist-subject reviews. To have profitable production, providing fish farmers with well-balanced feed at cost-effective prices is a prerequisite. Formulation issues, and in particular the provision of species-specific feeds that meet the nutritional requirements of different life stages of the farmed species, remain important topics for both commercial and farm-made feed production sectors.

1.1.6 Water quality

Fish is an inexpensive source of protein and an important cash crop in many regions of world and a foudamental physical substrata of aquatic ecosystems in which they carry out their life functions such as feeding, swimming, breeding, digestion and excretion (Bronmark and Hansson, 2005). Water quality is determined by various physic-chemical and biological factors, as they may directly or indirectly affect its quality and consequently its suitability for the distribution and production of fish and other aquatic

animals (Moses, 1983). Many workers have reported the status of aquatic ecosystems (lentic and lotic) after receiving various kinds of pollutants altering water quality characteristics (physical, chemical and biological). All living organisms have tolerable limits of water quality parameters in which they perform optimally. A sharp drop or increases within these limits have adverse effects on body functions (Kiran, 2010). So, good water quality is very essential for survival and growth of fish.

1.1.6.1 Water Quality Variables

Water qualities determine performance and subsequently influence survival rates. Fish influence water quality through processes like digestion interfering with the nitrogen balance in the water and respiration. Knowledge of testing procedures and interpretation of results are important. Some water quality factors are more likely to be involved with fish mortality such as dissolved oxygen, temperature, and ammonia. Others, such as pH, alkalinity, hard- ness and clarity affect fish, but usually are not directly toxic. Each water quality factor interacts with and influences other parameters, sometimes in complex ways. What may be toxic and cause mortalities in one situation can be harmless in another. The importance of each factor, the determination method and frequency of monitoring depends upon the type and rearing intensity of the production system used (Bhatanagar, 2013).

1.1.6.1.a Temperature

Salmonid do well in water temperatures ranging from 1°C to 20 $^{\circ}$C, but usually grow most rapidly between temperatures of 10°C and 18°C depending on the species and strain and life stage. The high levels of dissolved oxygen ideally close to saturation,

concentrations ranging from 8 parts per million to 12 parts per million depending on temperature and altitude is essential to them. Salmon are very different from eels, carp, bass, and many other fish because the hemoglobin in the salmonid's red blood cells is designed to work well at high levels of dissolved oxygen, but not at lower levels that create stress (Pannell, 2017).

1.1.6.1.b Salinity

Salmonids are euryhaline, meaning they can withstand and do well over a wide range of salinity, but they cannot have activity in marine waters except that certain conditions are met (this may involve smooth status, fish size, and temperature). As previously mentioned, most species are anadromous. There is a constant influx of water through the gills into the fish's tissues in fresh water. This is pumped back out by the kidney (located near the backbone of fish), which produces copious amounts of dilute urine (up to 1/3rd of the salmonid's body weight per day). Special cells on the gill filaments move salts into the bloodstream to create for salts washed out in the urine (Pannell, 2017).

1.1.6.1.c Dissolved oxygen

Dissolved oxygen (DO) levels in a culture system must be maintained above levels considered stressful to fish. Warm water fish can tolerate lower DO concentrations than cold water fish. DO tolerance for cool water fish is between that exhibited by warm and cold water fishes. Excessively low DO concentrations, less than 1 - 2 mg/L, will kill fish. Prolonged exposure to low, nonlethal levels of DO constitutes a chronic stress and will cause fish to stop feeding, reduce their ability to grow, and make them more susceptible to disease. In northern climates, iced- over ponds with deep snow cover can experience

winter kill as oxygen is depleted since diffusion and photosynthesis can't replenish DO used by fish and other organisms in winter Super saturation of oxygen (and nitrogen). Bubble gas disease, which is the accumulation of gas in the blood or tissues. Gas bubble disease can result in erratic behaviour, stress, and death (Bhatnagar, 2013).

1.1.6.1.d pH

Fish survive and grow best in waters with a pH between 6 and 9. If the pH is outside this range, fish growth is reduced. At a pH level below 4.5 (in fact salmonids are known to be acid-sensitive fish), or above 10, mortalities may occur. As pH increases, the proportion of the total ammonia nitrogen (TAN) in the toxic form, NH3, increases. As pH decreases, the solubility of heavy metals and, therefore, their toxicity also increases. Portable, hand-held pH meters are reasonably priced and facilitate quick, accurate measurements (Boyd, 1992).

1.1.6.1.e Carbon dioxide

An excess of carbon dioxide in the water can cause fish to become fatally sedated. In addition, free carbon dioxide can decrease the pH of the water. Only when using groundwater, transporting fish at high densities or in RAS with oxygenation (as opposed to aeration) are carbon dioxide problems likely to develop. At high concentrations, carbon dioxide causes fish to lose equilibrium, become disoriented, and possibly die (Tessa, 2014).

1.1.6.1.f (Nitrogenous) Wastes

Metabolic wastes include ammonia, nitrites (NO2-), and nitrates (NO3-). Most fish and freshwater invertebrates excrete ammonia as their principle nitrogenous waste. In culture systems, toxic ammonia co-exist with the nontoxic ammonium ion (NH4+) and their collective sum is expressed as Total Ammonia Nitrogen (TAN). The amount of TAN in the toxic form increases dramatically as the pH rises above 7.5 and less so as temperature increases. Fish continuously exposed to more than 0.02 mg/L of NH3 may exhibit reduced growth and increased susceptibility to disease. NH3 concentrations greater than approximately 0.02 mg/L can be lethal for some species, with cold-water fish generally being more susceptible to NH3 toxicity than warm water fish. When fish are cultured intensively and fed protein-rich diets they can produce high concentrations of ammonia in the water. Ammonia and other metabolic wastes are gradually removed by natural processes in ponds or through the use of biological filters in RAS. Ammonia is removed by bacteria that initially convert it into nitrite and subsequently into nitrate. Nitrite (NO2-) is toxic to fish at approximately 1 mg/L (though this varies greatly depending on the species, with the chloride content of the water) (Oliver, 2008).

1.1.6.1.g Biological oxygen demand

Biological oxygen demand (BOD) is a very important parameter in estimating the state of organic pollution of the water body. BOD is not a pollutant and exercises no direct harm but it may cause an indirect harm by reducing DO concentration levels detrimental to fish life and other beneficial uses. BOD represents that fraction of dissolved organic matter which is degraded and easily assimilated by bacteria. BOD indicates the presence of

biodegradable organic matter quantitatively, which consumes DO from water. The higher values of BOD produce obnoxious smell and unhealthy environment. A higher value of BOD is due to favourable environmental conditions for microbiological activities at higher temperature (Tamot et al., 2008) BOD is directly linked with decomposition of dead organic matter present in the water and hence the higher values of BOD can be directly correlated with pollution status and has an inverse relation with DO concentration. The BOD values were observed between 0 and 4.0 mg/Lin Hathaikheda reservoir in Bhopal (Namdev et al., 2011). Tamot et al. (2008) reported that the BOD value ranged from 3.2 to 6.8 mg/l in Halali Reservoir. High values of BOD were usually observed near the bottom of the cage aquaculture site where nutrients and organic matter from the fish, excess feed and waste accumulated, which resulted in high oxygen demand and in dry season, when water temperature increases, the rate of decomposition increases (Namdev et al., 2011).

1.1.7 Pathogens

The increasing demands in aquaculture systems world-wide has provided new opportunities for the transmission of aquatic viruses and the occurrence of viral diseases remains a significant limiting factor for aquaculture production and for the sustainability of biodiversity in the natural environment (Crane, 2011).A vast array of aquatic animal species is farmed in high density in freshwater, brackish and marine systems where they are exposed to new environments and potentially new diseases. On-farm stress may compromise their ability to combat infection, and farming practices facilitate rapid transmission of disease. Viral pathogens, whether they have been established for decades or whether they are newly emerging as disease threats, are particularly challenging since

there are few, if any, efficacious treatments, and the development of effective viral vaccines for delivery in aquatic systems remains elusive(Ramstad, 2008).

1.1.8 Stress in aquaculture

The ability of an organism to survive stems from its capacity to respond and adapt to challenges from the environment. Stress therefore, is the organism's response to these sensed changes. Without stress, there would be no coordination of an organism's response to changing environmental conditions. The concept of stress was first documented by the French physiologist Claude Bernard (1813-1878) in his description of milieu “intérieur” to describe an organism's physiological capacity to maintain a consistent "environment within" in the presence of changing external conditions. Later this concept was adopted to describe what we now know as the term homeostasis by American physiologist Walter Cannon (1871-1945). While previous descriptions of homeostasis explained the ability of organisms to maintain a consistent range of physiological set points, they did not include the elucidation of the adaptations required by an organism to maintain homeostasis. Sterling & Eyer (1988) then put forth the notion of allostasis to define how organisms respond to the perpetual encounter with physiological and behavioral variability, an ebb-and-flow, and thus stability of life is achieved through variability. Stress is classically known as "any challenge to homeostasis" as first defined by Hans Selye (1907-1982), but has since evolved to include the concept of allostasis to characterize how the processes of stress leads to changing homeostatic set points through physiological variability. Allostasis achieved through the stress response is essential to the maintenance of homeostasis throughout the numerous challenges to survival during an organism's life, which impacts how that

organism and future generations respond thereon. The considerable level of consistency of this general process among vertebrates implies that the coordinated effort of a stress response is a common link among species which is crucial for any organism's survival and fitness. Different sources of stress are recognized and include: physiological stress, activity or exercise induced stress, psychological stress, stress arising from pathological conditions, environmental stress, and acute vs. chronic stress. Stress has complex situation because of the hypothalamic-pituitary-adrenal (HPA) axis response to stress has evolved to aid vertebrates to cope with stressors, but animals kept by humans are subjected to stressors that they either would not normally encounter such as transportation, or are exposed to stressors over much longer periods than would normally be the case, such as restrictive housing conditions. To animal welfare stress is generally regarded as antipathetic. The above stressors are categorized as either acute or chronic depending on their duration and frequency. Acute stressors are events which the animal experiences for a short period of time such as handling. Chronic fish stressors are defined as a constant or recurring exposure that causes a prolonged physiological response. Stressors of both types can have severe negative effects on the vertebrates growth and health. In a review concerned with the scientific evaluation of animal welfare in general, and of laying hens in particular, Barnett and Hemsworth (2003) adopt the position of Broom (1986) that: ‘‘The welfare of an individual is its state as regards its attempts to cope with its environment’’. They define ‘‘its attempt to cope’’ as biological responses including but not limited to immunological, physiological and behavioral responses, which they term the ‘‘homeostasis approach’’. Anyway, they point out that, although

there is general acceptance of this approach for assessing welfare, a lack of biological responses to a welfare issue is often attributed to inadequate methodology.

1.1.9 Nets and seines

Using of nets, seines or hands for moving fish to new aquatic enclosures are common fish-handling practices. If handling, seining, and netting are not done properly, it disrupts the protective mucus and fish scales, thereby increasing the susceptibility to parasitic or pathogenic invasion (Conte, 2004). Fish-handlers should always use wet hands or wear soft, wet gloves when handling fish to avoid of disrupting of mucus, the appropriate makeup of netting varies with species, and depending on presence or absence of scales and scale type, either a continuous or knotted mesh should be used. Knotted mesh can dislodge fish scales resulting in parasitic and pathogenic invasion. Seining soil-based ponds can also disturb the substrate, and if not done correctly and with proper equipment can result in adverse water quality (Tucker, 1985; Tucker and Robb, 2003). The most stress results from rough handling. All fish-handling processes should be slow and deliberate so as not to increase the natural avoidance reactions of fish, which can lead to excessive activity and potential exhaustion. The impacts of fish exhaustion, rates of recovery and their relation to fish welfare are addressed by Schreck (2000, 1990) and Schreck et al. (1997).

1.1.10 Lift nets and fish pumps

When fish are loaded into lift nets and cages, and lifted from the water, distress and injury most often occur. Excessive gravitational weight loading on fish positioned at the bottom of the net can cause injuries from compression and spine injury from adjacent

fish. Load weight should be adjusted to prevent excessive stress and mechanical injuries. It is often more efficient and less stressful to move fish between tanks or ponds by moving the water along with the fish. Although fish can be safely moved and harvested using nets, harvesting and moving fish in water by use of fish pumps or transfer pipes appears to be the least invasive means of moving fish. This has been accomplished with crustacean culture (Parkeret al., 1974), and is also a common practice today with some finfish species (Conte, personal observation).

1.1.11 Acclimation and timing

When fish are transported, the characteristics of the receiving water should be matched as closely as possible to the source water. Stress can occur if changes in water temperature and quality are abrupt, even when values are within the tolerance range of the species; and definitely when they are outside of the range (Wedemeyer, 1997). To prevent abrupt changes, the fish can be acclimated by gradual water exchange between the source water and the receiving water. Temperature has a profound effect on response to handling. Seining and netting activities should be performed during the cooler period of the day, as water has less capacity to hold oxygen at higher temperatures (Wheaton, 1977; Lawson, 1995). In many large pond operations in desert areas, seining is initiated at night and finished in the early morning to avoid handling fish during periods of extreme daytime temperatures. If fish are handled during maximum heat, the associated stress often results in mortality in both the short and long-term. Temperature-related stress can occur any time fish are handled, but fish are especially vulnerable during periods of summer heat (Piper et al., 1982).

1.1.12 Transport

Multiple-phase operation that should be designed to minimize stress is fish transporting (Piper et al., 1982). Fish are stopped feeding for about 24 h prior to harvest and transport so that they do not void feces and foul the transport water. They are often kept in holding tanks during this period, which allows them to recover from any previous handling. Fish are then transferred to a tanker using lift nets or pumps, driven to and off-loaded at the delivery location. Transporting fish by tank truck requires special care to ensure that water quality and temperature requirements are maintained, or even compensated for in the event of elevation changes, which affect the oxygen holding capacity of water. Contemporary long distance tankers are insulated and equipped with chillers, carbon dioxide strippers, anti-foam agents, water buffers, circulation pumps, and oxygen sources. Short-haul tank trucks are usually equipped with ice, circulation pumps, and anti-foam agents. Much of the research on stress in fish was aimed at overcoming the challenges of harvest and transportation. Fish display a wide variation in physiological responses to stress, and genetic history appears to account for much of the inter-specific variation (Barton, 2002). Plasma cortisol elevations can differ by as much as two orders of magnitude among different species of fish following identical stressors. Thus, some fish transport easily and with few precautionary steps taken other than the fundamentals of maintaining water quality and temperature. Others species require specific precautionary steps to prevent ionic and osmotic imbalances that result in mortality (McDonald and Milligan, 1997). For example, species such as striped bass (Conte, personnel observation) and black bass (Carmichael et al., 1984a, b; Carmichael and Tomasso, 1988) require exposure to pre-salted water before and after transport to

withstand the combination of stressors and fully recover at final destination. Fish producers are aware that whenever fish are handled or transported, there is a temporary decline in weight (Conte, personal communication.) Therefore, they generally avoid repeated handling and allow fish a recovery period after handling and transport. The fish's primary physiological responses to acute netting, handling and transport recover in 6 h to 1 day. However, physiological recovery may take from 10 days to 2 weeks if the stressors persist, but are not lethal (Schreck, 1981; Schreck et al., 1997).

1.1.13 Breeding and reproductive technology

Stress adversely affects the reproductive performance of teleost fish (Billard et al., 1981). Commercial growers believe that the process of spawning fish is stressful, and post-spawn fish are given special feeding and environmental attention to assure recovery and reconditioning for use as future brood stock (Conte, personnel communication). Fish species vary in their reproductive behavior and success when placed in artificial habitats. Some species such as tilapia (i.e. Oreochromis spp.) reproduce so readily, even under high-density culture conditions, that systems and culture techniques are designed to retard maturation and spawning events (Belarian and Haller, 1982; Guerrero, 1982). Other species such as sturgeon (*Acipenseridae*) require spawning induction and assistance to achieve reproductive success, even when cultured at the lowest density (Conte et al., 1988). Consequently, different approaches are used to manage reproduction for different species of fish and some examples are presented in context. In nature, channel catfish spawn in natural crevices and the male guards the eggs. In culture, both sexes are stocked at very low density in pond systems containing dispersed sheltered spawning habitats and at a higher ratio of males to females. Males and females form pairs

and spawning and fertilization events are controlled by the fish. Eggs or fry are then collected from the spawning shelters (Busch, 1990). Wild rainbow trout usually migrates to specific riffle areas in side streams off of larger bodies of water to spawn. Since it is not practical to duplicate this in production aquaculture, reproduction of farmed trout is usually achieved by maintaining separate-sex populations in raceway systems. When ready to spawn, the fish are individually removed, hand stripped for their eggs and milt, and then milt from several males is used to fertilize the eggs from a single female (Stevenson, 1987). Breeding of other fish such as Atlantic salmon more closely resembles that of rainbow trout. Brood stock are usually maintained in net pens at moderate density, and later each sex is hand stripped of gametes that are used for fertilization (Sedgwick, 1988) Striped bass are usually induced to spawn using gonadotropin injections followed by strip spawning. However, when two mature males and a single mature female are placed in a 5 ft. diameter tank, they will exhibit courtship behavior, which will induce female spawning to release her eggs (Harrell et al., 1992; Conte, personal observation).

1.1.14 Physiological response to Stress

Generally, stress in fish is a non-specific response classified as three distinct responses: Primary response that is characterized by the activation and the secretion of the hormones, corticosteroids (cortisol) and catecholamine, into the blood. Secondary response which is the release of hormones triggers the secondary response which involves the release of glucose into the blood for energy production, followed by increases in heart rate, gill blood flow and metabolic rate which causes changes to blood lactate and hematocrit and tertiary response is the changes in blood physiology ultimately

cascade into a whole body change or the tertiary stress responses. Changes associated with the tertiary stress response include reduced growth rate, decreased disease resistance, altered behavior and reduced survivability (Bruce, 2002). Hans Selye once defined stress as ‘‘the nonspecific response of the body to any demand made upon it’’ (Selye, 1973). A common misconception among fishery biologists is that stress, in itself, is detrimental to the fish. This is, however, not necessarily the case. The response to stress is considered an adaptive mechanism that allows the fish to cope with real or perceived stressors in order to maintain its normal or homeostatic state. Quite simply, stress can be considered as a state of threatened homeostasis that is re-established by a complex suite of adaptive responses (Chrousos, 1998). If the intensity of the stressor is overly severe or long- lasting, however, physiological response mechanisms may be compromised and can become detrimental to the fish's health and well-being, or maladaptive, a state associated with the term ‘‘distress’’ (Selye, 1974; Barton and Iwama, 1991) and the important concern of managers and aquaculturists. Response differences to stressors are clearly evident among closely related fish species and such differences appear to be consistent. Barton (2000) and Ruane et al. (1999) both showed that brown trout (*Salmo trutta*) exhibited greater cortisol increases after brief handling and short-term confinement, respectively, than did rainbow trout (*Oncorhynchus mykiss*). This difference was also consistent with glucose responses between these two species. Similarly, both McDonald et al. (1993) and Barton (2000) found that lake trout (*Salvelinus namaycush*) were more sensitive to a transport stressor than brook trout (*Salvelinus fontinalis*), a closely related char species. A few studies have subjected fish to continuous severe stressors in an attempt to characterize maximum corticosteroid

responses to stress. Plasma cortisol in sturgeons and paddlefish reached maximum levels of about 13 and 60 ng/ml, respectively, when subjected to severe continuous confinement accompanied by handling (Barton et al., 1998, 2000), but in juvenile rainbow trout, this plateau was about 160 ng/ml using the same experimental protocol (Barton et al., 1980). In similar studies, peak plasma cortisol concentrations exceeded 500 ng/ml in juvenile chinook salmon (Strange et al., 1978) and approached 1,400 ng/ml in striped bass (Noga et al., 1994), further emphasizing the wide variations in stress responses apparent among fish species. Most fish species tested show their highest plasma increase in cortisol within about 0.5–1 hr after a stressful disturbance (Barton and Iwama, 1991), but there are exceptions to this general pattern. Vijayan and Moon (1994) found that circulating cortisol in the sea raven (*Hemitripterus americanus*), a sedentary, benthic marine fish, took about 4 hr to reach its peak level of about 260 ng/ml following an acute stressor. Those authors suggested that the slow rate of response to the stressor may help conserve energy in a normally in- active species having a slow metabolic rate. Differences in corticosteroid stress responses also exist among strains or stocks within the same species (Iwama et al., 1992; Pottinger and Moran, 1993), their hybrids (Noga et al., 1994), and between wild and hatchery fish (Woodward and Strange, 1987). Within a single strain or population, variation in stress responses also has a genetic component (Heath et al., 1993) and some fish may be predisposed to consistently exhibit high or low cortisol responses to stressors (Pottinger et al., 1992b; Tort et al., 2001), a pattern that appears to have a behavioral correlate (Øverli et al., 2002). The tendency for major differences in stress responses between and among taxa is a trait that ap- pears to be at least partly heritable (Fevolden et al., 1991; Fevolden and Røed, 1993; Pottinger et al., 1994). Fevolden et al.

(1999) estimated a heritability value of 0.56 for the plasma cortisol increases measured in adult rainbow trout after being exposed to three stressful events, each spaced more than 1 mo apart. Similarly, Tanck et al. (2001) recently attempted to calculate heritability estimates for stressor-induced plasma cortisol elevations in common carp (*Cyprinus carpio*) and determined, with reservation, a relatively high mean heritability value of 0.60 for an androgenic stock. It is unclear, however, whether fishes that display relatively high or low corticosteroid stress responses are actually ‘‘more or less stressed’’ than others or simply have different capacities to respond to stressors. Differences in physiological mechanisms that would account for wide variations remain largely unexplored, but Pottinger et al. (2000) found recently that high cortisol levels exceeding 1,500 ng/ml in chub (*Leuciscus cephalus*) following a disturbance were associated with low corticosteroid receptor affinity. The teleostean hypothalamic– pituitary– interrenal (hpi) axis is a system comparable with the mammalian stress axis (hypothalamus- pituitary-adrenal; hpa), as a result of convergent evolution (Wendelaar, 1997; Mommsen, 1999) and it is of utmost importance in stress regulation as well as for the adaptation and/or acclimation of fish to their dynamic environment. In teleostean fish, cortisol is the principal corticosteroid and plays an important role in a number of physiological processes including growth, immune regulation, maintenance of energy balance, and reproduction (De Jesus, 1991; Vazzana, 2002, Wedelaar, 1997). During HPI axis activation corticotropin-releasing factor (CRF), produced in the hypothalamic preoptic area (POA), stimulates the pituitary gland corticotropes to secrete adrenocorticotropic hormone (ACTH), which regulates cortisol synthesis and secretion. In teleosts, cortisol plays also a vital role in the maintenance of hydromineral balance, as fish cannot

synthesize aldosterone, and cortisol carries out this mineralocorticoid function (Mc Cormick, 2008; weselaar, 1997). Cortisol enters by passive diffusion into the cells where its action is mediated by the Glucocorticoid receptor(s) (GR) and the mineral corticoid receptor (MR) (Prunet, 2006), a classofligand-activated transcription factors. During larval development, marine teleosts undergo dramatic changes in morphology, growth and metabolism in order to accomplish their metamorphosis into juvenile fish. Throughout this period, cortisol regulates osmoregulatory function (Ayson, 1995; Lin, 1999) and is implicated in the metamorphosis from larvae to juveniles (Tsalafouta, 2014). The vertebrate neuroendocrine stress axis (hypothalamus–pituitary–adrenal or internal; hereafter the HPA axis) is an ancient physiological system that plays a pivotal role in mediating organismal responses to environmental change. In all vertebrates that have been studied, neurosecretory neurons in the hypothalamus synthesize neuropeptides of the corticotropin-releasing factor (CRF) family that acts on the pituitary gland to stimulate the release of corticotropin (adrenocorticotropic hormone; ACTH). Corticotropin is a small peptide hormone (39 amino acids) derived by proteolytic processing of the precursor protein proopiomelanocortin (POMC); it is secreted into the systemic circulation and binds to receptors expressed in adrenocortical cells to increase the biosynthesis of corticosteroids (Danver, 2009). Corticosteroids are the primary "stress hormones" of vertebrates and in mammals have been classified into two groups, the glucocorticoids (GCs) and the mineralocorticoids, owing to their often distinct physiological functions. Such distinctions are less clear in nonmammalian species (e.g., in teleost fishes cortisol, a classical GC in humans, plays important roles in hydro mineral balance) (McCormick, 2006). Corticosteroids have diverse actions in development,

physiology, and behavior in vertebrate species. They are well known to influence development of the brain, lungs, and other organ systems; to mobilize stored energy, inhibit energy storage, and stimulate gluconeogenesis; to stimulate feeding behavior in order to replenish depleted energy stores following a stress response; and to influence learning and memory consolidation. A major, and perhaps primitive, role for corticosteroids is in osmoregulation. In all vertebrate species studied, corticosteroids have been shown to influence plasma ion concentrations. In teleost fishes cortisol (fish do not make the classical tetrapod mineralocorticoid aldosterone) stimulates sodium transport across epithelia of the gills, gut, and kidney and is essential for promoting seawater adaptation (McCormick, 2006). Corticosteroids also function in negative feedback at the level of the brain and pituitary gland to inhibit the secretion of CRF and ACTH and thus return the system to basal following a stress response. The corticosteroids bind to two receptor subtypes belonging to the nuclear hormone receptor (NR) superfamily: the type I [the mineralocorticoid receptor (MR)] and the type II [the GC receptor (GR)]. The type I receptor has higher affinity than the type II for corticosteroids, is selective for aldosterone, and thus mediates actions of this mineralocorticoid on hydro mineral balance (thus it is named MR); whereas the type II receptor plays a principal role in mediating GC actions during a stress response (thus it is named GR). The two receptors show region-specific expression in the brain, with the GR being more widely expressed than the MR. The differential affinities, specificities, and expression patterns have led to the hypothesis that the MR maintains basal activity of the HPA axis while the GR mediates negative feedback under rising GC concentrations in response to a stressor (De kloet, 1998). There is also evidence for corticosteroid receptors (CRs) located in the plasma

membrane that mediates rapid actions of these hormones. Studies of extant species show that many of the genes of the HPA axis are present in urochordates and cephalochordates; in fact, many stress-related genes predate the chordates, thus showing their ancient origins. Furthermore, the major functions of this axis in mediating developmental, physiological, and behavioral responses to environmental change are ancient features of the chordate lineage. Strong positive selection has maintained the structure and functions of this axis owing to the pivotal adaptive role that stress hormones play in individual survival (McCormick, 2006). In response to stressors, the hypothalamus-pituitary-internal (HPI) axis responds in a coordinated manner leading to the release of cortisol into the circulation in teleosts (Wendelaar, 1997). The hypothalamus is the site of initial stressor recognition, resulting in the release of corticotropin-releasing factor (CRF), which acts on the anterior pituitary gland to synthesize and release adrenocorticotrophic hormone (ACTH) into the circulation. ACTH is produced by posttranslational modification of protein encoded by the gene proopiomelanocortin (pomc) (Mommsen, 1999) ACTH binds to the melanocortin (Machluf, 2011) receptor (MC2R) on the steroidogenic cells of the internal tissue and activates cortisol biosynthesis and secretion (Machluf, 2011; Nesan, 2012). The internal tissue is analogous to the adrenal gland in higher vertebrates and cortisol is the primary circulating glucocorticoid in fish. Cortisol has a variety of effects in teleosts, but its most well-studied function in response to stress is to enhance metabolic capacity and mobilize energy stores to restore homeostasis (Machluf, 2011; Nesan, 2016).

1.1.15 Endocrine responses to stress in fish

When fish are exposed to a stressor, the physiological stress response is initiated by the recognition of a real or perceived threat by the central nervous system (CNS). The sympathetic nerve fibers, which innervate the chromaffin cells, stimulate the release of catechol- amines via cholinergic receptors (Reid et al., 1996, 1998). The chromaffin tissue (adrenal medulla homo- logue) is located mainly in the anterior region of the kidney in teleostean fishes (Reid et al., 1998). Because catecholamines, predominantly epinephrine in teleostean fishes, are stored in the chromaffin cells, their release is rapid and the circulating levels of these hormones increase immediately with stress (Mazeaud et al., 1977; Randall and Perry, 1992; Reid et al., 1998). The release of cortisol in teleostean and other bony fishes is delayed relative to catecholamine release. The pathway for cortisol release begins in the HPI axis with the release of corticotropin-releasing hormone (CRH), or factor (CRF), chiefly from the hypothalamus in the brain, which stimulates the corticotrophin cells of the anterior pituitary to secrete adrenocorti-cotropin (ACTH). Circulating ACTH, in turn, stimulates the internal cells (adrenal cortex homologue) embedded in the kidney to synthesize and release corticosteroids into circulation for distribution to target tissues. The internal tissue is located in the anterior kidney in teleosts and exhibits considerable morphological variation among taxonomic groups (Nandi, 1962), but is found throughout the kidney in chon-drosteans (Idler and O'Halloran, 1970). Cortisol is the principle corticosteroid in actinopterygian (i.e., teleostean, other neopterygian and chondrostean) fishes (Sangalang et al., 1971; Barton et al., 1998) whereas 1a-hydroxycorticosterone is the major corticosteroid in elasmobranchs (Idler and Truscott, 1966, 1967). Cortisol synthesis and release from internal cells has a

lag time of several minutes, unlike chromaffin cells, and, therefore, proper sampling protocol can allow measurement of resting levels of this hormone in fish (Gamperl et al., 1994). As a result, the circulating level of cortisol is commonly used as an indicator of the degree of stress experienced by fish (Wendelaar Bonga, 1997). Control of cortisol release is through negative feedback of the hormone at all levels of the HPI axis (Fryer and Peter, 1977; Donaldson, 1981; Bradford et al., 1992). Regulation of the HPI axis is far more complicated than this description implies, however (Bruce, 2002).

1.2 Thesis Objectives

The research presented in this thesis aims to study innovative approaches in aquaculture for Conservation and management of particularly threatened fish species. Different strains of brown trout (*Salmo trutta* and *Salmo ghigii*) have been taken into account as experimental models.

Objectives of this study are to:

1. Reproduction potential (sperm Quality and motility) during mentioned season, as well as, to start new investigations on reproductive biology of Mediterranean trouts to be applied to future breeding strategies concerning sperm quantity and quality to be managed with cryopreservation.
2. Investigate the growth indicator of Salmonid (new food ingredient such as biochar to compare the rate of growth at the end of experimental period on *Salmo trutta* larvae).
3. Probe the Health status of Salmoind, as stress resistance between wild type trout (*Salmo trutta*) and albino Mediterranean trout (*Salmo ghigii*).

Chapter 2. Comparison of sperm concentration during spawning season of different strains of Salmo trutta complex (*Salmo trutta, Salmo ghigii, Salmo cettii*) *and marble trout* (*Salmo marmoratus*)

2.1 Abstract

In Salmonid species, direct factors that affect sperm concentration are culture conditions, stripping time, age, feeding and presence of males. It is reported that inside culture conditions, temperature of water can strongly influence quality of fish sperm, especially duration of motility .This research was carried out to evaluate the quantity of fish milt in different Salmonid lineages; Atlantic brown trout (*Salmo trutta*), an albino strain of Mediterranean brown trout (*Salmo ghighii*), a pure Mediterranean lineage (*Salmo cettii*) and marble trout (*Salmo marmoratus*) and the aim of present research was to start new investigations on reproductive biology of Mediterranean trouts to be applied to future breeding strategies concerning sperm cell quantity and quality to be managed with cryopreservation. The samples were collected during spawning season considering two different periods between mid-December 2015 and mid-January 2016. In this research, data on brown trout and marble trout sperm concentration in a fully mature brood stock (5 years and older) were quantified. Data had showed no significant difference ($P > 0.05$) in species during the first stripping and second stripping during spawning season. In the present study the survival values of larvae showed no changes during successive stripping in cultured males. This demonstrates that the viability of eggs is more under the influence of egg quality than sperm quality.

Key words: sperm concentration, spawning season, Salmo trutta, Salmo ghigii, Salmo cettii and Salmo marmoratus.

2.2 Introduction

Studies of sperm motility of fish have been limited to species with a commercial interest in aquaculture or species involved in conservation programmes (Alavi and Cosson, 2006). However, in the last years studies on male reproduction have considerably increased (Trippel, 2003). Control of reproductive function in captivity is essential for the sustainability of commercial aquaculture production, and in many fishes with manage of manipulating photoperiod, water temperature or spawning substrate it can be achieved (Daras et al, 2017., Dzyuba, 2014., Mylonas, 2010).The fish farming industry has been more focused on the quality or eggs or larvae rather than that of sperm (Williot et al., 2000; Rurangwa et al., 2004).. In some species poor quality can be a limiting factor in their culture, however even when fertilization success is high differences in sperm quality in farmed fish may be affected by different components of brood stock husbandry, during collection and storage of sperm prior to fertilization or the fertilization procedure. Although other approaches for quantification of sperm quality have been suggested, motility is most commonly used since high assessment of sperm motility historical relied on subjective estimates of motility characteristics the value of which is questionable in predicting fertility (Rurangwa et al, 2004). The cycle of fish reproductive is separated in the growth (gametogenesis) and maturation phase (oocyte maturation and spermiation), both controlled by the reproductive hormones of the brain, pituitary and gonad. Even though in most fishes the growth phase of reproductive development is concluded in captivity the major exemption being the freshwater eel (*Anguilla spp*.), oocyte maturation (OM) and ovulation in females, and spermiation in males may require exogenous hormonal treatments. These hormonal manipulations are used as a management tool to

enhance the efficiency of egg production and facilitate hatchery operations in some fishes, but in others exogenous hormones are the only way to produce fertilized eggs reliably (Mylonas, 2010). Hormonal manipulations of reproductive function in cultured fishes have focused on the use of either exogenous luteinizing hormone (LH) preparations that act directly at the level of the gonad, or synthetic agonists of gonadotropin-releasing hormone (GnRHa) that act at the level of the pituitary to induce release of the endogenous LH stores, which, in turn act at the level of the gonad to induce steroidogenesis and the process of OM and spermiation. After hormonal induction of maturation, brood stock should spawn spontaneously in their rearing enclosures; by the way, in aquaculture conditions the natural breeding behavior followed by spontaneous spawning may be lost (Costantinos, 2010). For that reason, it is also necessary to employ artificial gamete collection and fertilization for many species. At the end, a common question regarding hormonal treatments is their effect on gamete quality, compared to naturally maturing of spawning fish. The main factors that may have significant consequences on gamete quality mainly on eggs and should be considered when choosing a spawning induction procedure and include (a) the developmental stage of the gonads at the time the hormonal therapy is applied, (b) the type of hormonal therapy, (c) the possible stress induced by the manipulation necessary for the hormone administration and (d) in the case of artificial insemination, the latency period between hormonal stimulation and stripping for fertilization (Mylonas, 2010). by the reason of that males may produce a lower amount of milt with lower quality, dysfunctions of reproduction of captive fishes are not restricted to females, despite they do undergo complete spermatogenesis and spermiation in captivity (Mylonas and Zohar, 2001b, 2007; Zohar

and Mylonas, 2001b).A serious problem for those species in which hatchery production that is based on the artificial fertilization and the acquisition of gametes by manual stripping is lower amount of milt production represents. Production can be limited by difficulties in acquisition of adequate milt from male breeders and may necessitate the use of a much higher number of male breeders than if spawning could occur spontaneously (Mylonas et al, 2010). Also for species that spawn spontaneously in the tank, the production of highly viscous milt reduces the rapid dispersal of the spermatozoa and thus reduces the sperm fertilization capacity (Vermeirssen et al., 2003). The levels of Lower plasma, LH during the spermiation period have been suggested as the cause of the reduced amount of milt produced by some fishes (Mañanos et al., 2002; Mylonas and Zohar, 2001a). The amount of LH in the pituitary or the ability of the pituitary to synthesize LH in response to treatment with exogenous GnRHa is not affected in these fishes, suggesting that again the reproductive dysfunction in the males may be identified in the brain control of GtH synthesis and/or release. In some species, poor sperm quality can be a limiting factor in their culture, however, even when fertilization success is high, differences in sperm quality between males when mixed sperm from multiple males is used may severely reduce the apparent population size and may affect the future genetic integrity of the stock. Sperm quality in farmed fish may be affected by different components of brood stock husbandry, during collection and storage of sperm prior to fertilization or the fertilization procedure. Although other approaches for quantification of sperm quality have been suggested, motility is most commonly used since high motility is a prerequisite for fertilization and correlates strongly with fertilization success. The assessment of sperm motility has historically relied on subjective estimates of

motility characteristics, the value of which is questionable in predicting fertility (Rurangwa, 2004). At several stages of the fish farming and hatchery activities, there is a need for a reliable measure of sperm quality. However, although computer assisted semen analysis in fish has developed over the last 10 years, it is limited to a few institutions and mostly in research laboratories. The main reason may be related to the cost of the equipment, which hampers its access to fish breeders although good collaboration between institutes, laboratories and hatcheries could allow its broader use. Presently, the system can provide precise and accurate information on sperm motion characteristics and positive correlations have been found between fertilizing capacity and defined sperm motility characteristics in the fish species in which the system has been tested so far. Four central fields for CASA application in aquaculture can be proposed: (1) general evaluation of suitability of male brood stock for reproduction, (2) improvement of artificial fertilization, (3) optimization of dilution/extension media and cryopreservation protocols, (4) management of sperm cryobanks (Rurangwa, 2004). The motility of sperm is a key factor in allowing us to determine semen quality and fertilizing capacity. In 2004 Alavi researched on the effects of temperature and pH on the motility of spermatozoa in three fish species: Salmonids, Cyprinids and sturgeons. They examined motility, fertilizing ability and velocity of spermatozoa, as well as the duration of the motility period, depends on the temperature of the assay medium and also of that of the brood fish holding tank. In contrast, the pH of the swimming medium, and thus the intracellular pH of spermatozoa, has less influence on sperm motility parameters in cyprinids, Salmonids and sturgeons (Alavi, 2004). Another studies of Schlenk and Kahmann (1938), showed that, at 12.5 and 16 °C, the forward velocity of spermatozoa of

Salmo trutta fario was high (160 and 164 m/s) 4 s after their activation by freshwater. After 8, 16, and 26 s this value slows down to 85 and 91, 24 and 33 and 2 and 5 m/s, respectively. In a like manner forward movement of the lake trout spermatozoa, *Salvelinus namaycush*, ceases completely within 29 s at 12.5 and 16 and within 56 s at 2.25 °C (Schlenk and Kahmann, 1938, Dadras et al, 2017). Also the beat frequencies of flagella of rainbow trout spermatozoa have been measured at different temperatures (Billard and Cosson, 1988, 1992): the beating frequency was low at 5 °C, remains constant up to 10 °C, then increases rapidly at about 14 °C and stabilizes at values above 21 °C . Temperature affects the beat frequency of demembraned and ATP reactivated frequencies increased with temperature: 25 Hz at 5 °C, 45 Hz at 15 °C and more than 80 Hz at 25 °C (Billard and Cosson, 1988). Between 5 and 20 °C, these values were the same as in live spermatozoa, but at 25 °C, the beat frequency is 50 Hz in live spermatozoa, while it reaches 80 Hz in demembraned sperm reactivated in the presence of 1 mM ATP. Those observations suggested a negative effect of ambient temperature on spermatozoa in a similar manner. In addition, the rate of decrease in beat frequency as a function of time after activation (so-called slope in Hz per 10 s). They found that this rate is not affected by pH. Billard and Cosson (1992) used the measurement of percentage motile cells as an indicator of sperm motility in rainbow trout. The comparison of sperm motility parameters during the activation of sperm at different of motile spermatozoa and the total duration of forward movement decrease when the temperature of the swimming medium and the initial beat frequency increase. In (Bobe, 2010) Sperm quality was reviewed showing that sperm quality can be defined as its ability to successfully fertilize an egg and subsequently allow the development of a normal embryo. In the wild or under

aquaculture conditions, the quality of fish gametes can be highly variable and is under the influence of a significant number of external factors or brood stock management practices. For these reasons, the topic of gamete quality has received increasing attention. Despite the significant efforts made towards a better understanding of the factors involved in the control of gamete quality, the picture is far from being complete and the control of gamete quality remains an issue in the aquaculture industry (Bobe, 2010).

2.3 Materials and Methods

2.3.1 Experimental setup

In total one hundred sixty breeders of *Salmo trutta*, *Salmo marmoratus*, *Salmo cettii and Salmo ghigii* from three different hatcheries in three different regions, the first one Centro ittico valdastico in the Veneto region, North Italy, second one Monchio delle Corti fish hatchery in the Emilia-Romagna region, Parma province and third one was in Santa Fiora near mount Amiata in Tuscany. In this research, data on brown trout and marble trout sperm concentration in a fully mature brood stock (5 years and older) were measured. In addition, concentrations are reported for the first time on a primiparous albino strain of hybrid Mediterranean trouts. All fish were tagged with an intramuscular passive transponder (Biomark FDX-β PIT tags) to identify them in hatcheries. Each pit tag has a different barcode number that can be read by a proper detector. For every breeder transferred in the hatchery are recorded some data like sex, weight, length and personal barcode. Sperm motility and milt concentration were measured in the hatchery during the reproductive season from the 23rd of November 2015 until the 3rd of February 2016. PIT tags of male breeders were checked before the stripping operations.

2.3.2 Sampling procedure

Animals were sedated in a 30% ml/l phenoxyethanol solution and stripped manually by operators (Fig. 2.1 a, b).

2.3.3 Methods of analysis

Milt was collected in syringes without needle to avoid contaminations or in tissue culture flasks, preserved in ice and analyzed in the laboratory.

2.3.4 Motility assessment

Typically, the motility evaluation is assessed via computer-assisted sperm analysis (CASA) systems or cell motility analysis (CMA). These techniques, well known since the 1980 are developed to evaluate several characteristics of the spermatozoa motility such as speed, direction etcetera (Cosson et al., 1997; Kime et al., 2001; Rurangwa et al., 2004; Dietrich et al., 2005). In my dissertation work, I performed visual analyses with a phase-contrast or dark-field microscope (Billard et al., 1977, 1995; Cosson et al., 1999; Ingermann et al., 2002; Christen et al., 1987). 10 µl of AquaBoost Activator (Cryogenetics and Minitüb GmbH) were placed on a slide and a small drop of milt was mixed to it (Fig.2.1). Values assessed for motility ranged from 0 to 3: 0 for no motility at all or only few spermatozoa moving, 1 for the 20-40% of spermatozoa moving, 2 for 50-70% of spermatozoa moving and 3 for 80-100% of spermatozoa moving.

Figure 2.1: visual analyses with a phase-contrast or dark-field microscope. A small drop of milt from the culture flask was mixed, using a toothpick, with 10 µl of AquaBoost® Activator (Cryogenetics® and Minitüb GmbH) and placed on a slide for the motility assessment.

2.3.5Concentration assay

The sperm concentration in each sample of milt was measured with photometer SDM6 (Minitüb GmbH and Cryogenetics (Fig 2.4). Tipically sperm concentration is measured by spectrophotometric method of Ciereszko and Dabrowsky (1993) standardized by counting the sperm density in a cell counting chamber (Neubauer, Makler, Burker or Thoma chambers) and with spermatocrit determination (Foote, 1964). The photometer used in this work was developed for measure the dimensions of spermatozoa of different animals, including salmonids, for a more reliable evaluation of the sperm concentration in each sample. For the measuring were used 10 µl of milt diluted in 4 ml of NaCl 0.9% (Sodio Cloruro EUROSPITAL) in polystyrene disposable cuvettes (Sarstedt). A solution of 0.9% NaCl served as a blank.

A

B

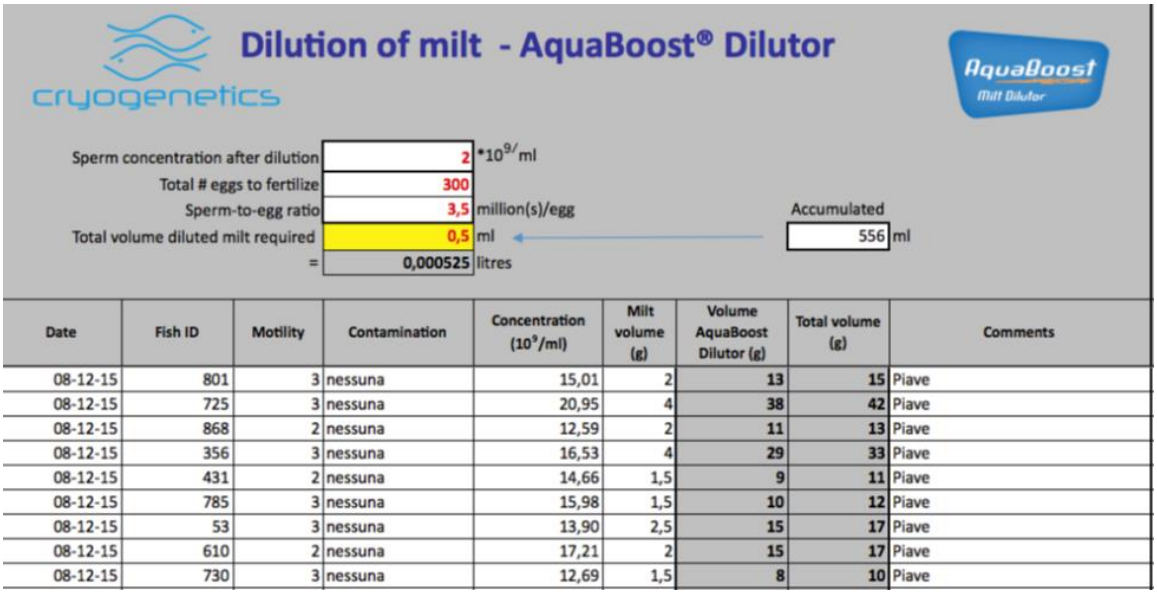
Figure 2.2: a) albino strain of Mediterranean brown trout (*Salmo ghighii*) **b**) Atlantic brown trout (*Salmo trutta*)

Figure 2.3: fingerlings of Atlantic brown trout (*Salmo trutta*) and albino strain of Mediterranean brown trout (*Salmo ghighii*)

Figure 2.4: picture of the photometer SDM6 (by Minitüb GmbH and Cryogenetics®) showing the data output

2.3.6 Concentration during reproductive season

During all the reproductive season, from the 23rd of November 2015 until the 3rd of February 2016, nine males of marble trout were kept separated in the same outdoor tank in order to measure every week the variations in milt concentration. This analysis was conducted in order to identify the time span when the milt concentration was maximum.



Dilution of milt - AquaBoost® Dilutor

cryogenetics

AquaBoost Milt Dilutor

Sperm concentration after dilution	2	$*10^{9/}$ml
Total # eggs to fertilize	300	
Sperm-to-egg ratio	3,5	million(s)/egg
Total volume diluted milt required	0,5	ml
=	0,000525	litres

Accumulated: 556 ml

Date	Fish ID	Motility	Contamination	Concentration (10^9/ml)	Milt volume (g)	Volume AquaBoost Dilutor (g)	Total volume (g)	Comments
08-12-15	801	3	nessuna	15,01	2	13	15	Piave
08-12-15	725	3	nessuna	20,95	4	38	42	Piave
08-12-15	868	2	nessuna	12,59	2	11	13	Piave
08-12-15	356	3	nessuna	16,53	4	29	33	Piave
08-12-15	431	2	nessuna	14,66	1,5	9	11	Piave
08-12-15	785	3	nessuna	15,98	1,5	10	12	Piave
08-12-15	53	3	nessuna	13,90	2,5	15	17	Piave
08-12-15	610	2	nessuna	17,21	2	15	17	Piave
08-12-15	730	3	nessuna	12,69	1,5	8	10	Piave

Figure 2.5 Example of Excel data sheet used in the dilution of milt. In the higher box the operator must insert the fertilization condition like the sperm concentration that wants to obtain after dilution, the total number of eggs to fertilize and the sperm-to-egg ratio. In the yellow box the algorithm result given in ml of diluted milt that has to be used. In the columns the operator has to write the date of sampling, the fish ID, the motility assessment, the contamination, the concentration measure obtained with SDM6, the milt volume obtained from the stripping. In the two following columns, in bold, the amount of dilutor that the operator must add to the fresh milt.

2.3.7 Calculations and Statistical analyses

The SPSS software was used for analyses of data. One-way analysis of variance (ANOVA) was employed to compare the means. When significant F-ratios were calculated by ANOVA, the Tukey test was applied to identify which means were different.

2.4 Results

2.4.1 Motility and milt concentration analyses

2.4.1.1 Motility assessment

The motility assessment was performed via visual analyses with a phase-contrast or dark-field microscope. One hundred and thirteen data from various males were collected during the four months of last winter reproductive season. Data of the motility were collected in a barplot (Fig. 24) and were analysed divided per month of sampling.

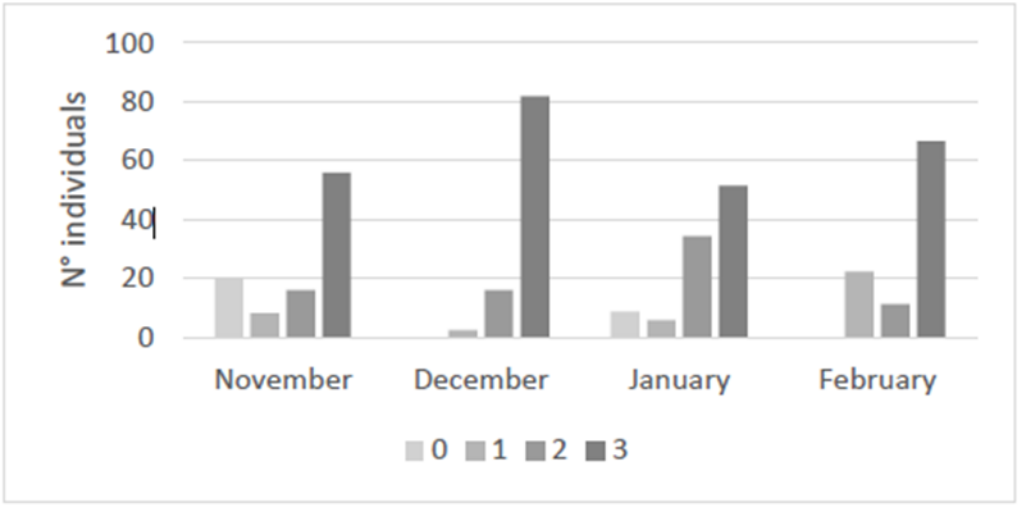


Figure 2.6: Barplot displaying data of the spermatozoa motility of males sampled during the reproductive season and divided per month of sampling. 0, 1, 2, 3 indicate the value of motility assessed via microscope. 0 for no motility at all or only few spermatozoa moving, 1 for the 20-40% of spermatozoa moving, 2 for 50-70% of spermatozoa moving and 3 for 80-100% of spermatozoa moving.

2.4.1.2 Concentration assay

The concentration assay was performed via photometer SDM6 (by Minitüb GmbH and Cryogenetics) on the same individuals of the motility assessment the same day of the stripping, output results are given in 10^9/ml (figure 2.7).

Figure 2.7: picture of the photometer SDM6 (by Minitüb GmbH and Cryogenetics®) showing the data output. In the example the measurement of the milt concentration is 17.148*109 /ml.

The data of sperm concentration of marble trout have been collected and the results were collected in the boxplot in Figure. 2.8.

Data in figure (2.9) showed no significant difference (P> 0.05) in species during the first stripping and second stripping during spawning (N R Bury1 2003) season. Milt value of marble trout was significantly (P < 0.05) higher than the other groups. Between the first stripping of albino Mediterranean trout (*Salmo ghigii*) we couldn't observed any significant difference (P> 0.05).

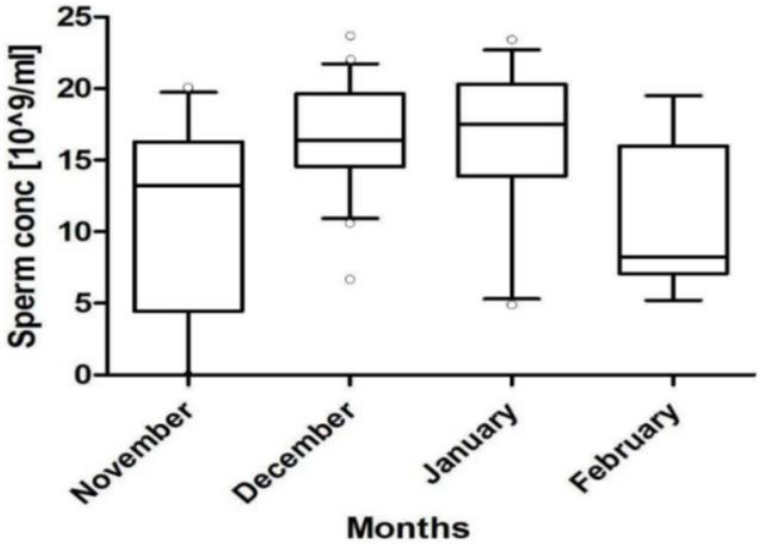


Figure 2.8: Boxplot displaying data from concentration assay divided per month of sampling. Higher values of the concentration are recorder in December and in January

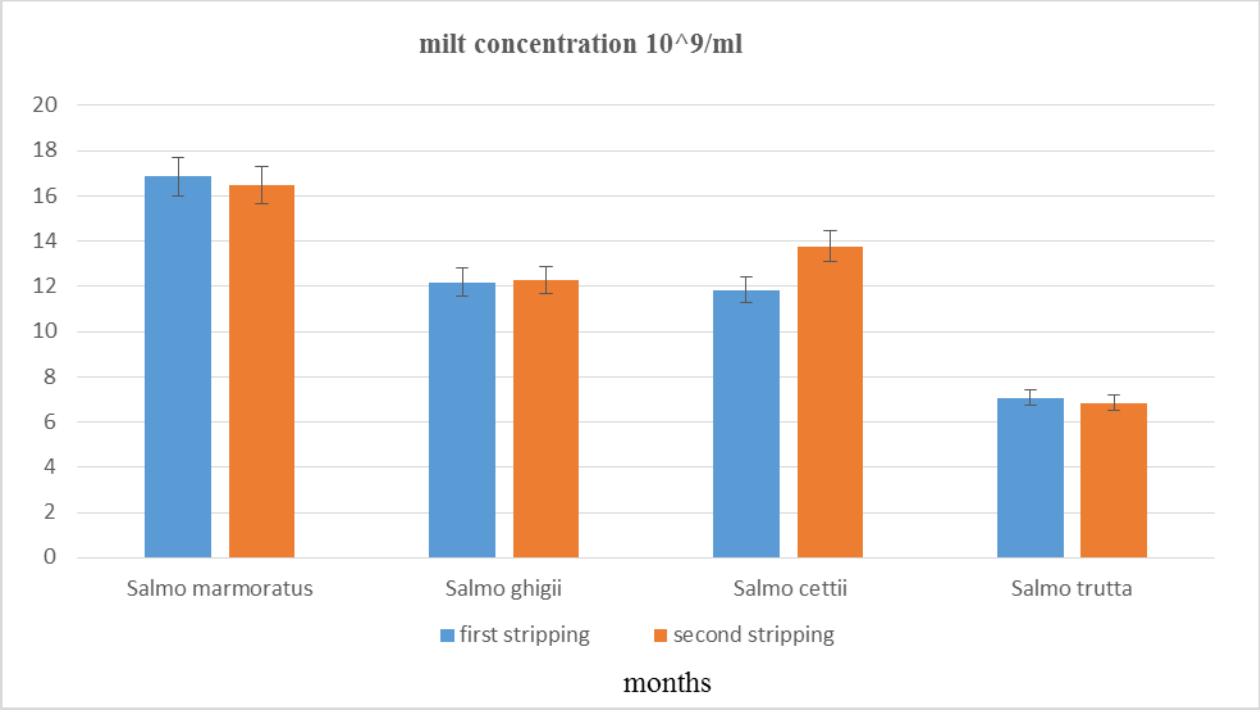


Figure 2.9: displaying data from concentration assay divided per first and second stripping. Higher values of the concentration are recorder for marbel trout during first and second stripping.

2.5 Discussion

As shown in the plot in Fig. 2.6, most of the milt samples, 66.97%, showed a high sperm motility (between 80-100% of spermatozoa moving) during the four months of sampling. In November and in February the number of individuals that showed as thenozoospermia, from 0% (0 in the plot) to 20-40% (1 in the plot), were higher than in the center period of the reproductive season. The mean value for the motility assessed during four month sampling is 2.51 (corresponding to 50-70% of spermatozoa moving). It is interesting to notice that in the short term, between collection of samples and analyses, urine and feces contamination when present in low quantity did not affect the motility of sperm. As shown in the plot in Fig.2.7, the higher values of the concentration were observed in December and in January, central months of this reproductive season while in November

and in February the variability of the data was higher. In the group of nine males monitored during all the reproductive season, to observe the seasonal variability of the milt concentration, was not possible to distinguish a trend in order to identify a specific period in which the value was maximum for all the breeders. Being able to identify a time lapse of high milt concentration would be very convenient both because the fertilization rate could be higher and because this could allow operators to strip males only in the best moment of the reproductive season. Stripping is equivalent to stress fishes: sedation, manipulation and deep stripping can compromise the health of the animals and, in some cases, lead to their death. From the data collected is possible to observe that the time lapse in which is recorded a higher milt concentration, for almost all the trout males, is from the first days of December until the first days of January. It is believed that, if it was possible to continue the analyses after the beginning of February, could be observed a bell-shaped curve illustrating the end of milt production after the reproductive season. From the tendency graphic of the seasonality is possible to observe that males have a very long timescale of high milt concentration (greater than 15*109 sperm/ml); this is indicative that they are not the limiting factor of a higher number of fertilization during the reproductive season. In effect, in hatcheries, the same male can be stripped more than once during reproductive period because his milt production is continuous and persist until the end of the season. For females is quite different because they produce and can lay eggs only once per year and they cannot be stripped again till the sequent reproductive season, the year after. In our study, milt concentration parameter of cultured trout was stable during successive stripping. This demonstrates that the viability of eggs is more under the influence of egg quality than sperm quality.

2.6 Conclusion

In our research trouts were submitted to an innovative approach based on new technologies in the field of reproductive biology. In particular, the use of a photometer dedicated to measuring the sperms concentration of milt belonging to different fish species has demonstrated his usefulness in artificial reproduction (Zuccon, 2016). A simple dark-field microscope and the SDM6 are time and space-savings tools. A trained technician can make evaluations, for the stripped males, in less than one hour while the operators in the hatchery are stripping the females and preparing the eggs for the fertilizations. The choice of the males with higher concentration of sperm and a good motility is important in order to obtain higher fertilization rate during the season, especially for the endangered species, whose offsprings are going to be released in rivers. Quantifying sperm quality throughout the spawning season is important for estimating a stock's reproductive potential (Trippel, 2003) and improvement of short-term and long-term storage (cryopreservation) techniques for many captivity-bred and endangered species (Rideout et al. 2004). In our study, milt concentration parameter of cultured trout was stable during successive stripping. Throughout the life cycle of cultured males, commercial dry feed was used .Several studies have shown that fish gamete quality varies depending on quality of food composition (Astuarino et al., 2001). The new technology has demonstrated to be the important tool useful for more efficient management plans for conservation of endangered salmonid species.

2.7 Acknowledgements

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Chapter 3: Interaction of dietary biochar (*black carbon*) *on the growth performance and survival rate of early stage larvae of brown trout* (*Salmo trutta*)

3.1 Abstract

Brown trout (*Salmo trutta*) have long been a favourite sport-fish among anglers, and have been introduced primarily on the basis of recreational fishing. A balanced diet and proper feeding practices are important in aquaculture. It is necessary to have a proper amount of feed with ingredients necessary for fish such as protein for normal tissue function, maintenance and renewal of the fish body, carbohydrates and lipids for energy source, vitamins and minerals for body functions, growth, reproduction and maintenance of fish metabolism. An experiment conducted at the University of Parma in Italy in the summer 2016, aimed to investigate the effects of black carbon (i.e. biochar) on growth performance and survival rate of brown trout (*Salmo trutta*). After eight weeks feeding trial, the highest final body weight was obtained by group which received diet supplemented with that dietaries of biochar (0.2, 0.3 per kg^{-1} diet) group significantly increased body weight (BWI), specific growth rate(SGR), feed conversion ratio (FCR) and condition factor (CF) ($P< 0.05$). Experimental diets had significant effect on percentage of survival rate in compare with control group ($P<0.05$). It is important to feed fish with proper feed containing the required nutrients, follow feeding instructions taking into consideration light and temperature factors, feed fish with a proper amount of feed and to choose a proper method of feeding to get optimal results in the development of the body weight and length of the fish. Under the experimental conditions, dietary Biochar had a synergistic effect on enhancing growth performance of brown trout (*Salmo trutta*).

Key words: Salmo trutta, Biochar, Growth performance, Survival rate

3.2 Introduction

In aquaculture industry various methods are commonly used to increase the growth rate and survival rate. A new methodology to enrich this aim is the use of black carbon that manufactured through pyrolysis of biomass has become known as 'biochar' (Lehmann et al. 2009). Black carbon can be produced from different sources such as woody materials, wastes of agricultural, (Demirbas 2004; Van Zwieten. 2010), green waste (Chan et al. 2007) animal manures and other waste products (Downie et al. 2007; Lima et al. 2008; Chan et al. 2008). In 2014, Surintorn researched on the effects of dietary activated charcoal (AC) on health status, intestinal morphology and fillet geosmin content of Nile tilapia(*Oreochromis niloticus*) prior to harvesting (2 and 4 weeks). Four dietary treatments (each diet in six replicates) were formulated to incorporate black carbon at levels of 0, 10, 20 and 30 g kg^{-1} of the dry diet. The result showed that there were not significant differences in growth performances among experimental treatments. The moisture and protein content in the fillet decreased and increased, respectively, as the incorporation level of AC increased. The hematological indices and several immune parameters did not differ significantly among treatment groups. Among the fifteen blood chemicals parameters examined, the significant reductions in protein and cholesterol and the changes in blood minerals were observed in fish fed dietary AC C20 g kg^{-1}. Dietary AC tended to increase the height of intestinal villi and goblet cell number. Dietary AC also influenced the reduction in geosmin in the fish fillet. Taken together, they examined that black carbon (at 10 g kg^{-1} diet) could be used as a feed supplement for Nile tilapia (*Oreochromis niloticus*) prior harvesting to reduce geosmin without negative effects (Pirarat, 2015; Surintorn, 2014). Other studies on the ability of AC to absorb a wide

range of noxious substances and gases (McFarland and Chyka 1993; American Academy of Clinical Toxicology 1999; Bacaoui et al. 2001) would most likely increase feed efficiency, thereby improving the growth performances of the animals. In Yoo et al.'s research (Yoo et al. 2005), were showed that the growth performance (WG, SGR, FCR) of Nile tilapia at the grow-out stage appeared to be similar in all diets. However, an improvement was observed in fish fed diet at 10 g kg^{-1} black carbon supplementation level, but differences were not significant because of the high standard deviations observed. In their experience, even though the experimental diets were formulated to contain similar levels of nutritive chemical composition, the incorporation of black carbon at high levels affected the physical characteristics of the extruded diets, including palatability, color and smell. These qualities could influence the fish appetites. The variable effects of black carbon diets on growth performance have been demonstrated in chicken and fish. For example, growth performances (FI, WG, FCR) were linearly improved as the supplementation level of wood charcoal increased from 0 to 100 g kg^{-1} in broiler chicken at 28 days of age; however, the positive effects were limited at the older ages (Kutlu et al, 2001). Charcoal supplementation up to 6 g kg^{-1} could improve growth performances in broilers during the 21st to 49th days of age (Kana et al, 2011). Supplementation with bamboo charcoal at 5 g kg-^{1} significantly improved the growth performances (FE, WG, and SGR) of flounder, *Paralichythys olivaceus*, at juvenile stages (Thu et al. 2010). In addition, a mixture of dietary black carbon and wood vinegar at 5 and 10 g kg^{-1} for 8 weeks was shown to increase feed efficiency and WG, respectively, of flounder at later stages (Yoo et al, 2005). Trinh Thi Lan in 2016 was determined the effects of biochar and charcoal on water quality and on growth

performance of striped catfish (*Pangasius hypophthalmus*) raised in tanks. The five treatments in a completely randomized design with 4 replicates were: NBC (no biochar or charcoal), BF (biochar in feed), CF (charcoal in feed), BW (biochar in water) and CW (charcoal in water). Growth rates were increased 36% by adding biochar to the feed and by 44% with charcoal. There were no benefits from adding either biochar or charcoal to the water. In contrast, adding black carbon to the water had a negative effect on feed intake growth rate and survival. The ratio of weight to length in the fish at the end of the experiment was 25% greater when biochar or charcoal was added to the feed, indicating an enhanced flesh to bone ratio due to the faster growth rate with addition of biochar or charcoal. Levels of ammonia nitrogen (TAN), nitrite (NO2-, phosphate (PO43-) and chemical oxygen demand (COD) in the tank water were reduced by adding biochar or charcoal to the feed, but not to the water. The role of biochar (and charcoal) in facilitating the formation of biofilms as habitat for gut microbiota could be the explanation for the improved growth rates recorded with biochar and charcoal added to the diet (Trinh Thi Lan,2016). In fact, it appeared that adding biochar or charcoal to the water had a negative effect on feed intake and as a result of reduced feed intake, growth rates were also reduced. Similar improvements in growth rate of *P. hypophthalmus* by adding bamboo charcoal to the feed were reported by Jahan et al (2014), with the optimum level at 1% of the feed.

3.3 Materials and Methods

Feeding trial was carried out at the University of Parma. The fish were acclimatized at ambient conditions for 15 days and starved for 24 h before the beginning of the feeding trial (Delsoz et al 2017). One hundred and eighty trout (initial body weight: 1.00± 0.53g)

in recirculation system were fed with three practical diets: the basal diet as the control diet supplemented with two levels of Bio char (0.2 and 0.3 g per kg^{-1} diet). Each tank was supplied with an air pump contacted with air stone for aeration. Water temperature (via palintest pool 9 kit-photometer) was daily measured and the average was between 8-10 °C during the experimental period. Water quality parameters such as pH, temperature, ammonium, ammoniac, nitrate and nitrite were measured weekly. The pH (using WTW, Model PH 330i -pH-meter) and dissolved oxygen (using WTW, Model PH 330i - dissolved oxygen meter) were also determined.

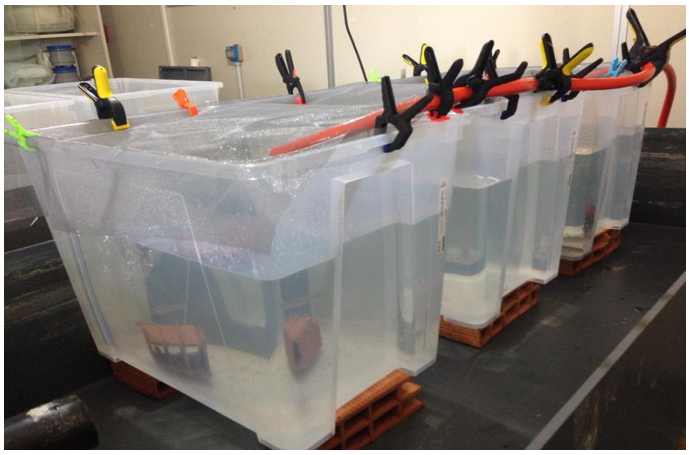


Figure 3.1: recirculation system with biofilter and UV-C sterilizing light

3.3.1 Food preparation

The commercial pallet has been used for experiment period and the composition of the experimental diet has been list in table 3.1.

Table3.1: Composition of the experimental diet (dry weight).

Ingredients	% kg^1 dry weight
Fish meal	40
Wheat meal	20
Corn meal	5
Fish oil	6.5
Soybean meal	20
Beet molasses	2
Salt	1
Vitamin mixture	2
Mineral mixture	1
Lysine	1.5
Methionine	1.5
Proximate composition	%
Crude protein	40
Crude lipid	9
Moisture	5.5
Ash	55.2
Crude Energy (kcal kg^1)	1897

Biochar was obtained by gasification in a fixed-bed, down-draft, 20 KW compact gasifier (All Power labs) at 800 °C provided by Department of Engineering "Enzo Ferrari", University of Modena Reggio in Italy. A mix of feedstock biomass was used: conifer wood and poplar wood.

Figure3.2: Pellet mixed with biochar (at the right side of picture) and without biochar (at the left side of picture).

3.3.2 Experimental setup

The fertilized eggs of brown trout (*Salmo trutta*) from fish farm in Monchio (region of Emilia Romagna in Parma province, Italy) were transferred to the recirculation system with bio-filter and UV-C light to sterilized, which has been built at the University of Parma. The eggs have been kept for three months and after two months of first feeding with starter food and reared to the fingerling size the experiment has been started. All the water parameters including pH, temperature, ammonium, ammoniac, nitrate and nitrite were measured regularly. Each Culture tank was containing 400 L recalculated (80%; 20% fresh input of water) at 12 ^{0}C. All the fish held under 12:12 h light: dark, fed commercial fish feed pellets (Skretting, Norway) 3 meals a day (08.30am,12:30 pm and 03.30 pm) at0.9% of bodyweight were kept at12.0±0.4°C for 8 weeks. 3 groups, with 2 tank replicates per group of 30 fish per tank each were set up. Fish of the control group has been fed with normal pallet. The biochars (0.2 and 0.3 g per kg^{-1} diet) groups received feed spiked during 60 days. One hundred and eighty individuals of *S. trutta* (initial body weight: 1.00± 0.53g) in recirculation system were fed. Biometry (measure

the total length and weight) has done every two weeks during the feeding trial. The daily amount of feed was calculated according 5% of body weight. Unfed feed and feces were daily removed after feeding.

3.3.3 Sampling procedures

Total period of experiment was two months but fish were adapted two weeks before start of experimental rearing, and biometry (measure length hand weight) has been done by the end of every two weeks.

3.3.4 Physical indices and survival rate

After the feeding trial, the growth parameters including body weight gain (BWI), specific growth rate (SGR), feed conversion rate (FCR), condition factor and survival rate (%) was individually determined by following equations (Tekinay and Davis 2001).

Condition factor (CF) was derived from growth indices of weight (W, g) and length (L, cm) as per Busacker et al. (1990):

(1) $CF = (W/L3) \times 100$

The specific growth rate (SGR) is the daily growth rate of fish body weight in percentage. The SGR can be calculated in the formula:

$$SGR = ((\ln W1 - \ln W0) / t) \times 100$$

The rate of growth in fish depends on different factors such as species, age, water temperature, quality and quantity of food. Young fish are capable of doubling their weight in a much shorter time than when they are older due to fast growth rate. Therefore it is useful to know specific growth rate in different age of the fish (Alyshbaer, 2013).

(3) Feed conversion ratio is a feed efficiency calculation which gives the amount of feed which taken to grow one kilo of fish (Anderson and Silva, 2003). The FCR can be calculated with the formula:

FCR = dry feed intake (g)/wet WG (g)

FCR is used to determine or guide one on how to efficiently use feed. It is important for the nutritionist that allows for the estimation of how much feed is required for the growing cycle (Anderson and Silva, 2003).

SGR (% day-1) = (Ln W_f - Ln W_i) × 100/t

CF = 100 × [wet weight (g)/TL (cm) 3]

Where W_f and W_i were final and initial fish weights, respectively; TL was total length and t is the experimental duration in day.

3.3.5 Calculations and Statistical analyses

Results are presented as mean ± SD. Differences among values were assessed by a one-way ANOVA followed by a Fisher protected least significant difference post- hoc test for multiple comparisons. Data presented in percentage were arcsine transformed prior to statistical analysis. If the transformed data still did not conform to the assumptions of ANOVA, a Kruskal-Wallis one-way ANOVA on ranks (R-ANOVA) was performed followed by Tukey test for multiple comparisons. All analyses were performed with (SPSS 18) using a significance level of $P < 0.05$ (Bryman, 2011).

3.4 Result

Data presented in table 3.1 indicated that the highest final body weight was obtained in T2 group which received the diet supplemented with 0.2 g biochar followed by T3; the lowest values were obtained in the control. The results showed that FCR, SGR, BWI and CF were significantly affected by black carbon supplementation (Table 3.1). During the whole experimental period, T2 fish produced the lowest FCR (0.480± 0.016) and also had highest SGR and BWI (1.801± 0.139 .146.40± 16.98). The values of survival rate and condition factor were higher in fish supplemented with black carbon compared with the control (Table 3, $p<0.05$).

Table 3.1: Effects of dietary Biochar (black carbon) on growth performance of *Salmo trutta*

Treatments	ADG	WG	BWI (%)	FCR	CF	SGR
T1(Control)	0.821 ± 0.232^{a}	0.342 ± 0.11^{a}	41.07 ± 11.58^{a}	0.741 ± 0.045^{a}	0.006 ± 0.0022^{a}	0.684 ± 0.168^{a}
T2	2.93 ± 0.339^{b}	0.971 ± 0.191^{b}	146.51 ± 16.98^{b}	0.480 ± 0.016^{b}	0.019 ± 0.0038^{b}	1.801 ± 0.139^{b}
T3	2.481 ± 0.145^{b}	0.899 ± 0.069^{b}	124.05 ± 7.23^{b}	0.549 ± 0.026^{b}	0.0179 ± 0.0014^{b}	1.613 ± 0.064^{b}

The values with different letters in the figure are significantly different ($p<0.05$).

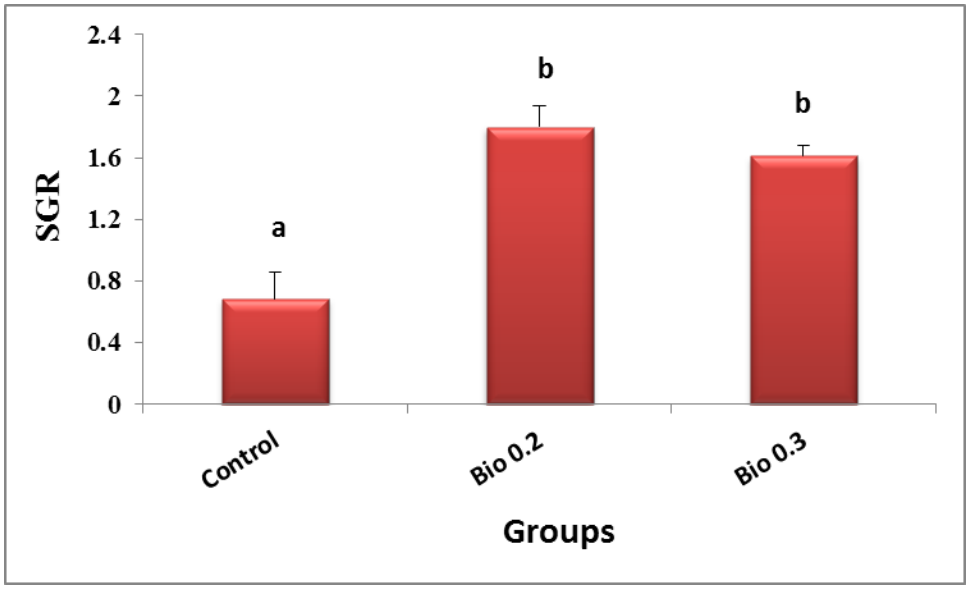


Figure 3.3: Specific Growth rate (SGR) of *Salmo trutta* fed the experimental diets for 60 days

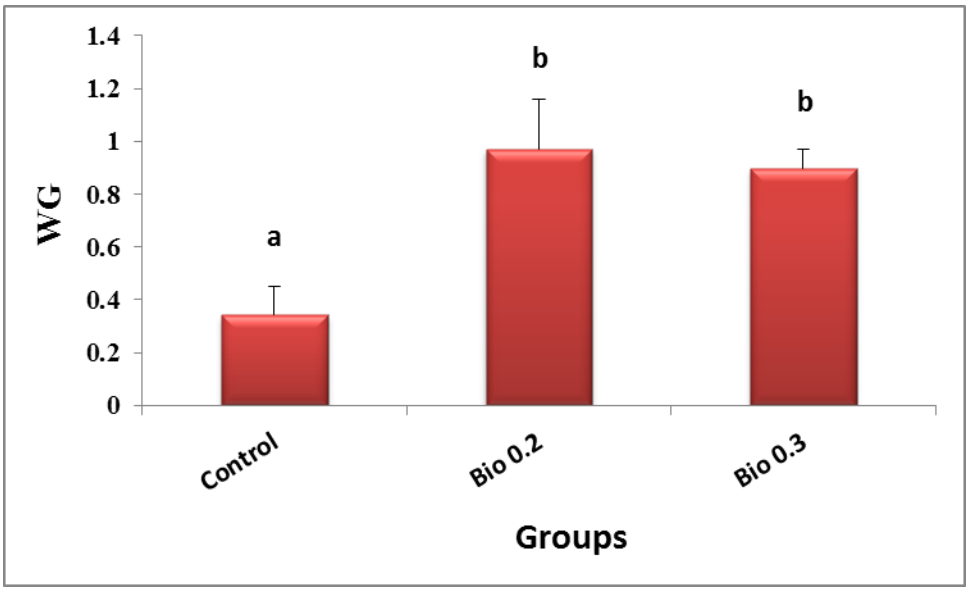


Figure 3.4: weight gain (WG) of *Salmo trutta* fed the experimental diets for 60 days

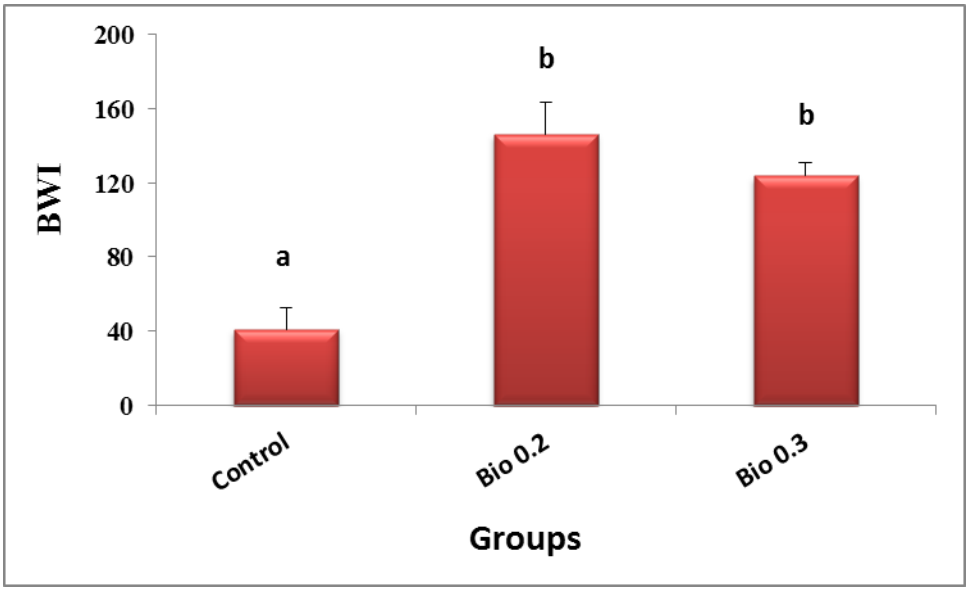


Figure 3.5: Body growth weight (BWI %) of *Salmo trutta* fed the experimental diets for 60 days

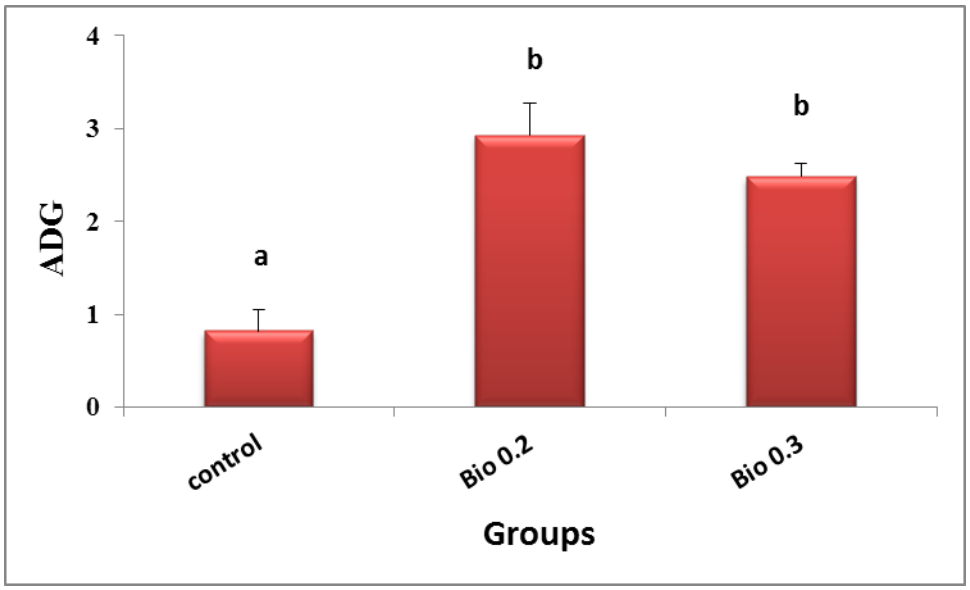


Figure 3.6: average daily growth (ADG) of *Salmo trutta* fed the experimental diets for 60 days

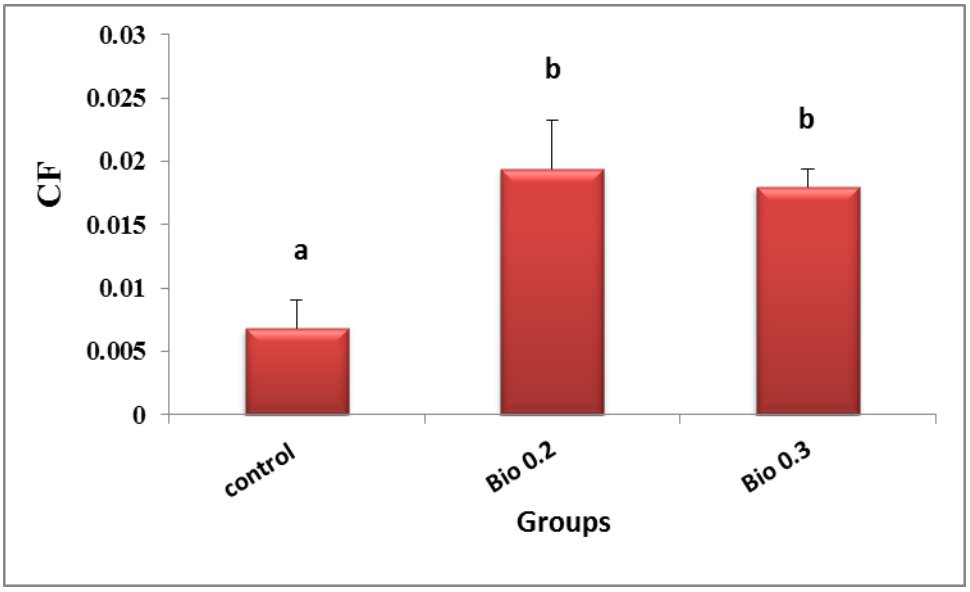


Figure 3.7: Condition factor (CF) of *Salmo trutta* fed the experimental diets for 60 days

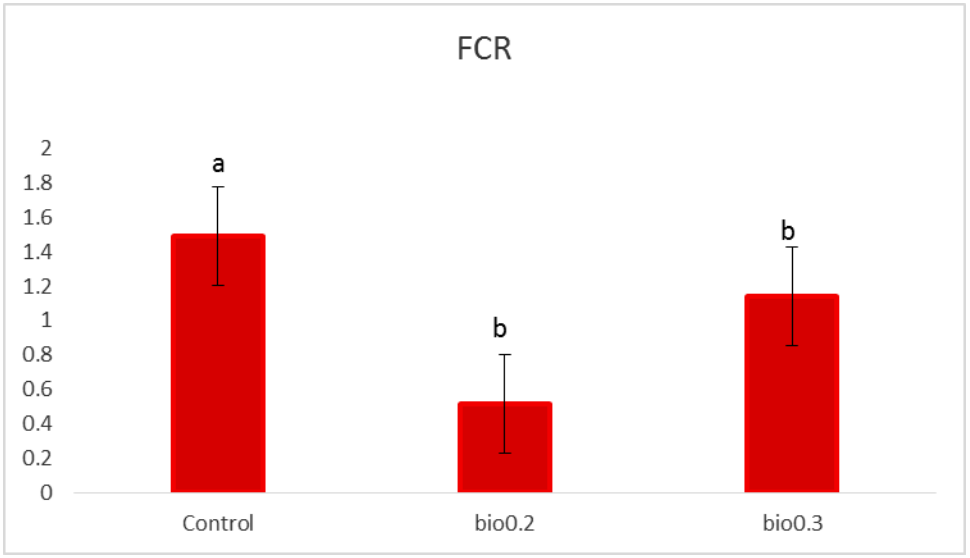


Figure 3.8: Food conversation ratio (FCR) of *Salmo trutta* fed the experimental diets for 60 days

3.4.1 Growth rate and Physical indices

The growth performance of *Salmo trutta* fed different experimental feeds was determined at the end of 4 and 8 weeks (Table 2). Data showed that the highest final body weight was obtained by groups which received diet supplemented with Biochar (0.2, 0.3 per kg-1 diet) group significantly increased body weight (BWI), specific growth rate (SGR), feed conversion ratio (FCR) and condition factor (CF) ($P<0.05$). The lowest values were obtained by control group and during the whole experimental period, fish fed with the control diet produced the lowest FCR, SGR and BWI, and were significantly different from the groups fed with other diets ($P> 0.05$). However, under the experimental conditions, dietary Biochar had a synergistic effect on enhancing growth performance of brown trout (*Salmo trutta*).

3.4.2 Survival rate

Experimental diets had significant effect on percentage of survival rate in compare with control group ($P<0.05$)

Table 2. Effects of dietary Biochar on CF and survival rate

Treatment	Condition factor (CF)	Survival rate (%)
T 1(Control)	0.006 ± 0.002^{a}	86 ± 0.01
T 2	0.019 ± 0.003^{b}	96 ± 0.00
T 3	0.017 ± 0.001^{b}	93 ± 0.00

The values with different letters in the figure are significantly different ($p<0.05$).

3.5 Discussion

The act of process of Supplementing of Biochar (Black Carbon) in feed has been known to enhance the growth performance and intestinal functionality of terrestrial and aquatic animal (Mekbungwan et al., 2004; Ven et al.2006; Thu et al., 2010). In this study, we observed a tendency in fish fed the Biochar-supplemented diet towards a better growth performance compared with the control group. Previous studies assessing the effect of on growth performance and nutrient utilization of aquatic animals have reported conflicting results (Thu 2010; Pirarat et al 2015). Our study revealed that the supplementation of Biochar (Black Carbon) in feed (2-3%) can increase the growth performance and survival rate in *Salmo trutta*. Data showed that the highest final body weight was obtained by groups which received diet supplemented with that dietaries of Biochar (0.2, 0.3 per kg-1 diet) group significantly increased body weight increase (BWI), specific growth rate (SGR), feed conversion ratio (FCR) and condition factor (CF) ($P<0.05$). The lowest values were obtained by control group and during the whole experimental period, fish fed with the control diet produced the lowest FCR, SGR and BWI, and were significantly different from the groups fed with other diets ($P > 0.05$). However, experimental diets had significant effect on percentage of survival rate in compare with control group ($P<0.05$). Under the experimental conditions, dietary Biochar had a synergistic effect on enhancing growth performance of brown trout (*Salmo trutta*).

3.6 Conclusion

Black carbon, a non-nutritive substance, has the potential to be used as feed additive due to its detoxifying effects and it has been widely used in medical, veterinary and wastewater treatments, as well as in water treatments for aquaculture. So far, studies in the utilization of AC in animal feed have been controversial (Thu 2010; Pirarat et al 2015).Supplementation of black carbon in feed has been known to enhance the growth performance and intestinal function of terrestrial and aquatic animals (Mekbungwan et al., 2004, Thu et al., 2010). In this study, we observed a tendency in fish fed the Biochar-supplemented diet towards a better growth performance compared with the control group. Our study revealed that the supplementation of Biochar (Black Carbon) in feed (2-3%) can increase the growth performance and survival rate in *Salmo trutta larvae.* Further studies regarding the application of Biochar (Black carbon) in aquatic animals, especially their appropriate inclusion levels in specific species in different life stages (Alevin, Fry, and Juvenile, adult) and specific rearing conditions are needed.

3.7 Acknowledgements

Firstly, I would like to express my sincere gratitude to my advisor Prof. Francesco Nonnis Marzano for the continuous support of my Ph.D study and related research, for his patience, motivation, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my Ph.D study. Besides my advisor, I express my sincere appreciation to Prof. Alessio Malcevschi and the people who gave their time, advice, and support for this study. I also thank the staff of the department of chemistry, life sciences and environmental sustainability, university of Parma, Italy for their technical help during this trial.

Chapter 4: stress management; is there difference in stress resistance between wild type of Atlantic brown trout (*Salmo trutta*) *and albino Mediterranean trout* (*Salmo ghigii*)*?*

4.1 Abstract

Different stress factors can all evoke non-specific responses in fish, which are considered adaptive to enable the fish to cope with the disturbance and maintain its homeostatic state. Fish faced with stressful stimuli launch an endocrine stress response through activation of the hypothalamic-pituitary-interrenal (HPI) axis to release cortisol in to the blood. Scientifically validated biomarkers to capture systemic cortisol exposure over longer periods of time are of utmost importance to assess chronic stress in governmental, wild life, aquaculture and scientific settings. Cortisol is the main corticosteroid hormone in teleost secreted in response to stressor exposure and plays a key role in stress adaptation. The aim of present research was to check difference in stress resistance between wild type trout, Atlantic brown trout (*Salmo trutta*) and albino Mediterranean trout (*Salmo ghigii*). An experiment conducted at Monchio fish hatchery of Parma province in Italy during the summer 2017. Scale cortisol was quantified with a validated ultra-performance liquid chromatography tandem mass spectrometry method. At the end of experimental period we observed all the albino type that has been received cortisol in their diet, return to blind. The cortisol content in ontogenetic is an innovative biomarker for chronic stress application in science and industry.

Key words: Stress, Management, Salmo trutta, albino Mediterranean trout, Salmo ghigii

4.2 Introduction

Attention to animal welfare because of neural complexity has been managed on vertebrates and now because of the size and visibility of that segment of the aquaculture industry is currently focused on the fish. The growers have forced to develop cultural practices designed to control and minimize animal stress due to the characteristics of the aquatic environment and their impact on the animal. This was because of necessity to keep fish alive and healthy and was not done as a result of social awareness; and managing of stress is a key to make sure animal welfare (Conte, 2004). Aquatic farmers have limited knowledge of the basic biological principles of animal stress and have little exposure to the linkages between these concepts and the issues critical to animal welfare but they are aware of the consequences of fish stress (Schreck, 2000). Even though aquaculture industry has many tools available for monitoring and preventing stress, not all growers have had exposure to the information that is available or know of its value when addressing issues of animal welfare (Conte, 2004). Stress happens when that all the requirements of fish such as specific physical happen and chemical requirements relative to the aquatic environment are not met, the animals are quickly stressed, and their health and survival jeopardized. Culturing animals in water is far less forgiving, and potential problems occur more frequently than with terrestrial animal culture. The inherent advantage of this with respect to animal welfare is that aquatic producers recognize that controlling animal stress is absolutely essential for their economic success; and that the development of specific stress management protocols is essential to animal health and survival (Conte, 2004). In 2007 Alsop research on larvae and embryos of zebra fish has examined patterns of cortisol and expression of genes involved in corticosteroid synthesis

and signaling. In his research, embryonic cortisol levels decreased 70% from 1.5 h post fertilization (hpf) to hatch (42 hpf) and then increased 27-fold by 146 hpf. The mRNA abundances of steroidogenic acute regulatory protein, 11-hydroxylase and 11-hydroxysteroid dehydrogenase type 2, increased several fold after hatch and preceded the rise of cortisol levels. In contrast to other teleosts that possess two glucocorticoid receptors (GRs) and one mineralocorticoid receptor (MR), only one GR and MR were identified in zebra fish, which were cloned and sequenced. Also GR mRNA abundance decreased from 1.5 to 25 hpf, rebounded, and then was stable from 49 to 146 hpf. MR transcripts increased continuously from 1.5 hpf and were 52-fold higher by 97 hpf. An acute cortisol response to a stressor was not detected until 97 hpf, whereas melanocortin type 2 receptor mRNA increased between 25 and 49 hpf. Generally, the patterns of cortisol and the expression of cortisol biosynthetic genes and melanocortin type 2 receptor suggest that the corticoid stress axis in zebra fish is fully developed only after hatch (Alsop, 2007). In another research has shown that Glucocorticoids hormones (GCs) are intuitively important for mediation of age-dependent vertebrate life-history transitions through their effects on ontogeny alongside underpinning variation in life-history traits and trade-offs in vertebrates. These concepts largely derive from the ability of GCs to alter energy allocation, physiology and behavior that influences key life-history traits involving age specific life history transitions, reproduction and survival. A lot of researches across vertebrates have shown that the neuroendocrine stress axis plays a role in the developmental processes that lead up to age-specific early life-history transitions. While environmental sensitivity of the stress axis allows for it to modulate the timing of these transitions within species, little is known as to how variation in stress axis function

has been adapted to produce interspecific variation in the timing of life-history transitions. Crespi in 2013 has assessed of the literature confirms that of previous reviews that there is only equivocal evidence for correlative or direct functional relationships between GCs and variation in reproduction and survival. He has concluded that the relationships between GCs and life-history traits are complex and general patterns cannot be easily discerned with current research approaches and experimental designs. Conceptual models of HPA/I axis actions, such as allostatic load and reactive scope, to some extent explicitly predict the role of GCs in a life-history context, but are descriptive in nature. He has proposed that GC effects on life-history transitions, survival probabilities and fecundity can be modeled in existing quantitative demographic frameworks to improve his understanding of how GC variation influences life-history evolution and GC-mediated effects on population dynamics (Crespi, 2013). In Hau et al.'s work in (2010) were synthesized relationships between breeding season length (a surrogate measure for number of annual breeding opportunities) and baseline and stress-induced GC levels, but they limited their analysis to passerines and they controlled for body mass and environment type. They have conducted multivariate analyses that account for other intrinsic and extrinsic factors affecting life-history evolution. A lot of studies of most researchers have proposed simple and direct relationships between the HPA/I axis and life history traits (e.g. the adaptive stress hypothesis of Boonstra & Boag 1992; the Cort-Fitness and Cort-Adaptation hypothesis of Bonier et al. 2009a), the evidence suggests that the relationship is more nuanced and complex. Although meta-analyses and interspecific comparative studies are complex, the use of multivariate analyses that control for phylogeny and methodological differences across studies offer a

powerful, quantitative means of testing hypotheses and resolving patterns relating GC levels and HPA/I axis function to variation in life histories. In 2015 Aerts research on common carp (*Cyprinus carpio* L.) has demonstrated that cortisol in scales is the long-sought biomarker for chronic stress. The methodology was to compare Undisturbed (CTR) and daily stressed (STRESS) of common carp. Dexamethasone (DEX) or cortisol (CORT) fed fish served as negative and positive controls. The Scale cortisol was measure with a validated ultra-performance liquid chromatography tandem mass spectrometry method. The result has shown an increase in scale cortisol content in STRESS and CORT but not in CTR and DEX fish. Also, Aerts has proved that Scale cortisol content reflects its accumulation in a stressor and time dependent manner and validates the scale cortisol content as biomarker for chronic stress. In terms of confirmation the Plasma analyses have been confirmed that (i) CTR, DEX and CORT treatments were effective, (ii) the results of plasma cortisol of STRESS fish showed no signs of chronic HPI-axis activation, and (iii) plasma cortisol is a poor predictor for chronic stress. He has concluded innovative biomarker for chronic stress offering ample applications in science and (Aerts, 2015).

4.2.1 Cortisol in ontogenetic scales of fish as biomarker for chronic stress

In Aerts et al., (2015) work a validated UPLC-MS/MS method has been used for cortisol analysis. All CTR and DEX fish showed scale cortisol values below the detection capability (CCβ), while most STRESS and CORT fish showed scale cortisol values above CCβ at both days. Upon comparing treatments, at day 21 a significant accumulation was found for CORT fish (CORT vs. CTR: $P<0.0001$); at day 42 a significant accumulation was found for STRESS fish (STRESS vs. CTR: $P = 0.0001$).

When comparing within treatments from 21 to 42 days, a significant increase in scale cortisol was found in STRESS ($P = 0.0031$) and CORT ($P = 0.0026$) fish, respectively. These findings were in line with the predicted incorporation and accumulation of cortisol in scales over time and validate the scale cortisol content as a biomarker for chronic stress. In line with the predicted effect of blocking endogenous cortisol production by DEX, they have found no cortisol incorporation in scales of DEX treated fish. On the other hand, feeding cortisol and physically stressing the fish enhanced scale cortisol levels. They have showed that the scale cortisol content is highly suitable for quantification of chronic stress in common carp, this notion makes scale cortisol an innovative and beyond state-of-the art biomarker with high potential impact in science and industries related to fish (e.g. physiology, toxicology, immunology, behavioral studies, aquaculture, etc.). In a broader sense placoid and ganoid scales are predicted to be suitable as well expanding the use of this biomarker to other actinopterygian and even chondrichtyan species. Their new tool for chronic stress monitoring has a huge valorization potential to be adopted in a broad range of governmental, academic and industrial settings such as decision making on animal friendly aquaculture (e.g. animal-based optimization of Recirculating Aquaculture Systems and improvement of product quality, Organic Aquaculture), monitoring of wild fish stocks (e.g. ecology based population dynamics), the welfare of fish in public aquaria and in animal trials (Aerts, 2015). The classic endocrine response to stress is the secretion of glucocorticoids (GCs). Despite that, it remains controversial as to what purpose GCs serve at such times. Report of Hans Selye of stretching back to the time, has been included that GCs help mediate the ongoing or pending stress response, either via basal levels of GCs permitting other facets

of the stress response to emerge efficaciously, and/or by stress levels of GCs actively stimulating the stress response. In contrast, a revisionist viewpoint posits that GCs suppress the stress response, preventing it from being pathologically over activated. In the review of Robert in 2000, they considered the findings regarding GC action and, based on them, generate criteria for determining whether a particular GC action permits, stimulates, or suppresses an ongoing stress response or, as an additional category, is preparative for a subsequent stressor. They have applied these GC actions to the realms of cardiovascular function, fluid volume and hemorrhage, immunity and inflammation, metabolism, neurobiology, and reproductive physiology (Robert, 2000).

4.3 Materials and Methods

Commercial fish pellets (Skretting, Stavanger, Norway) have been used 2 meals a day (09.30 am and 03.30 pm) and five days per week, at 0.9% of body weight were kept at 20.0 ± 0.4°C for 6 weeks. Tree groups, with 2 tank replicates per group, of 10 fish per tank each were set up. Fish of the CTR group were left undisturbed. The CORT group received feed spiked with 500 mg kg-1 cortisol (hydrocortisone, Sigma-Aldrich, St. Louis, MO, USA), for the entire 8-weeks duration of the experiment. Spiked feeds were prepared by uniformly spraying pellets with CORT (1 mg ml-1 in ethanol); pellets were left to dry overnight at room temperature (Barton, 1987). The stress group was stressed tree times per day (five days per week). The type, duration and timing of a subset of stressors were applied randomly and included: netting (15–60 min), air exposure (1–3 min), sudden temperature drop (up to 5°C), chasing (up to 10 min) and confinement (in a bucket with low water level).

Figure 4.1: Raceway concrete tank type, which has been used during experiment

4.3.1 Experimental animals

In this research, data on brown trout (*salmo trutta*) and albino trout (*salmo ghiggi*) brood stock (age 3+) were measured. In addition, concentrations are reported for the first time on a primiparous albino strain of hybrid Mediterranean trouts.

4.3.2 Experimental procedures

4.3.3 Feed preparation

4.3.4 CORTISOL SPIKED FEED

The procedure of preparation of cortisol spiked feed:

-Prepare a cortisol solution of exactly 1 mg/ml cortisol in ethanol (p.a.)

-Weigh exactly 0.001 g of cortisol in an Eppendorf tube on ananalytical balance

-Add exactly 1 ml of ethanol using an Eppendorf pipet of 100 - 1000 µl

-Close the tube

-Vortex shortly to homogenize

-Weigh the needed amount of feed on a tray on an analytical balance

-Spread the feed pellets uniformly across the tray making sure that the pellets are in 1 layer hereby touching each other (no gaps between pellets and no pellets on top of each other)

-Spray the needed volume of 1 mg/ml cortisol solution onto the feed to obtain cortisol spiked feed at 500 mg cortisol per kg of feed by using a plant sprayer e.g. for preparing 1 kg of feed 500 ml of the 1 mg/ml cortisol solution is sprayed

-Dry the feed onto the air during 24 h in a restricted area to prevent contamination

-Store the prepared feed in the fridge at 4 °C

Figure 4.2: Spraying the needed volume of cortisol solution onto the diet

4.3.5 Experimental setup and Sampling procedures

Twenty-one days after the start of the experiment, fish were anesthetized in 2phenoxy-ethanol (0.1%; v/v) and 10 scales were collected from the left flank of the fish. Fish were

allowed to recover and placed back in the respective tanks. At the day 42, Ontogenetic scales (from the right flank) as well as the 21-days regenerated scales (from the left flank) were collected. Fulton's condition factor (K factor; 100W/L3, where W is whole body wet weight in grams and L is standard length in centimeters) was calculated for each fish (Nash, 2006).

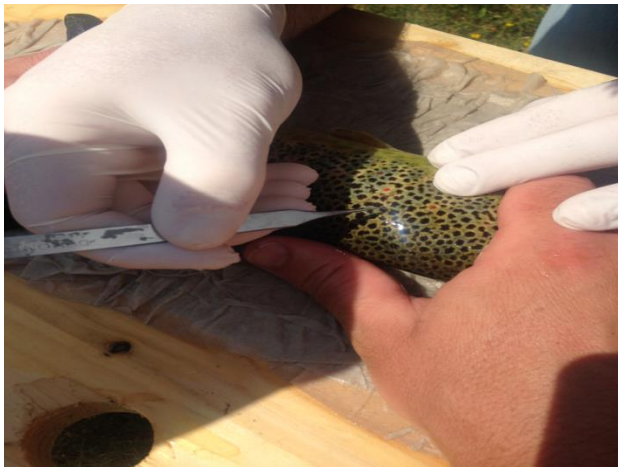


Figure 4.3: scale sampling of wild type (*Salmo trutta*).

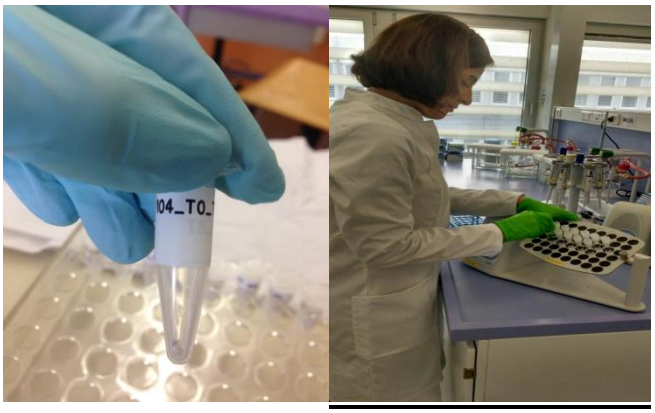
4.3.6 Scale cortisol analysis

An ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) quantification method for cortisol in fish scales was developed in an EN ISO/IEC 17025 accredited environment and validated according the requirements of the Commission Decision No. 2002/657/EC. Chromatographic analysis was performed on an Acquity UPLC-MS/MS Premier XE using an Acquity Ultra Performance LC BEH C18 (1.7 µm; 2.1 mm x 100 mm) column (Waters, Milford, USA). Samples were evaporated to dryness with a Turbovap nitrogen evaporator (Biotage, Sweden). Grace Pure SPE C18-Max (500 mg, 6 ml) columns for solid-phase extraction (SPE) were obtained from Grace

Davison Discovery Sciences (Lokeren, Belgium). High performance liquid chromatography (HPLC) gradient grade methanol (Hipersolv Chromanorm) as extraction solvent was obtained from VWR International BVBA (Leuven, Belgium). Methanol absolute LC-MS as well as formic acid ULC-MS grade from Biosolve BV (Valkenswaard, The Netherlands) and ultrapure water of a Milli-Q gradient Q-Gard 2 from Millipore (Billerica, USA) were used as mobile phase solvents. Only products with a certificate of analysis were used. Cortisol was from Sigma-Aldrich (Diegem, Belgium) and cortisol-d4 from CDN Isotopes (Pointe-Claire, Canada) was used as an internal standard. Individual stock standard solutions of 1 mg mL-1 of cortisol and internal standard were prepared in methanol and stored at 4°C. Calibration standards, ranging from 5 µg L-1 to 100 µg L-1, were prepared by addition of 10 µl of a cortisol-d4 solution of 0.5 µg L-1 to 0.5 µL, 1 µL, 2.5 µL, 5 µL, and 10 µL of a cortisol standard solution of 1 µg L-1 in 100 µL of H2O/MeOH (80:20; v/v), respectively. As 100 mg of sample were used, this corresponded to a range from 5 µg kg-1 to 100 µg kg-1 in fish scales. Scales were sampled from sacrificed fish deeply anesthetized with 2-phenoxy-ethanol (0.1%; v/v) (Sigma-Aldrich, St. Louis, USA). Scales from a standardized row dorsally to the lateral line were removed from the skin with fine tweezers. All samples were rinsed with ultrapure water and air dried on a paper tissue at room temperature. To obtain a homogenized sample scales were cut into fine pieces using scissors. Between samples, scissors were rinsed with ethanol followed by ultrapure water and dried with a paper tissue to avoid cross-contamination between samples. Of the homogenized sample 0.100 ± 0.001 g was weighed into a 10 ml test tube. Subsequently, 8 ml of methanol was added as extraction solvent and 10 µL of a cortisol-d4 solution of 0.5 µg L-1 was added as

internal standard. When lower amounts of scale were used, the volume of cortisol-d4 was adapted accordingly. The sample was vortex-mixed for 30 s, placed on an overhead shaker at 60 rpm for 1 h at room temperature, and centrifuged for 10 min at 3500 g at 7°C. All supernatant was taken, evaporated to dryness under nitrogen at 60°C using a nitrogen evaporator, and reconstituted in 5 mlH2O/MeOH (80:20; v/v). After conditioning a C18 SPE column with 3 ml of methanol followed by 3 ml of ultrapure water, the sample was loaded. The column was washed with 4.5 ml H2O/MeOH (65:35; v/v) and retained compounds were eluted with 2.5 ml H2O/MeOH (20:80; v/v) into a 10 ml test tube and evaporated to dryness under nitrogen at 60°C using a nitrogen evaporator. The sample was finally reconstituted in 100 µL H2O/MeOH (80:20; v/v) in a vial and analyzed by means of UPLC-MS/MS. Glucocorticoids were separated using a gradient elution of mobile phases A and B. Mobile phase A was a mixture of ultrapure water with 0.1% formic acid, while mobile phase B was a mixture of methanol with 0.1% formic acid. Initially, gradient elution started at 20% (v/v) of mobile phase B. Subsequently, mobile phase B was increased to 56% at 1.5 min, to 63% at 6.5 min, to 99.1% at 7.5 min after which it was kept at 99.1% to 8 min, and finally decreased to 20% at 9 min and kept in this way to 10 min. The flow rate was kept constant at 0.4 ml min-1, resulting in a 10 min running time. Samples were cooled at 7°C in the auto sampler. The injection volume was set at 40 µL, while the column temperature was maintained at 30°C. Chromatographic analysis was performed on a mass spectrometer used in the multiple reaction monitoring (MRM) mode in order to achieve optimal sensitivity and selectivity. For cortisol as well cortisol-d4 two precursor fragment ion transitions were determined. Instrumental parameters were optimized by direct infusion of a 10 µg L-1

standard solution in methanol/0.1% formic acid at a flow rate of 10 µLmin-1. The use of two fragment ion transitions allowed the determination of the ratio between both transitions, which was used together with the relative retention time for the identification and confirmation of the identity of each compound according the requirements of the Commission Decision No. 2002/657/EC. The mass spectrometer was used in positive electrospray ionization mode (ESI+). Both compounds were analyzed as their proton adducts [M+H] +. The MS detector settings were set at the following values: a source temperature of 120°C, a de-solvation temperature of 300°C at a gas flow of 800 L h-1, a cone gas flow of 50 L h-1, and a capillary voltage of 3 kV. Argon was used as collision gas at a pressure of 1.11 10–2 mbar. The optimized UPLC-MS/MS conditions with indication of retention time, precursor ion, fragment ions, cone voltage, and collision energy for both compounds are given in Table 1.Data analysis was performed using Quanlynx software from Waters; analysis results were reported as the value (µg kg-1) ± the expanded measurement uncertainty (µg kg-1) with a coverage factor (k) of 2 (95% confidentiality interval). The UPLC-MS/MS method for cortisol in fish scales was validated according the requirements of the Commission Decision No. 2002/657/EC.



A **B**

Figure 4.4: a) barcoded eppendorf tube with samples of scale **b)** scale cortisol analysis in stress physiology laboratory in Ostend (Belgium)

4.4 Calculations and Statistical analyses

All parameters were modeled using a linear mixed model in SAS 9.4 (SAS Institute Inc., Cary, NC) with treatment, day of sampling and their interaction (where appropriate) as fixed effects. A random intercept for tank was introduced in the model to correct for clustering of fish in tanks. Dunnett's test was performed to compare treatments with controls.

4.5 Results

Although I spent an entire month at the Department of Stress Physiology Research Group at the University of Ghent, ready to analyze the scale samples previously collected at Monchio hatchery and preliminarily processed at the University of Parma, no analyses were executed because of supposed unexpected problems reported by Dr. J Aerts and his collaborators. The results would have been delivered directly to University of Parma as preliminarily stated on the basis of a scientific collaboration between the two groups. However Dr. J. Aerts refused to deliver results on time for this submission of this thesis. I decided to insert this part despite the final unexpected ending because of the great effort and amount of work dedicated by several people of Prof. Nonnis Marzano group during spring and summer 2017.

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Chapter 5. General conclusion, discussion and future research

5.1 General conclusion and remarks

In this study, the use of a photometer dedicated to measuring the sperms concentration of milt in artificial reproduction during reproduction season. The choice of the males with higher concentration of sperm and a good motility is important in order to obtain higher fertilization rate during the season, especially for the endangered species, whose offsprings are going to be released in rivers. Quantifying sperm quality throughout the spawning season is important for estimating a stock's reproductive potential (Trippel, 2003) and improvement of short-term and long-term storage (cryopreservation) techniques for many captivity-bred and endangered species (Rideout et al. 2004).

Black carbon, a non-nutritive substance, has the potential to be used as feed additive due to its detoxifying effects and it has been widely used in medical, veterinary and wastewater treatments, as well as in water treatments for aquaculture. So far, studies in the utilization of AC in animal feed have been controversial. In this study, we observed a tendency in fish fed the Biochar-supplemented diet towards a better growth performance compared with the control group. Previous studies assessing the effect of on growth performance and nutrient utilization of aquatic animals have reported conflicting results (Thu 2010; Pirarat et al 2015). Our study revealed that the supplementation of Biochar (Black Carbon) in feed (2-3%) can increase the growth performance and survival rate in *Salmo trutta*.

Stress happens when that all the requirements of fish such as specific physical happen and chemical requirements relative to the aquatic environment are not met, the animals are quickly stressed, and their health and survival jeopardized. Culturing animals in water

is far less forgiving, and potential problems occur more frequently than with terrestrial animal culture. The inherent advantage of this with respect to animal welfare is that aquatic producers recognize that controlling animal stress is absolutely essential for their economic success; and that the development of specific stress management protocols is essential to animal health and survival (Conte, 2004). Knowledge and understanding of what constitutes stress in fish has increased immensely in the past few decades, notably in the area of physiological mechanisms and responses that lead to changes in metabolism and growth, immune functions, reproductive capacity, and normal behavior.

5.2 Recommendation for future research

Based on the work done so far, the following recommendation for future work are suggested for their importance in improving the approaches in aquaculture for Conservation and management of threatened fish species efficiency of the process:

1. Comparison of sperm concentration during spawning season has been conducted in this study to apply for future breeding strategies concerning sperm cell quantity and quality with cryopreservation.
2. Two different doses of biochar has been tested on early life stage of brown trout *(S. truta*), more studies must be used of other species with difference dose from other sources biochar with blood sampling at the end of experiment to check effect of bichar on immune system and health status of fish.
3. In this study chronic stress was synthesized on an albino type (*S. ghigii*) and wild type of brown trout (*Salmo trutta*). And future works are recommended to focus on other species.

References

Ackerman P, Trenholm M, Ewart D, MacWilliams C, MacKinla D. **2008**. Quinsam River Salmon Hatchery Fish Health Management Plan.

Aerts J, Metz JR, Ampe B, Decostere A, Flik G, DeSaeger S (2015) Scales Tell a Story on the Stress History of Fish. PLOS ONE **10**(4):e0123411. doi:10.1371/journal.pone.0123411.

Alavi SMH, Cossong. Sperm motility in fishes (II) Effects of ions and osmolality: a review. Cell bio1. Int 2006; **30**(1):1-14.

Alavi, S.M.H., Cosson J., Karami M., Amiri B.M. & Akhoundzadeh M.A. (2004) Spermatozoa motility in the Persian sturgeon, *Acipenser persicus*: effects of pH, dilution rate, ions and osmolality. Reproduction **128**, 819–828.

Alsop, A. Mathilakath M. Vijayan. 2007. Development of the corticosteroid stress axis and receptor expression in zebrafish. Am J Physiol Regul Integr Comp Physiol **294**: R711–719.

Aly, S.M., Mohamed, M.F. and John, G., 2013. Effect of probiotics on the survival, growth and challenge infection in Tilapia nilotica (*Oreochromis niloticus*). Aquaculture Research, **39**, 647–656.

Anon. **2004**. Atlantic Salmon in Maine: Committee on Atlantic Salmon in Maine. In: Press, T.N.A. (Ed.). National Research Council, pp. 23-46.

Anon. **1980**. University of California Division of Agriculture and Natural Resources; Trout and Salmon Culture (Hatchery Methods). California Fish Bulletin 164, 201.

Anderson, T. and Silva, D. S. 2003: Nutrition. –In Lucas, S. J., and Southgate, C. P. (ed) Aquaculture. Pages: 502. Blackwell publishing company.

Asche, F., 2008. Farming the sea. Marine Resource Economics **23** (4), 527–547.

Ayson, F. G., Kaneko, T., Hasegawa, S. & Hirano, T. Cortisol stimulates the size and number of mitochondrion-rich cells in the yolk-sac membrane of embryos and larvae of tilapia (*Oreochromis mossambicus*) in vitroand invivo.J.Exp.Zool. **272**, 419–425 (1995).

Bacaoui, A, Yaacoubi A, Dahbi A, Bennouna C, Phan Tan Luu R, Maldonado-Hodar FJ, Rivera-Utrilla J, Moreno Castilla C (2001) Optimization of conditions for the preparation of activate carbons from olive-waste cakes. Carbon **39**:425–432.

Barnett, V, Lewis T. Outliers in statistical data; 3rded.**1994**. ISOGUM.

Barton, BA, Schreck CB, Barton LD. Effects of chronic cortisol administration and daily acute stress on growth, physiological conditions, and stress responses in juvenile rainbowtrout .DisAquatOrg .1987; **2**:173–185.

Barton, B. A., C. B. Schreck, and L. G. Fowler. 1988. Fasting and diet content affect stress-induced changes in plasma glucose and cortisol in juvenile chinook salmon. Prog. Fishcult. **50**:16–22.

Barton, B.A., 2002. Stress in fish: a diversity of responses with particular reference to changes in circulating corticosteriods. Integ. Comp. Biol. **42**, 517–525.

Belarian,J.,Haller,R.D.,1982.Theintensiveculture oftilapiaintanks,racewaysandcages.In:Muir,J.F.,Roberts, R.J. (Eds.), Recent Advances in Aquaculture. Westview Press, Boulder, CO, USA, pp. 287–355.

Bernatchez, L., **2001**: The evolutionary history of brown trout (*Salmo trutta*) inferred from phylogeographic, nested clade, and mismatch analyses of mitochondrial DNA variation. Evolution **55**, 351–379.

Bhatnagar, A. Devi P. 2013. Water quality guidelines for the management of pond fish culture. International journal of environmental sciences,Volume **3**, No 6.

Bianco, P. G., **1995**a: Mediterranean endemic freshwater fishes of Italy. Biol. Conserv. **72**, 159–170.

Bianco, P. G., **1998**: Freshwater fish transfers in Italy: history, local modification of fish composition, and a prediction on the future of native populations. In: 'Stocking and Introductions of Fishes' New Fishing Books. J. Cowx (Ed.). Blackwell, Oxford, pp. 165–197.

Bianco, P. G.; Delmastro, G. B., **2011**: Recenti novita tassonomiche riguardanti i pesci d'acqua dolce autoctoni in Italia e descrizione di una nuova specie di luccio. Researches on wildlife conservation, vol 2 (suppl.), IGF publ., USA, pp. 14.

Billard, R. & Cosson M.P. (1992) some problems related to the assessment of sperm motility in freshwater fish. Journal of Experimental Zoology **261**, 122–131.

Billard, R., Dupont J. & Barnabé G. (1977) Diminution de la motilité et de la durée de conservation du sperme de Dicentrarchus labrax L. (*Poisson téléostéen*) pendant la période de spermiation. Aquaculture **11**, 363-367

Billard, R., Cosson J., Crim L.W. & Suquet M. (**1995**) Sperm physiology and quality. In broodstock management and egg and larval quality (ed. by N.R. Bromage & R.J. Roberts), pp. 53-76, Cambridge University Press, Cambridge.

Billard, R., Bry, B.O., Gillett, C., 1981. Stress, environment and reproduction in teleost fish. In: Pickering, A.D. (Ed.), Stress in Fish. Academic Press, London, pp. 185–208.

Bobe, J. Labbe, C. 2010. Egg and sperm quality in fish General land comparative endocrinology **165**. 535-548.

Bonier, F, Martin PR, Moore IT, Wingfield JC. Do baseline glucocorticoids predict fitness? 2009; **24**(11): 634-420.

Boonstra, R, Boag PT. spring Declines in *Microtus pennsylvanicus* and the Role of steroid hormones. Jornal of Animal Ecology. Volume 61.Issue **2**(1992), 339-352.

Bostock,J, **2010**.Foresight project on Global Food and Farming Futures, The application of Science and technology development in shaping current and Future aquaculture production systems.J. Agric. Sci.1-9..

Boyd, C.E. and C.S. Tucker. **1992**. Water Quality and Pond Soil Analyses for Aquaculture. Auburn University, AL. 183 pp.

Brönmark, C. and Hansson, L.-A. (2002). Environmental issues in lakes and ponds: current state and future perspective. Environmental Conservation, **29**, 290–306.

Bruce, A. Barton. 2002. Stress in Fishes: A Diversity of Responses with Particular Reference to Changes in Circulating Corticosteroids. Integ. And Comp.biol. **42**:517–525.

Bury N R, Sturm A, Rouzic P Le, Lethimonier C, Ducouret B, Guiguen Y, Robinson-Rechavi M, Laudet V, Rafestin-Oblin M E and Prunet P (2003). "Evidence for two distinct functional glucocorticoid receptors in teleost fish." Journal of Molecular Endocrinology **31**: 141–156.

Busch, R.L., **1990**. Channel catfish culture in ponds. In: Tucker, C.S., Robinson, E.H. (Eds.), Channel Catfish Farming Handbook. Van Nostrand Reinhold, New York, USA, pp. 19–84.

Bryman, Alan; Cramer, Duncan (**2011**). Quantitative Data Analysis with IBM SPSS 17, 18 and 19: A Guide for Social Scientists. New York: Routledge. ISBN 978-0-415-57918-6.

Carmichael, G.J., Tomasso, J.R., Simco, B.A., Davis, K.B., 1984a. Confinement and water quality-induced stress in largemouth bass. Trans. Am. Fish. Soc. 113, 767–777. Carmichael, G.J., Tomasso, J.R., Simco, B.A., Davis, K.B., 1984b. Characterization and alleviation of stress associated with hauling largemouth bass. Trans. Am. Fish. Soc. **113**, 778–785.

Caputo, V., **2003**: Le trote in Italia e nelle Marche: generalita su tassonomia, distribuzione e caratteristiche biologiche. Quaderni dell'Ambiente, Vol. 15, Provincia di Pesaro e Urbino, Italy, pp. 9–22.

Chan, KY, Van Zwieten L, Meszaros I, Downie A, Joseph S (2007) Agronomic values of greenwaste biochar as a soil amendment. Aust J Soil Res **45**:629–634.

Cho, C., 1997. Reduction of waste output from salmonid aquaculture through feeds and feeding. Prog. Fish- Culturist **59**, 155–160.

Christen, R., Gatti J.-L., & Billard R. (1987). Trout sperm motility: the transient movement of trout sperm is related to changes in the concentration of ATP following the activation of the flagellar movement. European Journal of Biochemistry, **166**, 667–671.

Chrousos, G. P. 1998. Stressors, stress, and neuroendocrine integra- tion of the adaptive response. Ann. N.Y. Acad. Sci. **851**:311– 335.

Ciereszko A., Dabrowski K. (1993). Estimation of sperm concentration of rainbow trout, whitefish and yellow perch using spectrophotometric technique. Aquaculture **109**, 367–373.

Conte. **2004**. "Stress and the welfare of cultured fish." Applied Animal Behavior Science 205-223.

Costantinos C. Mylonas, C. Fostier, A. Zany, S. 2010. Bloodstock management and hormonal manipulations of fish reproductions. General and Comparative Endocrinology **165**.516-534.

Cosson J., Billard R., Cibert C., Dreanno C., Linhart O. & Suquet M. (1997) Movements of fish sperm flagella studied by high speed videomicroscopy coupled to computer assisted image analysis. Polskie Archiwum Hydrobiologii **44**, 103-113.

Conte F.S. 2004. Stress and the welfare of cultured fish. Applied Animal Behaviour Science **86** .205–223.

Conte, F.S., Doroshov, S.I., Hung, S.I.O., Strange, E.M., 1988. Hatchery manual for the white sturgeon *Acipenser transmontanus* Richardson with application to other

North American acipenseridae. Pubication no. 3322. Division of Agricultural and Natural Resource, University of California, USA, p. **104**.

Conte F.S. 2004. Stress and the welfare of cultured fish. Applied Animal Behavior Science **86** (2004) 205–223.

Commission Decision No.2002/657/EC. Concerning the performance of analytical methods and the interpretation of results. OffJEurCommun. **2002**.

Crespi J. Tony D. Williams. Tim S. Jessop. Delehanty B. 2013. Life history and the ecology of stress: how do glucocorticoid hormones influence life-history variation in animals. Functional Ecology, **27**, 93–106.

Crane M. Hyatt A. 2011. Viruses of Fish: An Overview of Significant Pathogens. Viruses. Nov; **3**(11): 2025–2046.

Dadras,H. Dzyuba, H. Cosson, H. Golpour, A. Siddique, M. Linhart, O. Effect of water temperature on the physiology of fish spermatozoon function: a brief review. Aquaculture Research, 2017, **48**, 729–74.

Davidson WS, Koop BF, Jones SJ, Iturra P, Vidal R, Maass A, Jonassen I, Lien S, Omholt SW. Sequencing the genome of the Atlantic salmon (*Salmo salar*). Genome Biol. 2010; **11**(9):403.

Delsoz N, Khara H, Shenavar A.R, Mohseni M., 2017. Interaction of dietary *Pediococcus acidilactici* and folic acid on growth performance, haematological parameters and non- specific immune response of finger barbel, *Acipenser nudiventris*. Iranian Journal of Fisheries Sciences. **16**(3) 869-883.

De Jesus, E. G., Hirano, T. & Inui, Y. Changes in cortisol and thyroid hormone concentrations during early development and metamorphosis in Japanese flounder, *Paralichthys olivaceus*. Gen. Comp. Endocrinol. **82**, 369–376 (1991).

Dekloet, R. et al. 1998. Brain corticosteroid receptor balance in health and disease. Endocr. Rev.**19**: 269–301.

Demir, O. 2011. Aquaculture and Fish Feed Sectors of Turkey General View - II (in Turkish).Eğirdir Su Ürünleri Fakültesi Dergisi **7**(1):39-49. ISSN: 1300- 4891, E-ISSN: 1308-7517. Ergül.

Demirbas, A (2004) Effects of temperature and particle size on bio-char yield from pyrolysis of agricultural residues. J Anal Appl Pyrol **72**:243–248.

Denver, R. 2009. Structural and Functional Evolution of Vertebrate Neuroendocrine Stress Systems. Trends in Comparative Endocrinology and Neurobiology: Ann. N.Y. Acad. Sci. **1163**: 1–16.

Dietrich G. J., Kowalski R., Wojtczak M., Dobosz S., Goryczko K., & Ciereszko A. (2005). Motility parameters of rainbow trout (*Oncorhynchus mykiss*) spermatozoa in relation to sequential collection of milt, time of post-mortem storage and anesthesia. Fish Physiology and Biochemistry, **31**(1), 1–9.

Downie, A, Klatt P, Downie R, Munroe P (**2007**) slow pyrolysis: Australian Demonstration Plant successful on multi- feed stocks. In 'Bioenergy 2007 Conference'. Jyvaskyla, Finland.

Duarte, CM, M. N., Holmer M. 2007. "Rapid domestication of marine species." Science **316**: 382–383.

Dzyuba, V. Cosson, J. Motility of fish spermatozoa: from external signaling to flagella response: a review. Reproductive biology (**2014**). 145-175.

ENISO/IEC17025. General requirements for the competence of testing and calibration laboratories. CEN/CENELEC. **2005**.

Eurachem/CITACGuide. Quantify in gun certainty in analytical measurement (QUAM); 2nd ed.2000; Available:http://www.eurachem.org/.

Euro lab Technical Report No.1/2007. Measurement certainty revised: alternative approaches to uncertainty evaluation. **2007**; Available:http:// www.eurolab.org/.

FAO. **2016**. The State of World Fisheries and Aquaculture 2016. Contributing to food security and nutrition for all. Rome. 200 pp.

FAO. **2013**. Food and Agriculture Organization of the United Nations; Cultured Aquatic Species Information Programme: *Oncorhynchus kisutch* (Walbaum, 1792).

Fevolden, S. E., T. Refstie, and K. H. Røed. 1991. Selection for high and low cortisol stress response in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). Aquaculture **95**: 53–65.

Foote, R.H. and Boucher J.H. (1964). A comparison of several photoelectric procedures for estimating sperm concentration in dog semen. Am. J. Vet. Res., **25**: 558-56.

Gamperl, A. K., M. M. Vijayan, and R. G. Boutilier. 1994. Experimental control of stress hormone levels in fishes: Techniques and applications. Rev. Fish Biol. Fish. 4:215–255.

Gandolfi G., Zerunian S., Torricelli P., Marconato E. (**1991**). I pesci delle acque interne italiane. Istituto poligrafico e Zecca dello stato, Roma.

Gatlin, D., Hardy, R., **2002**. Manipulations of diets and feeding to reduce losses of nutrients in intensive aquaculture. In: Tomasso, J. (Ed.), Aquaculture and the Environment in the United States. U.S. Aquaculture Society, Baton Rouge, Louisiana.

Giuffra, E.; Bernatchez, L.; Guyomard, R., **1994**: Mitochondrial control region and protein coding genes sequence variation among phenotypic forms of brown trout *Salmo trutta* from northern Italy. Mol. Ecol. **3**, 161–171.

Giuffra, E.; Guyomard, R.; Forneris, G., **1996**: Phylogenetic relationships and introgression patterns between incipient parapatric species of Italian brown trout (*Salmo trutta* L. complex). Mol. Ecol. **5**, 207–220.

Guerrero, R.D., **1982**. Control of tilapia reproduction. In: Pullin, R.S.P., Lowe-McConnell (Eds.), The Biology and Culture of Tilapias. ICLARM, MCC, Makati, Metro Manila, Philippines, pp. 309–316.

Guide to the expression of uncertainty in measurement (GUM); 1st ed.1995; NBN-X-40-001.55.

Halvorsen, M.; Stabell, O. B., **1990**: Homing behaviour of displaced stream-dwelling brown trout. Anim. Behav. 39, 1089–1097.

Haussmann, MF. Marchetto MF. Telomeres: linking stress and survival, ecology and evolution. Cur Zool. 2010; **56**:714-27.

Heath, D. D., N. J. Bernier, J. W. Heath, and G. K. Iwama. 1993. Genetic, environmental, and interaction effects on growth and stress response of chinook salmon (*Oncorhynchus tshawytscha)* fry. Can. J. Fish. Aquat. Sci. **50**:435–442.

Hendry, AP, S. S. (**2004**). "Evolution Illuminated: Salmon and Their Relatives." New York: Oxford University Press.

Hendry, AP, Stearns SC. Evolution Illuminated: Salmon and Their Relatives. New York: Oxford University Press; **2004**.

Hinshaw,J.,Fornshell,G.,2002.Effluentsfromraceways.In:Tomasso,J.(Ed.),Aquaculturean dtheEnvironment in the United States. U.S. Aquaculture Society, Baton Rouge, Louisiana.

Hulata, G. (2001). "Genetic manipulations in aquaculture: a review of stock improvement by classical and modern technologies." Genetica **111**: 155–173.

Idaho Division of Environmental Quality (IDEQ), **1998**. Idaho Waste Management Guidelines for Aquaculture Operations. Idaho Department of Health and Welfare, Division of Environmental Quality, Twin Falls.

Idler, D. R. and M. J. O'Halloran. 1970. Steroids of a chondrostean: Identification of internal tissue in the American sturgeon, *Acipenser oxyrhynchus* Mitchill, by histological and histochemical methods. J. Endocrinol. **48**:621–626.

Ingermann, R.L., Holcomb M., Robinson M.L., Cloud J.G. (2002). Carbon dioxide and pH affect sperm motility of white sturgeon (*Acipenser transmontanus*). J Exp Biol 2002; **205**:2885e90.

ISO/DIS5725. Accuracy of measurement methods and results.1994.

ISO/TS21748. Guidance for the use of repeatability, reproducibility and trueness estimates in measurement uncertainty estimation. 2004.

Iwama, G. K., J. C. McGeer, and N. J. Bernier. 1992. The effects of stock and rearing density on the stress response in juvenile coho salmon (*Oncorhynchus kisutch*). ICES Mar. Sci. Symp. **194**: 67– 83.

Kana, JR, Teguia A, Mungfu BM, Tchoumboue J (2011) Growth performance and carcass characteristic of broiler chickens fed diets supplemented with graded levels of charcoal from maize cob or seed of *Canarium schweinfurthii* Engl. Trop Anim Health Prod **43**:51–56.

Kottelat M., Freyhof J. (**2007**). Handbook of European Freshwater Fishes. Kottelat, Cornol, and Freyhof, Berlin.

Kime, D.E., Van Look K.J. W., McAllister B.G., Huyskens G., Rurangwa E., & Ollevier F. (2001). Computer-assisted sperm analysis (CASA) as a tool for monitoring sperm quality in fish. Comparative Biochemistry and Physiology - C Toxicology and Pharmacology, **130**(4), 425–433. http://doi.org/10.1016/S1532-0456 (01)00270-8.

Kiran, GS, Anto Thomas T, Selvin J, Sabarathnam B, Lipton AP. Optimization and characterization of a new lipopeptide biosurfactant produced by marine *Brevibacterium aureum* MSA13 in solid state culture. Bioresour Technol. 2010; **101**:2389–2396. doi: 10.1016/j.biortech.2009.11.023.

Kutlu, HR, U ¨nsal I, Go ¨rgu ¨lu ¨ M (2001) Effects of providing dietary wood (oak) charcoal to broiler chicks and laying hens. Anim Feed Sci Technol **90**:213–226.

Lawson, T.B., **1995**. Fundamentals of Aquaculture Engineering. Chapman & Hall, New York, p. 355.

Lehmann, J, Czimczik C, Laird D and Sohi S, Stability of biochar in soil, in Biochar for Environmental Management, ed by Lehmann J and Stephen J. Earthscan, London, pp183–206 (**2009**).

Lima, IM, Mcaloon AJ, Boateng AA (2008) Activated carbon from broiler litter: process description and cost of production. Biomass Bioenergy **32**:568–572.

Lin, G. R.,Weng, C. F., Wang, J. I. & Hwang, P. P. Effects of cortisol onion regulation in developing tilapia (*Oreochromis mossambicus*) larvae on seawater adaptation. Physiol. Biochem. Zool. **72**, 397–404 (1999).

Liu, Y. Internal Organogenesis in the Zebrafish Model. Organogenesis 3, **44** (2007).

Lorenzoni, M.; Corboli, M.; Martin Door, A. J.; Mearelli, M.; Giovinazzo, G., **2002**: The growth of pike (*Esox lucius* L. 1758), in Lake Trasimeno. Fish. Res. **59**, 239–246.

Lorenzoni, M.; Maio, G.; Nonnis-Marzano, F., **2004**: Stato attuale delle conoscenze sulle popolazioni autoctone di trota in Italia: necessita di un approccio integrato. Quaderni ETP 33,1 –12

Lucentini, L.; Palomba, A.; Gigliarelli, L.; Lancioni, H.; Viali, P.; Panara, F., **2006**: Genetic characterization of a putative indigenous brown trout (*Salmo trutta* fario) population in a secondary stream of the Nera River Basin (Central Italy) assessed by means of three molecular markers. Ital. J. Zool. **73**, 263–273.

Machluf, Y., Gutnick, A. & Levkowitz, G. Development of the zebrafish hypothalamus. Annals of the New York Academy of Sciences **1220**, 93–105 (2011).

Mazeaud, M. M., F. Mazeaud, and E. M. Donaldson. 1977. Primary and secondary effects of stress in fish. Trans. Am. Fish. Soc. **106**:201–212.

McFarland, AF, Chyka PA (1993) Selection of activated charcoal products for the treatment of poisonings. Ann Pharmacother **27**:358–361.

McDonald, G., Milligan, L., **1997**. Ionic, osmotic and acid–base regulation in stress. In: Iwama, G.K., Pickering, A.D., Sumpter, J.P., Schreck, C.B. (Eds.), Fish Stress and Health in Aquaculture. Soc. Exp. Biol. Semin. Ser. 62. Cambridge University Press, UK, pp. 119–144.

McCormick, S. D., Regish, A., O'Dea, M. F. & Shrimpton, J. M. Are we missing a mineralocorticoid in teleost fish? Effects of cortisol, deoxy corticosterone and aldosterone on osmoregulation, gill Na1,K1-ATPase activity and isoform mRNA levels in Atlantic salmon. Gen. Comp. Endocrinal. **157**, 35–40 (2008).

McCormick, S.D.&D.Bradshaw.2006.Hormonal control of salt and water balance in vertebrates .Gen. Comp. Endocrinol. **147**: 3–8.

Mekbungwan, A,Yamakuchi k and T sakaida 2004.Intestinal Villus histological alterations in piglets fed dietry charcoal powder including wood vinegar compound liquid. Anat histol Embryol **33**:11-16.

Mommsen, T., Vijayan, M. & Moon, T. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. Rev. Fish Biol. Fisher. **9**, 211–268 (1999).

Moss,B.,Stephen,D.,Alvarez,C.,Becares,E.,van de Bund,W.,van Donk,E.,deEyto, E., Feldmann, T., Fernández-Aláez, C., Fernández-Aláez, M., Franken, R. J. M., García-Criado F., Gross, E., Gyllström, M., Hansson, L-A., Irvine, K., Järvalt, A., Jenssen, J-P., Jeppesen, E., Kairesalo, T., Kornijow, R., Krause, T., Künnap, H., Laas,A., Lill, E., Luup, H., Miracle, M. R., No˜ges, P., N˜oges, T., Nykannen, M., Ott, I., Peeters, E.T.H.M., Phillips, G., Romo, S., Salujo˜e, J., Scheffer, M., Siewertsen, K., Tesch, C., Timm, H., Tuvikene, L., Tonno, I., Vakilainnen, K., and Virro, T. (2002). The determination of ecological quality in shallow lakes – a tested expert system (ECOFRAME) for implementation of the European Water Framework Directive. Aquatic conservation. **13**. 507–49.

Mylonas, C. C., Zohar, Y., 2001a. Endocrine requlation and artificial induction of oocyte maturation and spermitation in basses of the genus Morone. Aquaculture **202**. 205-220.

Mylonas, C. C., Zohar, Y., 2001b.use of GnRHa. Delivery systems for the control of reproduction in fish. Rev. fish Biol. Fish. **10**. 463-491.

Mylonas, C.C., Zohar. Y. **2007**. Promoting oocyte Maturation, ovulation and spawning in farmed fish. In: Babin, P.J., Creda. G., Lubzens, E. (Eds), the fish Oocyte: From Basic Studies to Biotechnological Applications. Kluwer Academic publishers, Dordrecht, The Netherlands, PP. 433-470.

Namdev GP, Shrivastava A, Awasthi PK. Constraints and opportunity for organic paddy production in Jabalpur district of Madhya Pradesh. Indian Journal of Fundamental and Applied Life Sciences. 2011; **1**(3):141-145.

Nandi, J. 1962. The structure of the internal gland in teleost fishes. Univ. Calif. Publ. Zool. **65**:129–212.

Nash, RDM. Valencia AH. Geffen AJ. The origin of Fulton's condition factor setting the record straight. Fisheries 2006; 31:236–238.

Neil, D.M., Thompson, J. and Albalat, A. **2013**. Freshwater culture of salmonids in recirculating aquaculture systems (RAS) with emphasis on the monitoring and control of key environmental parameters. Technical report.

Nesan, D. Mathilakath M. Vijaya. **2016**. Maternal Cortisol Mediates Hypothalamus-Pituitary-Interrenal Axis Development in Zebrafish. Scientific RepoRts 6:22582 DOI: 10.1038/srep22582.

Nesan, D. & Vijayan, M. M. Role of glucocorticoid in developmental programming: Evidence from zebrafish. Gen. Comp. Endocrinol. 1–10 (**2012**).

Nord test Technical Report TR537. Handbook for calculation of measurement uncertainty in environmental laboratories; 2nd ed. **2004**; Available: http://www.nordtest.org/.

Noga, E. J., J. H. Kerby, W. King, D. P. Aucoin, and F. Giesbrecht. 1994. Quantitative comparison of the stress response of striped bass (*Morone saxatilis*) and hybrid striped bass (*Morone saxatilis 3 Morone chrysops and Morone saxatilis 3 Morone americana*). Am. J. Vet. Res. **55**:405–409.

Oliver, A. **2008**. Quantifying and modeling of the nitrogenous wastes associated with the commercial culture of Atlantic cod (*Gadus morhua L*.).

Pankhurst, N. W. and M. Dedual. 1994. Effects of capture and re- covery on plasma levels of cortisol, lactate and gonadal steroids in a natural population of rainbow trout. J. Fish Biol. **45**:1013– 1025.

Pennell, W. Prouzet P. 2017 .Salmonid fish: biology, conservation status, and economic importance of wild and cultured stocks. Fisheries and aquaculture .vol. III.

Piper, R.G., McElwain, I.B., Orme, L.E., McCraren, J.P., Fowler, L.G., Leonard, J.R. (Eds.), **1982**. Fish Hatchery Management. American Fisheries Society, Bethesda, MD, USA, p. 517.

Pirarat, N. Boonananthananasarn S. krongpong L. katagiri T. masashi M. 2015. Effect of Activated Charcoal- Supplemented Diet on Growth performance and intestinal morphology of Nile Tilapia (*Oreochromic niloticus*). **45**(1): 113-119.

Post, G.B. **1987**. Text Book of Fish Diseases. TFH Publications, Neptune, New Jersey, p. 287.

Pottinger, T. G., A. D. Pickering, and M. A. Hurley. 1992b. Consistency in the stress response of individuals of two strains of rainbow trout, *Oncorhynchus mykiss*. Aquaculture **103**:275– 289.

Prunet,P.,Sturm,A.&Milla,S.Multiplecorticosteroidreceptorsinfish:fromold ideas to new concepts. Gen. Comp. Endocrinol. **147**, 17–23 (2006).

Ramseyer, L., Garling, D., 1997. Fish Nutrition and Aquaculture Waste Management. Illinois–Indiana Sea Grant Program, Publication CES-305.

Ramstad, A., Midtlyng P.J. Strong genetic influence on IPN vaccination-and-challenge trials in Atlantic salmon *Salmo salar* L. J. Fish Dis. 2008; **31**:567–578.

Ramussen, R. a. (1998). "Extinction Rates of North American Freshwater Fauna." Conservation Biology 13: 1220–1222.

Rand Weaver, M., T. G. Pottinger, and J. P. Sumpter. 1993. Plasma somatolactin concentrations in salmonid fish are elevated by stress. J. Endocrinol. **138**:509–515.

Randall, D. J. and S. F. Perry. **1992**. Catecholamines. In W. S. Hoar and D. J. Randall (eds.), Fish physiology, Vol. 12B, pp. 255– 300. Academic Press, New York.

Reid, S. G., M. M. Vijayan, and S. F. Perry. 1996. Modulation of catecholamine storage and release by the pituitary-internal axis in the rainbow trout (*Oncorhynchus mykiss*). J. Comp. Physiol. **165**B:665–676.

Robert, m. Sapolsky, l. M. R., and allan u. Munck (2000). "How Do Glucocorticoids Influence Stress Responses? Integrating Permissive, Suppressive, Stimulatory, and Preparative Actions." The Endocrine Society **21**(1): 55-89.

Robb, D.H.F., Roth, B., 2003. Brain activity of Atlantic Salmon (*Salmo salar*) following electrical stunning using various field strengths and pulse durations. Aquaculture **216**, 363–369.

Robert, M. Sapolsky. Romero M. Allan u. Munck. 2000. How Do Glucocorticoids Influence Stress Responses? Integrating Permissive, Suppressive, Stimulatory, and Preparative Actions. Endocrine Reviews **21**(1): 55–89.

Roger, C. Viadero Jr.a, James H. Cunningham a, Kenneth J. Semmens b, Aislinn E. 2005. Tierney Effluent and production impacts of flow-through aquaculture operations in West Virginia Aquacultural Engineering **33** (2005) 258–270.

Rosamond Naylor, K. H., Ian A. Fleming, Rebecca Goldburg, Susan Williams, John Volpe, Fred Whoriskey, Josh Eagle, Dennis Kelso, And Marc Mangel (**2005**).

"Fugitive Salmon: Assessing the Risks of Escaped Fish from Net-Pen Aquaculture." BioScience 55.

Ruane, N. M., S. E. Wendelaar Bonga, and P. H. M. Balm. 1999. Differences between rainbow trout and brown trout in the reg- ulation of the pituitary-interrenal axis and physiological perfor- mance during confinement. Gen. Comp. Endocrinol. **113**:210– 219.

Rurangwa, E. Kime, D. E., Ollevier, F., Nash, J. P., 2004. The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture **234**, 1-28.

Royal Decree of the Belgian State of January 18th 2012 published in the Belgian Government Gazette of January 27th **2012**.

Ryman, N.; Allendorf, F.; St ahl, G., **1979**: Reproductive isolation with little genetic divergence in sympatric populations of brown trout (*Salmo trutta*). Genetics 92, 247–262.

Sangalang, G. B., M. Weisbart, and D. R. Idler. 1971. Steroids of a chondrostean: Corticosteroids and testosterone in the plasma of the American Atlantic sturgeon, *Acipenser oxyrhynchus* Mitch- ill. J. Endocrinol. **50**:413–421.

Schindler, DE, Scheuerell MD, Moore JW, Gende SM, Francis TB, Palen WJ. Pacific salmon and the ecology of coastal ecosystems. Front Ecol Environ 2003; **1**(1):31-7.

Schindler, S. M., Moore JW, Gende SM, Francis TB, Palen WJ (2003). "Pacific salmon and the ecology of coastal ecosystems." Front Ecol Environ **1**(1): 31-37.

Schlenk, W. & Kahmann H. (1938) the chemical composition of seminal fluids and their physiological importance study with trout sperm. Biochemical Zoology **295**, 283–301.

Schreck, C.B., **2000**. Accumulation and long-term effects of stress in fish. In: Moberg, G.P., Mench, J.A. (Eds.), the Biology of Animal Stress: Basic Principles and Implications for Animal Welfare. CABI Publishing, CAB International, Oxon and New York, pp. 147–158.

Schreck, C.B., Olla, B.L., Davis, M.W., **1997**. Behavior responses to stress. In: Iwama, G.K., Pickering, A.D., Sumpter, J.P., Schreck, C.B. (Eds.), Fish Stress and Health in Aquaculture. Soc. Exp. Biol. Semin. Ser. 62. Cambridge University Press, UK, pp. 145–170. Schwedler, T. E. Tucker, C.S., Beleau, M.H.,

1985.Non infectious diseases .In: Tucker, C. S. (Ed.), Channel Cat fish Culture. Elsevier, Amsterdam, pp. 497–541.

Schreck, C.B., **1981**. Stress and compensation in teleostean fishes: response to social and physical factors. In: Pickering, A. (Ed.), Stress and Fish. Academic Press, London, pp. 295–321.

Schreck, C.B., Olla, B.L., Davis, M.W., **1997**. Behavior responses to stress. In: Iwama, G.K., Pickering, A.D., Sumpter, J.P., Schreck, C.B. (Eds.), Fish Stress and Health in Aquaculture. Soc. Exp. Biol. Semin. Ser. 62. Cambridge University Press, UK, pp. 145–170.

Selye, H. 1973. The evolution of the stress concept. Am. Sci. **61**: 692–699.

Selye, H. **1974**. Stress without distress. McClelland Stewart, Toronto.

Sedgwick, S.D., **1988**. Salmon Farming Handbook. Fishing News Books Limited, Farnham, Surrey, England, p. 207.

Selye, H. 1973. The evolution of the stress concept. Am. Sci. **61**: 692–699.

Selye, H. 1974. Stress without distress. McClelland Stewart, Toronto.

Steven, T. Summerfelt, John W. Davidson, Thomas B. Waldrop, Scott M. Tsukuda, Julie Bebak-Williams, 2004. A partial-reuse system for coldwater aquaculture. Aquacultural Engineering **31**.157–181.

Stevenson, J.P., 1987. Trout Farming Manual. Fishing News Books Limited, Farnham, Surrey, England, p. 259.

Surintorn, B. Pranorm K. Taratip P. Yanling Hua. 2014. The effects of the supplementation of activated charcoal on the growth, health status and fillet composition-odor of Nile tilapia (*Oreochromis niloticus*) before harvesting. Aquacult Int **22**:1417–1436.

Tacon, agj. And halwart, m. 2007. Cage aquaculture: a global overview. In halwart, m., soto, d. And artur, jr., orgs. Cage aquaculture regional reviews and global overview. Roma: FAO, FISHERIES technical. No. **498**, p. 3-16.

Tamot, P. Moshra R. Smodutt. 2008. Water quality monitoring of halali reservoir with reference to cage aquaculture as a modern for obtaining enhanced fish production. The 12th work lake conference: 318-324.

Tanck, M. W. T., K.-J. Vermeulen, H. Bovenhuis, and H. Komen. 2001. Heredity of stress-related cortisol response in androgenic common carp (*Cyprinus carpio L.*). Aquaculture **199**:283– 294.

Tekinay, A.A. and Davies, S.J., 2001. Dietary carbohydrate level influencing feed intake, nutrient utilization and plasma glucose concentration in the rainbow trout, *Oncorhynchus mykiss.* Turkish Journal Veterinary and Animal Science, **25**, 657–666.

Tessa L. Getchis. 2014. Aquaculture management guide.

Thu M, Koshio S, Ishikawa M, Yokoyama S (2010) Effects of supplementation of dietary bamboo charcoal on growth performance and body composition of juvenile Japanese flounder, *Paralichthys olivaceus*. J World Aquac Soc **41**:255–262.

Tort, L., D. Montero, L. Robaina, H. Ferna´ndez-Palacios, and M. S. Izquierdo. 2001. Consistency of stress response to repeated handling in the gilthead sea bream *Sparus aurata* Linnaeus, 1758. Aquacult. Res. **32**:593–598.

Tsalafouta, A., Papandroulakis N. Gorissen M. Katharios P. Flik G. Pavlidis M.2014. Ontogenesis of the HPI axis and molecular regulation of the cortisol stress response during early development in Dicentrarchus labrax. Physiology molecular biology 4:5525 DOI: 10.1038/srep05525.

Tucker, C.S. (Ed.), 1985. Channel Catfish Culture. Elsevier, Amsterdam, p. 657.

Van Zwieten, L. Kimber S. Morris S. Chan K. Y. Downie A. Rust J. Joseph S. Cowie A. 2010. Effects of biochar from slow pyrolysis of papermill waste on agronomic performance and soil fertility. Plant Soil **327**:235–246.

Vazzana, M., Cammarata, M. & Parrinello, N. Confinement stress in sea bass (*Dicentrarchus labrax*) depresses peritoneal leukocyte cytotoxicity. Aquaculture **210**, 231–243 (2002).

Vermeirssen, E. L. M. Mazorra de Quero, C. Shields, R. J.,Norberg, B., Kime, D.E., Scott, A. P., 2003. Fertility and motility of sperm from Atlantic halibut (*Hippoglossus hippoglossus*) in relation to dose and timing of gonadotropin-releasing hormone agoinst implant.Aquaculture **230**, 547-567.

Waples, RS, Hendry AP. Special Issue: Evolutionary perspectives on salmonid conservation and management. Evol Appl. 2008; **1**(2):183-8.

Waples, RS, H. A. (2008). "Evolutionary perspectives on salmonid conservation and management." Evol Appl **1**(2): 183-188.

Wedemeyer, G.A., 1997. Effects of rearing conditions on the health and physiological quality of fish in intensive culture. In: Iwama, G.K., Pickering, A.D., Sumpter, J.P., Schreck, C.B. (Eds.), Fish Stress and Health in Aquaculture. Soc. Exp. Biol. Semin. Ser. 62. Cambridge University Press, UK, pp. 35–71.

Wendelaar Bonga, S. E. The stress response in fish. Physiol. Rev. 77, 591–625 (1997). Mommsen, T. P., Vijayan, M. M. & Moon, T. W. Cortisol in teleosts: dynamics, mechanisms of action, and metabolicregulation.Rev.FishBiol.Fisher.**9**, 211–268 (1999).

Wendelaar Bonga, S. The stress response in fish. Physiol. Rev. **77**, 591–626 (1997).

Wheaton, F.W., 1977. Aquaculture Engineering. Wiley, New York, p. 708.

Williot, P., Kopeika E.F. & Goncharov B.F. (2000) Influence of testis state, temperature and delay in semen collection on spermatozoa motility in the cultured Siberian sturgeon (*Acipenser baeri* Brandt*)*. Aquaculture.

Woodward, C. C. and R. J. Strange. 1987. Physiological stress responses in wild and hatchery-reared rainbow trout. Trans. Am. Fish. Soc. **116**:574–579.

Yavuzcan, H., Pulatsü, S., Demir, N., Kırkağaç, M., Bekcan, S., Topçu, A., Doğankaya, L. and Başçınar, N. 2010. Sustainable Aquaculture in Turkey. (In Turkish). TMMOB ZiraatMühendisliği VII. Teknik Kongresi, Proceedings Book-2, 767-789.

Yoo JH, Ji SC, Jeong GS (2005). Effect of dietary charcoal and wood vinegar mixture (CV82) on body composition of olive flounder *Paralichthys olivaceus*. J World Aquac Soc **36**:203–208.

Zerunian, S. (**2002**). Condannati all'estinzione? biodiversità, biologia, minacce e strategie di conservazione dei pesci d'acqua dolce indigeni in Italia. Ministero dell'ambiente e della tutela del territorio, Rome.

Zuccon, G. 2016. Integrating genetic analyses and morpho-cellular approaches to sustainably conserve the marble trout (*Salmo marmoratus* Cuvier, 1817) populations. PhD thesis. Università degli Studi di Ferrara.

Zohar, Y,Tal, Y,Schreier,H, H.J., steven, C.R., Stubble Field. J, place, A, R., 2005. Commercially Feasible urban recirculating aquaculture: addressing the marine sector in: costa pierce. B., Desbonnet. A., Edwards,P., (Eds.) Urban Aquaculture. CABI publishing, walling ford, PP 159-171.

LIST OF PUBLICATIONS

Academic journal

1. **Negin Delsouz khaki**, Hossein Khara, Alireza Shenavar, Mahmmoud Mohseni., 2017. "Interaction of dietary *Pediococcus acidilactici* and folic acid on growth performance, haematological parameters and non- specific immune response of finger barbel, *Acipenser nudiventris"*. Iranian Journal of Fisheries Sciences.2017. 16(3) 869-883.

Conference paper (Opral peresentation)

1. **Negin Delsouz khaki,** Hossien Khara, Mahmmud Mohseni, Alireza Shenavar Masouleh and Francesco Nonnis Marzano., 2017. "Interaction of dietary *Pediococcus acidilactici* and folic acid on the growth performance, haematology parameters and non-specific immune response of finger barbel, (*Acipenser nudiventris*)". Aquaculture Europe 17. Dubrovnik, Croatia.

Conference Proceeding

1. **N. Delsouz Khaki,** F. Nonnis Marzano (**2015**). "Effects of bactocell probiotic (*Pediococcus acidilactici*) and folic acid on immune system on ship sturgeon fingerling", 4th International Conference and Exhibition on Probiotics, Functional and Baby Foods, page (53), Valencia, Spain.

2. **N. Delsouz Khaki**, A. Piccinini, G. Zuccon and F. Nonnis Marzano (**2016**). "Comparison of sperm concentration during spawning season of different brown trout (*Salmo trutta, Salmo ghigii, Salmo cetti*) and marble trout (*Salmo marmoratus*) strains with first description of a brown trout albino lineage", Aquaculture Europe 16, Page (67), Edinburgh, Scotland.

3. **N. Delsouz Khaki,** A. Malcevschi, Andrea Voccia, F. Marzano (**2017**). "Interaction of dietary biochar (black carbon) on the growth performance and survival rate of early stage larvae of brown (*Salmo trutta*)", Aquaculture Europe 17, Page (85), Dubrovnik, Croatia.