Research for determination factors of fish life span

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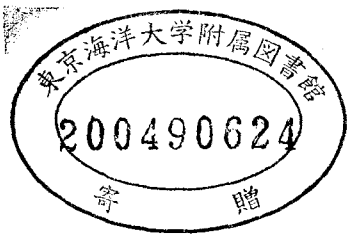

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CONTENTS

ABSTRACT

Some fish species show parental death shortly after their first spawning. The well-known examples are ayu (*Plecoglossus altivelis*) which dies in only one year. Although the mechanisms for such a short life span are still unclear, there have been proposed some hypotheses. Since it is shown clearly that ayu produced ROS higher than other species, it is supposed that high ROS production strongly related in aging advances, resulting in shortened life span. Homeostasis disturbances by maturation, debility for exhausting energy of spawning and decreasing of feeding activities during spawning and after spawning are also considered to be factors which ayu dies in only one year. Along these hypotheses, this study dealed with carp as a model fish with long life span and ayu as a model fish with short life span and was performed for disclosing whether 1) influence of the oxidative stress on biomembrane, 2) apoptosis related factors, 3) caloric restriction, and/or 4) feeding activity would be relevance to short life span or not. Clarification of fish life span determination factors will also contribute to the stable fish culture techniques including 'programmed' fish culture on the basis of mechanism elucidations.

This thesis is composed of five chapters. Chapter I deals with introduction and general discussion. It was given for gaining insight into aging and senescence studies. The free radical theory of aging, telomere theory of aging, programmed cell death, p53 tumor suppressor protein induced apoptosis and caloric restriction were reviewed comprehensively. Regulatory peptides and control of food intake were also described.

In Chapter II, it was investigated the influence of partial oxidative stress on permeability and fluidity of nucleated fish red blood cells for simulating nucleated somatic cells. Peroxide value indicating lipid hydroperoxide level in nucleated red blood cells of common carp (*Cyprinus carpio*) increased with increasing body size. It was detected that oxidation of nucleated red blood cells led to the degraded PUFA compositions and accelerated the permeability of calcein and ATP in the nucleated red blood cells restrictedly oxidized with AAPH treatment. Using fluorescence probes, PC_3P, it was found that oxidative stress reduced the membrane fluidity of nucleated red blood cells. It was also observed that AAPH had no significant influence on the osmotic fragility and electrophoretic profiles of red blood cell proteins. These results suggest that partial oxidative-stress, even if failure to fragment the membrane, may affect membrane permeability of fish nucleated red blood cells for an important energy molecule, ATP.

It is well known that ayu (*Plecoglossus altivelis*) die after spawning and the life span is only one year. One possible cause is that enhanced oxidative stress might induce DNA damage and subsequent DNA repair systems as phosphorylated p53 in ayu, leading to apoptosis and relating to their short life span. Telomeres, the non-coding sequences at the ends of chromosomes, shortening of telomeres can induce cell cycle arrest and apoptosis.

Chapter III was, then, addressed to the p53 and its phosphorylation in ayu brain, the oxidative DNA damage by measuring the levels of 8-OHdG and the induction of apoptosis by measuring the levels of caspase-9/6, -3 with aging in brain and liver. It was also investigated that age related changes in telomere length in the ayu. The findings indicate that oxidative stress activated caspase-9/6, -3 in brain and liver, and activated p53 through the phosphorylation of p53 and p53 with aging in ayu brain. There was no significant change in telomere length through life span. It was suggested that the age-related of apoptosis might be involved in increasing of DNA damage and mutations in brain and liver, and could partially explain the short life span of ayu.

The effects of caloric restriction on post-spawning death of ayu were investigated in Chapter IV. Caloric restriction is the only established intervention that extends life span in mammals, insects and nematodes. One of the hypotheses suggested that most of the effects of CR on aging may be due to reduced oxidative stress at the cellular level. It was known that ayu produced ROS higher than other fish and that the life span of ayu is only one year. It was attempted to quantify age-associated changes of the degree of attenuation on oxidative damage and hormonal homeostasis in CR. The oxidative DNA damage by measuring the levels of 8-OHdG and the induction of apoptosis by measuring the levels of caspase-9/6, -3 with aging in brain and liver were surveyed. Changes in major sexual hormones were also investigated. Caspase activities in brain and liver were reduced by CR, although CR was no influence to DNA damage level. Plasma testosterone levels of CR ayu were significantly higher and progesterone and 17 β-estradiol levels were lower than the control ayu. However, life span of ayu was not prolonged by CR. These results suggest that there would be factors determining life span of ayu other than CR and apoptosis.

Chapter V deals with roles of leptin in post-spawning death of ayu. It is well known that ayu dye after spawning and the life span is only one year. The determinants for such a short life span are probably involved in spawning and some accompanied changes in hormonal homeostases. It is one of the accompanied changes that feeding activity of ayu decreases during spawning and after spawning. Then, it was investigated the relationships among leptin and ghrelin, they are regulators for food intake, and other major hormones, 17 β-estradiol and prolactin. Leptin levels were significantly higher during spawning, associated with decrease in appetite. Leptin levels were also synchronized with levels of 17 β-estradiol and prolactin. Ghrelin levels were no significant difference. Therefore, one possible explanation for decrease in appetite during ayu spawning is that the alteration of 17 β-estradiol homeostasis induced the secretion of leptin. The inability to reduce the leptin level into the basal after spawning would be in part responsible for a short life span of ayu.

In conclusion, it was revealed that the mechanisms governing life span of ayu were through at least two pathways. One is apoptosis induced by oxidative stress with aging.

This pathway is probably, however, an alleyway, since CR could afford to down-regulate apoptosis pathway but did not extend the life span of ayu. Another is decreasing appetite during and after spawning induced by leptin in ayu. Reproduction induced physiological anorexia: it is beginning to death.

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CHAPTER I

INTRODUCTION AND GENERAL BACKGROUNDS

It is mentioned that the control system for maintaining the constancy of the homeostases of animals is always working. Although it is becoming clear that these are controlled by the network of immunity systems, nervous systems and internal secretion systems, the regulation mechanisms in fish is hardly clear, and becomes one of the big research subjects.

Now, the view of individual aging of animals has common theory of "programmed" aging. A programmed theory connects shortening of the telomere by reduction of telomerase activity to a cell life. The telomerase activities of cells of fish are, however, very high, and it is hard to explain in a programmed theory for fish aging.

The life span of fish is mainly prescribed by the grade of aging and debility like other animals. This research is focused to the destruction of homeostasis with fish aging and aimed at dynamic understandings of interactions among oxidative stress, feeding action, aging, etc. The following sections will deal with comprehensive backgrounds associated with those factors and brief summaries of this thesis.

Section 1

General Background

The study of aging, by nature multidisciplinary, has been characterized by a dizzying variety of theories, a huge phenomenological literature, and the absence of firmly established primary causes. The diverse life histories of animal species, which manifest aging in very different ways, have been an obstacle to testing unified theories. For experimental gerontology to provide more than a catalog of age-related changes, it has been necessary for biologists to define the alterations that are common to most old cells, tissues, and animals, simultaneously respecting that there is not a single phenomenon of aging or a single cause. Many theories have been proposed to explain the basis of aging. They have been classified into organ theories (immune or neuroendocrine), physiological theories (free radical, dross-linking and waste-product accumulation) and genome-based theories (somatic mutation, error theory and program theory) of aging.

1. THE FREE RADICAL THEORY OF AGING

1.1 Origins of the Free Radical Theory

In 1956, Denham Harman suggested that free radicals produced during aerobic respiration cause cumulative oxidative damage, resulting in aging and death. He noted parallels between the effects of aging and of ionizing radiation in both of them causing mutagenesis, cancer, and gross cellular damage (Harman, 1956). At that time, the presence of hydroxyl radical (•OH) in living matter had been just identified (Commoner et al., 1954). Harman (1956), therefore, hypothesized that endogenous oxygen radical generation would occur *in vivo*, as a by-product of enzymatic redox chemistry. He ventured that the enzymes involved would be those involved in the direct utilization of molecular oxygen, and that particularly those would contain iron. Finally, he hypothesized that traces of iron and other metals would catalyze oxidative reactions *in vivo* and that peroxidative chain reactions would make progress, analogous to the principles of *in vitro* polymer chemistry. All of these predictions have been confirmed during the past 40 years.

The theory gained credibility through the identification in 1969 of the enzyme superoxide dismutase (SOD) (McCord and Fridovich, 1969), which provided the first compelling evidence of *in vivo* generation of superoxide anion (O_2^{-}•), and from the subsequent elucidation of elaborate antioxidant defenses (Yu, 1994). The use of SOD as a tool to locate subcellular sites of O_2^{-}• generation led to a realization that buttressed up the free radical theory, namely, that mitochondria are a principal source of endogeneous ROS (Chance et al., 1979). Gerontologists had long observed that species with higher metabolic rates have shorter maximum life span potential; they age faster. The accumulation of cardiac lipofuscin in the monkey related with its cumulative O_2 consumption at a sexual

maturation stage (Nakano et al., 1989). The realization that energy consumption by mitochondria may result in $O_2^{-}\bullet$ production linked the free radical theory with the rate of living theory irrevocably: a faster rate of respiration, associated with a greater generation of oxygen radicals, hastens aging.

1.2. Oxidants and evolutionary theories of aging

The intracellular generation of oxidants capable of limiting life span may appear paradoxical. It seems reasonable to expect that natural selection might have devised aerobic cells that do not leak toxic by-products. Evolutionary biologists have contributed to the free radical theory by suggesting why physiologically harmful generation of oxygen radicals occurs. They have argued that natural selection favors genes that act to preserve nongerm cells, a principle called antagonistic pleiotropy (Kirkwood, 1977, 1992; Kirkwood and Rose, 1991; Williams and Nesse, 1991). The concept of antagonistic pleiotropy stresses that reproductive success is principally a function of external factors. With the exception of modern-day humans, individuals do not usually die of old age, but are eaten, parasitized, or out-competed by others. Kirkwood and Cremer (1982) contributed a physiological perspective, expressed as the 'disposable soma theory' which states that although it is theoretically possible to invest sufficiently in somatic maintenance and repair to fend off harmful age changes, natural selection may favour a balance that falls short of immortality and results in senescence. The limiting resources would take many forms and include energy and nutrition, DNA repair and replacement of defective proteins, and response to cellular stress. The positive correlation between resistance to physiological stressors and life span in a range of mammalian species is consistent with the disposable soma theory (Kapahi et al., 1999). The theory was proposed to account for somatic aging, but it can also apply to germ cells. Indeed, similar age changes and protective mechanisms might be expected in both germ and somatic cells, even if the expression varies among specific cell types. In terms of natural selection, the tremendous cost of death before reproductive age, the constantly compounding probability of death from external threats, and the cost of failing to reproduce all ensure that selective pressure is strongest at young ages. Any novel mutations that decrease oxidative damage first have to satisfy the criteria of youthful reproduction. In short, the selective pressure to compete effectively at an early age may guarantee a certain degree of O_2 toxicity and work against the conservation of the soma in the long run.

1.3. Oxidants and the somatic mutation theory of aging

The somatic mutation theory holds that the accumulation of DNA mutations is responsible for degenerative senescence (Bohr and Anson, 1995; Evans et al., 1995; Miquel, 1992; Morley, 1995; Vijg and Gossen, 1993). In the case of cancer, which results from both point mutations in oncogenes and the loss of tumor suppressor gene function (often by deletion), the role of mutations are unquestionable (Ames et al., 1995). It remains to be seen whether or not the argument is valid for nonproliferative senescence. For instance, whereas significant age-related increases in somatic mutations in a reporter transgene (*lacZ*) have been measured in a mitotic tissue of transgenic mice, no increase was detected in the largely postmitotic brain of the same animals (Dolle et al., 1997), suggesting that neurodegeneration, at least, is unlikely to be the result of accumulated somatic mutations in nuclear DNA. Moreover, the accumulation of mutations in the liver tissues was not dramatic, suggesting that mutagenesis may be of little functional consequence to mitotic tissue as well (Warner and Johnson, 1997). A compelling argument for the somatic mutation theory of aging was provided years ago through the discovery that DNA repair ability correlates with species-specific life span (Hart and Setlow, 1974) and has been recently reconfirmed by Cortopassi and Wang (1996). However, they have also noted that DNA repair, which is necessary for the prevention of tumorigenesis, is necessary but not sufficient for longevity. Ultimately, arguments about the physiological significance of somatic mutations hinge on how disruptive a given mutational burden is to a cell or animal; this is currently an unanswerable question.

In any case, it has been demonstrated in numerous studies with prokaryotes, yeast, and mammalian cells that oxidants are mutagens, against which cells protect their genetic material (Feig et al, 1994; Grollman and Moriya, 1993). Although it is not yet clear what function of mutations can be attributed to oxidative damage, the identification and characterization of defense genes against oxidative mutagenesis (Beckman and Ames, 1997) and the development of *in vivo* mutagenesis assays (Martus et al., 1995) have finally opened up avenues for definitive experiments.

1.4. Oxidants and mitochondrial theories of aging

The mitochondrion has also long attracted attention as one of the cell's weak links, an organelle whose dysfunction has profound negative pleiotropic effects (Luft, 1994). Mitochondria supply ATP and also sequester potentially toxic Ca^{2+}, yet because of their generation of $O_2^{-}\bullet$ and H_2O_2, they are on the front lines of respiratory oxidative stress. The idea that the mitochondrion is therefore uniquely vulnerable was embraced early on by proponents of the free radical theory (Harman, 1972). In the early 1980s, it was proposed

that oxidative damage to mitochondrial DNA (mtDNA) inpostmitotic cells would lead to mutations and blocks of replication, and consequently to mitochondrial dysfunction and physiological decline (Fleming et al., 1982; Miquel et al., 1980). This mtDNA mutation hypothesis of aging, which incorporates free radicals, somatic mutations, and the central role of mitochondria in homeostasis, is presently under intense scrutiny (Arnheim and Cortopassi, 1992; Bandy and Davison, 1990; Bittles, 1992; Cortopassi and Liu, 1995; Hagen et al., 1997; Muller, 1992; Nangley et al., 1992; Ozawa, 1995; Richter, 1992, 1995; Richter et al., 1988; Schapira and Cooper, 1992; Shigenaga et al., 1994; Wallace et al., 1995; Wei, 1992).

2. TELOMERE THEORY OF AGING

Mammalian cells have evolved complex mechanisms for regulating cellular life span. Normal cells demonstrate a strictly limited growth potential and senescence after a defined number of cell divisions. Cellular senescence is one of the bases of organismal aging. In contrast, tumor cells often exhibit an apparently unlimited proliferation potential and are called "immortalized". Some investigators have proposed that the progressive shortening of the tips of the eukaryotic chromosomes, the telomeres, are important component of senescence and is involved in control of the cell cycle. The enzyme telomerase adds TTAGGG repeated onto mammalian telomeres, which prevent their shortening. Telomerase is ordinarily inactive in most somatic cells but can be detected in tumor cells. The activation of telomerase in malignant cancers seems to be an important step in tumorigenesis, whereby the cell gains the ability of indefinite proliferation to become immortal. As detailed information accumulates about how telomeres dynamics are involved in the regulation of cell cycle events, one can expect new opportunities for application to gerontology.

2.1. Telomere and telomerase

The extreme ends of eukaryotic chromosomes- the telomeres- are special structures that provide protection from enzymatic end-degradation and maintain chromosome stability (Dahse et al., 1997). Chromosomes with truncated telomere tips fuse with other chromosome ends or become lost during cell division. Telomeres also play a role in organization of the cellular nucleus by serving as attachment points to the nuclear matrix (de Lange, 1992). Apart from providing stabilization and protection to the chromosomes, telomeres carry out another important function in replicating cells: Their structure allows the end of linear DNA to be replicate completely.

Telomeres are composed of a DNA component and multiple protein components (Graeber, 1996). The telomeric DNA consists of noncoding tandemly repeated sequences, with the exact repeat sequence varying from one species to the other. In humans and other vertebrates, the repeat unit is the hexanucleotide TTAGGG (5' →3' direction). Although telomeres are generally considered to be localized structures at the ends of chromosomes, such sequences are also being identified at internal positions in chromosomes (Katinka and Bourgain, 1992). The length of telomeres also varies among different species. Human have telomeres 8-14 kilobasepairs (kbp) long, whereas the mean telomeric repeat lengths in some ciliates are as little as 36 bp, and those in mice may be as much as 150 kbp (Rao, 1996). In human chromosomes, telomeres are adjoined centromerically by a subtelomeric region consisting of degenerated telomeric DNA sequences and unique repeats (Brown et al., 1990)

All chromosomes lose a small amount of telomeric DNA during each cell division, a natural consequence of the nature of the cellular DNA replication machinery. DNA polymerases replicate only in a 5' →3' direction by extending exist polynucleotide chains. The mechanism of DNA replication differs for the leading and the lagging DNA strands. The leading strand is replicated continually. To replicate the lagging strand, DNA polymerization starts from several RNA primers, which are elongated to create DNA fragments, termed Okazaki fragments. These RNA primers are finally degraded and replaced by DNA sequences. Removal of the terminal RNA primer on the lagging strand leaves a gap that ordinary is filled in by extension of the next Okazaki fragment. Because there is no template for the last Okazaki fragment beyond the 5' end of the chromosome, one strand cannot be synthesized to its very end. This reduction of chromosomal DNA at the 3' ends during multiple cell cycles.

The loss of genomic sequences at each replication cycle can be compensated by addition of terminal sequences through various mechanisms: e.g., in yeast by recombination events (Lundblad and Blackburn, 1993) or *Drosophila* by transposition (Biessmann and Mason, 1992). Moreover, organisms possess the ability to transfer species-specific terminal sequences onto DNA: Shampay et al. (1984) demonstrated that telomeric DNA from *Tetrahymena* introduced into yeast became elongated with yeast telomeric sequences. The crucial experiments came from Greider and Blackburn (1985, 1987), who detected in *Tetrahymena* extracts an activity that added telomeric repeats to single-stranded telomeric DNA oligonucleotide primers; they also found that this process is inactivated by treating the extract with a RNA-degrading enzyme. Therefore, this RNA-dependent activity, named terminal transferase or telomerase, was found to be a ribonucleoprotein complex that utilizes sequences of its own RNA component as a template for the *de novo* synthesis of telomeric DNA sequences. Both RNA and several protein components of telomerase are needed for enzymatic activity (Collins, 1996; Feng et al., 1995; Kim et al., 1994; Singer

and Gottschling, 1994).

Although the bulk of telomeric DNA is double stranded, the extreme terminus is a single-stranded G-rich 3' overhang that serves as a template for elongation and forms a telomeric 'T-loop'. This loop is stabilized by certain telomere-binding proteins, notably TRF1 and TRF2 (Zakian, 1996). The functions of telomeres appear to include protection of chromosomes from illegitimate fusion, the localization of chromosomes in the nucleus and the selective silencing of proximal subtelomeric genes (Greider, 1994). The telomeric repeat sequences are added on by the enzyme telomerase (Greider and Blackburn, 1985; Yu et al., 1990), which present compensates for the loss of DNA from the end of chromosomes due to incomplete replication.

In human, telomeres are up to 20 kb in length (Brown, 1989). In contrast, rodent telomeres have been reported to be heterogeneous in length (Zijlmans et al., 1997). *Mus musculus* has been reported to have telomeres up to 150 kb in size (Prowse and Greider, 1995). *Mus spretus*, however, has telomeres with similar length to humans (up to 30 kb in size) (Zijlmans et al., 1997), whereas rat telomere length ranges from 20 to 100 kb (Golubovskaya et al., 1999; Jennings et al., 1999).

In humans, both *in vivo* and *in vitro*, telomere shortening appears to be a major component of cell senescence and aging (Campisi et al., 1996; Harley, 1997). Telomeres have been reported to shorten during post-natal development and aging in liver (Aikata et al., 2000; Takubo and Kaminishi, 2001), kidney (Melk et al., 2000) and lymphocytes (Benetos et al., 2001). However, this is less apparent in mice because of the very long telomeres (30-150 kb). Telomere shortening has been extensively studied in mice, especially in telomerase-deficient knockout mice (Artandi and DePinho, 2000; Blasco et al., 1999; Herrera et al., 1999). Moreover, it was reported that relationships between kidney telomere shortening and longevity in the rat (Jennings et al., 1999).

2.2. Telomere theory of aging in mammals

In 1998, after endless efforts to get to the bottom of an intriguing mechanism of original aging, it became clear at least in the rough. Bodnar et al. (1998) has given a brilliant evidence for the hypothesis. According to the hypothesis, telomere shortening in every subsequent cell division is responsible for the limited cell proliferation in culture (the so called 'Hayflick's limit' found 40 years ago by Hayflick and Moorhead, 1961). It was quite natural to attribute organismal aging to the same cause. The most complicated problems of cellular and organismal aging have got the following explanation. Shorter telomeres, found in cells of patients with inherited premature aging, progerias (Allsopp et al., 1992) were consistent with the sharply restricted Hayflick's limit in these cells and provided a distinct evidence for an existing correlation between agings of an organism with that of its cells.

Discovery of telomerase activity in malignant tumor cells explained the mechanism of tumorigenesis. The same activity in gametes clarified why our children always started their aging from zero level and not from the level reached by the parents (by its cell) during conception. Therefore, it was logical to consider organismal aging to be a result of telomere shortening and limited cell proliferation.

However, some facts instantly appeared which did not go in this line of telomere theory of aging. First, proliferative capacity of human cells appeared to demonstrate a very insignificant, if any, decrease with age (Francheschi et al., 1999). Moreover, skin fibroblasts of human at very old age never exhaust their Hayflick's limit (Cristofalo et al., 1998). Additionally, some other contradictions against the telomere theory of aging have been intensity discussed earlier.

In 1998, Hayflick proposed a new hypothesis, according to which telomere shortening, leading to the loss of replicative capacity, determined only the species life span, whereas aging itself was caused by the accumulation of some cell damages, as it had been suggested earlier by Orgel (1973) though these damages still need to be clarified.

Dr. Hayflick's hypothesis appears to be a misconception. First, the theory is at variance with the following observations.

1. The Hutchinson-Gilford progeria patients demonstrate the real accelerated aging and not a simple reduction in the life span (Allsopp et al., 1992). Similar phenomena are observed in the other forms of hereditary premature aging-Werner's syndrome (Wyllie et al., 2000).
2. Telomeres examined in cells of people extremely old at age never reach the critical length. Blocking normal cellular proliferation (Mondello et al., 1999).

Nevertheless, all old people die at an age nearing hundred years, and their telomeres by no means are exhausted. Thus, organisms die precisely because of aging, though they never live till all their cells stop proliferating.

It was assumpted that aging and consequent death need not necessarily reach Hayflick's limit in all tissues of an organism. Exhausted proliferative potential of cells in some areas of organ tissue might be sufficient to promote one of the age-dependent diseases. Combination of these disorders, gradually increasing with age, is aging. At this point, again there is a contradiction with Dr. Hayflick's new hypothesis.

Developing his idea, Hayflick (1998) considers that it is necessary to distinguish aging from disease, in other words, to distinguish diseases from age-related changes and try to pick out pure aging. Such attempts seem unpromising in principle, because age-related diseases, to the common person are aging. Nobody dies just because of age.

Telomere shortening in certain human tissues might promote disorders such as essential hypertension, non-insulin-dependent diabetes mellitus, atherosclerosis, and cancer (Aviv and Aviv, 1998). There are evidences for a possible role of telomere shortening in

malignancies, compared with the surrounding tissues, (Morin, 1997). Zeichner et al. (1999) found that accelerated cell division in children and hence higher rate of telomere shortening may provide an explanation for a more rapid progression to acquired immunodeficiency syndrome in infected infants.

On the other hand, Omura et al. (1998) has shown that an average telomere length decrease with the advance of age in cells of all human tissues, except for heart, brain, retina and sex glands. It also appears that chronic degenerative diseases are accompanied by unusual low telomeres. It is noteworthy that the exceptions mentioned above might be well explained within the hypothesis suggesting irregular telomere shortening in different tissues of an organism. The absence of telomere shortening in germ cells is, apparently, because of telomerase activity in these cells. Brain neurons stop dividing at the time of birth although significant telomere shortening is not observed, which contradicts the proposed idea at first sight. The point is that cells reach their proliferative limit irregularly, and does not guarantee the rapid and full cessation of mitoses in the tissue. Meanwhile, brain cells need rapid and full cessation, because links between neurons must be settled from the time of birth, and if neurons continue to divide as other cells, these links will be surely destroyed. Therefore, it is the brain than just by telomere shortening, because the cessation of mitotic activity is probably provided by some additional and more effective mechanisms. The retina may operate in the same mechanism.

Thus, there are many reasons to believe that the telomere theory of aging is in principle correct. All the above difficulties may easily find their explanation with regard to the phenomenon of uneven telomere shortening in different tissues and organs of the organism. Francheschi et al. (1999) truly claimed that any living organism might be regarded as a mosaic of cells with different replicative histories and potentials. According to these authors, this particular fact throws doubt on the validity of the telomere theory of aging. By contrast, it is this fact that may explain all the apparent misunderstandings and difficulties within the frames of the telomere theory.

2.3. Telomere theory of aging in teleosts

In fish, high telomerase activity has been detected in several normal organs of the rainbow trout *Oncorhynchus mykiss* (Klapper et al., 1998). Telomerase activity of the normal organs has been detected in both fry and adult fish, being 10 - 100-fold higher than that in the human tumor cell line L-428. In contrast, no telomerase activity has been detected in the differentiated organs of mammals. Greider (1998) has described the correlation between telomerase activity and the proliferation potential of cells. In general, rainbow trout grow continuously throughout their life and, therefore, the high telomerase activity detected in their normal organs is postulated to lead to cell proliferation and organ

growth. In previous investigation (Yoda et al., 2002), relative telomerase activity per cell in eyed embryos of rainbow trout was 19.3 - 50.7-fold higher than in Hela cells (a human cervical carcinoma cell line), which are well known to express a high level of telomerase activity (Morin, 1989). Therefore, it is assumpted that aging and consequent death of fish need not to reach Hayflick's limit in all tissues of an organism.

3. PROGRAMMED CELL DEATH

Cells have a built-in cell death program, apoptosis (programmed cell death), which protects the organism by removing potentially damaged cells and unnecessary cells after differentiation. Apoptosis is induced in a wide range of physiological settings that are regulated by cell growth and differentiation in normal biological processes and in pathogenesis in vertebrates (Cohen et al., 1992; Ellis et al., 1991; Fernandes-Alnemri et al., 1994; Jacobson et al., 1997; Nicholson and Thornberry, 1997; Steller, 1995).

3.1. Defining features of programmed cell death

Despite the tremendous impact of research in apoptosis upon the understanding both of cellular and molecular mechanisms of cell demise, as well as mechanisms of degenerative diseases, the confusion between apoptosis and programmed cell death has been somewhat obscured. Regardless of whether this paradox is attributable either to disconnection of modern science from its philosophical foundations (Sloviter, 2002) or to a more trivial neglect of classical papers (Lockshin and Zakeri, 2001), it is likely that progress in the identification and understanding of nonapoptotic forms of programmed cell death may have been unnecessarily delayed.

Indeed, well before the upsurge in the understanding of mechanisms of apoptosis, a clear warning had been issued to avoid confusion between the form of cell death called apoptosis, and the concept of programmed cell death as a sequence of events, but not necessarily those that led to the morphology of apoptosis. However, the modern science is recently identifying apoptosis as the programmed cell death. Therefore, apoptosis is used as the same term of the programmed cell death in this thesis.

3.2. Multiple mechanisms of apoptosis

Programmed cell death with apoptotic morphology can be triggered by several stimuli, including intracellular stress and receptor-mediated signaling. These signals feed into an evolutionarily conserved intracellular machinery of execution (Green, 2000; Hengartner,

2000), the mechanisms of which have mainly been traced to the activity of the caspase family of cysteine-proteases (Cryns and Yuan, 1998; Yuan et al., 1993; Zhivotovsky et al., 1997). Caspase-mediated apoptotic cell death has been extensively reviewed, e.g. Green, 2000; Hengartner, 2000; Leist and Jaattela, 2001; Martin, 2002; Ravagnan et al., 2002; Wajant, 2002. The caspases are synthesized as zymogens and upstream signals convert these precursors into mature proteases. Inhibitor caspases; caspase-1, -2, -4, -5, -8, -9, -10 and -14 are activated via oligomerization-induced autoprocessing (Butt et al., 1998; Li et al., 1997; Martin et al., 1998; Muzio et al., 1996; Srinivasula et al., 1998; Yang et al., 1998), while effector caspases; caspase-3, -6 and -7 are activated by other proteases, including initiator caspases and granzyme B. Proteolytic cleavage of cellular substrates by effector caspases largely determines the features of apoptotic cell death (Green, 1998; Liu, et al., 1998; Sakahira et al., 1998; Stroh and Schulze-Osthoff, 1998; Wolf and Green, 1999; Zhang et al., 1998).

Three major pathways have been identified according to their initiator caspase: the death receptor pathway involving caspase-8 (Medema et al., 1997), the endoplasmic reticulum stress pathway attributed to activation of caspase-12 (Nakagawa et al., 2000), and the mitochondrial pathway, in which various signals can trigger the release of harmful proteins by mitochondria into the cytoplasm, leading to activation of caspase-9 and down-stream cleavage of caspase-3, -7 or -6 (Green and Reed, 1998; Grutter, 2000; Li et al., 1997, 1998a; Luo et al., 1998).

Although caspase-3 is widely involved in the execution of apoptosis (Stennicke et al., 2002), its effector functions may be dispensable for apoptotic-like cell death (Kuida et al., 1996; Miyashita et al., 1998). The use of either pharmacological inhibitors or knockout animals further showed that cells could trigger alternative mechanisms of cell demise. For example, sympathetic and dorsal root ganglion neurons deprived of nerve growth factor (NGF) die in a caspase-2-dependent manner, but the same neurons derived from caspase-2 knockout mice still die following nerve growth factor deprivation. The death depends on activation of caspase-9, which does not occur in wild-type mice (Troy et al., 2001). Thus, rather than a single linear mechanism, alternative caspase-mediated pathways may be activated for apoptotic cell death, depending on whether a preferential caspase is blocked. It is likely that the network of intrinsic regulatory pathways that impinge upon the activity of caspases, such as the inhibitors of apoptosis (IAPs) and IAP-binding proteins (Salvesen and Duckett, 2002), may regulate the choice between alternative pathways in normal cells, dependent on metabolic state, stage of differentiation and other conditions.

In addition, caspase inhibition fails to block programmed cell death with apoptotic morphology in several experimental models (Assefa et al., 2000; Carmody and Cotter, 2000; Lorenzo et al., 1999; Mateo et al., 1999; Mathiasen et al. 1999). For example, the ultrastructural features of apoptosis inducing factor (AIF)-induced cell death represent an

example of a slight variation from the standard pattern of apoptosis morphology, which appears to be independent of caspase activation (Arnoult et al., 2003; Joza et al., 2001). Cell death pathways independent of caspase activation have been described, for example, even in some forms of cell death induced either by the Bcl-family protein Bax (Jürgensmeier et al., 1998), as well as I cell death involving the activation of other proteases, such as calpain (Squier et al., 1994), proteasome (Hirsch et al., 1998) and serine proteases.

Recent reports shows that the serine protease Omi/HtrA2 is a mitochondrial direct X-chromosome-linked inhibitor of apoptosis protein (XIAP)-binding protein, which is released from mitochondria upon induction of apoptosis together with cytochrome *c* and Smac/Diablo (Hegde et al., 2002; Martins, 2002; Martins et al., 2002), and its release can be inhibited by Bcl-2 (van Loo et al., 2002). These reports suggest that in some cases there may be a cooperative action between serine proteases and caspases in the execution of cell death.

The previous studies show that the classically defined apoptotic morphology can be achieved either by activation of caspases, or through the mediation of other families of proteases, although the exact cytological features of cell demise may vary slightly among these various forms of apoptosis.

3.3. Apoptotic pathway in fish

The characterization of genes that are involved in apoptosis has been pursued intensively, and has led to the identification of several classes of genes, the Bcl-2 family, apoptosis-inducing factor Bax, and the caspase family. Many genes with homology to mammalian apoptosis regulators have been identified in zebrafish DNA databases (Inohara and Nunez, 2000), suggesting that most apoptotic pathways are evolutionally well conserved between fish and higher vertebrates.

3.3.1. The caspase family genes

Cell death genes were first identified in the nematode *Caenorhabditis elegans*. The *ced*-3 gene encodes a cysteine protease that has a key role in the cell death-signaling pathway (Hengartner and Horvitz, 1994; Yuan et al., 1993). Vertebrate cells also possess several cysteine proteases belonging to the caspase family, which are homologous to ced-3 cysteine protease (Alnemri et al., 1996). Caspase is produced as an inactive precursor composed of four distinct domains: the prodomain, large subunit, and small subunit, and a linker region between the two subunits flanked by aspartic residues (Nicholson and Thornberry, 1997). The pro-caspase is activated by hydrolysis between the large and small

subunits, resulting in removal of the prodomain and linker region and the large and small subunits form an active mature enzyme (Nicholson and Thornberry, 1997). X-ray crystal structural analysis of caspase-1 and caspase-3 revealed that mature caspase forms a tetramer with two catalytic sited that interacts via the small subunit (Rotonda et al., 1996; Walker et al., 1994; Wilson et al., 1994). Multiple forms of caspase have been found in other vertebrates, e.g. 12 type in humans (Nicholson et al., 1995; Yaoita and Nakajima, 1997), 10 type in mice (Hu et al., 1998; Kumar et al., 1994; Van de Craen et al., 1997; Wang et al., 1996), and eight types in *X. laevis* (Kumar, 1999; Nakajima et al., 2000). Cascade reactions of proteolytic processing of caspases induce apoptosis. Class I caspases, such as caspase-2, -8, -9 and -10, promote the upstream part of the cascade reaction via N-terminal prodomains, bound to specific death adaptor molecules (Colussi and Kumar, 1999; Cryns and Yuan, 1998). Class II caspases with short N-terminal prodomains, e.g. caspase-3, -6 and -7, act as effectors caspase in proapoptotic signaling from the caspase cascade to cell death by porteolytic processing of proteins, such as the inhibitor of caspase-activated DNase (Sakahira et al., 1998), poly-ADP-ribose polymerase (Earnshaw et al., 1999), and protein kinase Cδ (Emoto et al., 1995). In fish, caspase-3 was recently cloned and characterized (Yabu et al., 2001).

The full-length cDNA sequence of zebrafish caspase was isolated from cDNA library of zabrafish 12-h embryos (Yabu et al., 2001). This clone had an 846 bp ORF encoding a protein of 282 amino acids with a predicted molecular mass of 31.5 kDa. The amino acid sequence identities of the zebrafish caspase-3 with chicken, hamster, human, rat, mouse, and *X. laevis* caspase-3 were 64, 62, 62, 62, 61 and 58%, respectively. According to the X-ray crystal structure of human caspase-3 (Rotonda et al., 1996), Cys-166, His-124, and Gly-125 in the catalytic center, and Arg-97, Gln-164, Arg-243 and Ser-256 located in the binding pocket in the S1 subsite, are conserved in the zebrafish caspase-3. The pentapeptide motif QAC (R/Q/G) G around the active center Cys-166 is conserved in zebrafish caspase-3 (Yabu et al., 2001).

A recombinant zebrafish caspase-3 lacking the prodomain showed high activity toward the mammalian caspase-3 and -7 substrate, Ac-DEVD-MCA. However, the enzyme had only low activity against caspase -1 substrate Ac-YVAD-MCA, caspase-6 and -8 substrate Ac-IETD-MCA, and caspase -9 substrate Ac-LEHD-MCA. Therefore, zebrafish caspase-3 has strict substrate specificity, which is similar to that of known members of the caspase-3 subfamily, such as human caspase-3 and caspase-7 (Thornberry et al., 1997).

By homology analysis using current nucleotide databases of zebrafish and *Takifugu rubripes*, several fish genes encoding caspases are available (Inohara and Nunez, 2000). Functional assays by overexpression of zebrafish caspase-3 in fathead minnow tailbud cells and zebrafish embryo (Yabu et al., 2001) and zebrafish caspy and caspy2 in human 293T cells showed their induction of apoptosis (Masumoto et al.,2003).

3.3.2. Expression of caspase-3 mRNA in fish

Zebrafish caspase-3 transcripts were expressed at all developmental stages examined by Northern blotting (Yabu et al., 2001). At the 4- and 1000- cell stages, high levels of caspase-3 mRNA were present in fertilized eggs as a maternal factor. Furthermore, caspase-3 mRNA was expressed in the shield, 1-somite, pharyngula, and hatching periods, which coincided with zygotic gene expression after gastrulation. In situ hybridization demonstrated that caspase-3 mRNA was expressed throughout the embryo at every developmental stage. In the pharyngula period, caspase-3 mRNA is present at higher levels in the pectoral fin bud, otic vesicle, and hindbrain.

When caspase-3 was overexpressed by introducting its cDNA into fish cultured cells and embryos, extensive apoptosis and ceramide generation were induced (Yabu et al., 2001). This suggests that the tissue-specific, developmental expression patterns of the caspase-3 gene regulate the spatial and temporal distribution of apoptotic cells. In mammals, caspase-3-knockout mice are born infrequently, die after only a few weeks, and have brain defects (Colussi and Kumar, 1999; Kuida et al., 1996; Woo et al., 1998). Therefore, both mammalian and fish caspase-3 may have important functions modulating a proapoptosic signal during development.

3.3.3. Death receptors

Plasma membrane death receptors, belonging to the tumor necrosis factor (TNF) family, have been cloned in fish (Hirono et al., 2000). TNF receptor containing an intracellular death domain is associated with cellular inframmatory and immune responses in mammals. Interaction of extracellular ligands, such as Fas ligand and TNFα, to death receptors is considered to induce apoptosis by activation of caspase-8 signaling. When a ligand binds to death receptors, the receptor-specific adapter protein Fas-associated death domain (FADD) is recruited, and then caspase-8 is activated by autolytic processing. Activated caspase-8 is known to promote the apoptotic signal by directly cleaving and activating downstream caspases. In the caspase-8-knockout mouse, caspase-8 is found to be required for killing induced by the death receptors Fas, tumor necrosis factor receptor 1 and death receptor 3 (Varfolomeev et al., 1998; Yeh et al., 1998). The heart muscle and fewer hematopoetic progenitor cells suggest that the FADD/caspase-8 pathway is required for growth and developmental roles for this death receptor pathway remain to be identified in zebrafish embryos.

3.3.4. The Bcl-2 and Bax families

Pro- and anti-apoptotic members of the Bcl-2 family (ced-9 homologs) regulate mitochondrial participation in cell death (Bernardi et al., 2001; Gottlieb, 2001). Current models are proposed that the release of cytochrome *c* from mitochondria triggers activation of caspase-9 in a complex with dATP and Apaf-1 (Yoshida et al., 1998). Activated caspase-9 then activates further downstream caspases. Bcl-2 family proteins, such as ZfMcl-1α (Chen et al., 2000), Bcl-XL (Chen et al., 2001), MCL-1 (Hong et al., 1999), have been cloned in zebrafish. They are expressed in oocytes and early embryos (Chen et al., 2000, 2001; Hong et al., 1999). In contrast, ced-4 homolog Bax triggers a mitonondrial proapoptotic pathway by promotion of mitochondrial release of cytochrome *c* (Bernardi et al., 2001; Gottieb, 2001). Functional analysis of the Bcl-2 and Bax families is required to establish experimental models regulating chemical and oxidative stress responses.

4. p53 TUMOR SUPPRESSOR PROTEIN INDUCED APOPTOSIS

Mutation in the p53 tumor suppressor gene occur in about 50 % of all human tumors, making it the most frequent target for genetic alterations in cancer (Agarwal et al., 1998; Almog and Rotter, 1998; Hansen and Oren, 1997; Levine, 1997; Prives and Hall, 1999). Such mutations probably facilitate carcinogenesis primary through abrogating the tumor suppressor activities of the wild type p53 protein, although at least some forms of tumor-associated mutant p53 proteins may also contribute overt oncogenic activities, gain of function. Excessive wild type p53 can reduce cancer incidence through elimination of cancer-prone cells from the replicative pool. However, such effects might become very undesirable if occurring in a normal, unperturbed cell. p53 activity must be, therefore, kept under tight control, being unleashed only when a cell accumulates lesions the may otherwise drive it into a cancerous state.

4.1. p53-activating signals

Under normal conditions, p53 is most probably latent. Consequently, it does not interfere with cell cycle progression and cell survival. Moreover, p53 knock-out mice appear in most cases to undergo proper development and maturation (Donehower et al., 1992), suggesting that p53 is not essential for the normal performance of cells within the body. However, a variety of conditions can lead to rapid induction of p53 activity. The common denominator of these conditions is that they represent various types of stress, which are likely to favor the emergence of cancer-bound cells. Such conditions include direct DNA damage (Huang et al., 1996; Kastan et al., 1991; Maltzman and Czyzyk, 1984)

as well as damage to components genetic material (*e.g.* the mitotic spindle (Cross et al., 1995)), ribonucleotide depletion (Linke et al., 1996), hypoxia (Graeber et al., 1996), heat shock (Ohnishi et al., 1996), and exposure to nitric oxide (NO) (Forrester et al., 1996). Accumulation of genomic aberrations is a key carcinogenic mechanism; the rapid induction of p53 activity in response to genomic damage thus serves to ensure that cells carrying such damage are effectively taken care of. Furthermore, p53 may also contribute, directly or indirectly, to particular DNA repair processes (Offer et al., 1999; Smith et al., 1995). The pivotal role of p53 in maintaining genomic integrity has earned it the guardian of the genome (Lane, 1992). In addition, p53 activity is triggered by a variety of oncogenic proteins, including Myc, Ras, adenovirus EIA, and β-catenin (Damalas et al., 1999; Debbas and White, 1993; Hermeking and Eick, 1994; Serrano et al., 1997), providing a direct link between oncogenic processes and the tumor suppressor action of p53.

4.2. Regulation of p53 gene expression

Induction of the p53 response upon stress occurs largely through alteration in the p53 protein. Changes in the rate of transcription of the p53 gene play a minor role, if any, in such induction. Consequently, the transcriptional regulation of p53 gene has received very little attention during recent years. This need not imply that the regulation of p53 gene expression is totally irrelevant. In fact, it was observed long ago that p53 mRNA levels rised substantially upon serum stimulation (Reich and Levine, 1984). This rise may be because of the presence of binding sites for serum-induced factors in the p53 promotor (Ginsberg et al., 1990) as well as to the ability of the p53 gene to bind the c-Myc (Reisman et al., 1993). The induction of an anti-proliferative gene, p53, by serum and growth factors may at first glance seem paradoxical. Cell undergoing DNA replication and extensive proliferation are, however, at higher risk of acquiring DNA damage and giving rise to multiple cancer-prone progeny than quiescent cells. Without DNA damage or other stress, p53 remains latent and once those conditions emerge call for a p53 response, the high levels of p53 mRNA ensure that such a response will be rapid and effective.

4.3. Activation of p53 by post-transcriptional mechanisms

Exposure of cell to p53-activating signals can lead within a relatively short time to a marked elevation in p53 protein. To some extent, this can be achieved by increased translation of the p53 mRNA, probably involving relief of a translational repression mechanism operating through the 3'-untranslated region of this mRNA (Fu et al., 1996). There also exists evidence that p53 itself can inhibit p53 synthesis through binding to its own mRNA (Fontoura et al., 1997; Mosner et al., 1995). Yet, it is generally accepted that

the accumulation of active p53 in response to stress occurs mainly through post-transcriptional mechanisms. Pivotal is the increase in the protein half-life of p53, since p53 is usually a very labile protein, turning over with a half-life sometimes as short as a few minutes (Rogel et al., 1985). In response to DNA damage and other type of stress, p53 was markedly stabilized (Kastan et al., 1991; Maltzman and Czyzyk, 1984). A rapid increase in p53 concentration without a need for *de novo* transcription is particularly advantageous in cells with severely damaged genomes. In addition, there is most probably a qualitative conversion of p53 from latent to active form. The best documented change concerns the sequence-specific DNA binding activity of p53. p53 operates as a gene-specific transcriptional activator, which relies on its ability to bind defined sequence elements within target genes (Agarwal et al., 1998; Almog and Rotter, 1998; Hansen and Oren, 1997; Levine 1997; Prives and Hall, 1999). The sequence-specific DNA binding activity of p53 is subject to constitutive negative regulation, primary through its inhibitory C-terminal domain (Bayle et al., 1995; Hupp et al., 1992; Wolkowicz et al., 1995). Relief of this inhibition upon exposure to stress results in increased DNA binding (Gu and Roeder, 1997; Hupp and Lane, 1995; Waterman et al., 1998) and consequently increased biochemical and biological activity. The transcriptional activity of p53 may also be induced by changes in other regions, *e.g.* modifications within its N-terminal transactivation domain, enabling a more efficient recruitment of components of the transcription machinery (Lambert et al., 1998). Finally, p53 activation may also involve a change in subcellular localization; whereas latent p53 may often be cytoplasmic, at least during part of the cell cycle (Shaulsky et al., 1990), exposure to stress results in its accumulation in the nucleus, where it is expected to exert its biochemical activities.

4.4. The p53-Mdm2 loop

A key player in the regulation of p53 is the Mdm2 protein. Mdm2 is the product of an oncogene, whose excess activity facilitates several types of human cancer (Lozano et al., 1998; Freedman et al., 1999; Juven-Gershon and Oren, 1999). Mdm2 exhibits a unique relationship with p53. On the one hand, the Mdm2 protein binds to p53 and inactivates it (Chen et al., 1996; Haupt et al., 1996; Momand et al., 1992). The binding occurs right within the p53 transactivation domain, interfering with recruitment of basal to p53 transactivation domain, interfering with recruitment of basal transcription machinery components (Lu and Levine, 1995; Thut et al., 1995). Moreover, Mdm2 actively represses transcription when tethered to p53 (Thut et al., 1997). Importantly, Mdm2 binding can also lead to complete elimination of p53 through proteolytic degradation. On the other hand, p53 binds specifically to the mdm2 gene and stimulates its transcription (Barak et al., 1993; Wu et al., 1993). This duality defines a negative feedback loop, which probably

serves to keep p53 in tight check and to terminate the p53 signal once the triggering stress has been effectively dealt with. In some situations, mdm2 transcription is induced later than that of other p53 target genes (Perry et al., 1993; Wu and Levine, 1997); this may set a time window within which p53 is allowed to exert freely its biochemical and biologiacal effects. The critical importance of the p53-Mdm2 loop is best illustrated by the analysis of mdm2 knockout mice. Inactivation of the mdm2 gene results in early embryonal lethality, but this is completely prevented by simultaneous inactivation of p53 (Jones et al., 1995; Montes et al., 1995). Conceivably, in the absence of functional Mdm2 protein, p53 becomes strongly deregulated to the extent that its excess activity leads to embryonic death. The other side of the coin is revealed in certain human cancers; excessive Mdm2 expression, achieved through mdm2 gene amplification (Oliner et al., 1992) or other mechanisms (Landers et al., 1994), can lead to constitutive inhibition of p53 and thereby promote cancer without a need to alter the p53 gene itself. It should be kept in mind, however, that excess Mdm2 can also promote cancer independently of p53 (Lundgren et al., 1997; Sun et al., 1998).

4.5. Covalent Modification of p53

Rapid post-translational activation of signaling protein is often achieved through covalent modifications, particularly protein phosphorylation. It was thus conceivable that the rapid stabilization and activation of the p53 protein upon stress also involves stress-induced covalent modifications of p53. Indeed, there is mounting evidence in support of this conjecture. p53 becomes phosphorylated on multiple sites *in vivo* in response to various types of stress, and many stress-activated kinase can phosphorylated p53 *in vitro* (Fuchs et al., 1998; Giaccia and Kastan, 1998; Jayaraman and Prives, 1999; Meek, 1998). A potential outcome of such phosphorylation is the stabilization of p53 through inhibition of p53 ubiquitination and degradation. The pivotal role of Mdm2 to p53 (Haupt et al., 1997; Kubbutat et al., 1997), phosphorylation of residues positioned within the binding interface of either protein may interfere with binding and lead to p53 stabilication. In the case of p53, several candidate sites within its Mdm2-binding domain have been identified which are modified in response to DNA damage and whose phosphorylation reduces the affinity of p53 for Mdm2 (Shieh et al., 1997, 1999; Unger et al., 1999). Of particular interest are serines 15 and 20 and threonine 18 of human p53, which locate within or very close to the Mdm2-binding domain of p53. Serine 15 particularly is the site of p53 phosphorylation by the ATM kinase (Banin et al., 1998; Canman et al., 1998), whose activity is required for p53 stabilization in response to ionizing radiation and some other types of DNA damage (Kastan et al., 1992; Khanna et al., 1995). It should be noted that although the idea that such a phosphorylation events are

responsible for p53 stabilization is very attractive, the *in vivo* relevance of this idea has been challenged recently (Ashcroft et al., 1999; Blattner et al., 1999). Hence, the effect of p53 phosphorylation on stability may depend on the intracellular context and particularly on the availability of alternative mechanisms for p53 degradation.

4.6. p53 and apoptosis in fish

Tumor suppressor gene, p53, was cloned from zebrafish and its expression was examined during embryogenesis (Cheng et al., 1997; Langheinrich et al., 2002; Thisse et al., 2000). Mdm2 and p53 was functionally analyzed in zebrafish by generating early embryonic knockdowns and examined the involvement of p53 in DNA damaged-induced apoptosis. Double knockdowns of p53 and Mdm2, induced by injection of antisense morpholinos, showed that p53-deficiency rescued Mdm2-deficient embryos completely, similar to observations in mice. p53-deficiency markedly decreased DNA damage-induced apoptosis, elicited by ultraviolet irradiation or by the anti-cancer compound camptothectin (Langheinrich et al., 2002). Thus, p53 may play a key role in DNA damage induced apoptosis by irradiation and chemicals.

5. CALORIC RESTRICTION

For almost 70 years, caloric restriction has been known to extend life span. Despite the extensive physiological characterization of this dietary regimen, the molecular basis for the slowing in aging remains unsolved. Recent findings have pinpointed a few molecular pathways that appear to regulate the aging process.

Caloric restriction (CR) refers to a dietary regimen low in calories without undernutrition. It was first noted in the 1930s that food restriction significantly extends the life span of rodents (McCay et al., 1989). This longevity results from the limitation of total calories derived from carbohydrates, fats, or proteins to a level 25-60 % below that of control animals fed ad libitum (Weindruch et al., 1986). The extension in life span can approach 50 % in rodents (Sohal and Weindruch, 1996). CR extends life span in a remarkable range of organisms, including yeast, rotifers, spiders, worms, mice, and rats. Emerging data show that its effect may also apply to nonhuman primates (Lane et al., 2001).

CR delays a wide spectrum of diseases in different experimental animals; for example, kidney disease, a variety if neoplasias, autoimmune disease, and diabetes (Engelman et al., 1990; Fernandes and Good, 1984; Fernandes et al., 1976; Johnson et al., 1997; Kubo et al., 1984; Sarkar et al., 1982; Shield et al., 1991). CR reduces age-associated neuronal loss in

most mouse models of neurodegenerative disorders such as Parkinson's disease (Duan and Mattson, 1999) or Alzheimer's disease (Zhu et al., 1999). However, beneficial effects in a mouse model for amyotrophic lateral sclerosis were not observed (Pedersen and Mattson, 1999). The CR regimen also prevents age-associated declines in psychomotor and spatial memory tasks (Ingram et al., 1987) and loss of dendritic spines necessary for learning (Moroi-Fetters et al., 1989) and improves the brain's plasticity and ability for self-repair (Mattson, 2000).

5.1. Mechanisms of aging and classical views of how CR works

It has been documented that oxidative damage is reduced in CR animals (Lee and Yu, 1990). If CR were to slow metabolism, the production of reactive oxygen species (ROS) would decrease as a simple consequence. However, studies measuring metabolic rate in CR animals give conflicting results. The weight of evidence in rodents indicates that metabolism, measured with oxygen consumption normalized to the reduced body mass of the animal, does not slow down (McCarter et al., 1985). Because the balance of existing data does not support a long-term overall reduction in metabolic rate, more subtle explanations must be adduced. One possibility is that a more efficient transport of elections through the respiratory chain might reduce the production of ROS and slow aging (Duffy et al., 1989, 1990; Weindruch et al., 1986). Another is that an increased ability to detoxify ROS slows oxidative damage in CR. The data relating CR to detoxification of ROS is again conflicting. On the one hand, organisms tend to be more resistant to an acute challenge by an exogenous oxidative stressor. For example, life-long CR seems to increase expression of SOD in rat liver (Semsei et al., 1989). On the other hand, in genetically altered strains of mice, there is no consistent correlation in the expression levels of SOD and life span (Hauck and Bartke, 2000).

Another theory suggests that lack of protein turnover may cause aging. Multiple studies of aging organisms have shown accumulation of aberrant (e.g., oxidatively damaged) proteins and a reduction in protein turnover (Gracy et al., 1985; Lavie et al., 1982). CR may slow down accumulation of these potentially harmful and abnormal proteins by speeding up protein turnover (Sohal and Weindruch, 1996; Taylor et al., 1989). As the body runs out of fat during CR, it may trigger the degradation of proteins, thereby increasing their turnover. The age-associated accumulation of oxidized proteins indeed declines with CR (Aksenova et al., 1998; Dhahbi et al., 1999), and the activity of the liver 20S proteosome may increase during CR (Scrofano et al., 1998). Microarray study of mouse skeletal muscle also showed an increase in protein synthesis and degradation during CR (Lee et al., 1999). However, the elevated turnover during CR is not uniform; although some damaged proteins were degraded, others continued to accumulate (Scrofano et al.,

1998). Briefly, the data suggest an increase in protein turnover during CR, but it is uncertain whether this change has an impact on the rate of aging.

The covalent modification of proteins by derivatives of glucose has also been shown to increase with aging (Cefalu et al., 1995; Masoro et al., 1989; Sell et al, 1996; Smith et al., 1994). These modified adducts in macromolecules, termed advanced glycation end products (AGE), have been linked to age-related pathologies (Lee and Cerami, 1992). A reduction of AGE during CR has been demonstrated (Cefalu et al., 1995; Masoro et al., 1989). The blood profile of CR animals predicts this reduction, because both glucose and insulin levels are reduced in CR animals (Masoro et al., 1983, 1992). However, a lower percentage of AGE during CR does not clearly explain the multiple other effects that are known occur. It is unlikely that a decrease in AGE is responsible for the long life span in CR because AGE is one of many degenerative changes in aging.

5.2. Regulation of yeast replicative life span by CR

In budding yeast, mother cells divide asymmetrically, giving rise to a newly made daughter cell and an aging mother cell. The mother cell adopts phenotypes of aging, including an enlarged size and sterility, and senesces after ~20 divisions. This aging has been linked to the repeated rDNA genes, which encode the large and small subunits of ribosomal RNA (Sinclair and Guarente, 1997). Aging mother cells accumulate extrachromosomal rDNA instability has not been observed in other organisms, and is evidently an idiosyncratic feature of yeast aging.

The SIR2 gene regulated the life span in yeast mother cells; mutations that inactivate SIR2 shorten the life span, and overexpression of SIR2 extends it (Kaeberlein et al., 1999). SIR2 functions to silence chromatin by deacetylating the histons in targeted regions of the yeast genome, including the rDNA. The silences chromatin is structurally less accessible to RNA polymerase and to recombinational enzymes, thereby reducing gene expression and stabilizing repeated DNA. The Sir2p deacetylase is unusual because it requires NAD as a consubstrate (Imai et al., 2000; Landry et al., 2000; Smith et al., 2000). NADH, NADP, and NADPH neither activate nor inhibit the enzyme (Imai et al., 2000).

CR can be imposed in yeast by reducing the glucose concentration in the media from the usual 2 to 0.5 % (Lin et al., 2000). Because cells continue to feed on yeast extract plus peptone, which are rich in amino acids, nucleotides, and vitamins, the growth rate remains rapid as glucose levels are lowered. Thus, the reduction in glucose from 2 % to 0.5 %, although modest, likely imposes a state of partial energy (ATP) limitation. Other dietary restriction protocols, which also limit amino acids and other nutrients (Jiang et al., 2000, 2002), drastically slow the growth rate and may make it more difficult to impose energy limitation.

When the glucose level in the media is lowered, yeast cells respond to that through shunting more of the carbon to the TCA cycle to generate ATP by respiration (Lin et al., 2002). This comes at the expense of fermentation, which is the preferred pathway of carbon use when glucose levels are high. This metabolic shift makes sense because cells harvest much more ATP by metabolizing the glucose to CO_2 in the TCA cycle than by fermenting it to ethanol.

The shift toward respiration is necessary and sufficient to extend the life span in yeast. It is still not certain how this shift activates Sir2p to provide greater longevity. One possibility is that the activation of respiration converts more NADH to NAD and the resulting increase in the NAD/NADH ratio activates Sir2p. It has also been suggested that nicotinamide, which is generated during the deacetylation reaction and can inhibit Sir2p in vitro, is a negative regulator of Sir2p in vivo (Bitterman et al., 2002). There is still no direct evidence for either of these models. Another possibility is that the increase in respiration during CR slows glycolysis, and this metabolic change activates Sir2p. Any mechanism for this latter effect is at present unknown.

The important lesson from the yeast studies is that the extension in life span by CR is not a mechanical consequence of a reduction in ROS or AGE. The extension is indeed a regulated response requiring SIR2. This regulation must involve a qualitative shift in metabolism that can be sensed by Sir2p. The deacetylase activity of the enzyme must then slow any degenerative processes that limit the life span.

5.3. Links between CR, aging, and apoptosis

Several recent studies suggest that apoptosis may limit mammalian life span. Mice with a targeted disruption in the p66shc gene exhibit a longer life span than wild-type animals (Migliaccio et al., 1999). Importantly, cells derived from the p66shc mice are resistant to DNA-damage-induced apoptosis in culture. Further, p66shc is one of the down-stream targets of the key regulator of damage-induced apoptosis, the tumor suppressor p53 (Trinei et al., 2002). In the cell culture studies, p66shc cells were resistant to oxidative stress or ionizing radiation, which both kill cells by the p53-dependent cell death pathway. This finding suggests that apoptosis may be a two-edged sword, providing critical tumor surveillance during the reproductive yeast, but contributing to organ dysfunction and aging in a postreproductive period.

The second finding may also implicate apoptosis in mammalian aging. The yeast SIR2 gene appears to promote survival in a wide range of organisms. In yeast, this gene promotes long life span in mother cells, and is also crucial to the generation of the long-surviving, specialized cell type termed spores. In *C. elegans*, an organism that diverged from the yeast lineage a billion years ago, the SIR2 ortholog sir-2.1 also promotes

long life in adult animals and regulates the formation of dauers during development (Tissenbaum and Guarente, 2001). It has been recently shown that a cytoplasmic Sir2p homolog can promote survival in the protozoan parasite *Leishmania* by preventing apoptosis (Vergnes et al., 2002). The mammalian ortholog of SIR2, SIRT1, represses the activity of p53 and therefore down-regulates apoptosis (Luo et al., 2001; Vaziri et al., 2001). If the survival function of SIR2 genes observed in yeast, worms, and protozoans extends to mammals, apoptosis may be, thus, important in limiting mammalian life span.

Furthermore, a hyperactive allele of p53 has been described that confers enhanced tumor surveillance on transgenic mice (Tyner et al., 2002). Interestingly, these mice develop early organ degeneration and signs of premature aging. These phenotypes further support the idea that apoptosis may limit mammalian life span, because its enhancement apparently speeds up the aging process.

The above studies raise the possibility that any process extending mammalian life span would have toe slow down apoptosis. However, in some organs with rapidly dividing cells, apoptosis actually increases during CR, for example, in the liver (James et al., 1998) and the gut (Holt et al., 1998). This increase, along with the known shrinkage of cells during CR (Birchenall-Sparks et al., 1985), may both contribute to the down-sizing of these organs in the restricted animal. The increased rate of apoptosis may minimize the risk of cancer during CR (James et al., 1998). The increase in apoptosis in these organs appears at odds with any central role for SIR2. However, it is possible that neuroendocrine changes are dominant in up-regulating apoptosis in this subset of organs.

The brain is one organ that does not shrink during CR (Keenan et al., 1995; Weindruch and Sohal, 1997). It would be of interest to determine whether CR slows cell death of neurons. This may be difficult to visualize in animals, because apoptosis is transient and the number of apoptotic cells at any given time will be low. However, it may be possible to test whether interventions that slow aging, such as CR, result in less apoptosis when neuronal cells are harvested and cultured.

6. REGULATORY PEPTIDES AND CONTROL OF FOOD INTAKE

The control of food intake is highly complex and involves numerous external and internal factors. The past decade has witnessed an upsurge in our understanding of the hypothalamic regulation of appetite. Expression of appetite or the motivational drive toward an energy source is a highly regulated phenomenon in vertebrates. It is considered a cornerstone for maintenance of energy homeostasis and for rigidly guarding the body weight around a set point. Abnormalities in the onset, periodicity, duration, and magnitude of eating episodes generally underline argument appetite (Kalra, 1997; Stunkard, 1996).

Some peptide participating in the regulation of appetite or feeding behavior have been discovered by the recent advancement of biological techniques. Many neuropeptides, such as neuropeptide Y (NPY), corticotrophin-releasing factor, agouti-related protein (AgRP), α-melanocyte stimulating hormone (α-MSH), cocaine- and amphetamins- regulated transcript (CART) peptides, melanin concentrating hormone, orexins, and leptin, interact with each other to regulate appetite and energy balance (Elmquist et al., 1999; Schwartz et al., 1999). Furthermore, various lines of evidence suggest that these peptides participate in cardiovascular and sympathetic regulations as well as in the regulation of appetite and feeding behavior (Elmquist et al., 1999; Schwartz et al., 1999). Physiological effects of these peptides are extensive and complex. This thesis, therefore, also focuses on the effects of leptin and ghrelin and sympathetic regulations within the hormonal system.

6.1. Leptin

In 1953, Kennedy proposed that body weight was maintained by the regulation of body content (Kennedy, 1953). His adipostat mechanism anticipated the presence of an unknown circulating factor that provided the hypothalamus with information on the extent of body fat stores. Although several adipostat factors were proposed in the intervening years, the discovery of leptin in 1994 revolutionized the field (Zhang et al., 1994). In accordance with its putative adipostat role, leptin is expressed and secreted by adiposites in white adipose tissue and circulates in plasma at concentrations proportional to fat mass, with a relatively long half-life. Peripheral or central nervous system administration of leptin to rodents reduces food intake and body weight and increase energy expenditure (Friedman and Halaas, 1998). Much lower dose are required with central nervous systems (CNS) administration, and peripheral leptin administration activates hypothalamic neurons expressing the leptin receptor, suggesting that these effects are mediated via the hypothalamus., Peripherally secreted endogenous leptin enters the CNS by either active uptake or simple diffusion in areas outside the blood-brain barrier. Leptin directly inhibits orexigenic ARC (arcuate nucleus) NPY/AgRP neurons and stimulates anorectic ARC POMC (pro-opiomelanocortin) neurons (Sahu, 2003). Leptin, therefore, acts as the afferent limb of a body fat regulation feedback loop.

The hyperphagic and obese ob/ob mouse lacks functional leptin (Zhang et al., 1994). Administration of exogenous leptin ameliorates these abnormalities in mice and men (Farooqi et al., 2002; Pelleymounter et al., 1995). However, the vast majority of obese humans have normally functioning ob genes and high plasma leptin levels reflecting their high fat mass, suggesting leptin resistance in obese individuals (Considine et al., 1996). The mechanism may involve reduced passage and capillary transport of leptin into the CNS and reduced leptin receptor expression and /or suppressed intracellular signaling may

occur. These factors would be responsible for the limited efficacy of leptin as an antiobesity drug in human trials to date (Mantzoros and Flier, 2000). While the absence of circulating leptin, communicating low or nonexistent body fat stores, has profound effects on appetite, body weight and fertility, raised leptin levels have much less dramatic results. Leptin may, therefore, play an important role during periods of starvation, but be less significant when food is freely available.

Leptin also plays important roles in neuroendocrine signaling and reproduction (Auwerx and Staels, 1998). Although leptin or leptin receptor has not been yet characterized in fish, heterologous Southern blotting (Zhang et al., 1994) and immunological screenings (Johnson et al., 2000; Yaghoubian et al., 2001) suggested fish would also express leptin-like proteins. Although some investigators, however, stated that mammalian leptin had no marked effect in immature coho salmon (Baker et al., 2000) or catfish (Silverstein and Plisetskaya, 2000), some leptin-administration studies suggest that leptin is able to modulate the fish food intake activity and other physiological responses. It was demonstrated that leptin stimulated luteinizing hormone (Peyon et al., 2001) and somatolactin releases (Peyon et al., 2003) in European sea bass. Weil et al. (2003) have recently revealed that the high concentration of human leptin at the pituitary level directly stimulated FSH and LH releases in female rainbow trout. Volkoff et al. (2003) have recently demonstrated that murine leptin injection reduced food intake activity of goldfish and that the leptin function was antagonized by orexin A, a food intake enhancing hormone. It is, therefore, supposed that fish also have a functional leptin system for modulating food intake activity and some physiological signalings. Investigations in rodents indicate that sex hormones may be important in determining plasma leptin. Frederich et al. (1995) found that at any given body fat content, female rats had higher leptin levels compared to male rats. In woman of reproductive age, leptin and estradiol showed similar profiles throughout the menstrual cycle (Cella et al., 2000; Mannucci et al., 1998). The primary ovarian signal responsible for regulating body weight and adiposity has been suggested to be 17 β-estradiol (Czaja et al,. 1983; Wade, 1975) and it has been shown that ovaries expressed leptin receptor messenger RNA (mRNA) (Cioffi et al., 1996; Karlsson et al., 1997). The administration of leptin also antagonized ovarian hormone secretion (Zachow et al., 1999). In mammals, 17 β-estradiol regulated leptin secretion (Kikuchi et al., 2001). However, studies on the relationships between leptin and 17 β-estradiol are limited in fish.

Prolactin (PRL) is considered as a primary an osmoregulatory hormone in fish (Manzon, 2002). Some studies also suggest that PRL may be associated with production of steroid hormones in the gonads, the onset of gonadal development, and reproductive behavior (De Ruiter et al., 1986). The result that PRL stimulated leptin secretion in mammalian (Gualillo et al., 1999) bethinks us of a possible role for PRL in the regulation of food intake. On the other hand, 17 β-estradiol enhances PRL production by directly stimulating PRL gene

transcription, leading to increased synthesis of PRL mRNA and PRL (Maurer, 1982). In teleosts, it is also suggested that 17 β-estradiol is involved in expression of PRL and PRL receptor mRNA of the gilthead seabream (Cavaco et al., 2003).

6.2. Ghrelin

Ghrelin is the only peripherally active appetite-stimulating hormone so far discovered. Ghrelin potently stimulates food intake and growth hormone secretion following peripheral administration in man and rats (Tschöp et al., 2000; Wren et al., 2000, 2001a). Plasma ghrelin levels are inversely correlated with body weight and rise following weight loss in humans (Cummings et al., 2002). The major source of circulating ghrelin is the stomach, though ghrelin mRNA and immunoreactivity are also found in other regions of the gastrointestinal tract (Date et al., 2000). Ghrelin is composed of 28 aminoacids with an acyl sidechain attached to the serine residue at position 3. This acyl group is crucial to ghrelin's orexigenic and growth hormone-releasing actions, which are mediated through the growth hormone secretagogue receptor (GHS-R; Kojima et al., 1999). The GHS-R is highly expressed in the hypothalamus, including the ARC, but also found in the brainstem, pituitary, gastrointestinal tract, adipose tissue and other peripheral tissues (Petersenn, 2002). It has been suggested that desacylated ghrelin has other biological functions mediated by separate GHS-R subtypes (Baldanzi et al., 2002).

Circulating ghrelin concentrations rise during fasting and fall rapidly after a meal. Ghrelin may be, therefore, involved in meal initiation (Cummings et al., 2001), though a recent work has shown that circulating ghrelin levels do not predict intermeal interval in humans (Callahan et al., 2004). Although calorie intake appears to be the primary regulator of plasma ghrelin levels (Tschöp et al., 2000), the exact mechanisms mediating ghrelin release are unknown. There is some suggestion that glucose and /or insulin suppress ghrelin release (Yoshihara et al., 2002), but another study has shown that physiological levels of either appear to have little effect on plasma ghrelin concentrations (Schaller et al., 2003). Circulating ghrelin levels are lower in obese individuals, perhaps reflecting a feedback mechanism to reduce appetite (Tschöp et al., 2001).

The orexigenic effects of peripheral ghrelin are mediated via the CAN. Peripheral administration of ghrelin activates neurons in the ARC and the paraventricular nucleus (Ruter et al., 2003) and intracerebroventricular administration of ghrelin antibodies into the rat brain inhibits fasting-induced feeding. The orexigenic effects of ghrelin are thought mediated via NPY and AgRP. Central injection of ghrelin activates NPY/AgRP neurons and increases hypothalamic NPY and AgRP. In NPY/AgRP double knockout mice, the orexigenic action of ghrelin are abolished (Chen et al., 2004)., Ghrelin has been recently found to be expressed in a previously uncharacterized neuronal population adjacent to the

third ventricle (Cowley et al, 2003). These hypothalamic ghrelin neurons may be involved in another hypothalamic appetite circuit, though the relationship between central and peripheral ghrelin signaling is currently unknown. It is interesting that the patterns of neuronal activation following peripheral and central ghrelin administration differ (Lawrence et al., 2002; Ruter et al., 2003).

Chronic peripheral ghrelin administration causes hyperphagia and obesity in rats (Tschöp et al., 2000; Wren et al., 2001b). The ghrelin system, therefore, offers a potential target for long-term antiobesity therapy. There is little change in body weight or food intake in ghrelin or GHS-R knockout animal models, but this may be due to compensatory developmental changes in other appetite regulatory systems (Sun et al., 2003, 2004).

A ghrelin-like ligand was detectable in the blood of a teleost as predicted by Shephred et al. (2000). Ghrelin cDNA has been also identified and characterized in some teleosts. Goldfish (*Carassius auratus*) ghrelin has 47 % similarity with the amino acid sequence of human ghrelin (Unniappan et al., 2002). Tilapia preproghrelin is a polypeptide of about 107 amino acids, consisting of a signal peptide (26 amino acids), the mature peptide (22 amino acids) and a C-terminal peptide (59 amino acids). Comparison of amino acid sequence of the mature peptide of tilapia with known sequences of other species show a 50-70 % homology between both teleost and avians, and about 40 % homology between bullfrog and mammals. The C-terminal portion rather than its N-terminal end of the mature peptide has high variability (Parhar et al., 2003). This is noteworthy because the N-terminal region is the biologically active segment of the ghrelin. The first four amino acids "GSSF" considered to be the active core of the of the ghrelin peptide in mammals (Bednarek et al., 2000) are conserved in tilapia but are different from those of bullfrog (GLTF: Kaiya et al., 2001) and goldfish (GTSF: Unniappan et al., 2002). The goldfish have two alternatively spliced ghrelin molecules (Unniappan et al., 2002) but tilapia and other vertebrates have a single ghrelin molecule because of the presence of a single cleavage site in their preproghrelin structure. In the Japanese eel (*Anguilla japonica*), the overall similarity is the same but the first seven amino acids are 100 % identical to mammalian ghrelins and eel ghrelin has the ability to stimulate growth hormone (GH) and prolactin release from the pituitary (Kaiya et al., 2003). The same effect can be induced *in vitro* in the tilapia (*Oreochromis mossambicus*) with rat ghrelin (Riley et al., 2002). This suggests that ghrelin peptide and its function in GH secretion are evolutionarily quite conserved. The tilapia ghrelin gene consists of four exons and three introns, and this structural organization resembles those of the goldfish ghrelin gene (Unniappan et al., 2002) but differs from those of the mouse and rat ghrelin, which contains an additional non-coding exon of 19 bp in the 5'-untranslated region (Tanaka et al., 2001). Furthermore, the sizes of introns in the ghrelin gene vary among animal species. Phylogenic variations in the organization of ghrelin molecules are not unexpected because the metabolic needs of each

animal species may have required the ghrelin protein to perform subtly different functions. In tilapia, RT-PCR analysis revealed a strong signal derived from ghrelin mRNA in the stomach but no signal could be detected in other tissues (Parhar et al., 2003). These are consistent with the fact that the stomach is the major ghrelin-producing site in the rat, human, chicken amphibians.

Section 2

The purpose and brief summaries of this research

Some fish species show parental death shortly after their first spawning. The well-known examples are ayu (*Plecoglossus altivelis*) which dies in only one year. Although the mechanisms for such a short life span are still unclear, there have been proposed some hypotheses. Since it is shown clearly that ayu produced ROS higher than other species, it is supposed that high ROS production strongly related in aging advances, resulting in shortened life span. Homeostasis disturbances by maturation, debility for exhausting energy of spawning and decreasing of feeding activities during spawning and after spawning are also considered to be factors which ayu dies in only one year. Along these hypotheses, this study dealed with carp as a model fish with long life span and ayu as a model fish with short life span and was performed for disclosing whether 1) influence of the oxidative stress on biomembrane, 2) apoptosis related factors, 3) caloric restriction, and/or 4) feeding activity would be relevance to short life span or not. Clarification of fish life span determination factors will also contribute to the stable fish culture techniques including 'programmed' fish culture on the basis of mechanism elucidations.

This thesis is composed of five chapters. Chapter I deals with introduction and general discussion. It was given for gaining insight into aging and senescence studies. The free radical theory of aging, telomere theory of aging, programmed cell death, p53 tumor suppressor protein induced apoptosis and caloric restriction were reviewed comprehensively. Regulatory peptides and control of food intake were also described.

In Chapter II, it was investigated the influence of partial oxidative stress on permeability and fluidity of nucleated fish red blood cells for simulating nucleated somatic cells. Peroxide value indicating lipid hydroperoxide level in nucleated red blood cells of common carp (*Cyprinus carpio*) increased with increasing body size. It was detected that oxidation of nucleated red blood cells led to the degraded PUFA compositions and accelerated the permeability of calcein and ATP in the nucleated red blood cells restrictedly oxidized with AAPH treatment. Using fluorescence probes, PC_3P, it was found that oxidative stress reduced the membrane fluidity of nucleated red blood cells. It was also observed that AAPH had no significant influence on the osmotic fragility and electrophoretic profiles of red blood cell proteins. These results suggest that partial oxidative-stress, even if failure to fragment the membrane, may affect membrane permeability of fish nucleated red blood cells for an important energy molecule, ATP.

It is well known that ayu (*Plecoglossus altivelis*) die after spawning and the life span is only one year. One possible cause is that enhanced oxidative stress might induce DNA damage and subsequent DNA repair systems as phosphorylated p53 in ayu, leading to apoptosis and relating to their short life span. Telomeres, the non-coding sequences at the ends of chromosomes, shortening of telomeres can induce cell cycle arrest and apoptosis. Chapter III, then, was addressed to the p53 and its phosphorylation in ayu brain, the oxidative DNA damage by measuring the levels of 8-OHdG and the induction of apoptosis

by measuring the levels of caspase-9/6, -3 with aging in brain and liver. It was also investigated that age related changes in telomere length in the ayu. The findings indicate that oxidative stress activated caspase-9/6, -3 in brain and liver, and activated p53 through the phosphorylation of p53 and p53 with aging in ayu brain. There was no significant change in telomere length through life span. It was suggested that the age-related of apoptosis might be involved in increasing of DNA damage and mutations in brain and liver, and could partially explain the short life span of ayu.

The effects of caloric restriction on post-spawning death of ayu were investigated in Chapter IV. Caloric restriction is the only established intervention that extends life span in mammals, insects and nematodes. One of the hypotheses suggested that most of the effects of CR on aging may be due to reduced oxidative stress at the cellular level. It was known that ayu produced ROS higher than other fish and that the life span of ayu is only one year. It was attempted to quantify age-associated changes of the degree of attenuation on oxidative damage and hormonal homeostasis in CR. The oxidative DNA damage by measuring the levels of 8-OHdG and the induction of apoptosis by measuring the levels of caspase-9/6, -3 with aging in brain and liver were surveyed. Changes in major sexual hormones were also investigated. Caspase activities in brain and liver were reduced by CR, although CR was no influence to DNA damage level. Plasma testosterone levels of CR ayu were significantly higher and progesterone and 17 β-estradiol levels were lower than the control ayu. However, life span of ayu was not prolonged by CR. These results suggest that there would be factors determining life span of ayu other than CR and apoptosis.

Chapter V deals with roles of leptin in post-spawning death of ayu. It is well known that ayu dye after spawning and the life span is only one year. The determinants for such a short life span are probably involved in spawning and some accompanied changes in hormonal homeostases. It is one of the accompanied changes that feeding activity of ayu decreases during spawning and after spawning. Then, it was investigated the relationships among leptin and ghrelin, they are regulators for food intake, and other major hormones, 17 β-estradiol and prolactin. Leptin levels were significantly higher during spawning, associated with decrease in appetite. Leptin levels were also synchronized with levels of 17 β-estradiol and prolactin. Ghrelin levels were no significant difference. Therefore, one possible explanation for decrease in appetite during ayu spawning is that the alteration of 17 β-estradiol homeostasis induced the secretion of leptin. The inability to reduce the leptin level into the basal after spawning would be in part responsible for a short life span of ayu.

In conclusion, it was revealed that the mechanisms governing life span of ayu were through at least two pathways. One is apoptosis induced by oxidative stress with aging. This pathway is probably, however, an alleyway, since CR could afford to downregulate apoptosis pathway but did not extend the life span of ayu. Another is decreasing appetite

during and after spawning induced by leptin in ayu. Reproduction induced physiological anorexia: it is beginning to death.

The contents of this thesis are partly submitted and have been published or will be published soon as follows.

1. Partial oxidative-stress perturbs membrane permeability for energy molecules and membrane fluidity of fish nucleated red blood cells, Comp. Biochem. Physiol. C, 139 (2004) 259-266. (collaboration with N. Okamoto and H. Ushio)
2. Elevated levels of oxidative DNA damage activate p53 and caspases in brain of ayu with aging, submitted to Journal of Applied Ichtiology (collaboration with N. Okamoto and H. Ushio)
3. Leptin is one of determining factors for post-spawning death of ayu (*Plecoglossus altivelis*), submitted to Journal of Experimental Zoology (collaboration with N. Okamoto and H. Ushio)
4. Enhanced oxidative damages and apoptosis in aging ayu liver, submitted to Aquaculture Research (collaboration with N. Okamoto and H. Ushio)
5. Effects of caloric restriction on post-spawning death of ayu, submitted to Experimental Gerontolgy (collaboration with N. Okamoto and H. Ushio)

CHAPTER II

OXIDATIVE STRESS OF CARP RED BLOOD CELLS

1. INTRODUCTION

In many ways, the red blood cells (RBCs) of non-mammalian vertebrates are very different from those of the mammalian. One of the most obvious differences is that non-mammalian RBCs, for instance, the fish RBCs are nucleated whereas mammalian RBCs extrude their nuclei before entering the circulation. In addition, in nucleated RBCs ribosomes are retained (Lane and Tharp, 1980; Lane et al., 1982; Sekhon and Beams, 1969), which enable them to synthesize proteins such as hemoglobin after their maturation (Keen et al., 1989; Speckner et al., 1989). They also retain functional mitochondria and maintain higher rates of metabolism than their mammalian counterparts (Boutilier and Ferguson, 1989). RBCs of lower vertebrates experience many structural and functional changes throughout their 4-6-month life span, including loss of mitochondria (Keen et al., 1989; Lane, 1984; Lund et al., 2000; Phillips et al., 2000; Speckner et al., 1989; Tiano et al., 2000, 2001).

Fish biological membranes are rich in polyunsaturated fatty acids such as docosahexaenoic acid, and would be sensitive to oxidative stress, directly or indirectly causing many fish illnesses (Ito et al., 1999, 2000; Sakai et al., 1989; Slater, 1982). Moreover, the RBCs, because of its role in the transport of oxygen via hemoglobin, are constantly exposed to reactive oxygen species (ROS) during life span. Hence, it is thought that nucleated fish RBCs suffer more oxidative stress than do mammalian RBCs.

Although there is repair systems against oxidative stress in cells, RBCs are constantly exposed to ROS during their life span, oxidative damages might be accumulated with aging. The protective responses against oxidative stress may decline with aging, thus predisposing cells and organisms to oxidative damages. Accumulation of membrane lipid peroxidation is one of the oxidative damages. We have recently demonstrated that sweet smelt RBCs accumulated lipid hydroperoxides with aging (Kaewsrithong et al., 2001). Although it is probable that whole body aging might go with such damages, the details for such damage are still ambiguous.

In this study, we explored the effects of the partial oxidative stress on the membrane permeability in the nucleated RBCs of the carp. The restricted oxidation of the biological membrane of fish nucleated RBCs with a free radical initiator, 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), was carried out as the model system for membrane lipid peroxidation associated with aging and other physiological abnormalities. This investigation could be of use for a better understanding of the molecular mechanisms of the aging process in aquatic organisms.

2. MATERIALS AND METHODS

2.1. Fish and handling of blood

The carp, *Cyprinus carpio* (about 30-400g), were obtained from Yoshida Research and Training Station of Tokyo University of Marine Science and Technology. Fish were fed a commercial diet until being sacrificed. Blood was collected from caudal vessels of carp with heparinized plastic syringes. Blood was centrifuged at 700 *g* for 5 min at 4°C to separate plasma from RBCs. The RBCs were washed three times with buffered isotonic solution containing 128 mM NaCl, 3 mM KCl, 1.5 mM $MgCl_2$ and 10 mM Hepes (Tiihonen et al., 1995). The buffy coat was removed and the resulting RBCs were immediately used for the following analyses.

2.2. Restricted oxidation of RBC membrane lipid with AAPH

The RBCs collected from carp (approximately 200 g) suspended in a buffered isotonic solution (about 2.0×10^9 cells/ml) were oxidized by the addition of 1 mM 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH; Wako Pure Chemicals, Tokyo, Japan) and incubated for 30 min at 21 °C in the dark. This condition was set for matching lipid peroxidation levels of the AAPH-treated RBC with those of larger carp RBCs as described below. After incubation with AAPH, the RBCs were separated by centrifugation at 700 *g* for 5 min at 4 °C and washed 3 times with the buffered isotonic solution.

2.3. Determination of hydroperoxides

Total lipids of carp RBCs obtained from various body sizes of carp (about 30-400g), and total lipids of the control and the AAPH treated (final concentration of 1 mM) RBCs were extracted according to the procedure of Bligh and Dyer (1959). Peroxide value of lipid extract from RBCs was determined according to Akasaka et al. (1992). Briefly, 50 μl of 250 μM diphenyl-1-pyrenoylphosphine (DPPP, DOJINDO Laboratories, Kumamoto, Japan) methanol solution was added to one hundred microliter of sample lipid in a glass tube with a screw cap under cooling in an ice bath. The tube was capped tightly and left standing for 60 min at 60 °C in the dark. Then, it was cooled in an ice bath and 2-propanol was added before the measurement. The fluorescence intensity at 380 nm derived from DPPP oxide was measured with an excitation wavelength of 352 nm. The hydroperoxide concentration was calculated from the resulting fluorescence intensity with a calibration curve using peroxide values estimated by the iodometry according to the Japan Oil Chemists' Society Standard Method for Analysis of Fats and Oils (Chapman and Mackay,

1949).

2.4. Determination of fatty acid composition

Total lipids of the control and the AAPH-treated RBCs were separately extracted according to the procedure of Bligh and Dyer (1959). An aliquot of the total lipid was saponified with 0.5 M methanolic sodium hydroxide and subsequently methyl-esterified by 14% BF_3 in methanol to obtain the corresponding fatty acid methyl esters, according to the A.O.C.S. method. Composition analysis of the fatty acid methyl esters was carried out through gas-liquid chromatography (GLC) with a Shimadzu GC-14B instrument equipped with a Supelcowax-10 fused silica open tubular column (0.25mm i.d. x 30m, 0.25 μm in film thickness; Supelco, PA) and a flame ionization detector. The column oven temperature was programmed from 140 to 240 °C at a rate of 1.2 °C/min. Peak identification was accomplished by reference to Equivalent Chain Length standard (Ackman, 1990).

2.5. Permeability measurement

RBCs (about 2.0×10^9 cells/ml) were incubated in the buffered isotonic solution containing 70 nM Calcein-AM (Dojindo Lab) at 21 °C for 30 min. After washing three times with the buffered isotonic solution, RBCs were incubated in the presence of 1 mM AAPH at 21 °C for 30 min. After washing three times with the buffered isotonic solution, the fluorescence intensity was measured at the excitation and emission wave lengths, 490 and 515 nm, respectively. Control experiments were carried out in the absence of AAPH.

2.6. Determination of ATP and its related compounds

One hundred microliter of the control or the AAPH treated RBCs (about 2.0×10^9 cells/ml) was homogenized with the same volume of 10 % (v/v) perchloric acid solution and centrifuged at 18,000 *g* for 5 min. The resulting supernatant was neutralized with KOH, centrifuged at 18,000 *g* for 5 min, and filtered with a piece of Millipore filter. The resulting filtrate was subjected to the following HPLC analyses. ATP and its related compounds were determined according to the method of Matsumoto and Yamanaka (1990) on a HPLC system (LC-10A system, Shimadzu, Kyoto, Japan) equipped with an Asahipak GS-320 column (Asahi Chemical Industry Co.). Mobile phase of 200 mM NaH_2PO_4 (pH 2.9 at 25 °C) with the flow rate of 0.6 ml/min was used. Eluted compounds were monitored with the absorbance at 260 nm.

2.7. Determination of Osmotic Fragility

Fifty microliter of the control or the AAPH-treated RBCs (about 2.0×10^9 cells/ml) was added to 1 ml of 0—0.7 % NaCl solution, then incubated at 4 °C for 30 min. After centrifugation at 700 *g* for 5 min the absorbance of supernatant was measured at 540 nm. The NaCl concentration causing 50 % of hemolysis was determined.

2.8. Membrane fluidity analysis

The membrane fluidity of RBCs was determined using a fluorescence probe, 1,3-bis(1-pyrenyl)propane (PC_3P; Dojindo Lab, Kumamoto) (Zachariasse et al., 1982). The final probe concentration was less than 5.0×10^{-7} M, and the incorporation of the probes was carried out according to the method of Almeida et al. (1982).

After incubation for 18 hr at 4°C with PC_3P, RBC samples were separated by centrifugation at 700 *g* for 5 min at 4°C and washed 3 times with the buffered isotonic solution. RBCs were, then, subjected to an oxidative challenge with AAPH as described above.

The fluorescence intensity was measured with a Shimadzu RF-1500 spectrofluorophotometer at the excitation wavelength of 330 nm. The parameter of fluidity, the excimer to monomer fluorescence intensity ratio (I'/I), was calculated from the emission signal intensities at 490 and 378 nm, respectively.

2.9. SDS-PAGE

RBCs (about 2.0×10^9 cells/ml) were oxidized by the treatments with 1, 100, 200, and 300 mM (final concentration) AAPH and incubated for 30 min at 21 °C in the dark. After incubation with AAPH, the RBCs were separated by centrifugation at 700 *g* for 5 min at 4 °C and washed 3 times with the buffered isotonic solution.

To prepare samples for SDS-PAGE, samples were boiled in a solution containing 10 % (v/v) glycerol, 125 mM Tris-HCl, 10 % (v/v) 2-mercaptoethanol, 4 % (w/v) SDS, and 0.004 % (w/v) bromophenol blue for 10 min at 100 °C, frozen in liquid nitrogen, and boiled again for 10 min at 100 °C for complete solubilization of membrane proteins after AAPH treatments. Samples were separated on a 2-15 % gradient gel (Daiichi Pure Chemicals, Tokyo) at the electrophoresis current of 50 mA. All buffers and gel systems were those of Laemmli (1970). Gels were stained with a silver staining kit for proteins (Amersham Biosciences, Tokyo).

2.10. Statistical analyses

Data obtained were analyzed using one-way ANOVA.

3. RESULTS

3.1. Hydroperoxides in RBC

DPPP is known to react with hydroperoxide to give fluorescent DPPP oxide. Therefore, the fluorescence intensity of DPPP oxide is directly correlated with the amounts of hydroperoxide. Figure II-1 shows lipid peroxide value in RBCs of various body sizes of carp. A high correlation was observed between carp body size and the peroxide value in carp RBCs (r=0.61, P<0.01). The present result indicates that carp body size is correlated closely with lipid hydroperoxide levels in RBCs.

Lipid peroxide values of the control and the AAPH-treated RBCs were 1.3±0.5 and 3.8±1.9 meq/2.0×10^6 cells, respectively (Figure II-2). Lipid peroxide value of the AAPH-treated RBCs was significantly higher (P<0.05) than the control value and was almost similar to that of large carp RBCs in Figure II-1.

3.2. Fatty acid composition

Changes in saturated fatty acid (SFA), monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA) compositions of the carp RBCs are shown in Table II-1. No significant difference in MUFA compositions was observed. On the other hand, PUFA composition of the AAPH-treated RBCs (19.2 %) was significantly lower (P<0.01) than those of the control (31.5 %), and SFA composition of the AAPH-treated RBCs (61.7 %) was significantly higher (P<0.01) than those of the control (48.1 %).

3.3. Permeability

Changes in fluorescence intensity of Calcein in RBCs are shown in Figure II-3. The initial value, 1.7±0.4, suggested that the carp RBCs should have no autofluorescence at this excitation wave length. The remaining fluorescence intensity of the treated RBCs, 213.6±76.9, was significantly lower than that of the control RBCs, 331.3±95.3 (P<0.01).

3.4. ATP and its related compounds

Effect of AAPH treatment on ATP levels of RBCs was examined. The control and the AAPH-treated RBCs showed ATP levels of 0.68±0.035 and 0.66±0.017 fmol/cell, respectively (Figure II-4a). Thus, no significant difference in ATP levels between the control and the AAPH-treated RBCs was observed in this study. On the other hand, the ratio value of ATP to ATP+ADP for the AAPH treated RBCs (0.431±0.01) was significantly lower than those for the control (0.567±0.01) (Figure II-4b) ($P<0.01$).

3.5. Osmotic fragility

Figure II-5 shows the hemolysis curves of the control and the AAPH-treated RBCs. No change in the hemolysis curve through AAPH treatment was observed. The 50 % hemolysis occurred at the NaCl concentration of 0.45±0.02 and 0.44±0.00 % for the control and the AAPH-treated RBCs, respectively. Thus, no significant difference in osmotic fragility between the control and the treated RBCs was observed in this study.

3.6. Membrane fluidity

Figure II-6 shows the membrane fluidity of the control and the AAPH-treated RBCs determined from PC_3P excimer to monomer fluorescence intensity ratios. The fluidity value of the treated RBCs, 62.3, was markedly lower than that of the control RBCs, 118.4 ($P<0.01$), suggesting that the RBCs membrane became more rigid after exposure to AAPH.

3.7. SDS-PAGE

Protein profiles of the 1, 100, and 200 mM AAPH-treated and the control RBCs were almost similar to one another as shown in Figure II-7, while the 300 mM AAPH-treated RBC exhibited different patterns. Stacked protein aggregates were observed around 200,000 Da in molecular mass. Thus, such a strong oxidative stress would induce decomposition and/or aggregation of proteins.

4. DISCUSSION

We confirmed that lipid hydroperoxides are accumulated in RBCs of older carp, as demonstrated by the significant augmentation in RBC lipid hydroperoxide, probably because of diminished removal rates of damaged cell components. The results also indicate

that the partial oxidative stress without obvious hemolysis led to 1) accumulation of hydroperoxide, 2) loss of small molecules, such as calcein and ATP, 3) reduction of membrane fluidity and 4) degradation of PUFAs of carp RBC membrane.

AAPH, a relatively acute radical initiator, is generally used in the concentration of over 30 mM at 37 °C. Sato et al. (1995) demonstrated that the formation of DMPO-AAPH radical adduct was highly dependent on temperature and concentration. The ratio of the radical formation level at 25 °C to that at 37 °C calculated from their data was about 1/4 in the presence of 30 mM AAPH. Their data also suggested that 30 mM AAPH gave over two-fold DMPO-AAPH radical adduct, compared with the case of 1 mM AAPH. The condition of 1 mM AAPH for 30 min at 21 °C used in the present study is, therefore, much milder than the generally adopted conditions. This mild oxidation is probably responsible for very low values of lipid hydroperoxide and no obvious protein aggregation obtained in the present study.

Calcein-AM is converted into a non-membrane permeable form, calcein, and is well retained in cytoplasm. This fluorescence probe is emitted by collapse of a cell membrane or rise in cell membrane permeability (Miller et al., 1997; Petronill et al., 1999; Yano et al., 1996). In this study, the oxidization by AAPH did not cause RBC burst, but the calcein fluorescence decreased as shown in Figure II-3. It is suggested that the RBC membrane was permeated by the AAPH oxidative stress and that such an oxidized biological membrane might also become leaky for ions and ATP, much smaller than calcein molecules. However, no significant decrease of ATP levels after AAPH treatment was observed as shown in Figure II-4a. One possible explanation of such a discrepancy is as follows: some ions and/or ATP would leak out through cell membrane permeabilized by lipid peroxidation as suggested by Deuticke and Haest (1987), Deuticke et al. (1987, 1991) and Ney et al. (1990). Cells must activate metabolisms in order to maintain the homeostasis of ions and ATP levels. This hypothesis would be also confirmed by a significant decrease in the ratio of ATP to ATP+ADP under the AAPH-elicited oxidative stress as shown in Figure II-4b. There are, however, possibilities that other oxidative damages such as protein cross-linking might be responsible for increase in membrane permeability as reported by Deuticke et al. (1983). In the present study, no obvious cross-linking pattern was observed in protein profiles of SDS-PAGE. Further investigations are required for disclosing the mechanism of the membrane permeability increase by the AAPH treatment. Thus, the oxidized fish nucleated RBCs might be always exposed to the risk for the loss of bioenergy. That might be a reason why the fish PUFA-rich RBCs retain functional mitochondria and maintain higher rates of metabolism but not mammalians. On the other hand, Phillips et al. (2000) reported that the rate of O_2 consumption declined in older rainbow trout RBCs by at least 50 %, compared to the younger. Rabini et al. (1997) reported that aging causes a reduction in the RBC ATP content. RBCs of older carp would also show a lower

respiration rate to meet their energy requirements adequately. On the other hand, there are also some evidences that the glycolytic production of ATP increases with aging (Lane, 1984; Phillips et al., 2000). We also believe that the alternative route of ATP production, glycolysis, is likely to be up-regulated in older carp as suggested by Phillips et al. (2000) for rainbow trout.

The composition of SFA and MUFA slightly increased and PUFA decreased in the AAPH-treated RBC, compared with control. The decrease in PUFA composition is probably due to lipid peroxidation by the AAPH treatment. It is known that once the unsaturation is removed by addition of peroxyl or hydroxyl groups, the membrane becomes more rigid (Borst et al., 2000). The present result that the fluorescence intensity I'/I ratio significantly decreased by the AAPH treatment suggests that membrane lipid oxidation would reduce membrane fluidity with decreasing PUFA composition.

The membrane fluidity is mainly determined by their lipid composition. The cholesterol/phospholipids molar ratio is not only a determinant of membrane fluidity; but the phospholipid composition and the length and the degree of unsaturation of the phospholipid fatty acyl chains also affect membrane fluidity. Many clinical studies have suggested that impaired RBCs deformability in humans has pathological consequences (Owen et al., 1982; Shiraishi et al., 1993; Zicha et al., 1999; Zubenko et al., 1996). However, there have been few previous studies of the rheology of nucleated RBCs. The fish RBCs are larger than human RBCs, have a stiffer membrane (more resistant to shear and bending) and contain a large nucleus that is absent from the human cells. Regardless of their large size, the fish RBCs do have sufficient membrane surface area to enable them to adapt their shape to traverse capillaries. Nash and Egginton (1993) noted that calculations and direct observation show that trout RBCs can enter cylindrical apertures down to 3 μm in diameter. This limiting size was similar to that in human RBCs. However, near this limiting diameter, their resistance to pore entry is about a thousand times higher than that of human RBCs. The relatively poor overall deformability of nucleated RBCs could arise from their decreased membrane fluidity, larger size and the presence of a larger nucleus. The results indicate that RBCs accumulating lipid hydroperoxides would be less deformable with membrane rigidity. RBCs with hydroperoxides are, therefore, hard to go through microcirculation and to perform satisfactory oxygen supply. Larger carp RBCs accumulating lipid hydroperoxides in Figure II-1 might also perturb oxygen supply and related homeostases.

Several series of studies demonstrated that aging of fish nucleated RBC was accompanied with many events, such as increase in hemoglobin concentration, decreases in metabolic enzymes such as citrate synthase, decrease in O_2 consumption (Phillips et al., 2000), accumulation of DNA damage (Moretti et al., 1998), increase in intracellular ROS and decline in mitochondrial membrane potential (Tiano et al., 2001). This study, focusing

on effects of very low levels of lipid hydroperoxide in the fish nucleated RBC membrane, has raised the additional viewpoint. It deserves further attention whether abnormalities in nucleated RBC functions are indeed related to changes in membrane lipid composition and membrane fluidity, since it may lead to a clearer understanding of metabolic abnormalities and mechanisms of fish RBCs. From the view of diagnostics, we had better give attention to not only changes in osmotic fragility and primary structure of proteins but changes in membrane permeability and fluidity. This and further studies in this area will be helpful to clarify how fish responds to oxidative stress.

ABSTRACT

We investigated the influence of partial oxidative stress on permeability and fluidity of nucleated fish red blood cells for simulating nucleated somatic cells. Peroxide value indicating lipid hydroperoxide level in nucleated red blood cells of common carp (*Cyprinus carpio*) increased with increasing body size. We detected that oxidation of nucleated red blood cells led to the degraded PUFA compositions and accelerated the permeability of calcein and ATP in the nucleated red blood cells restrictedly oxidized with 1 mM AAPH treatment for 30 min 21 °C in the dark. Using fluorescence probes, PC_3P, we found that oxidative stress reduced the membrane fluidity of nucleated red blood cells. It was also observed that AAPH had no significant influence on the osmotic fragility and electrophoretic profiles of red blood cell proteins. These results suggest that partial oxidative-stress, even if failure to fragment the membrane, may affect membrane permeability of fish nucleated red blood cells for an important energy molecule, ATP.

Table II-1. Changes in saturated, monounsaturated and polyunsaturated fatty acid compositions of the carp RBCs by the AAPH treatment

(Area %)

	Control	AAPH treated
Total saturated fatty acid	48.1±4.7	61.7±7.4**
Total monounsaturated fatty acid	20.4±3.0	19.2±4.9
Total polyunsaturated fatty acid	31.5±2.6	19.2±5.0**

**The asterisk denotes that there are significant differences ($P<0.01$) between the control and the AAPH-treated RBCs.

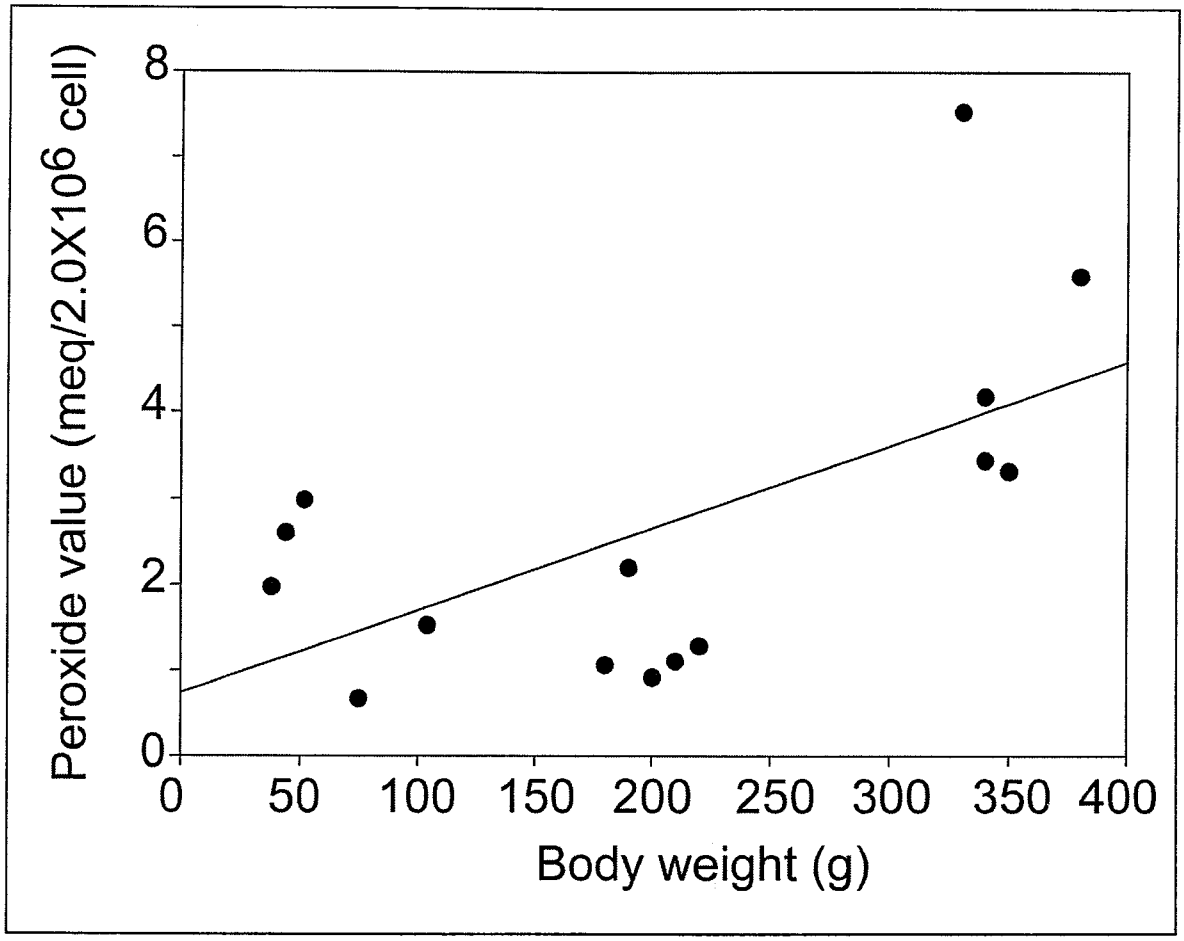

Figure II-1. Peroxide value in RBCs of carp with various body sizes. A high correlation was observed between body size and peroxide value (r=0.61, P<0.01).

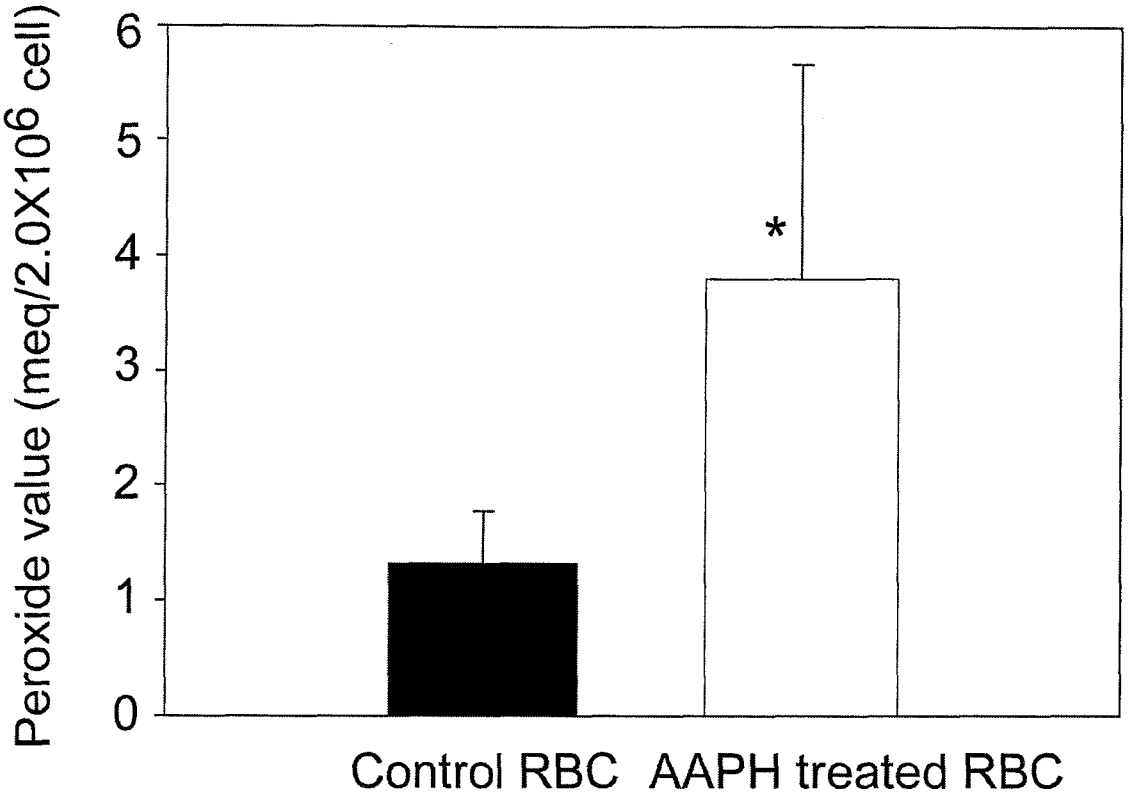

Figure II-2. Peroxide value in the control and the AAPH-treated RBCs. The mean values were represented with SD bars (n=5). The asterisk denotes that there are significant differences ($P<0.01$) between the control (black column) and the AAPH-treated (white column) RBCs.

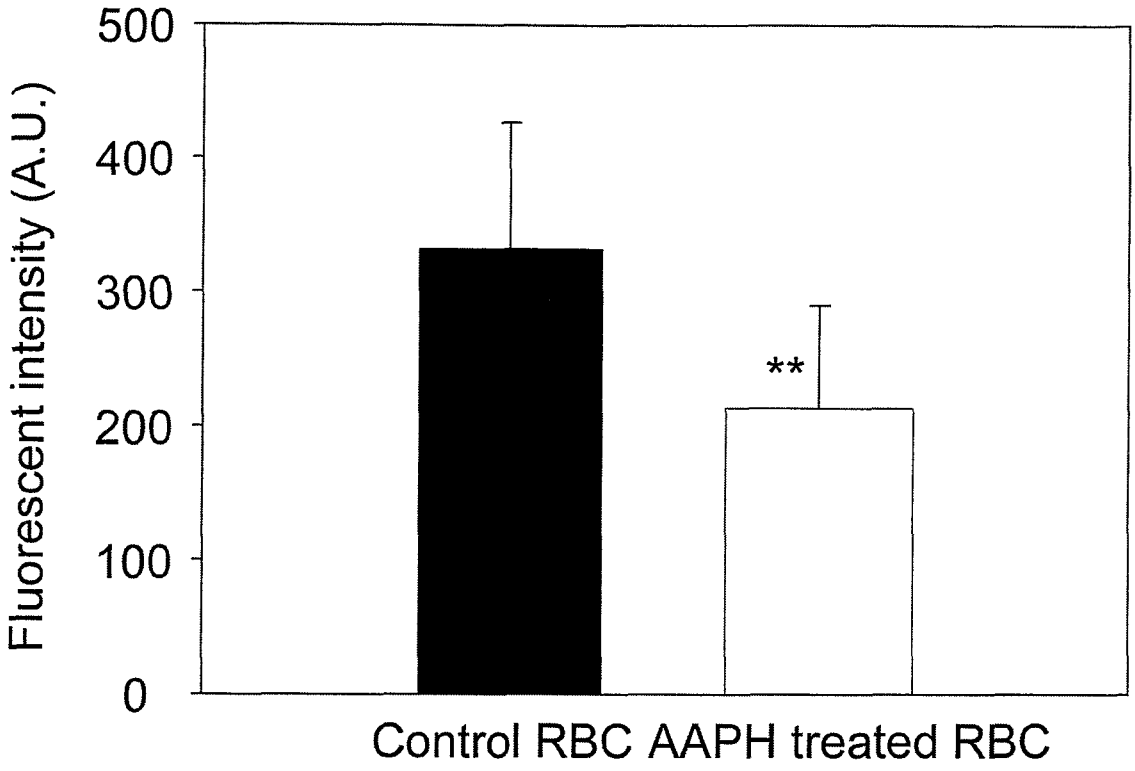

Figure II-3. Effect of mild oxidative stress on calcein holding capacity in carp RBCs. The mean values were represented with SD bars (n=15). The asterisk denotes that there are significant differences ($P<0.01$) between the control (black column) and the AAPH-treated (white column) RBCs.

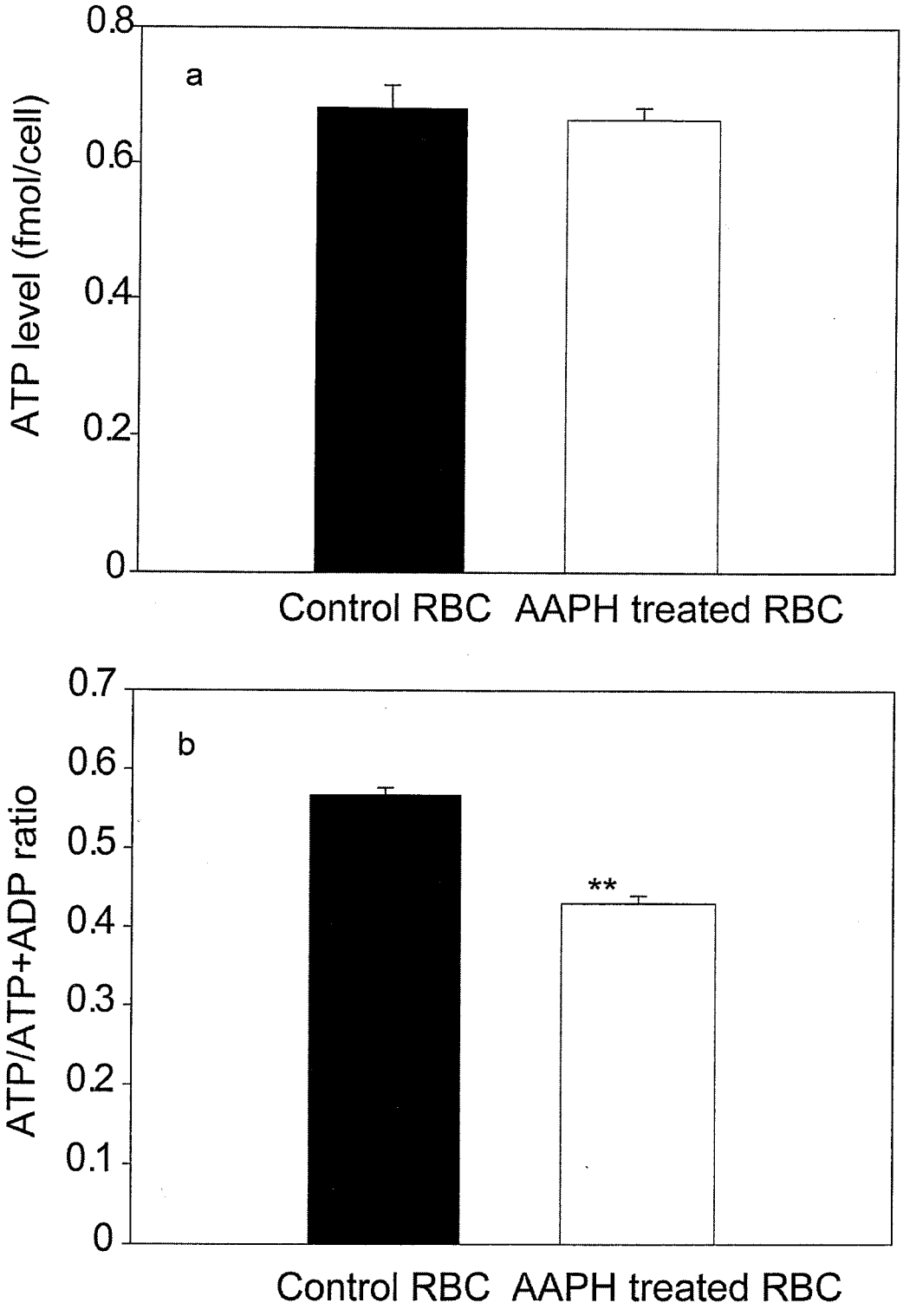

Figure II-4. ATP amount (a) and ATP/ATP+ADP ratio (b) of carp RBC of the control (black column) and the AAPH-treated (white column) RBCs. The mean values were represented with SD bars (n=10). The asterisk denotes that there are significant differences ($P<0.01$).

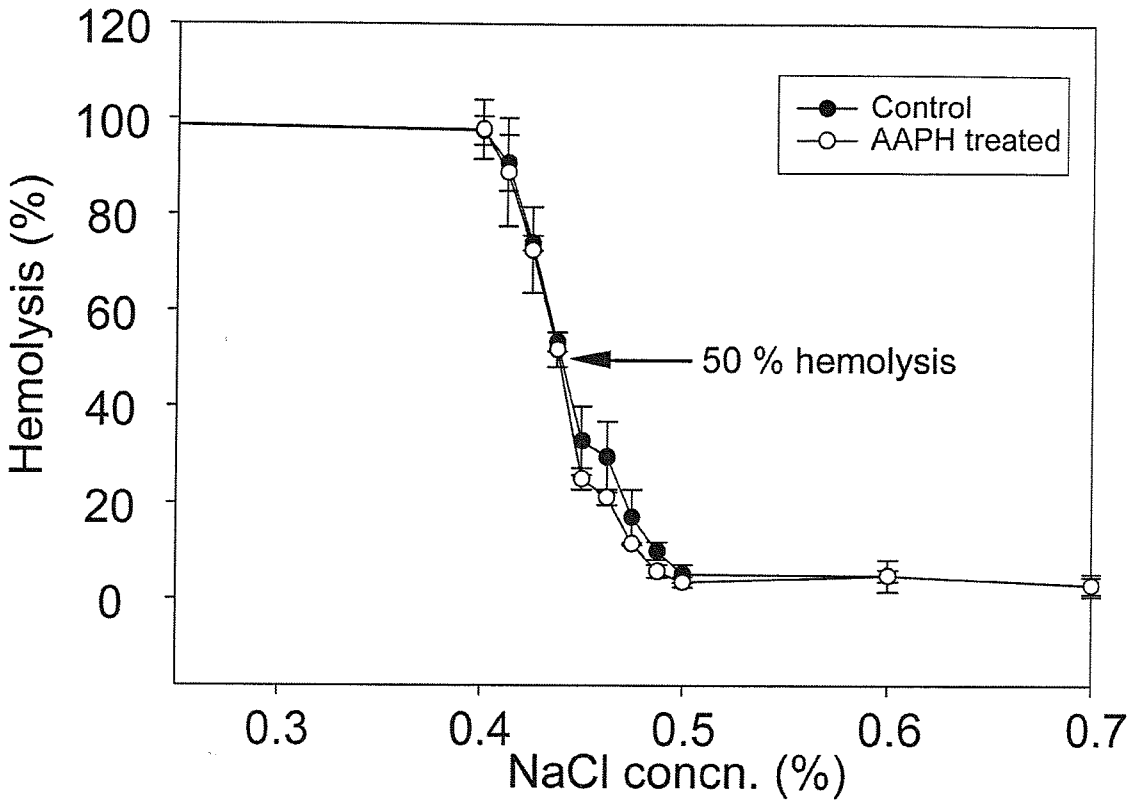

Figure II-5. The hemolysis curves of the control (black circle) and the AAPH-treated (white circle) RBCs No significant difference in hemolysis curves between control and AAPH-treated RBC were observed.

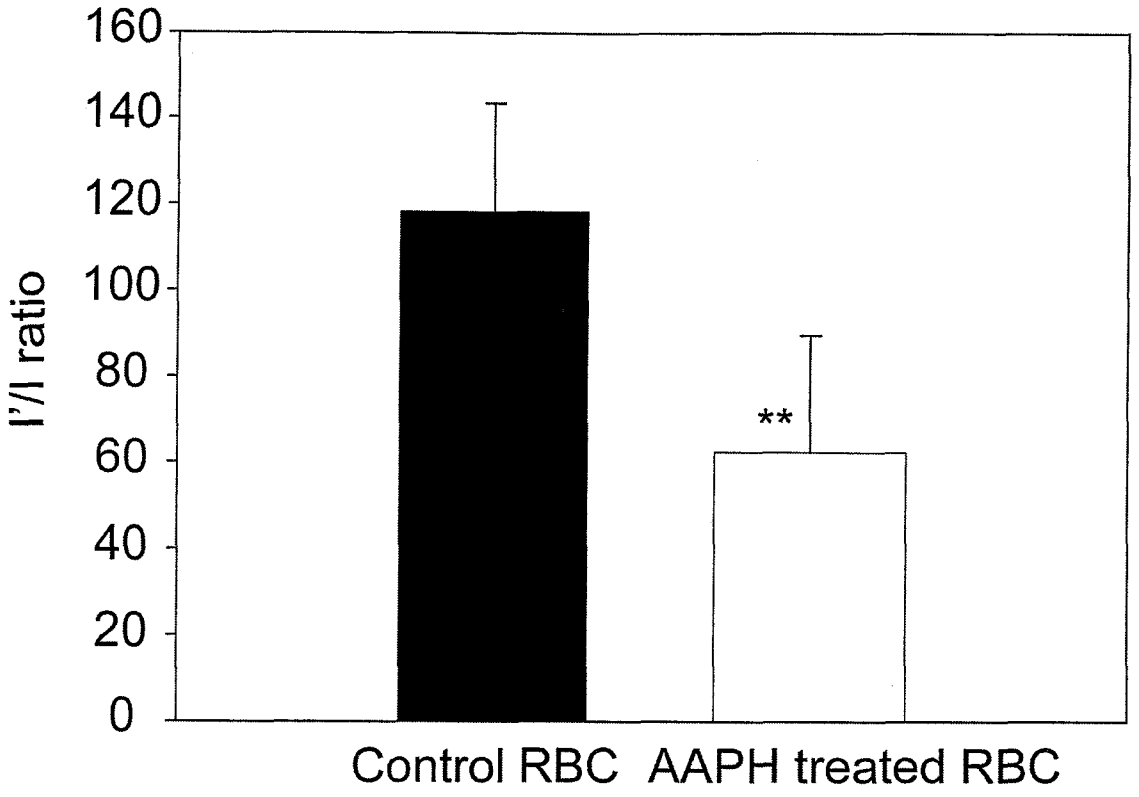

Figure II-6. Excimer (I') to monomer (I) fluorescence intensity ratio, I'/I, of PC_3P in carp RBCs. The fluidity value of the AAPH treated (white column) RBCs and that of control (black column). The mean values were represented with SD bars (n=24). The asterisk denotes that there are significant differences ($P<0.01$).

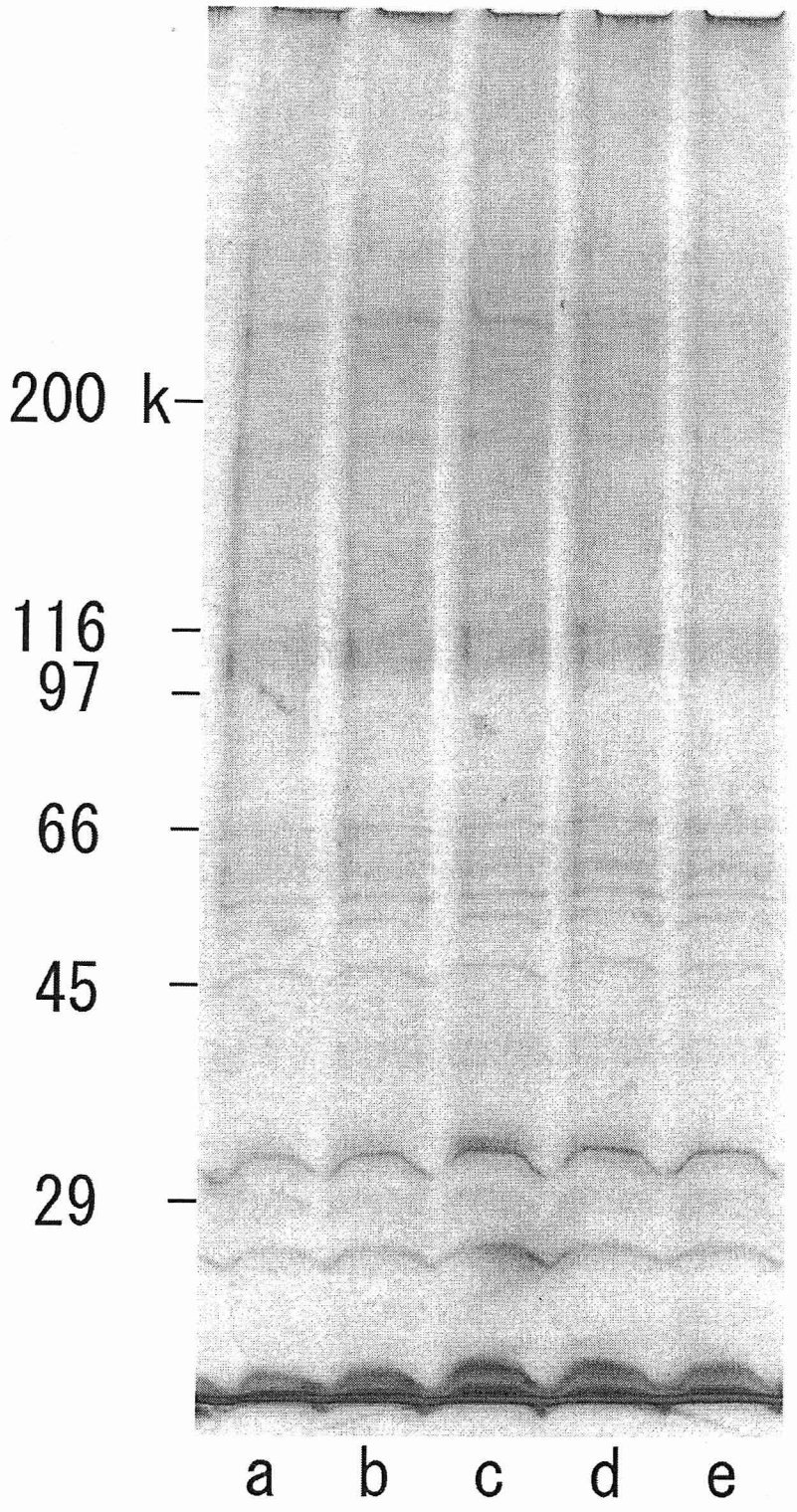

Figure II-7. The protein profiles of RBCs separated on a 2-15% SDS-PAGE gradient gel. Control (a) and AAPH-treated RBCs at final concentration of 1 (b), 100 (c), 200 (d) and 300mM (e).

CHAPTER III

APOPTOSIS IN AYU BRAIN AND LIVER

Oxidative stress elicits a wide variety of cellular events such as apoptosis (Ueda, et al., 1998), cell cycle arrest (Ueno et al., 1999), and induction of antioxidant enzymes (Kim et al., 2001). Several lines of evidence suggest that aging is accompanied by alterations in the apoptotic processes and the DNA repair systems. Shimohama et al. (1998, 2001) reported differential expressions of caspase family proteins during aging in rat brain. Beckman and Ames (1998) proposed one theory that aging is characterized by increasing production of reactive oxygen species (ROS) in somatic tissues. In general, fish have 5-7 years of life span, while the life span of ayu (*Plecoglossus altivelis*) is only one year. Moritomo et al. (2003) have recently demonstrated that ayu produced ROS higher than other species and suggested that high levels of ROS might relate to their short life span. In this chapter, the oxidative DNA damage in ayu brain and liver by measuring the levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG), the most abundant oxidative products of DNA (Takeuchi et al., 1994) was examined. This chapter also surveyed the levels of caspase-9/6 and -3, apoptosis induction factors.

Telomeres are the physical ends of linear chromosomes. In mammals, telomeres are composed of a variable number of tandem repeats of DNA that are made up of (TTAGGG)n repeats (Meyne et al., 1989). Although the bulk of telomeric DNA is double stranded, the extreme terminus is a single-stranded G-rich 3' overhang that serves as a template for elongation and forms a telomeric 'T-loop'. This loop is stabilized by certain telomere-binding proteins, notably TRF1 and TRF2 (Zakian, 1996). The functions of telomeres appear to include protection of chromosomes from illegitimate fusion, the localization of chromosomes in the nucleus and the selective silencing of proximal subtelomeric genes (Greider, 1994). The telomeric repeat sequences are added on by the enzyme telomerase (Greider and Blackburn, 1985; Yu et al., 1990), which present compensates for the loss of DNA from the end of chromosomes due to incomplete replication. In normal human somatic cells, because of inherent limitations in the mechanics of DNA replication, telomeres are shortening at each cell division. In the absence of telomerase, when telomere shortening reaches a critical limit, cells are susceptible to chromosomal aberrations such as end-to-end fusion and aneuploidy. In such a situation, the cells cease to divide and reach replicative senescence. Telomere length in a given cell thus may serve as a marker of its replicative history and of the residual capacity for further cell division.

Although the telomeric sequence was shown to be highly conserved among eukaryotic vertebrates throughout evolution (Meyne et al., 1989), the length of telomeres differs between species. In human, telomeres are up to 20 kb in length (Brown, 1989). In contrast, rodent telomeres have been reported to be heterogeneous in length (Zijlmans et al., 1997). *Mus musculus* has been reported to have telomeres up to 150 kb in size (Prowse and Greider, 1995). *Mus spretus*, however, has telomeres with similar length to humans (up to

30 kb in size) (Zijlmans et al., 1997), whereas rat telomere length ranges from 20 to 100 kb (Golubovskaya et al., 1999; Jennings et al., 1999).

In humans, both *in vivo* and *in vitro*, telomere shortening appears to be a major component of cell senescence and aging (Campisi et al., 1996; Harley, 1997). Telomeres have been reported to shorten during post-natal development and aging in liver (Aikata et al., 2000; Takubo and Kaminishi, 2001), kidney (Melk et al., 2000) and lymphocytes (Benetos et al., 2001). However, this is less apparent in mice because of the very long telomeres (30-150 kb). Telomere shortening has been extensively studied in mice, especially in telomerase-deficient knockout mice (Artandi and DePinho, 2000; Blasco et al., 1999; Herrera et al., 1999). Moreover, it was reported that relationships between kidney telomere shortening and longevity in the rat (Jennings et al., 1999).

In fish, high telomerase activity has been detected in several normal organs of the rainbow trout *Oncorhynchus mykiss* (Klapper et al., 1998). Telomerase activity of the normal organs has been detected in both fry and adult fish, being 10~100-fold higher than that in the human tumor cell line L-428. In contrast, no telomerase activity has been detected in the differentiated organs of mammals. In general, rainbow trout grow continuously throughout their life and, therefore, the high telomerase activity detected in their normal organs is postulated to lead to cell proliferation and organ growth. In previous investigation (Yoda et al., 2002), relative telomerase activity per cell in eyed embryos of rainbow trout was 19.3~50.7-fold higher than in Hela cells (a human cervical carcinoma cell line), which are well known to express a high level of telomerase activity (Morin, 1989). Hence, it was assumpted that aging and consequent death of fish need not to necessarily reach Hayflick's limit in all tissues of an organism.

Ayu have a short life span compared other fish, it therefore seemed important to extend these observations to a wider study of telomere length in ayu. Therefore, another aim of this chapter was to investigate telomere changes during ayu life span in brain and liver in order to reveal whether there is an effect of aging on the rate of telomere shortening.

Section 1

Elevated levels of oxidative DNA damage activate p53 and caspases in brain of ayu with aging

1. INTRODUCTION

Aging is characterized by an increased production of reactive oxygen species (ROS) in somatic tissues (Beckman and Ames, 1998), and it has been demonstrated that oxidants produced endogenously accelerate cell aging and death (Beckman and Ames, 1998; Tatton and Olanow, 1999) and function as signaling factors resulting in activation of transcription factors such as p53 (Ueno et al., 1999). The p53 tumor suppressor protein plays an important role in the cellular response to various stresses in mammals (Levine, 1997) and also cloned from zebrafish (Cheng et al., 1997). The p53-deficiency in zebrafish markedly decreased DNA damage-induced apoptosis elicited by ultraviolet irradiation or by the anti-cancer compound camptothecin (Langheinrich et al., 2002). Thus, p53 may also play a key role in DNA damage-induced apoptosis in fish. Phosphorylation of p53, especially at serine 15, represents an early cellular response to a variety of genotoxic stresses and promotes functional activation of p53 (Shieh et al., 1997). Several lines of evidence suggest that aging is accompanied by alterations in the apoptotic processes and the DNA repair systems. Shimohama et al. (1998, 2001) reported differential expressions of caspase family proteins during aging in rat brain. In this section, we surveyd that the oxidative DNA damage by measuring 8-OHdG, the levels of caspase-9/6 and -3, apoptosis induction factors and telomere length in ayu brain. This section also examined the levels of p53 and its phosphorylation form in ayu brain, which may play a key role in DNA damage-induced apoptosis.

2. MATERIALS AND METHODS

2.1. Fish

About 50 individuals of healthy ayu (*Plecoglossus altivelis*) were obtained from the Tochigi Prefectural Fisheries Experimental Station, Japan. Fish were stocked in outdoor ponds (14.8 m^2, 0.9 m in depth), where water was supplied from a natural stream. The fish were fed with commercial pellets for ayu (Oriental Yeast Co., Ltd., Tokyo, Japan). From July to September, ayu were used for 8-OHdG, p53, caspase and telomere length assays without determination of sex. We determined sex of fish and divided fish into two groups from October to December for p53 and caspase assays. Whole brain was dissected out and used for subsequent assay.

2.2. Measurement of 8-OHdG in brain

DNA samples of brain were obtained using Genelute Mammalian Genomic DNA kit (SIGMA, USA). After complete digestion of DNA with nuclease P1 (Calbiochem, USA) and alkaline phosphatase (Nippon Gene, Japan), 8-OHdG levels were determined using a competitive ELISA kit (High Sensitive 8-OHdG Check; Japan Institute for the Control of Aging, Shizuoka, Japan).

2.3. p53 and phosphorylated p53 analyses

For protein analyses, brain homogenates were prepared with phosphate-buffered saline (PBS, 10 % w/v) containing 0.2 % (v/v) PMSF. Protein concentration was estimated by using BCA Protein Assay Kit (Pierce, USA). Samples were separated on a 10-20 % polyacrylamide gradient gel in the presence of SDS (Laemmli, 1970). Proteins separated in gels were transferred to polyvinylidenedifluoride (PVDF) membrane (Immobilon-P^{SQ} Transfer Membrane, Millipore, USA) with semi-dry electroblotter. The blots were blocked in Tris-buffered saline (TBS) containing 5 % skimmed milk (w/v) separately and incubated for one hour with mouse anti-p53 antibody (Genzyme Techne, USA) for determination of the total p53 level, or alternatively rabbit anti-p53 antibody, phospho-specific (Ser^{15}) (Ab-3) (Oncogene, USA) for determination of the phosphorylated form. The region around the phosphorylation site of Ser15 is highly conserved from fish to human (Cachot et al., 1998). These antibodies successfully recognized one band around 50 k in molecular weight. After washing, the membrane was incubated with peroxidase-conjugated goat anti-rabbit IgG (H+L) (Pierce, USA) for the total p53, or alternatively peroxidase-conjugated rabbit anti-mouse IgG+A+M (H+L) (Zymed Laboratories Inc. USA) for the phosphorylated form. Detection with a FAST DAB with metal kit (SIGMA, USA) was performed according to the manufacturer's instructions. The membranes were digitally scanned and the signal densities were quantified using ImageJ (National Institutes of Health, USA).

2.4. Assay for caspase activities

For caspase assay, brain was dissected immediately after decapitation, washed once with cold PBS (400 × *g* for 5 min), and frozen at -80 °C. The brains were separately homogenized in 50 µl of chilled Cell Lysis Buffer. After incubation on ice for 10 min, samples were centrifuged at 36,000 × *g* for 10 min at 4 °C. Supernatants were incubated with an appropriate caspase substrate at 37 °C for 1 hour in a water bath. Substrates of caspase-9/6 and caspase-3 were used LEHD-AMC (250 µM in final concentration) and DEVD-AFC (50 µM in final concentration), respectively (BD ApoAlert Caspase

Fluorescent Assay kits; BD biosciences, Japan). Fluorescent intensities were used for quantification of protease activities for caspase-9/6 and caspase-3 as the amount of liberated AFC /g brain and liberated AMC /g brain, respectively, according to the manufacturer's instructions.

2.5. DNA isolation and Southern blot analysis

The DNA samples of whole brain were obtained using Genelute Mammalian Genomic DNA kit (SIGMA, USA). Equal amounts of DNA (10 μg) were digested by the restriction enzyme *Hinf*I (TOYOBO, Tokyo, Japan). Samples and λ*Hind*III used as a maker were loaded on a 0.8 % (w/v) agarose gel. The gels were run by 25 V for 16 hours to separate long telomeric DNAs (Kipling and Cooke, 1990; Norwood and Dimitrov, 1998; Kozik et al., 1998). Separated DNA was then transferred to nylon membranes (Hybond +; Amersham Biosciences, Tokyo, Japan) by a standard Southern blotting procedure. DNA was cross-linked to the membrane with 1200μJ of ultraviolet light.

2.6. Hybridization

The telomere-specific oligonucleotide $(TTAGGG)_4$ (Invitrogen, Japan) was end-labeled 37 °C for 15 min using terminal deoxynucleotidyl transferase from DIG Oligonucleotide 3'-End Labeling Kit (Roche, Mannheim, Germany). The blotted nylon membrane were prehybridized in 40 ml of DIG Easy Hyb (Roche) for 30 min at 62 °C, and then were hybridized in 20 ml of DIG Easy Hyb containing 10 pM of end-labeled, telomere-specific probe for 16 hour at 62 °C. Membranes were washed 2 times with 50 ml of 2 × standard saline citrate (SSC; SSC: 1 × SSC: 0.15 M NaCl plus 0.015 M sodium citarate) containing 0.1 % SDS solution for 5 min at 20 °C, and washed 2 times for 15 min at 62 °C. Chemiluminescence was performed using the DIG Luminescent Detection Kit (Roche) and used CSPD. The signals exposed to X-ray film (RX-U; Fuji Photo Film, Minamiashigara, Japan) and the mean terminal restriction fragment (TRF) length was quantified by using Densitograph (ATTO).

2.7. Statistical analyses

Data obtained were analyzed using one-way ANOVA.

3. RESULTS

3.1. Oxidative DNA formations in the brain of ayu

Oxidative stress was evaluated by the formation of 8-OHdG in the DNA of ayu throughout their life span. As shown in Figure III-1-1, the levels of 8-OHdG increased to maximal amount of 53.6±10.9 ng/μg DNA at October significantly higher than other levels ($p<0.01$).

3.2. Phosphorylation of p53 in brain of ayu

Western blots revealed increasing phosphorylation of p53 at Ser 15. Figure III-1-2 shows the phosphorylation ratio in brain of ayu. The ratios were gradually increased for both sexes and reached maximal levels in October, 2.6±0.1 for female and 2.6±0.2 for male, which were significantly higher than those in July, August and November ($p<0.05$). No significant difference was observed between male and female on each month.

3.3. Caspase-9/6 and -3 activities in brain of ayu

The activity of every caspase increased in October. Figure III-1-3 shows the activities of caspase-9/6 in brain. There were significant differences between male and female in October ($p<0.01$). The caspase-9/6 activity of female ayu ($1.3\pm0.2 \times10^{-3}$ caspase activity; relative fluorescence per g brain) and brain male ayu in October ($0.9\pm0.1 \times10^{-3}$ caspase activity; relative fluorescence per g brain) was significantly higher than other levels ($p<0.01$). There were significant differences between male and female in October ($p<0.01$)

As shown in Figure III-1-4, caspase-3 activity of female ayu in October (1.2±0.1 caspase activity; relative fluorescence per g brain) was significantly higher than other levels ($p<0.01$). In male, caspase-3 activity in October (0.8±0.01 caspase activity; relative fluorescence per g brain) was significantly higher than that in August, September and November ($p<0.01$). There were significant differences between male and female in October ($p<0.01$).

3.4. Telomere length in brain of ayu

Telomere length changes with age in brain of ayu were analyzed (Figure III-1-5). The terminal restriction fragment (TRF) length of DNA from normal somatic cells from young adults is typically in the 8-10 kbp range in adults. Generally, 8 or less kbp are called telomere shortening (de Lange et al., 1990; Harley et al., 1990; Hastie et al., 1990; Vaziri et

al., 1993). Although, there were individual differences in TRF length, no age-related telomere shortening was detected in the brain of ayu.

4. DISCUSSION

The results of the present study provide the first detailed description that elevated levels of oxidative DNA damage in ayu brain with age. It was also indicated that oxidative stresses induce activation p53 through the phosphorylation, and p53 induces apoptosis accompanied with caspase-9/6 and -3 activation. These observations suggest that the age-related of apoptosis might be involved in increasing of DNA damage and mutations in brain with age, and could partially explain the short life span of ayu. Besides, these findings indicate that telomere did not shorten in ayu brain in an age-dependent manner. These data also provide a novel mechanism for the age-related differences in life span and suggest a teleost specific regulation of telomere length during life span.

DNA damage cannot be tolerated in mammals if left un-repaired. Therefore, cells have developed many defense systems to prevent DNA damage. One major repair mechanism for DNA damage, including 8-OHdG, is the base excision repair pathway (Frosina, 2000; Lindahl and Wood, 1999). Under normal physiological conditions, the ROS generated by the respiratory chain can be scavenged by enzymatic and nonenzymatic antioxidant systems to prevent deleterious oxidative damage to the cell. However, as a result of aging-associated increase of ROS generation in the respiratory chain, the accompanied decreases in the intracellular concentrations of antioxidants and in activities of free radical scavenging enzymes, an elevation of ROS and oxidative stress is inevitable and mortal for cells (Ames et al., 1993). Increased levels of 8-OHdG in DNA of ayu brain were observed with aging in the present study, suggesting that brain DNA damage induced by oxidative stress and ROS would increase with ayu aging . The present study also supposes that DNA damage triggered by oxidative stress would be associated with the activation of p53. Oxidative stress stimuli and DNA-damaging agents stabilize p53, which promotes cell-cycle arrest to enable DNA repair or apoptosis to eliminate defective cells (Levine, 1997). It has been shown that phosphorylation plays important roles for regulation of biological p53 activation (Giaccia and Kastan, 1998; Prives, 1998). The mechanism for p53-induced apoptosis is involved in activation of mitochondrial Apaf-1/caspase-9 pathway (Soengas et al., 1999), death receptor signaling, (Bennett et al., 1998; Munsch et al., 2000; Müller et al., 1997), and cleavage of downstream caspases (Li et al., 1999). An additional route by which p53 may signal apoptosis is through the production of ROS (Johnson et al., 1996; Li et al., 1999). p53 itself was also shown to cause caspase activation through a mechanism independent on transcription or presence of Bax or cytochrome c

(Ding et al., 1998). Thus, it is likely that p53 can be also a transducer for apoptotic signals through modulating p53-dependent caspase in fish. Johnson et al. (1999) reported that p53 is required for caspase activation in response to some forms of neuronal injury, and indicated that caspase activation in response to DNA damage was dependent on the presence of a functional p53 gene. The activation of caspases during neuronal development appears to be essential for regulating the number of neurons surviving in the postdevelopmental brain. The failure to eliminate dying cells efficiently during development has been recently reported for mice deficient in either caspase-3 (Kuida et al., 1996) or caspase-9 (Hakem et al., 1998; Kuida et al., 1998). Also in fish, activation of caspases may have important roles for the apoptosis in a brain. Until recently, cell death had been thought to be absent from the brain of fish in stages beyond embryogenesis (de Caprona and Fritzsch, 1983; Fine, 1989; Fox and Richardson, 1982; Galeo et al., 1987; Waxman and Anderson, 1985). However, such an interpretation was in conflict with the apparent fate of the newborn cells after they have reached the cerebellum of *Apteronotus leptorhynchus* (Zupanc, 1999). The persistence of mitotic activity of secondary matrix cells during adult life has been also suggested in the trout (Pouwels, 1978a, b). The present result for ayu would support the previous researches that apoptosis occurred also in adult fish of the brain and was used as an efficient mechanism for the removal of cell damaged through injury in the adult fish brain.

Indeterminate growth of fish and the very slow occurrence of senescence were accompanied by high telomerase activities in all investigated fish tissues (Klapper et al., 1998). It seems possible that prevention of telomere erosion can prevent senescence not only at the cellular level but also in adult animals. However, there was no direct proof that avoidance of senescence in fish is caused by switching off the mitotic clock as a consequence of the high telomerase activity. Since the mechanism of senescence is not known, we have to consider the possibility of mechanisms of senescence in fish. Some fish species show the parental death shortly after first spawning like ayu, these species may represent an accelerated form of aging. Changes in the aging brain observed in the present study can be regarded as an age-related change, senescence. The mechanisms of senescence of fish need further investigation, which will influence on the direction of the entire study of senescence in fish.

ABSTRACT

It is well known that ayu (*Plecoglossus altivelis*) die after spawning and the life span is only one year. It is one of the causes that enhanced oxidative stress might induce DNA damage and subsequent DNA repair systems as phosphorylated p53 in ayu, this might be involved with apoptosis relating to their short life span. Telomeres, the non-coding sequences at the ends of chromosomes, shortening of telomeres can induce cell cycle arrest and apoptosis. This chapter, then, surveyed the p53 and its phosphorylation, the oxidative DNA damage by measuring the levels of 8-OHdG and the induction apoptosis by measuring the levels of caspase-9/6, -3 with aging in brain. Besides, it was investigated that age related changes in telomere length in the ayu. The findings indicate oxidative stress activates caspase-9/6, -3 activation, and activates p53 through the phosphorylation of p53 and p53 with aging in ayu brain. There was no significantly change in telomere length in brain. It was indicated that telomere did not shorten in ayu brain in an age-dependent manner. This chapter first reported that oxidative stress specifically induces the phosphorylation of p53 (Ser 15) with aging in fish brain.

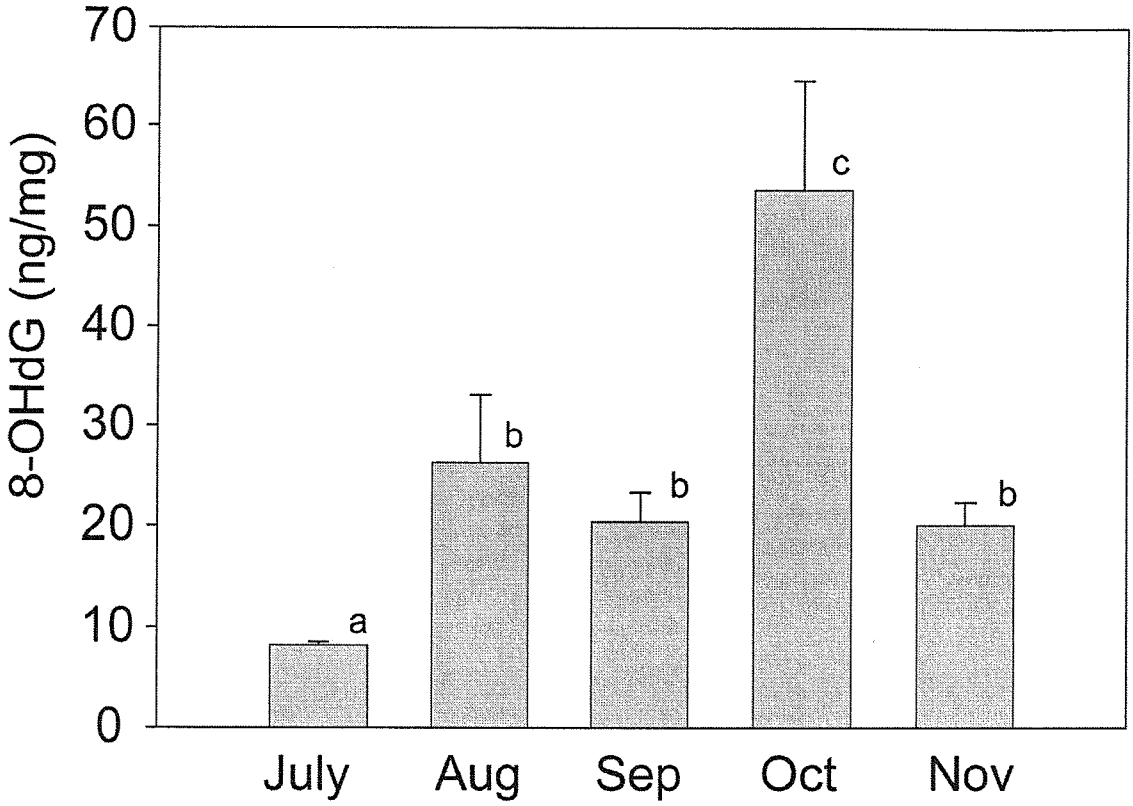

Figure III-1-1. The levels of 8-OHdG in ayu brain. The mean values were represented with bars of standard deviations (n=5). The different characters represent significant differences ($P<0.01$).

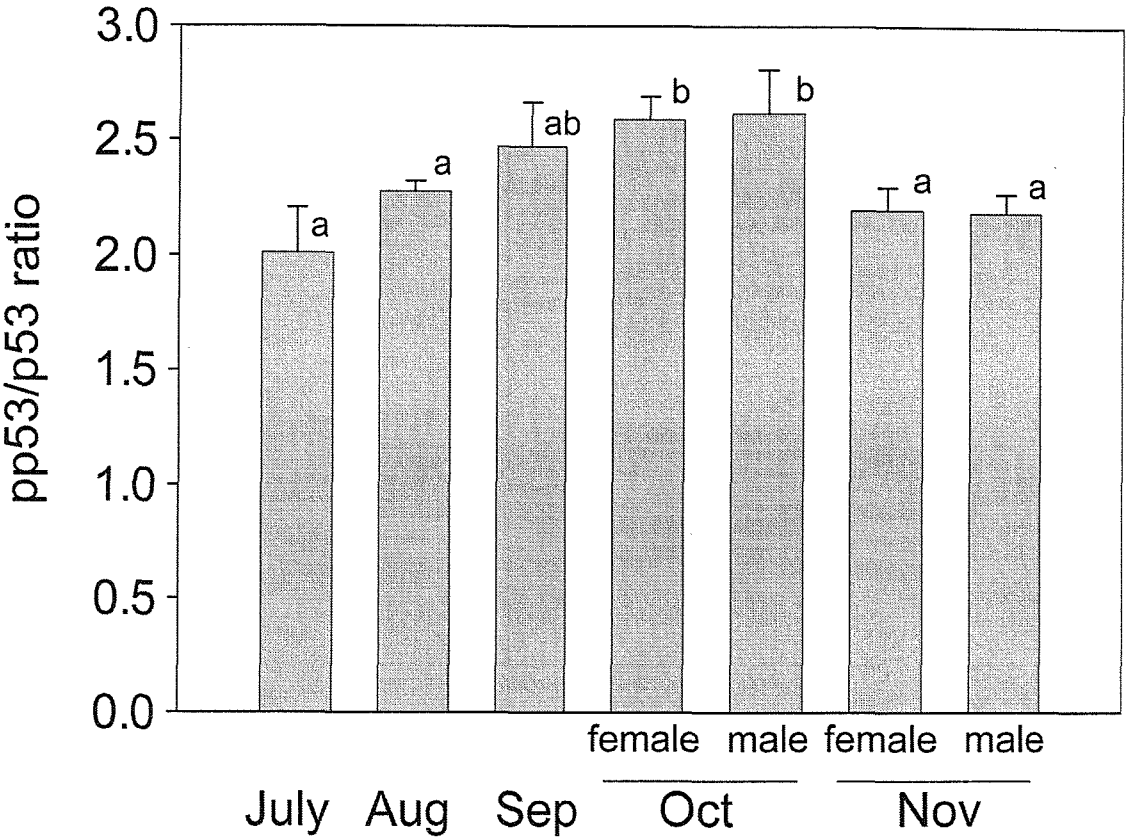

Figure III-1-2. The phosphorylation ratio of p53 at serine 15 in brain of ayu. The mean values were represented with bars of standard deviations (n=3). The different characters represent significant differences ($P<0.05$).

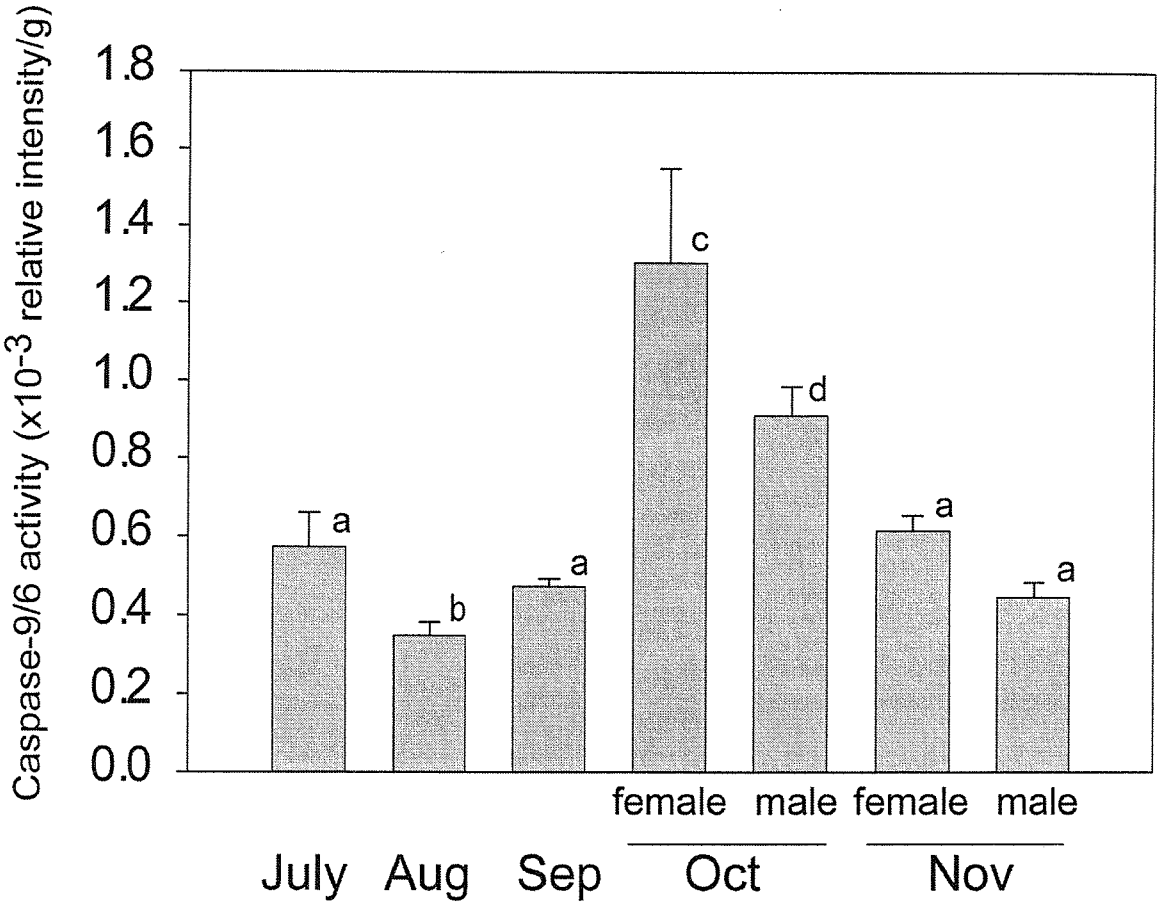

Figure III-1-3. The caspase-9/6 activities in brain of ayu. The mean values were represented with bars of standard deviations (n=3). The different characters represent significant differences ($P<0.01$).

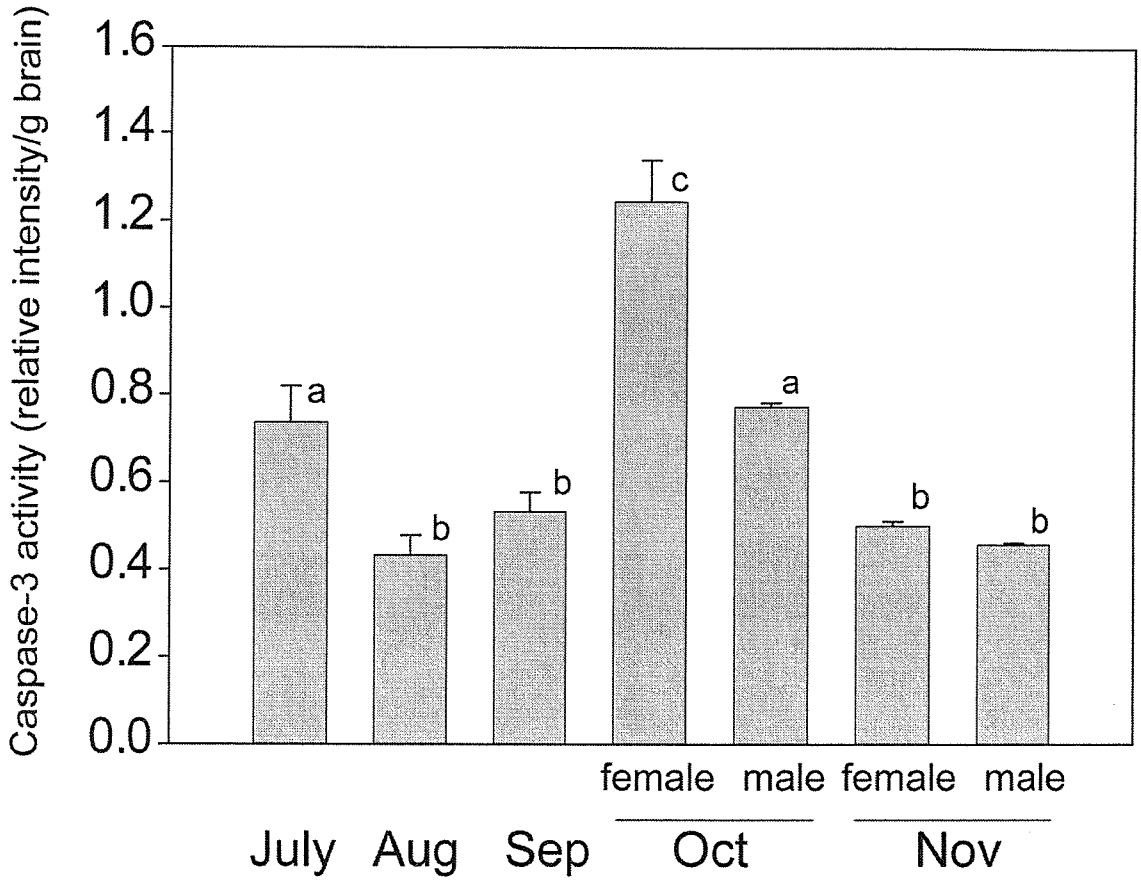

Figure III-1-4. The caspase-3 activities in brain of ayu. The mean values were represented with bars of standard deviations (n=3). The different characters represent significant differences ($P<0.01$).

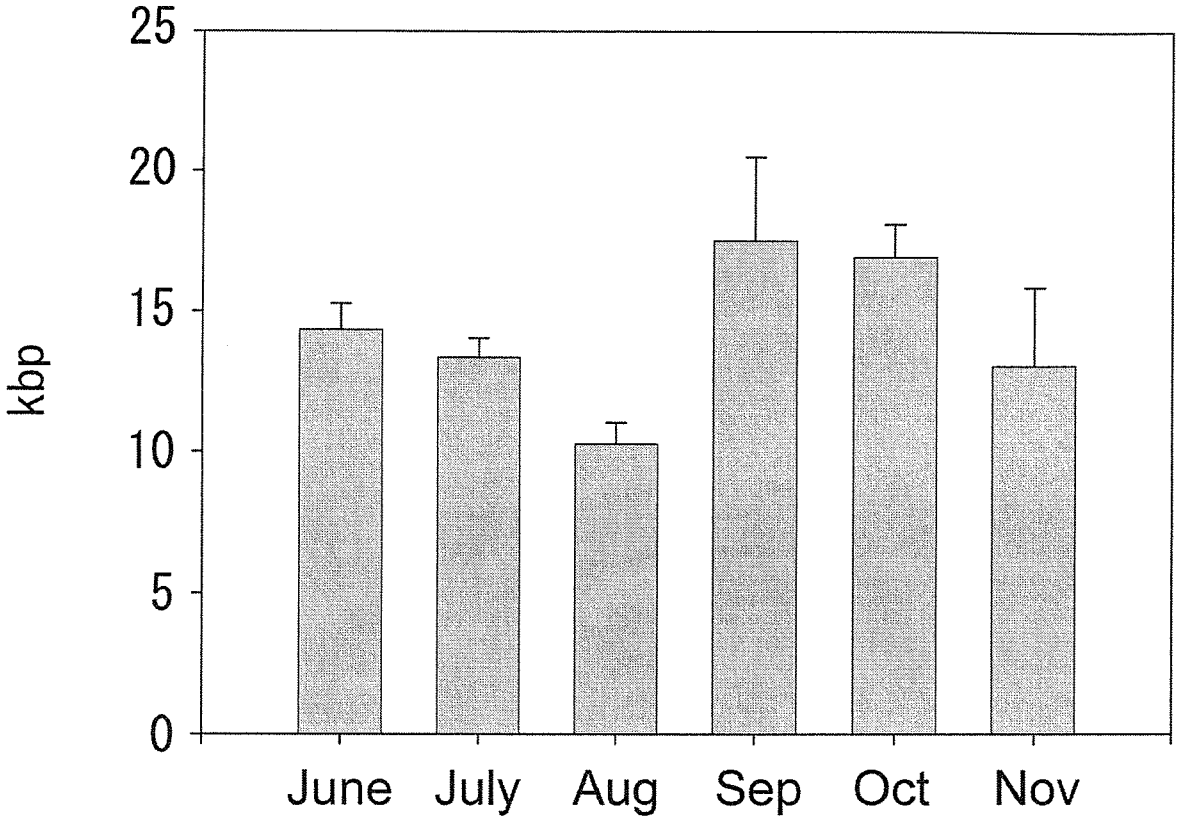

Figure III-1-5. Telomere length changes with age in brain of ayu. The mean values were represented with bars of standard deviations (n=3). No age-related telomere shortening was detected in the brain of ayu.

Section 2

Enhanced oxidative damages and apoptosis in aging ayu liver

1. INTRODUCTION

Aging is characterized by an increased production of reactive oxygen species (ROS) in somatic tissues (Beckman and Ames, 1998), and an increase in the production of ROS may promote the induction of apoptosis. Recently, it was found that splenocytes and thymocytes undergo apoptosis with aging in rats and the apoptosis was associated with enhanced expression of p53, Bax, and caspase-3 (Kapasi and Singhal, 1999). It was also demonstrated that aging attenuates apoptosis in the colonic mucosa of Fischer 344 rats (Xiao et al., 2001). However, the biological significance of the alteration of oxidants and these enzymes involved in the execution and regulation of apoptosis during aging remains to be defined.

The liver is an important metabolic organ, and is susceptible to a wide variety of disorders, possibly because it is constantly exposed to potentially harmful agents. It is generally recognized that oxidative end-products accumulate with age and therefore free radical-mediated damage to liver cells occurs. Thus, the liver was selected as a model organ for this study in recognition of the significant aging. It was shown that the livers of old rats are resistant to a moderate dose of genotoxic stress compared with the younger rat (Suh et al., 2002). However, no studies have demonstrated that oxidative stress relates to apoptosis during aging in ayu liver. In this section, we examined the oxidative DNA damage in liver of ayu by measuring the levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG), the most abundant oxidative products of DNA (Takeuchi et al., 1994). We also surveyed the levels of caspase-9/6 and -3, apoptosis induction factors and telomere length in liver.

2. MATERIAL AND METHOD

2.1. Fish

About 100 individuals of healthy ayu (*Plecoglossus altivelis*) were obtained from the Tochigi Prefectural Fisheries Experimental Station, Japan. Until used in experiments, fish were stocked in outdoor ponds (14.8 m^2, 0.9 m in depth), where water was supplied from a natural stream. The fish were fed with commercial pellets for ayu (Oriental Yeast Co., Ltd., Tokyo, Japan) by an automatic feeding machine four times per day. From June to September ayu were used for 8-OHdG, caspase and trlomere length assays without determination of sex. We determined sex of fish and divided fish into two groups for the following assays from October to December. Whole liver was dissected out and used for subsequent assay.

2.2. Measurement of 8-OHdG in liver

DNA samples of liver were obtained using Genelute Mammalian Genomic DNA kit (SIGMA, USA). Samples were digested to deoxyribonucleotide levels by treatment with nuclease P1 (Calbiochem, USA) and alkaline phosphatase (Nippon Gene, Japan). After appropriate dilution of the DNA, 8-OHdG levels were determined using a competitive ELISA kit (High Sensitive 8-OHdG Check; Japan Institute for the Control of Aging, Shizuoka, Japan).

2.3. Assay for caspase activity

Caspase activity was measured using fluorescent peptide substrates (BD ApoAlert Caspase Fluorescent Assay kits; BD biosciences, Japan). Briefly, liver was dissected immediately after decapitation, washed once with cold PBS (400 × *g* for 5 min), and frozen at -80 °C. The livers were separately homogenized in 50 μl of chilled Cell Lysis Buffer. After incubation on ice for 10 min, samples were centrifuged at 36,000 × *g* for 10 min at 4 °C. Supernatants were incubated with an appropriate caspase substrate at 37 °C for 1 hour in a water bath. Substrates of caspase-9/6 and caspase-3 were used LEHD-AMC (250 μM in final concentration) and DEVD-AFC (50 μM in final concentration), respectively. Fluorescent intensity at 460 nm was measured in a spectrofluorophotometer (Shimadzu RF-1500) with 380 nm of excitation for caspase-9/6. The excitation and emission wave lengths of 400 and 505 nm, respectively, were used for determination of caspase-3 activity. Quantification of protease activity for caspase -9/6 and caspase-3 were calculated as the amount of liberated AMC/g liver and liberated AFC/g liver, respectively.

2.4. Telomere length mesurement

Southern blotting to measure telomere length was performed for *Hinf*I-digested genomic DNA by using DIG Oligonucleotide 3'-end labeled $(TTAGGG)_4$ probe as described previously (Chapter III, Section 1).

2.5. Statistical analyses

Data obtained were analyzed using one-way ANOVA.

3. RESULTS

3.1. Oxidative DNA formations in the liver of ayu

Oxidative stress was evaluated by the formation of 8-OHdG in the DNA of ayu throughout their life span. As shown in Figure III-2-1, the levels of 8-OHdG gradually increased and the levels of September (35.8±0.4 ng/μg DNA) and October (36.6±0.4 ng/μg DNA) were higher than other levels ($p<0.01$).

3.2. Caspase activity in liver

Figure III-2-2 shows the activities of caspase-9/6 in liver. The caspase-9/6 activity of female ayu (31.0±2.1 $\times 10^{-3}$ caspase activity; relative fluorescence per g liver) and male ayu in November (30.9±1.7 $\times 10^{-3}$ caspase activity; relative fluorescence per g liver) was significantly higher than other levels ($p<0.01$). There were no significant differences between male and female.

As shown in Figure III-2-3, caspase-3 activity of female ayu in November (21.7±0.3 caspase activity; relative fluorescence per g liver) was significantly higher than other levels ($p<0.01$). In male, caspase-3 activity in October (19.1±0.2 caspase activity; relative fluorescence per g liver) was significantly higher than other levels ($p<0.01$) except female in October. There were significant differences between male and female in October and November ($p<0.01$).

3.3 Telomere length in liver of ayu

Telomere length changes with age in liver of ayu were analyzed (Figure III-2-4). No age-related telomere shortening was detected in the liver of ayu. The terminal restriction fragment (TRF) length of DNA from normal somatic cells from young adults is typically in the 8-10 kbp range in adults. Generally, 8 or less kbp are called telomere shortening (de Lange et al., 1990; Harley et al., 1990; Hastie et al., 1990; Vaziri et al., 1993). Although, there are individual differences in TRF length, no age-related telomere shortening was detected in the liver of ayu.

4. DISCUSSION

This observation suggested that the age-related of apoptosis might be involved in increasing of DNA damage and mutations in liver, and could partially explain the short life

span of ayu. Besides, it was indicated that telomere did not shorten in ayu liver in an age-dependent manner. These data also provide a novel mechanism for the age-related differences in life span and suggest a teleost specific regulation of telomere length during life span.

Present results gave us an opportunity to reveal the correlative change in oxidative stress, proliferation, apoptosis, DNA damage and its repair, which occur in the ayu liver during their life span. DNA damage cannot be tolerated in mammals if left un-repaired. Therefore, cells have developed many defense systems to prevent DNA damage. One major repair mechanism for DNA damage, including 8-OHdG, is the base excision repair pathway (Frosina, 2000; Lindahl and Wood, 1999). Under the normal physiological conditions, the ROS generated by respiratory chain can be scavenged by enzymatic and non-enzymatic antioxidant systems to prevent deleterious oxidative damage to the cell. However, as a result of aging-associated increase of ROS generation in the respiratory chain and decrease in the intracellular concentrations of antioxidants and activities of free radical scavenging enzymes, an elevation of ROS and oxidative stress is inevitable, and oxidative damage and apoptosis might just occur in the cell (Ames et al., 1993). ROS appears to react preferentially with one of the primary products of its own oxidation with guanine, 8-OHdG. Therefore, the increasing 8-OHdG is one of the indices for accumulating ROS as a DNA damaging agent. Migliore and Coppede (2002) demonstrated that increases in ROS-induced DNA damage were correlated with cell cycle arrest. Cumulative DNA damage caused by endogeneous free radicals has been suggested to underline cancer and other age-related disorders, including neurodegeneration (Migliore and Coppede, 2002; Turker, 2000). Bogdanov et al. (1999) provided further strong evidence that oxidative DNA damage accompanies normal aging. Increased levels of 8-OHdG in DNA of ayu liver were observed with aging in the present study, suggesting that liver DNA damage induced by oxidative stress and ROS would increase with aging of ayu. Recently, the longer maintenance of high levels of 8-OHdG in liver DNA is explained by the exhaustion and/or disturbance of the DNA repair system by the administration of carcinogens (Nakae et al., 1997). It is thus conceivable that early increment of 8-OHdG in the liver, induced by oxidative stress, might influence the apoptosis potential of initiated cells with already disrupted DNA repair producing stronger damage to DNA and promoting apoptosis.

However, the observation that oxidative DNA damage is mostly observed in the apoptotic cells could also mean that the oxidative degradation of DNA is the consequence rather the cause of apoptosis. This examination for apoptosis indicated that the induction of caspases might be related oxidative DNA damage, which occurred in liver cells with aging ayu. However, it remains to be answered as to whether these results are tissue specific and characteristic of tissues. Whether the alterations in the sensitivity to pro-apoptotic conditions observed during aging are part of the consequences of aging, and whether

apoptosis in liver of ayu per se participates in the normal aging process warrant further investigation.

ABSTRACT

It is well known that ayu (*Plecoglossus altivelis*) die after spawning and the life span is only one year. It is one of the causes that enhanced oxidative stress might induce DNA damage in ayu, this might be involved with apoptosis relating to their short life span. Telomeres, the non-coding sequences at the ends of chromosomes, shortening of telomeres can induce cell cycle arrest and apoptosis. This section, then, surveyed the oxidative DNA damage by measuring the levels of 8-OHdG and the induction apoptosis by measuring the levels of caspase-9/6, -3 with aging in liver. Besides, it was investigated that age related changes in telomere length in the ayu liver. Our findings indicate oxidative stress activates caspase-9/6, -3 activation, and accumulation 8-OHdG in ayu liver with aging. There was no significantly change in telomere length in liver. This observation suggested that the age-related of apoptosis might be involved in increasing of DNA damage and mutations in liver, and could partially explain the short life span of ayu. Moreover, it was indicated that telomere did not shorten in ayu liver in an age-dependent manner. The present results gave me an opportunity to reveal the correlative change in oxidative stress, proliferation, apoptosis, DNA damage and its repair, which occur in the ayu liver during their life span.

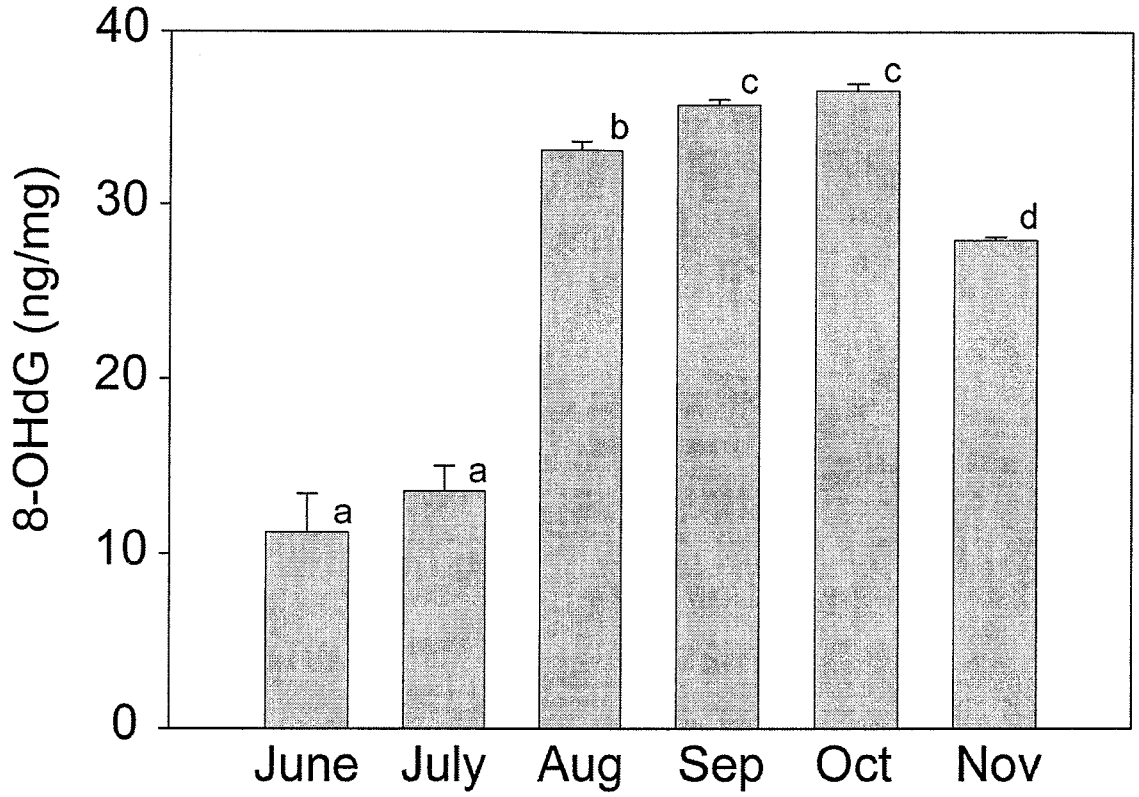

Figure III-2-1. The levels of 8-OHdG in ayu liver. The mean values were represented with bars of standard deviations (n=5). The different characters represent significant differences ($P<0.01$).

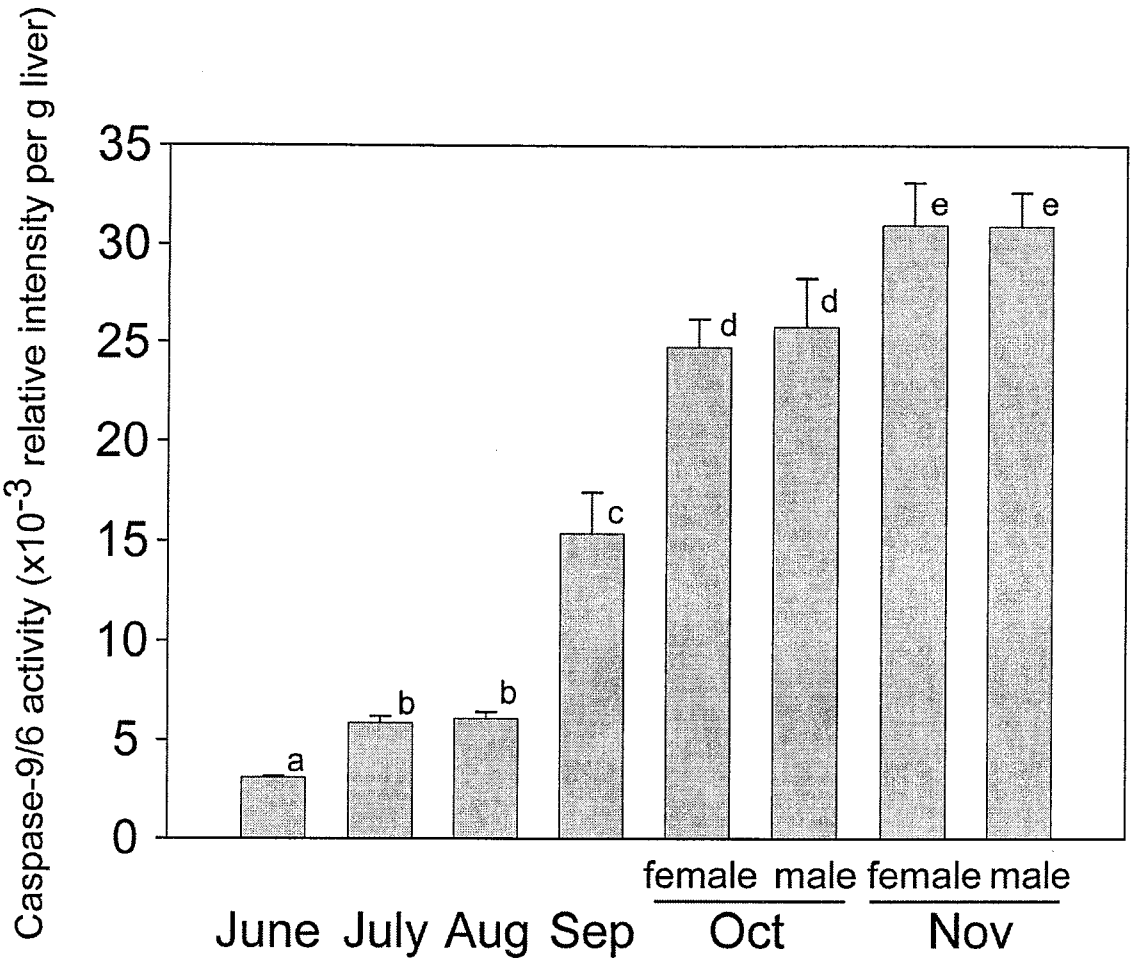

Figure III-2-2. The caspase-9/6 activities in liver of ayu. The mean values were represented with bars of standard deviations (n=3). The different characters represent significant differences ($P<0.01$).

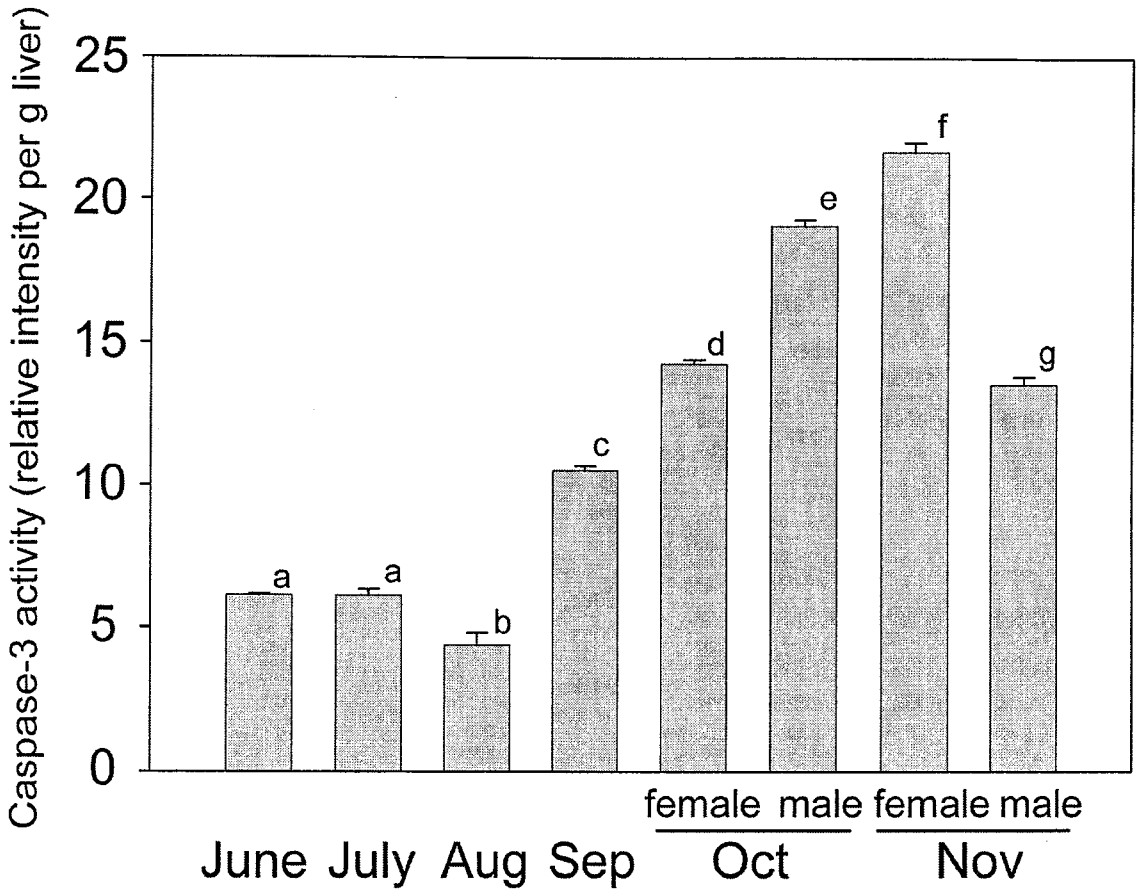

Figure III-2-3. The caspase-3 activities in liver of ayu. The mean values were represented with bars of standard deviations (n=3). The different characters represent significant differences ($P<0.01$).

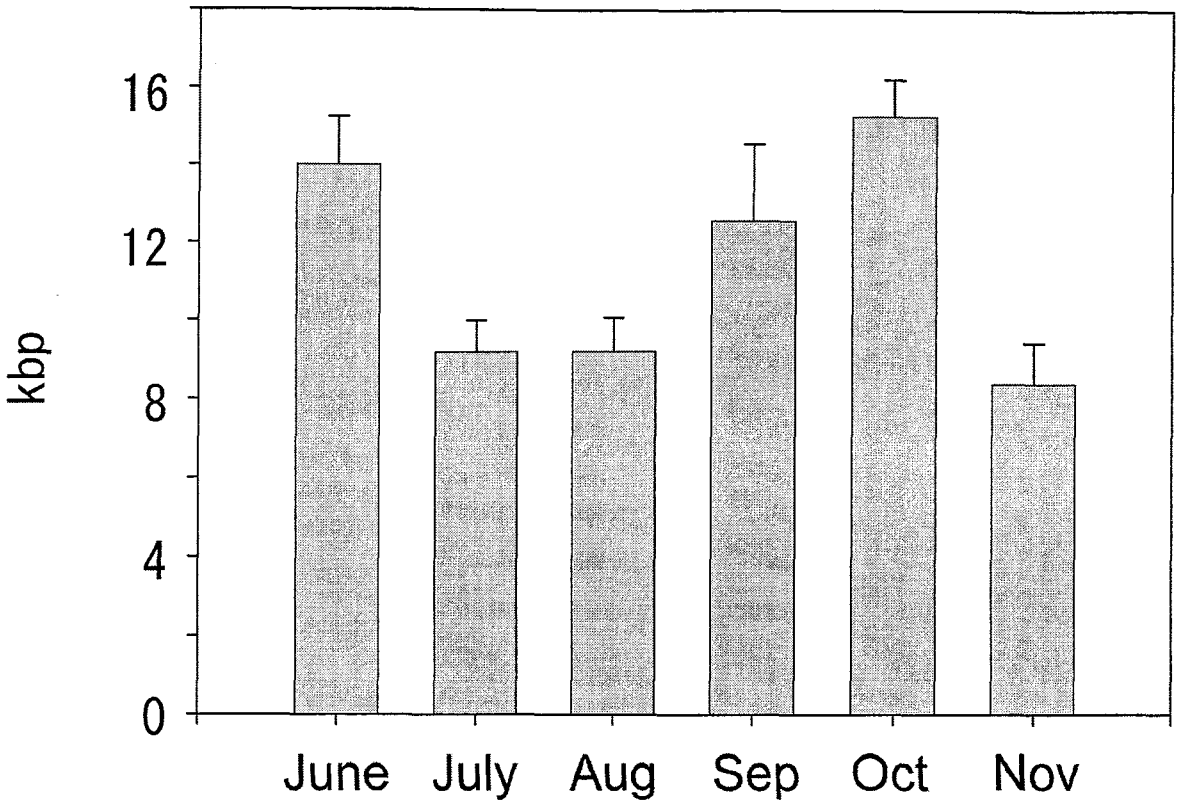

Figure III-2-4. Telomere length changes with age in liver of ayu. The mean values were represented with bars of standard deviations (n=3). No age-related telomere shortening was detected in the liver of ayu.

DISUCUSSION

In this chapter, it was revealed that the age-related of apoptosis might be involved in increasing of DNA damage and mutations in brain and liver, and could partially explain the short life span of ayu. It was also indicated that oxidative stresses induce apoptosis accompanied with caspase-9/6 and -3 activation in brain and liver. Especially in brain, oxidative stresses also induce activation p53 through the phosphorylation, and p53. Besides, these findings indicate that telomere did not shorten in ayu brain and liver under an age-dependent manner. These data also provide a novel mechanism for the age-related differences in life span and suggest a teleost specific regulation of telomere length during life span.

Recently, DNA damage has been shown to influence several genes involved in the cell-cycle checkpoint responses, including p53 (Kastan et al., 1991), $p21^{WAF1/Cip1}$(El-Deiry et al., 1994) and ATM (Kastan et al., 1992). In this chapter, it was revealed that caspase and p53 were activated during aging. However, in teleost, links among oxidative stress and formation of DNA base modifications, cell-cycle regulation and DNA repair are not clearly understood. The consequences of this damage in relation to aging and longevity should be considered within the idea that multiple mechanisms cause aging.

It has been well documented that the rate of production of superoxide anions and hydrogen peroxide in mitochondria is increased with age in animal tissues (Sohal and Sohal, 1991; Sohal et al., 1994; Perez-Campo et al., 1998). It was found that the increase in hydrogen peroxide production of *D. melanogaster* to mtDNA and membrane lipids of mitochondria (Sohal and Dubey, 1994). Sohal et al. (1995a) further demonstrated that the average life span of dipteran flies is inversely correlated with the rate of production of super oxide anions and hydrogen peroxide in mitochondria and with the level of protein carbonyls in the tissue cells. Moreover, the age-related increase in the rate of generation of hydrogen peroxide in mitochondria was observed to decrease 40 % in the fruit flies overexpressing Cu/ZnSOD and catalase as compared with the wild-type flies (Sohal et al., 1995b). Therefore, the rate and amount of hydrogen peroxide generated by mitochondria is an important determinant of the oxidative damage sustained by mitochondria. Richter et al. (1988) first demonstrated that oxidative damage to mtDNA is much more extensive than that to nuclear DNA. The specific content of 8-OHdG, and index of oxidative damage to DNA, of mtDNA was about 16 times higher than that of nuclear DNA in the liver of 3-month-old rats. Furthermore, the 8-OHdG level in liver mtDNA of 24-month-old rat was three times higher than that of the 3-month-old rats. Moreover, the levels of oxidative stress and proteins with oxidative modification and lipid peroxides in mitochondria have been shown to increase with age (Stadtman, 1992; Sohal et al., 1993). In the mammalian cell, the proper assembly and functioning of mitochondria are effected through the

coordination between gene products encoded by the nuclear and mitochondrial genomes (Poyton and McEwen, 1996). Communication between the nucleus and mitochondria is essential for delicate regulation of synthesis of protein in the cytoplasm and their subsequent import into mitochondria. ROS and some metabolites that regulate the activation of specific transcription factors, which may exert their functions in the nucleus, have been proposed to be among the signals for communication between mitochondria and the nucleus (Scarpulla, 1997).

It has been appreciated only very recently that mitochondria are not only the major metabolic energy supplier, but are also the main intracellular source and target of ROS and free radicals generated by the respiratory chain. The age-related decrease in the transcripts of mtDNA may result from a decline in the efficiency of mitochondrial transcription or reduction in the copy number of mtDNA in tissue cells. In this chapter, it was not surveyed the 8-OHdG level in mtDNA, however, it was suggested that age-dependent mtDNA mutations were more accumulation than nuclear DNA mutations resulting from damage by ROS. Therefore, it may be functional decline of mitochondria in aging ayu. Some experimental data have provided ample evidence to support the notion that mutation and oxidative damage to mtDNA and mitochondrial respiratory function decline in tissue cells are important contributors to human aging (Wei, 1998; Lee and Wei, 2001). In teleost, it was also guessed that oxidative damage to nuclear and mitochondrial DNA was important response and mechanisms during aging process.

Although a causal relationship between mutation of DNA and aging has emerged, the detailed mechanisms by which these molecular and biochemical events cause teleost aging are not clear. Understanding of the age-related changes in the structure and function of nuclear and mitochondria DNA in the aging process is critical for the elucidation of the molecular basis of aging and for the better management of aging and age-related diseases. Further studies on the interaction of signal pathways may change the scientific direction of the study of aging in teleost.

CHAPTER IV

EFFECTS OF CALORIC RESTRICTION ON POST-SPAWNING DEATH OF AYU

1. INTRODUCTION

The aging process causes a multitude of detrimental changes in the organism at all levels of biological organization, especially limiting maximum functional capacities, decreasing homeostasis and increasing the probability of death. All those changes are thought to originate from a smaller number of causes continuously operated throughout life. The life span for most species is genetically regulated during the aging process within the limit of maximum length.

Aging refers to a set of processes that lead over time to a gradual increase in vulnerability to be damaged and the probability of death. One of the major theories regarding a molecular mechanism governing these processes is the free radical theory (Harman, 1956). This theory postulates that overproduction of reactive oxygen species (ROS) during normal metabolic processes, or a loss of protective systems that reduce the ability of the organism to withstand oxidative challenge, is intricately connected to aging and lifespan (Finkel and Holbrook, 2000).

Genetic studies have establishes that aging is, at least in invertebrates, regulated by specific genes, whose mutations allow different organisms to extend life span (Guarente and Kenyon, 2000). Some of these genes provide an enhanced resistance to oxidative stress and may be among the causes of increased longevity in these lower organisms (Larsen, 1993; Melov et al., 2000; Migliaccio et al., 1999).

Only caloric restriction (CR) is the established intervention that extends life span in mammals. It reduces the incidence and delays the onset of age-related pathologies, and retards numerous age-related biological processes, resulting in maintenance of cellular function. The mechanisms underlying the robust protective effects of CR remain to be identified. One of the hypotheses suggested that most of the effects of CR on aging may be due to reduced oxidative stress at the cellular level. CR has been shown to suppress age-related oxidative damages in lipids, DNA and proteins, and also to increase the resistance of cells to oxidative stress (Guo et al., 2001; Leon et al., 2001; Li et al., 1998b; Zainal et al., 2000). CR is also known to alter fundamental mitochondrial bioenergetics, thus acting to reduce proton leaks, electron transport and free radical production and thereby protecting against free radical-induced macromolecular damage (Barja, 2002).

Previous study indicated that ayu (*Plecoglossus altivelis*) produced ROS higher than other species (Moritomo et al., 2003). Moreover, the life span of ayu is only one year. It seems likely that high levels of ROS relate to their short life span. The present chapter attempts to quantify age-associated changes of the degree of attenuation on oxidative damage and hormonal homeostasis in CR. Among the oxidative damages, base modifications, such as oxidation of deoxyguanosine to 8-hydroxy-2'-deoxy-guanosine (8-OHdG) have received increasing attention in recent years. 8-OHdG is one of the most

abundant oxidative products of DNA (Takeuchi et al., 1994). The effect of this mutation on aging was examined. The levels of caspase-9/6 and -3, apoptosis induction factors, and major sexual hormone change by CR were also surveyed in order to reveal the relation between CR and life span of ayu.

2. MATERIAL AND METHOD

2.1. *Fish*

Healthy ayu were obtained from the Tochigi Prefectural Fisheries Experimental Station, Japan. Until used in experiments, fish were stocked in outdoor ponds (14.8 m^2, 0.9 m in depth) water was supplied from a natural stream through an inlet mouth. The fish a were fed commercial pellets for ayu (Oriental Yeast Co., Ltd., Tokyo, Japan) by autofeeder four times per day. The fish were divided into two groups: the first group (control) and the other group (CR), fish of sample group were fed 70 % dietary compared with control. Total length, body weight, weight of brain and liver and gonadal organ of the fish were calculated in every month from July to November. From June to September ayu were used for following assays without determination of sex. We determined sex of fish and divided fish into two groups for the following assays except 8-OHdG assay from October to December. Whole brain and liver were dissected out and used for subsequent assay.

2.2. Measurement of 8-OHdG in brain and liver

DNA of liver and brain were obtained using Genelute Mammalian Genomic DNA kit (SIGMA, USA). 8-OHdG measurement was carried out as described in Section 1 of Chapter III.

2.3. Assay for caspase activity

Caspase activities in brain and liver were measured as described in Section 1 of Chapter III, using fluorescent peptide substrates (BD ApoAlert Caspase Fluorescent Assay kits; BD biosciences, Japan). Quantification of protease activity for caspase -9/6 and caspase-3 were calculated as the amount of liberated AMC/g each organ, and liberated AFC/g each organ, respectively.

2.4. Plasma progesterone, testosterone and17 β-estradiol measurement

Ayu for each group were separately used for measurements of plasma progesterone, testosterone and 17 β-estradiol. The progesterone EIA Kit (Cayman Chemical Company, USA) was used to evaluate progesterone concentrations. The plasma testosterone concentrations were determined with the Testosterone EIA Kit (Cayman Chemical Company, USA). The Estradiol EIA Kit (Cayman Chemical Company, USA) was used to evaluate 17 β-estradiol concentrations.

2.5. Statistical analyses

Data obtained were analyzed using one-way ANOVA.

3. RESULTS

3.1. Rearing fish and fish body condition

Water temperature throughout rearing is shown in Figure IV-1. Total length and body weight of control and CR ayu were shown in Figures IV-2 and IV-3, respectively. Total length and body weight were gradually increased from July to October. Total length and body weight of ayu in CR were lower than control levels. In total length, there were significant differences between control and CR in August ($P<0.05$), September, and November for both sexes ($P<0.01$). Body weight of CR ayu were significantly different from that of control fish in August ($P<0.05$), September ($P<0.01$), female in October ($P<0.05$), male in October ($P<0.01$), and both sexes in November ($P<0.01$).

There were significant differences in brain weight of ayu except August ($P<0.01$) between control and CR (Figure IV-4). In liver weight, significant differences were observed only in July and male of November ($P<0.05$) (Figure IV-5). Brain weight per body weight and liver weight per body weight are shown in Figures IV-6 and IV-7, respectively. In relative brain weight, there were significant differences in August, male of October and both sexes in November ($P<0.01$). No significant difference was observed in relative liver weight except for male in November ($P<0.05$). The relative brain and liver weights of CR ayu tended to surpass those of control ayu.

Gonadal weight from August to November was measured (Figure IV-8). There were significant differences in September, female in October and male in November ($P<0.01$) between control and CR ayu. There were significant differences in gonadal weight per body weight for female in October ($P<0.05$) and both sexes in November ($P<0.01$) (Figure

IV-9). Gonadal weights were relatively low in CR ayu, compared with control ayu.

3.2. Oxidative DNA formations in the brain and liver of ayu

Oxidative stress for DNA was evaluated by the formation of 8-OHdG throughout their life span. As shown in Figure IV-10, the levels of 8-OHdG in brain increased to the maximal at October for both control and CR ayu. There were significant differences in July ($P<0.01$) between control and CR groups. The levels of 8-OHdG in liver gradually increased from August in both groups, and significant differences were observed in July, November ($P<0.01$) and September ($P<0.05$) between control and CR ayu (Figure IV-11).

3.2. Caspase activity in brain and liver

Figure IV-12 shows the activities of caspase-9/6 in brain. The caspase-9/6 activities in brain of CR ayu were lower than those of control ayu until October. The levels in female of October ($P<0.05$), male of October and male of November ($P<0.01$) of CR ayu were significantly different from those of control ayu.

As shown in Figure IV-13, caspase-3 in brain activity of female ayu between CR and control showed significant differences in October and November ($P<0.01$). The level of control male in October was significantly higher than that of CR male ($P<0.05$).

Figure IV-14 shows the activities of caspase-9/6 in liver. The caspase-9/6 activities in liver of CR ayu were almost low compared with control ayu. There were significant differences in the levels in female of October ($P<0.05$), male of October and male of November ($P<0.01$) between control and CR.

As shown in Figure IV-15, caspase-3 activity in liver of CR female ayu in October and November were significantly different ($P<0.01$) from CR female ayu. The level of control male in October was significantly higher than CR male ($P<0.05$).

3.3. Sex hormone concentration

Figure IV-16 shows progesterone levels in control and CR ayu. Progesterone concentration in control ayu of June and August were significantly higher than CR ayu ($P<0.01$). However, control ayu in July, both sexes of October and female of November were significantly lower than CR ayu ($P<0.01$).

Plasma testosterone levels in control ayu were higher than CR except for June, July and female of November (Figure IV-17). There were significant differences between control and CR in July, August, female in October and male in November ($P<0.01$), and in June, September and male in October ($P<0.05$).

As shown in Figure IV-18, there were significant differences in plasma 17β-estradiol level in July between control and CR ayu ($P<0.05$). In other month, there was no difference between two divisions.

3.4. Transition of the number of survival

No significant difference was shown in number of survival as Figure IV-19, suggesting that caloric restriction would not affect life span of ayu.

4. DISCUSSION

The results of this chapter show that caloric restriction decreased caspase activities in brain and liver, and affected hormonal homeostasis in ayu. However, it was indicated that caloric restriction did not extend ayu life span.

A common methodology frequently employed to produce long-lived CR rodents is to feed the same diet as consumed by the control animals, but restricted in quantity. Hence, CR model system in this chapter also belongs to the general dietary restriction. Although, fish body size of CR was significant smaller than control, the levels of important organs weight (e.g. brain, liver and gonadal organ) per body weight of CR ayu were relatively high throughout ayu life span. It was suggested that essential nutrients were supplied in this study.

One mechanism responsible for life span extension by CR would be involved in the reduction of ROS production. CR has been shown to inhibit or delay age-related increases in oxidatively damaged proteins (Sohal et al., 1994), DNA (Kaneko et al., 1997), and lipids (Lass et al., 1998). The cellular changes were responsible for these decreases in oxidative damage and ROS. In the present chapter, oxidative damages to nuclear DNA were measured. DNA damage in brain and liver were elevated with aging, but the value of 8-OHdG did not decrease by CR against the previous reports. This discrepancy is probably because of the markedly high levels of ROS production in ayu. The produced ROS levels of ayu were about 3-7 times higher than that of trout and carp. (Moritomo et al., 2003). On the other hand, every caspase activity of CR ayu was relatively low compared with control ayu. It is suggested that cellular caspase-induced apoptosis might be controllable by CR.

In the male primate, fasting or caloric restriction significantly decrease serum testosterone concentrations (Veldhuis et al., 1993). As shown in Figure IV-17, plasma testosterone level of CR ayu also decreased. It is putatively due to the secondary hypogonadotrophism by CR. It has been reported that in women, visceral obesity is associated with elevated levels of total testosterone and free testosterone (Glass, 1989).

Progesterone and 17β-estradiol levels of CR ayu were relatively high compared with control ayu. It was reported that the plasma and tissue levels of estrogens and progesterone seemed to be related to low body fat accumulation (Kirschner et al., 1990). In adult men, plasma levels of estrone and estradiol are not correlated with the levels of testosterone, and dihydrotestosterone are negatively correlated with body total body fat (Bouchard et al., 1991). Thus, low energy availability is associated with both decrease in body weight and changes in the activity of the reproductive axis. In this study, it was thought that CR ayu had lower body fat than control ayu, resulting in higher progesterone and 17β-estradiol levels of CR ayu compared with control ayu.

The results were almost similar to the previous CR studies except for oxidative DNA damage accumulation. However, life span of ayu was not prolonged by CR. Therefore, there are something determination factors governing life span of ayu, however, are still open to speculation.

ABSTRACT

Caloric restriction is the only established intervention that extends life span in mammals, insects and nematodes. One of the hypotheses suggested that most of the effects of CR on aging may be due to reduced oxidative stress at the cellular level. It was known that ayu (*Plecoglossus altivelis*) produced ROS higher than other fish and that the life span of ayu is only one year. The present chapter attempts to quantify age-associated changes of the degree of attenuation on oxidative damage and hormonal homeostasis in CR. The oxidative DNA damage by measuring the levels of 8-OHdG and the induction of apoptosis by measuring the levels of caspase-9/6, -3 with aging in brain and liver were surveyed. Changes in major sexual hormones were also investigated. Caspase activities in brain and liver were reduced by CR, although CR was no influence to DNA damage level. Plasma testosterone levels of CR ayu were significantly higher and progesterone and 17β-estradiol levels were lower than the control ayu. However, life span of ayu was not prolonged by CR. These results suggested that there would be factors determining life span of ayu other than CR and apoptosis.

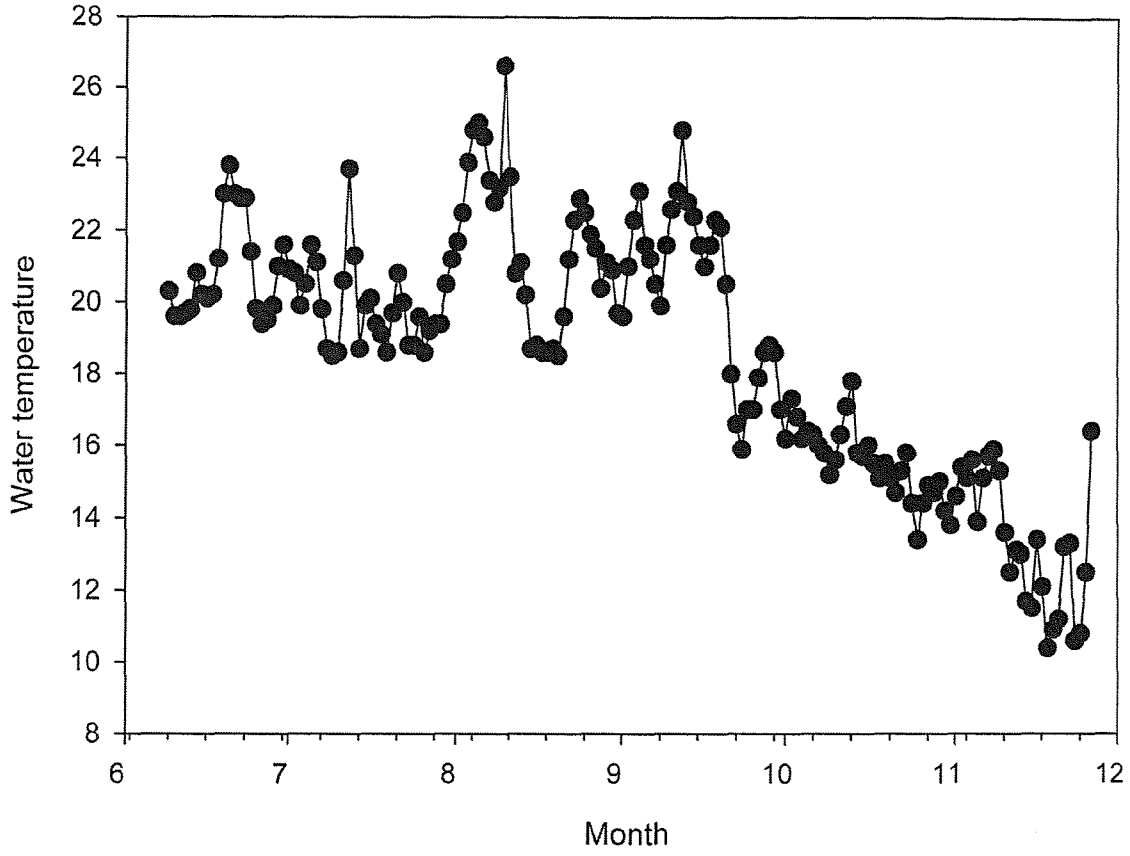

Figure IV-1. Water temperature throughout rearing ayu.

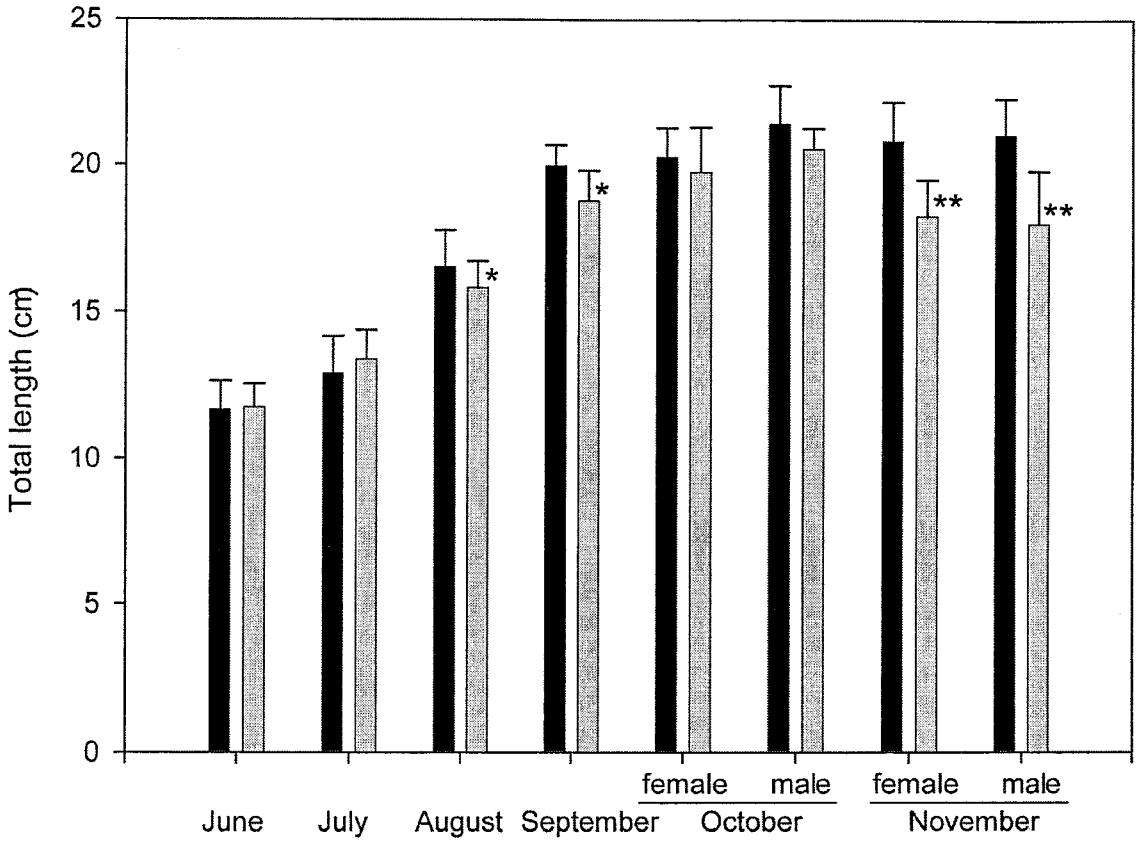

Figure IV-2. Total length of control and CR ayu. The mean values were representd with standard deviation (n=20). The asterisk denotes that there are significant differences (**P<0.01, *P<0.05) between the control (black column) and the CR (grey column).

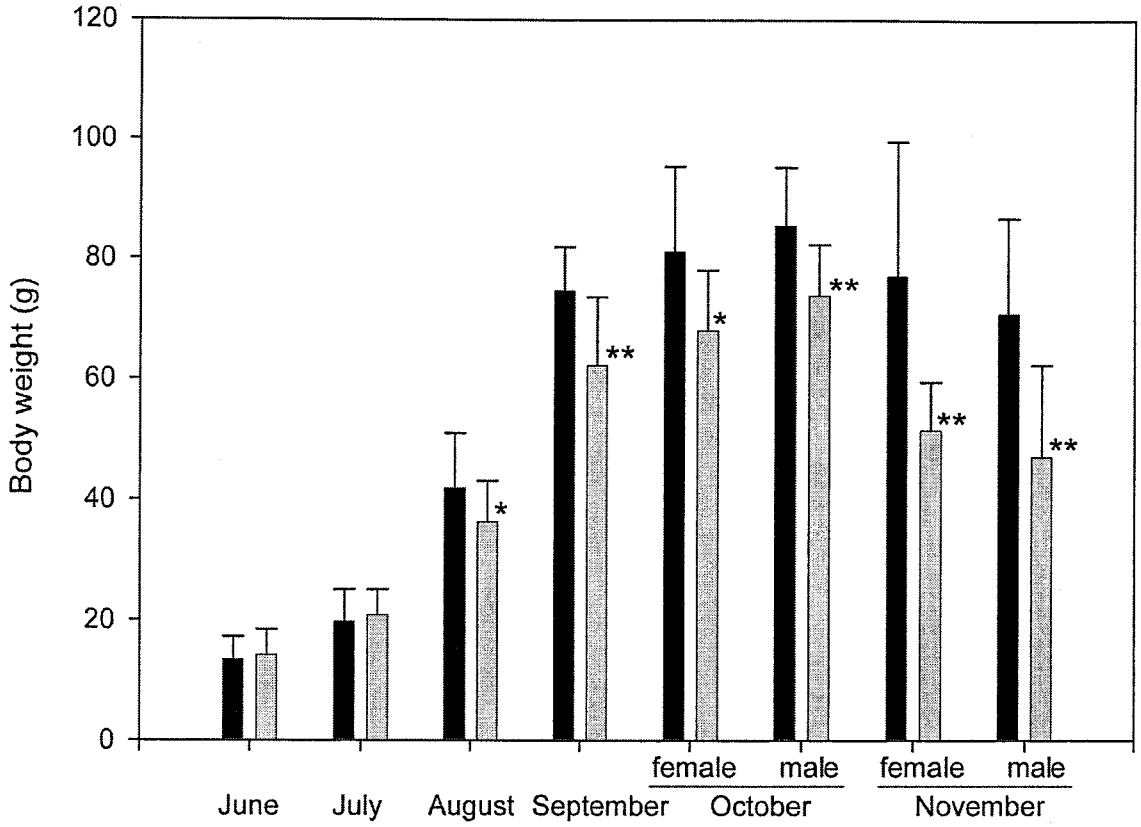

Figure IV-3. Total body weight of control and CR ayu. The mean values were represented with standard deviation (n=20). The asterisk denotes that there are significant differences (**P<0.01, *P<0.05) between the control (black column) and the CR (grey column).

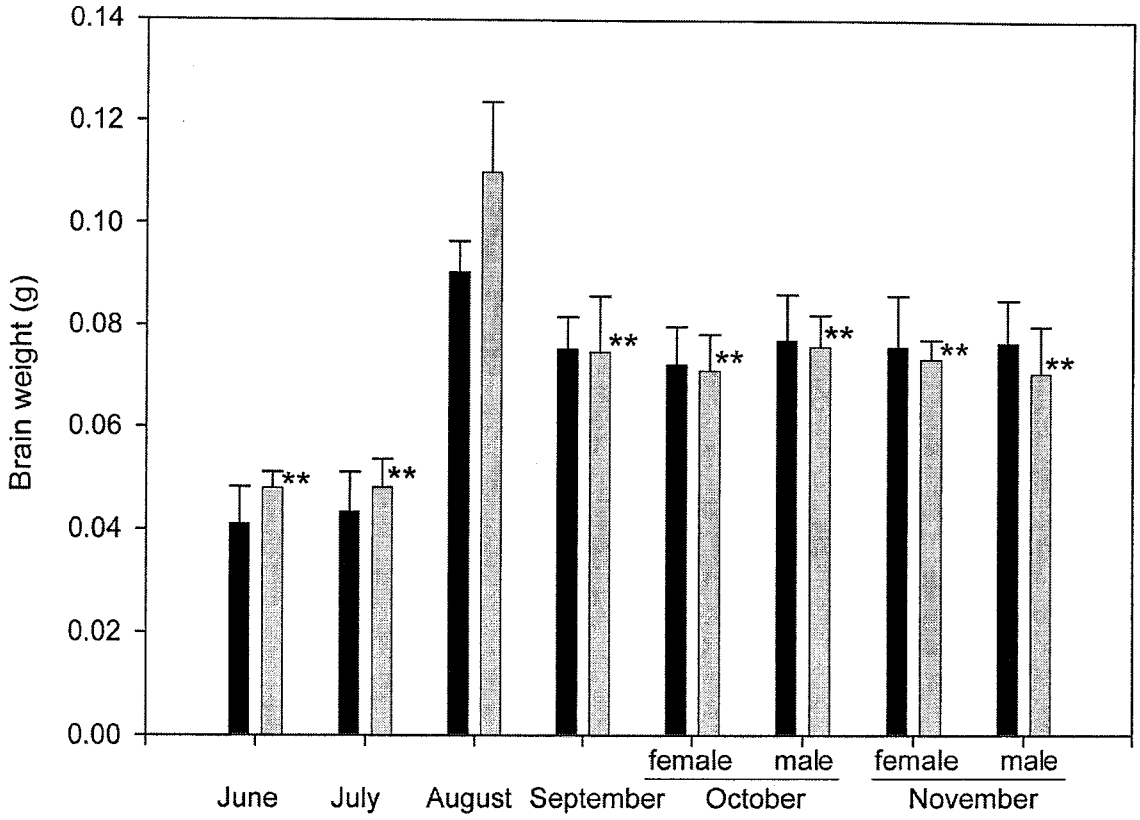

Figure IV-4. The brain weight of control and CR ayu. The mean values were represented with standard deviation (n=20). The asterisk denotes that there are significant differences (**P<0.01) between the control (black column) and the CR (grey column).

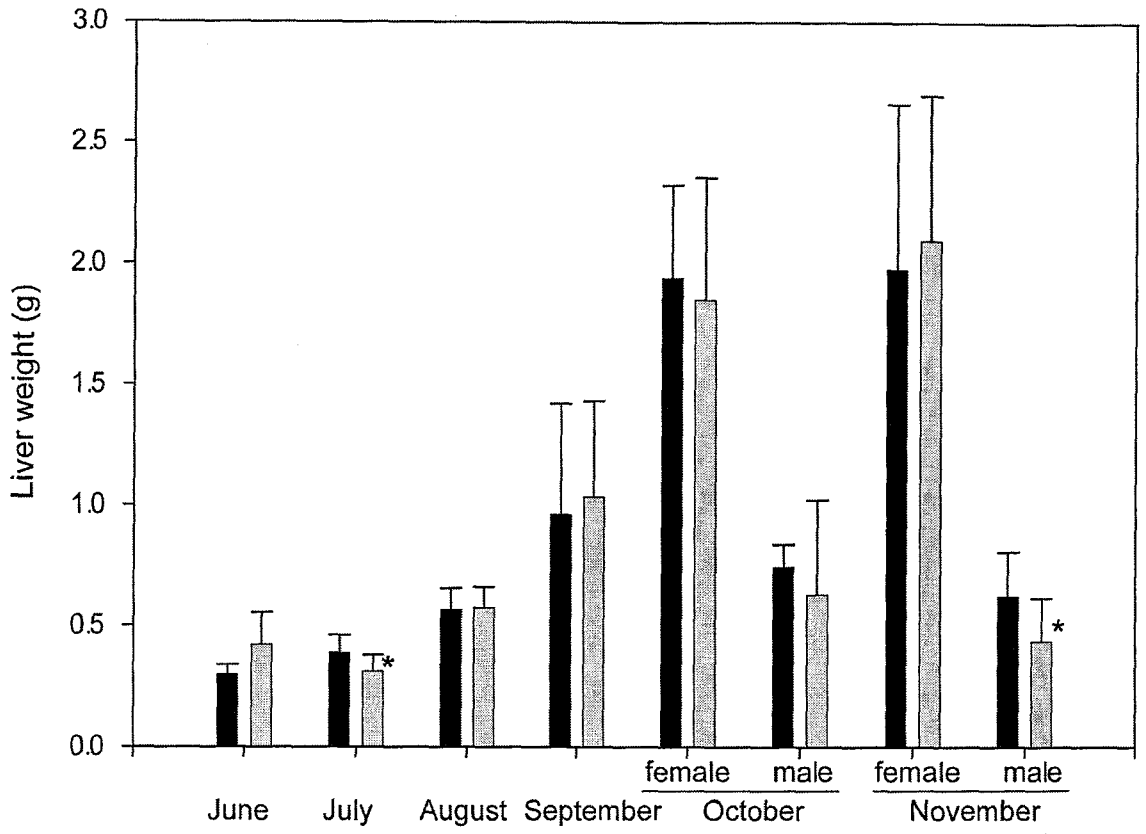

Figure IV-5. The liver weight of control and CR ayu. The mean values were represented with standard deviation (n=20). The asterisk denotes that there are significant differences (*P<0.05) between the control (black column) and the CR (grey column).

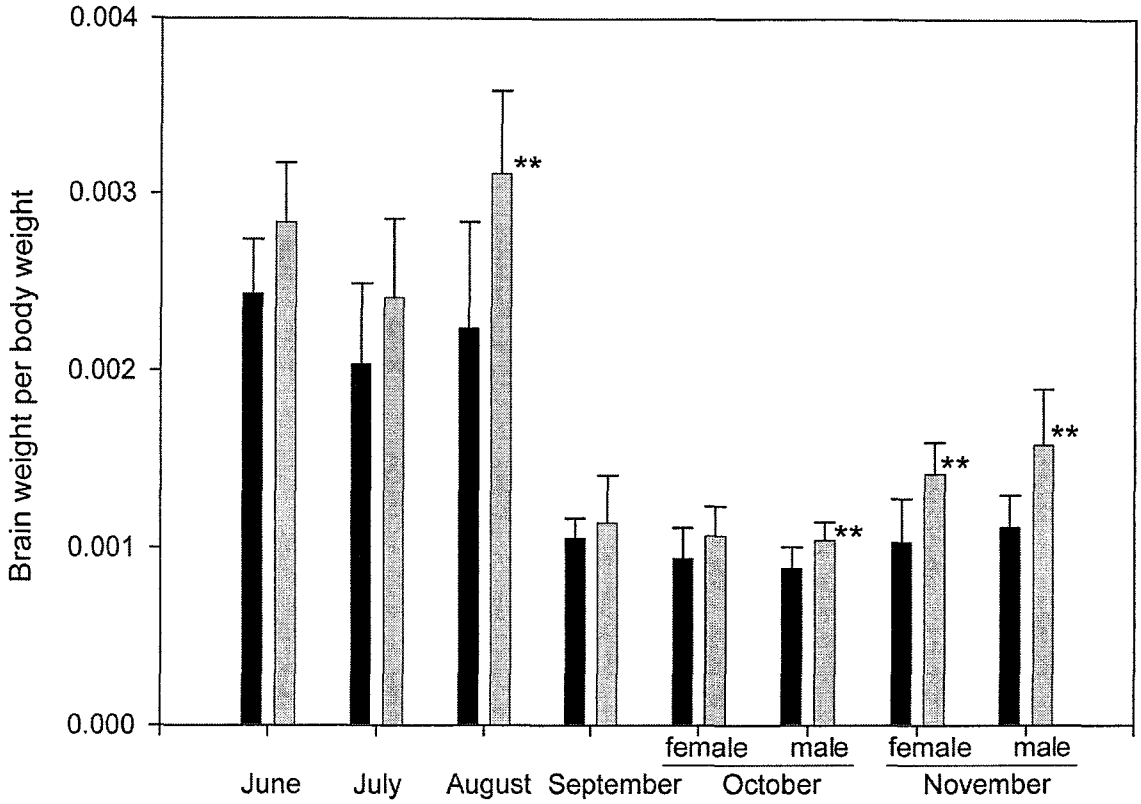

Figure IV-6. Brain weight per body weight of control and CR ayu. The mean values were represented with standard deviation (n=20). The asterisk denotes that there are significant differences (**P<0.01) between the control (black column) and the CR (grey column).

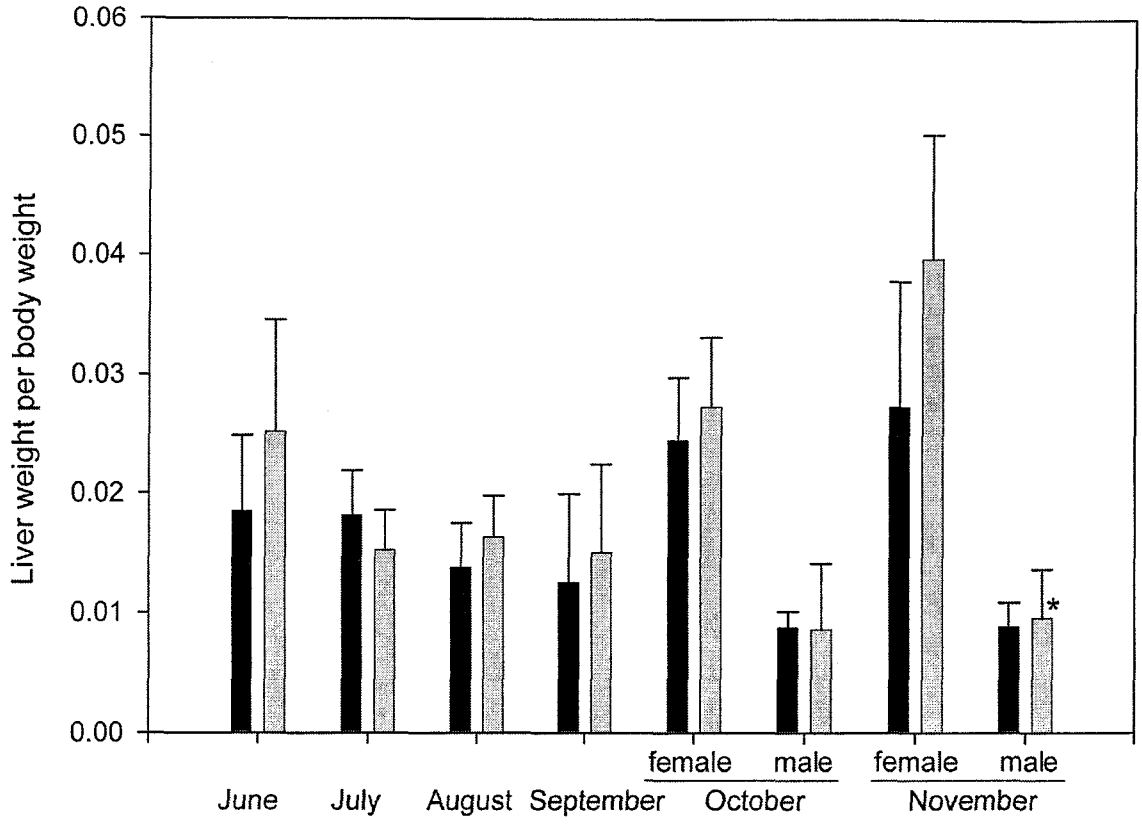

Figure IV-7. Liver weight per body weight of control and CR ayu. The mean values were represented with standard deviation (n=20). The asterisk denotes that there are significant differences (*P<0.05) between the control (black column) and the CR (grey column).

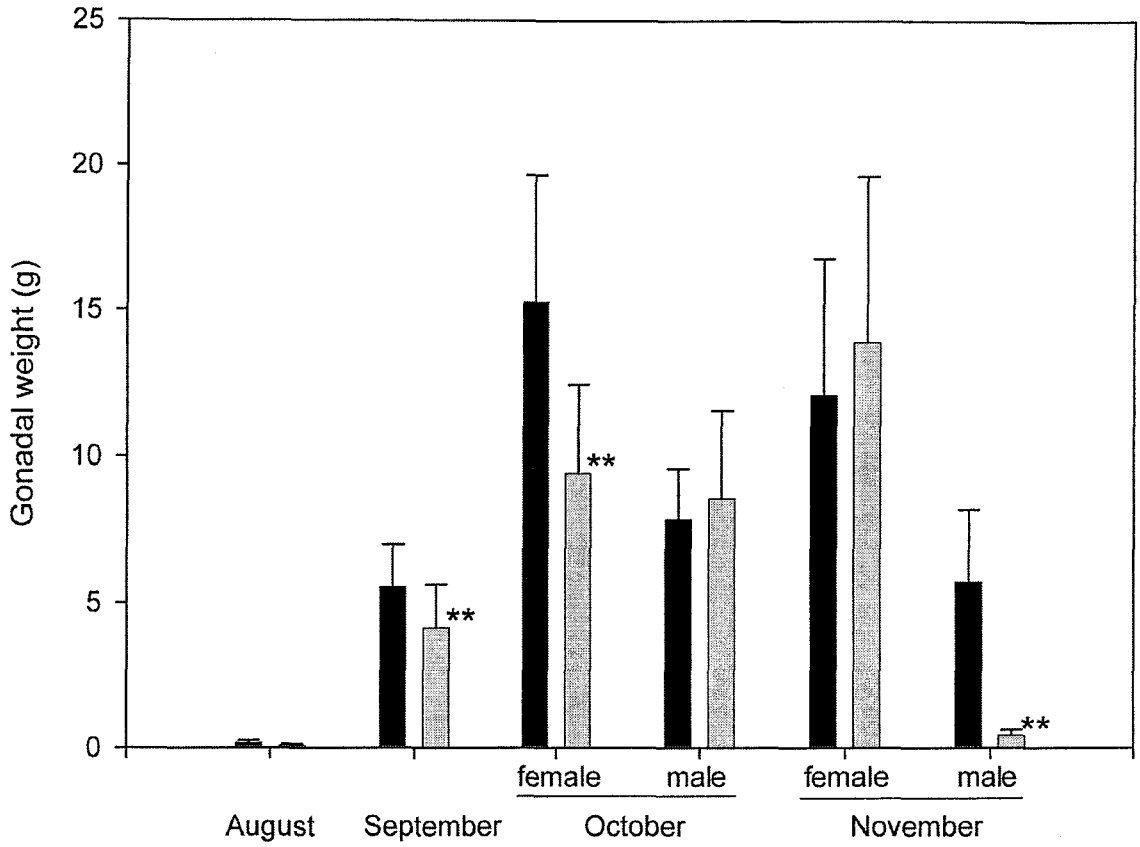

Figure IV-8. The gonadal weight of control and CR ayu. The mean values were represented with standard deviation (n=20). The asterisk denotes that there are significant differences (**P<0.01) between the control (black column) and the CR (grey column).

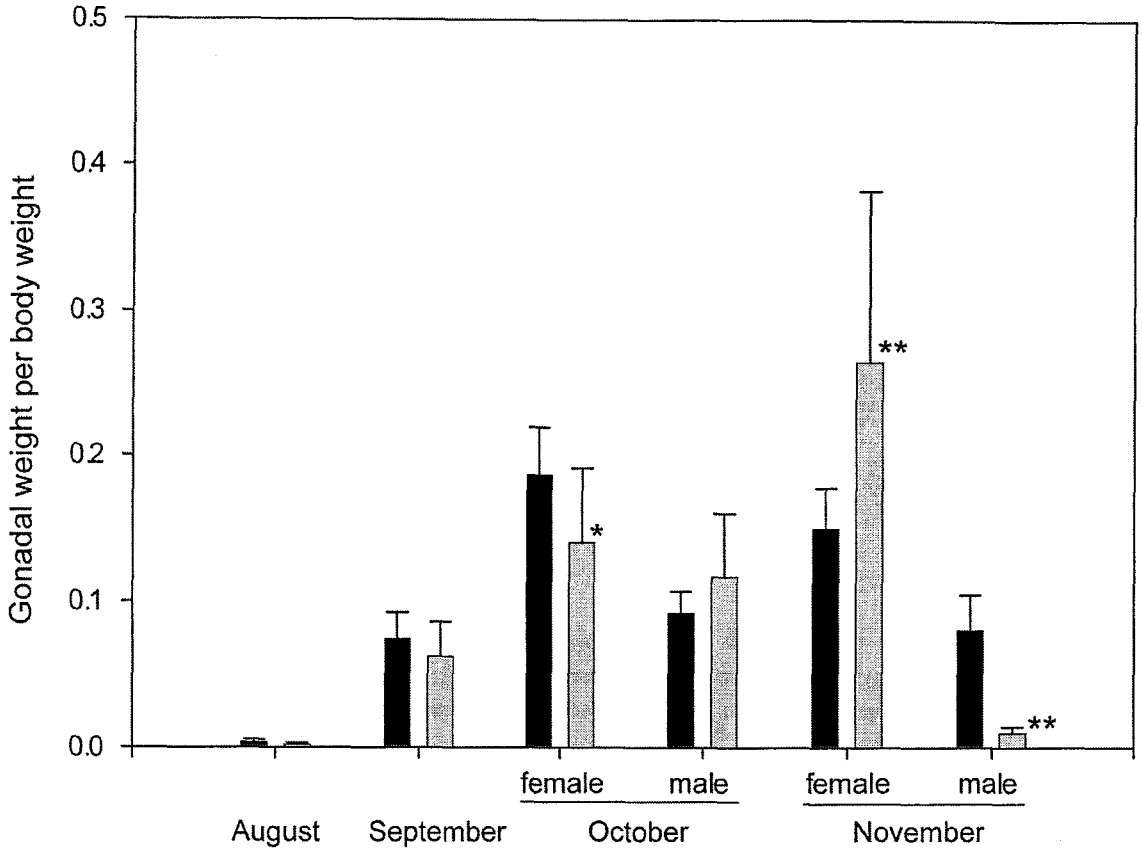

Figure IV-9. Gonadal weight per body weight of control and CR ayu. The mean values were represented with standard deviation (n=20). The asterisk denotes that there are significant differences (**P<0.01, *P<0.05) between the control (black column) and the CR (grey column).

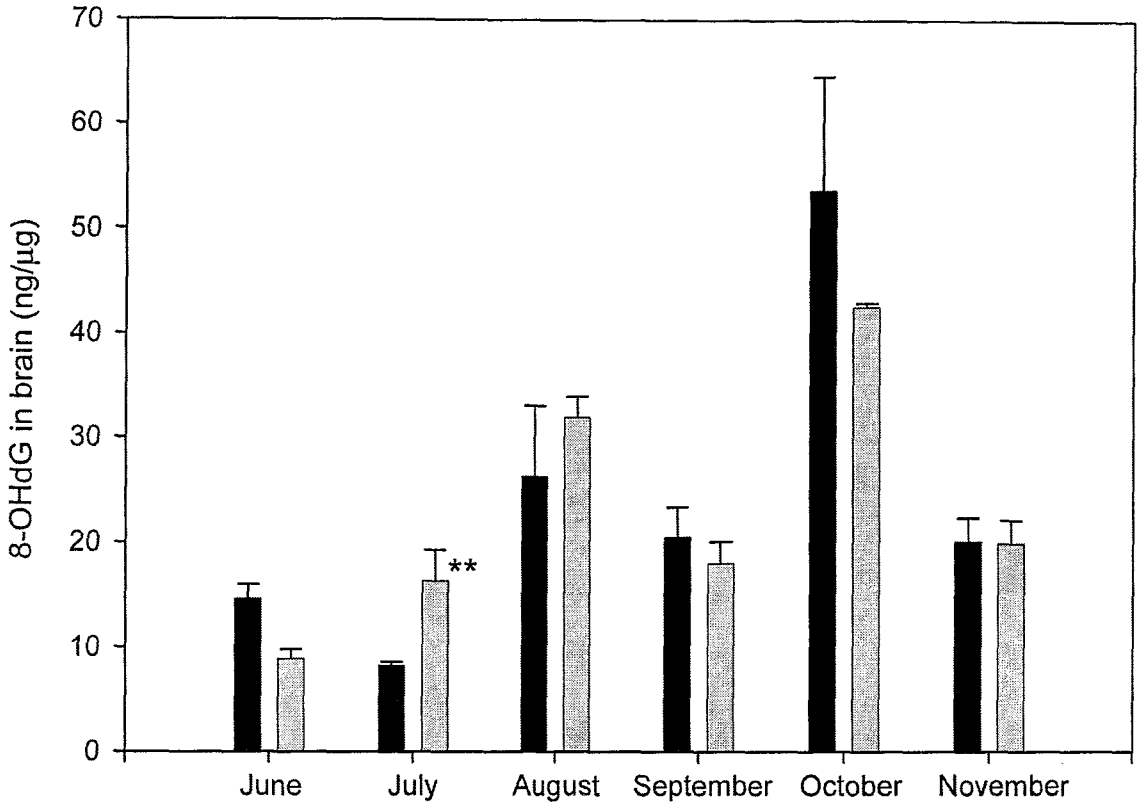

Figure IV-10. The levels of 8-OHdG in ayu brain. The mean values were represented with standard deviation (n=5). The asterisk denotes that there are significant differences (**P<0.01) between the control (black column) and the CR (grey column).

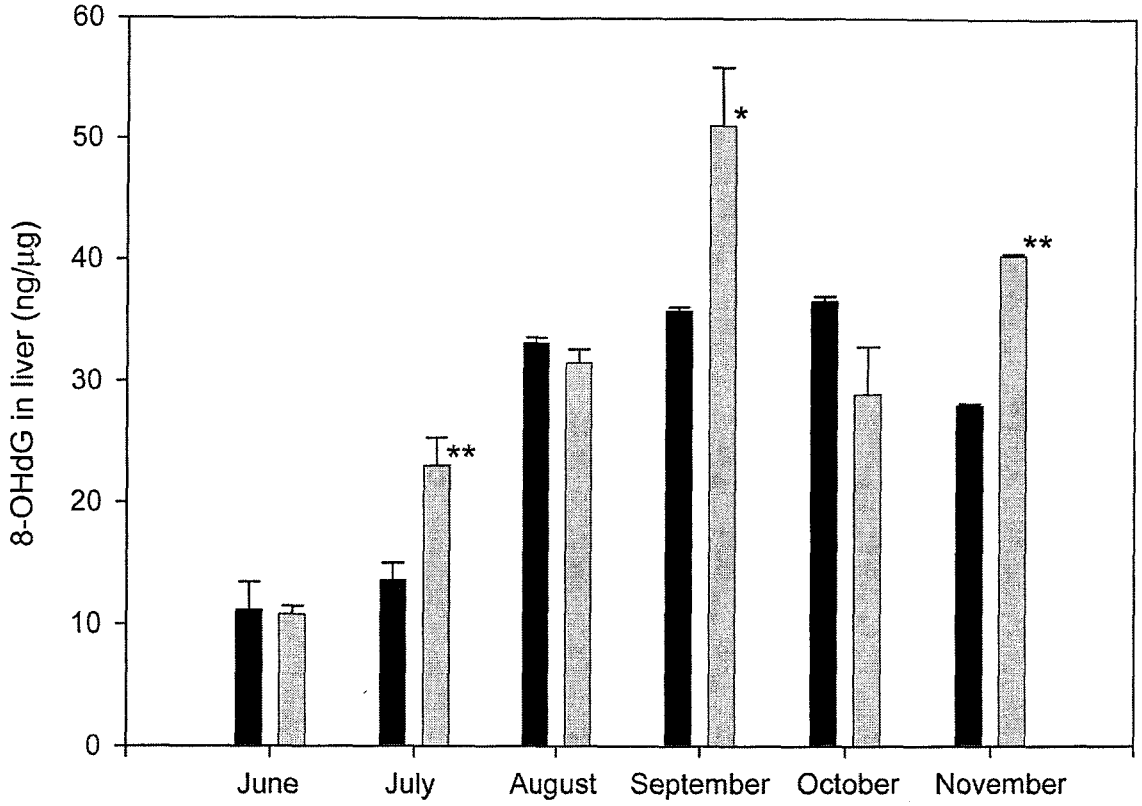

Figure IV-11. The levels of 8-OHdG in ayu liver. The mean values were represented with standard deviation (n=5). The asterisk denotes that there are significant differences (**P<0.01, *P<0.05) between the control (black column) and the CR (grey column).

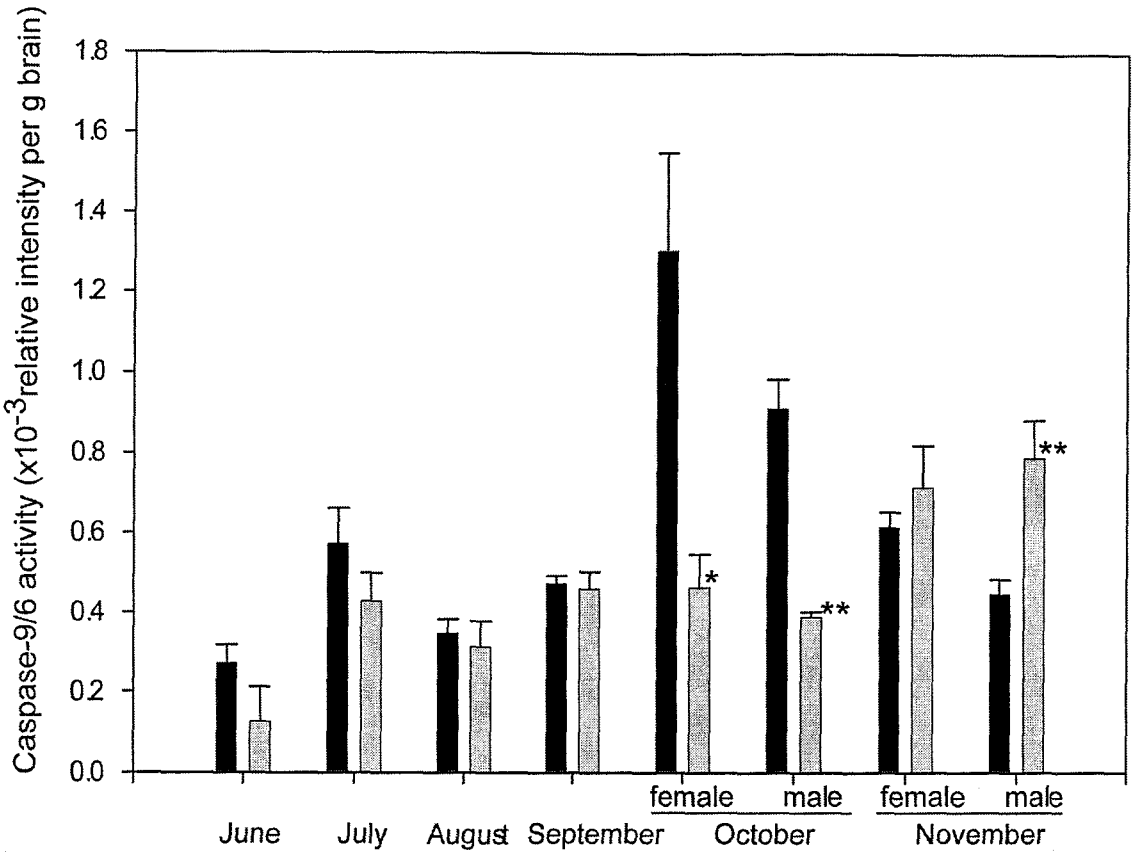

Figure IV-12. The caspase-9/6 activities in brain of ayu. The mean values were represented with standard deviation (n=3). The asterisk denotes that there are significant differences (**P<0.01, *P<0.05) between the control (black column) and the CR (grey column).

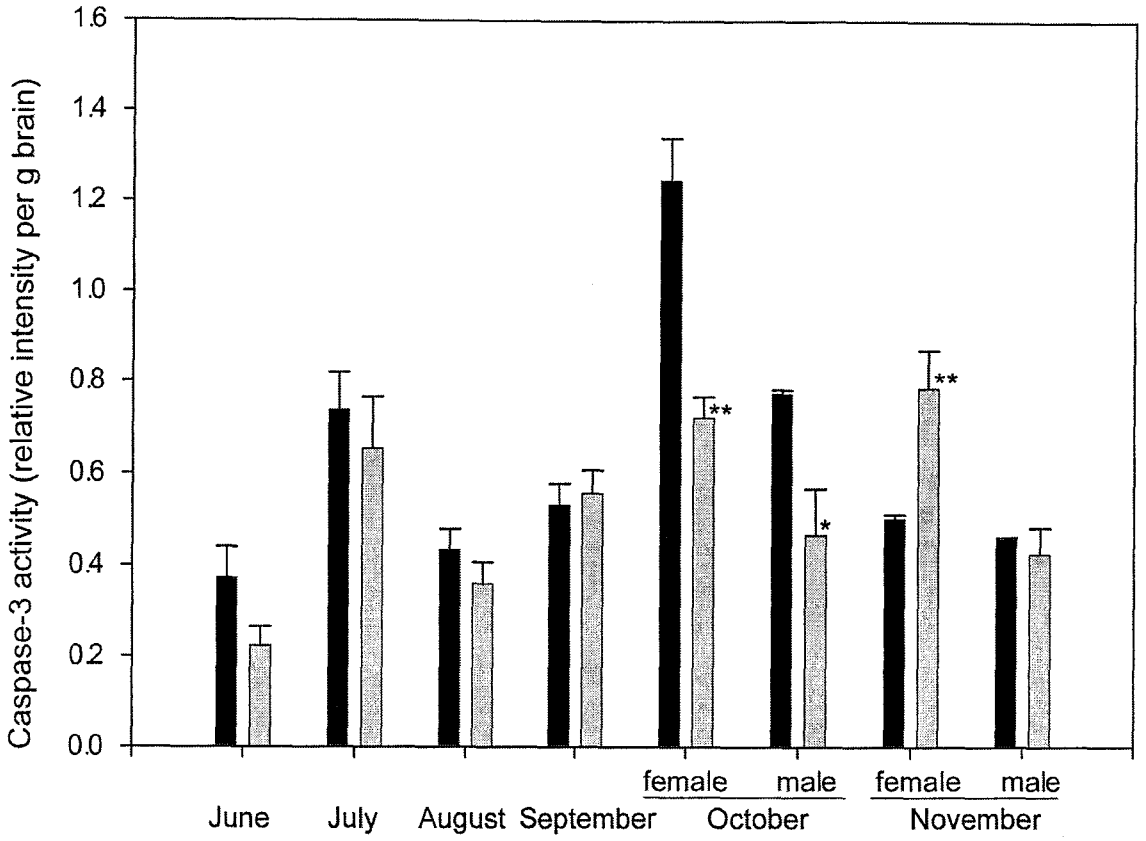

Figure IV-13. The caspase-3 activities in brain of ayu. The mean values were represented with standard deviation (n=3). The asterisk denotes that there are significant differences (**P<0.01, *P<0.05) between the control (black column) and the CR (grey column).

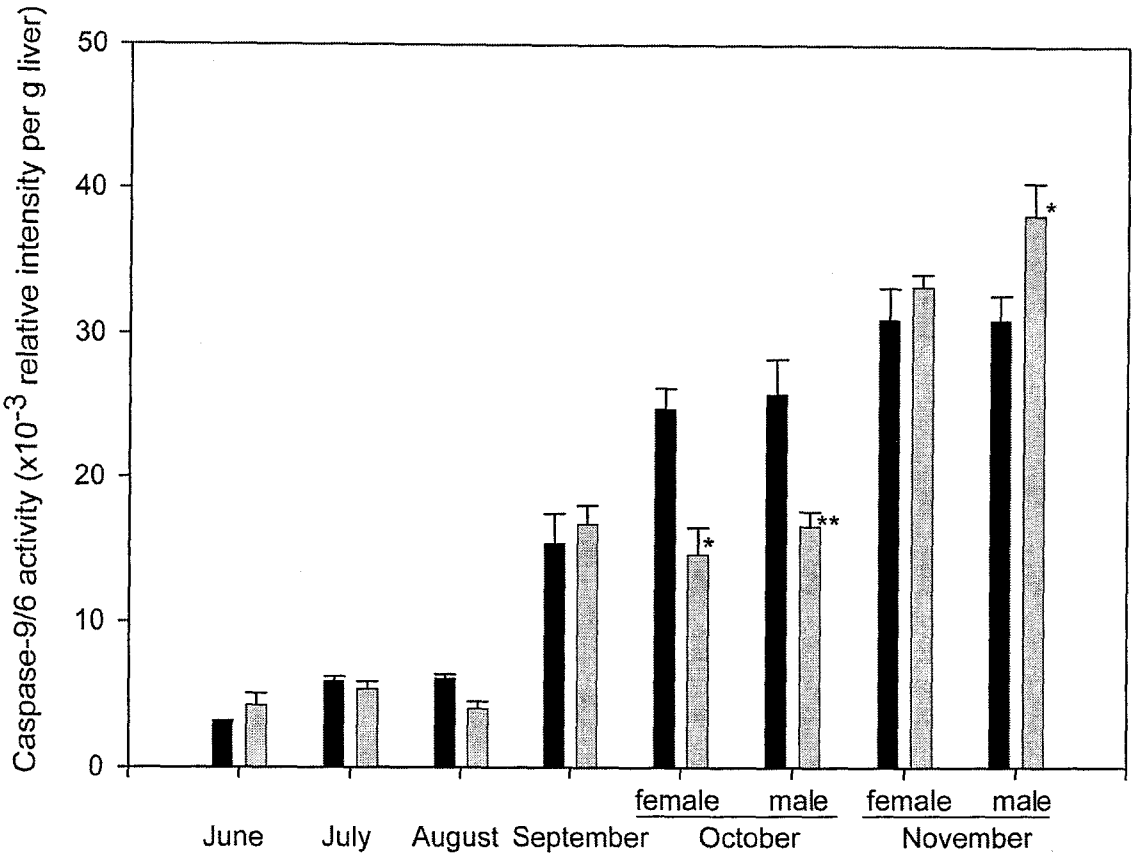

Figure IV-14. The caspase-9/6 activities in liver of ayu. The mean values were represented with standard deviation (n=3). The asterisk denotes that there are significant differences (**P<0.01, *P<0.05) between the control (black column) and the CR (grey column).

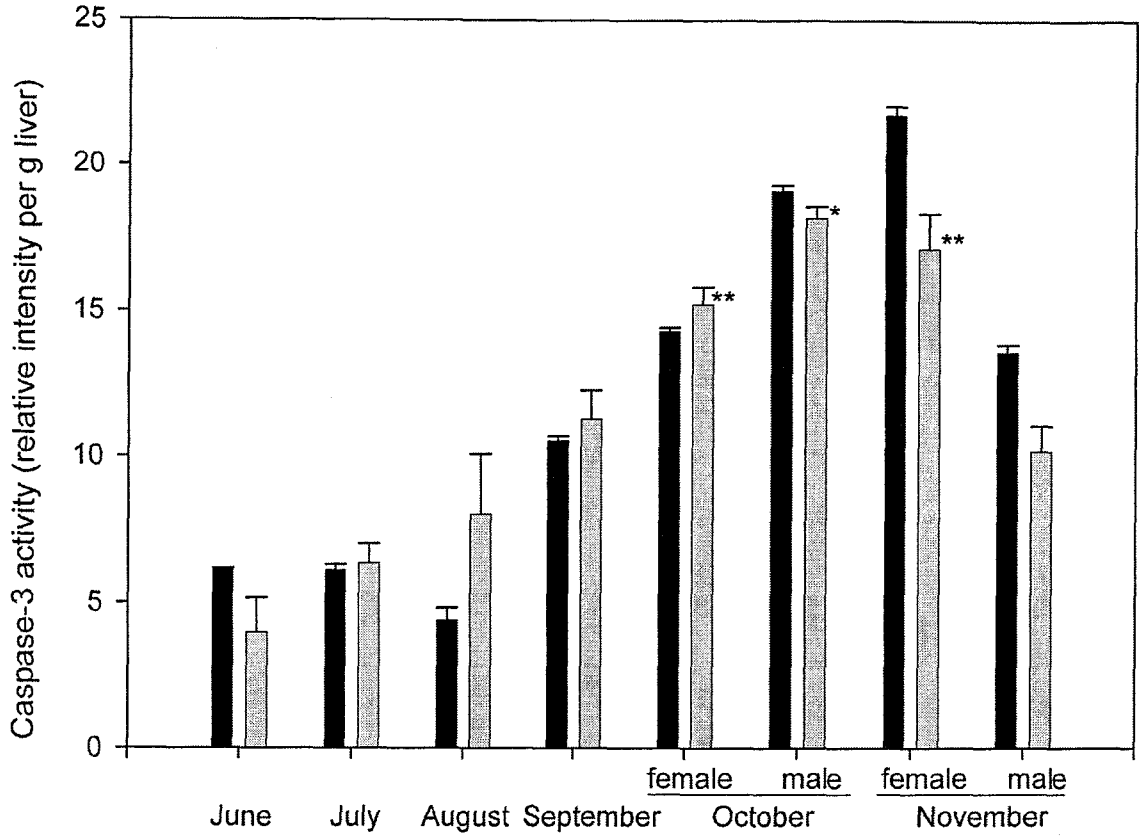

Figure IV-15. The caspase-3 activities in liver of ayu. The mean values were represented with standard deviation (n=3). The asterisk denotes that there are significant differences (**P<0.01, *P<0.05) between the control (black column) and the CR (grey column).

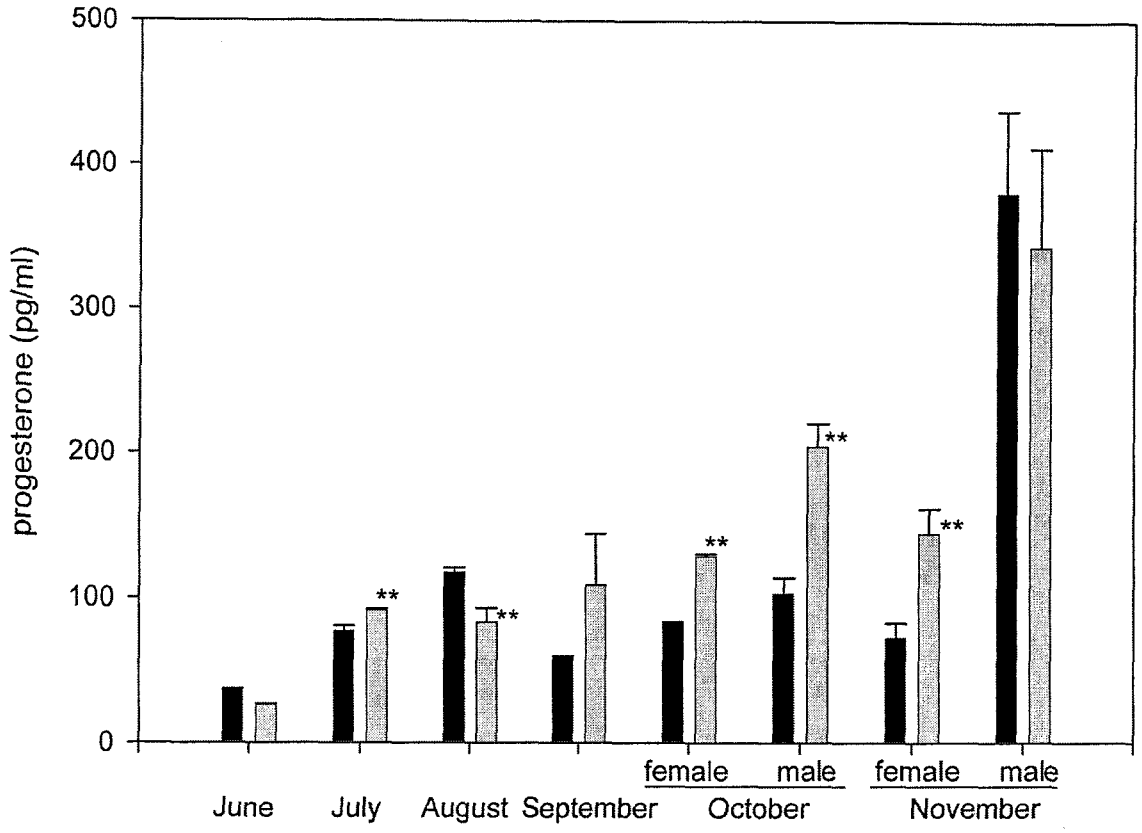

Figure IV-16. Plasma progesterone levels in control and CR ayu. The mean values were represented with standard deviation (n=3). The asterisk denotes that there are significant differences (**P<0.01) between the control (black column) and the CR (grey column).

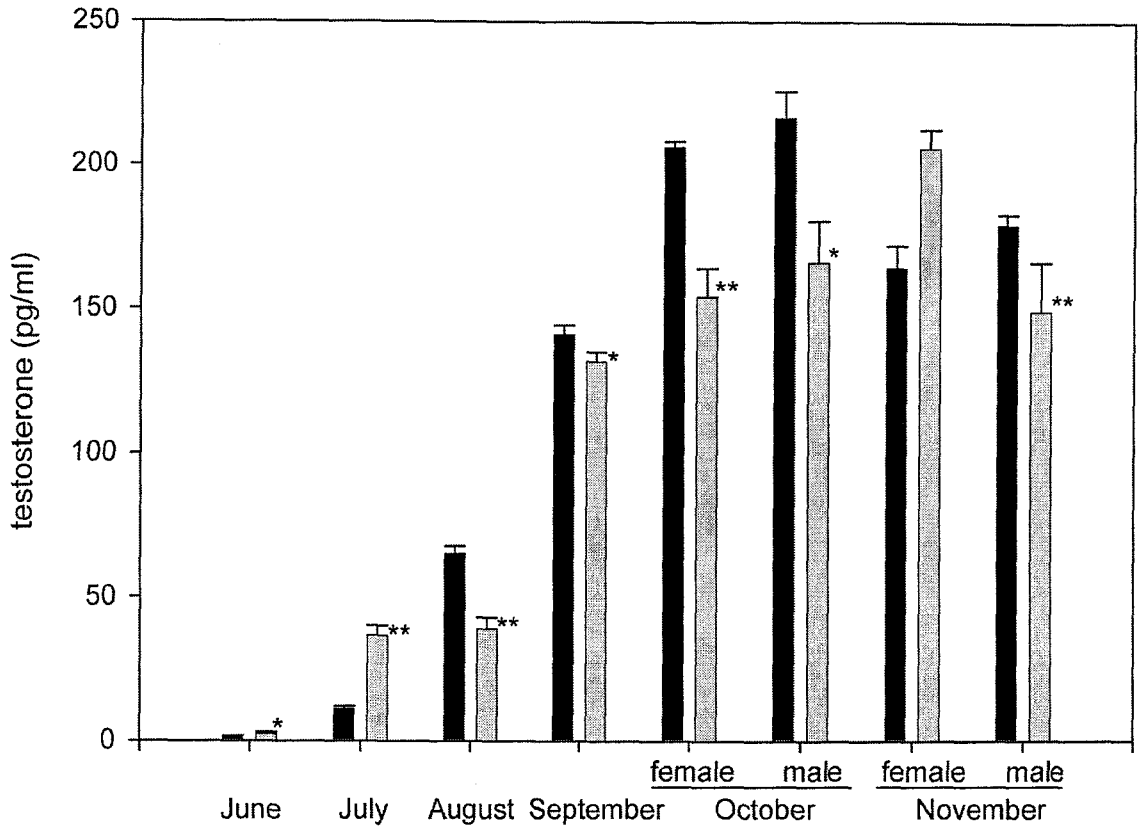

Figure IV-17. Plasma testosterone levels in control and CR ayu. The mean values were represented with standard deviation (n=3). The asterisk denotes that there are significant differences (**P<0.01, *P<0.05) between the control (black column) and the CR (grey column).

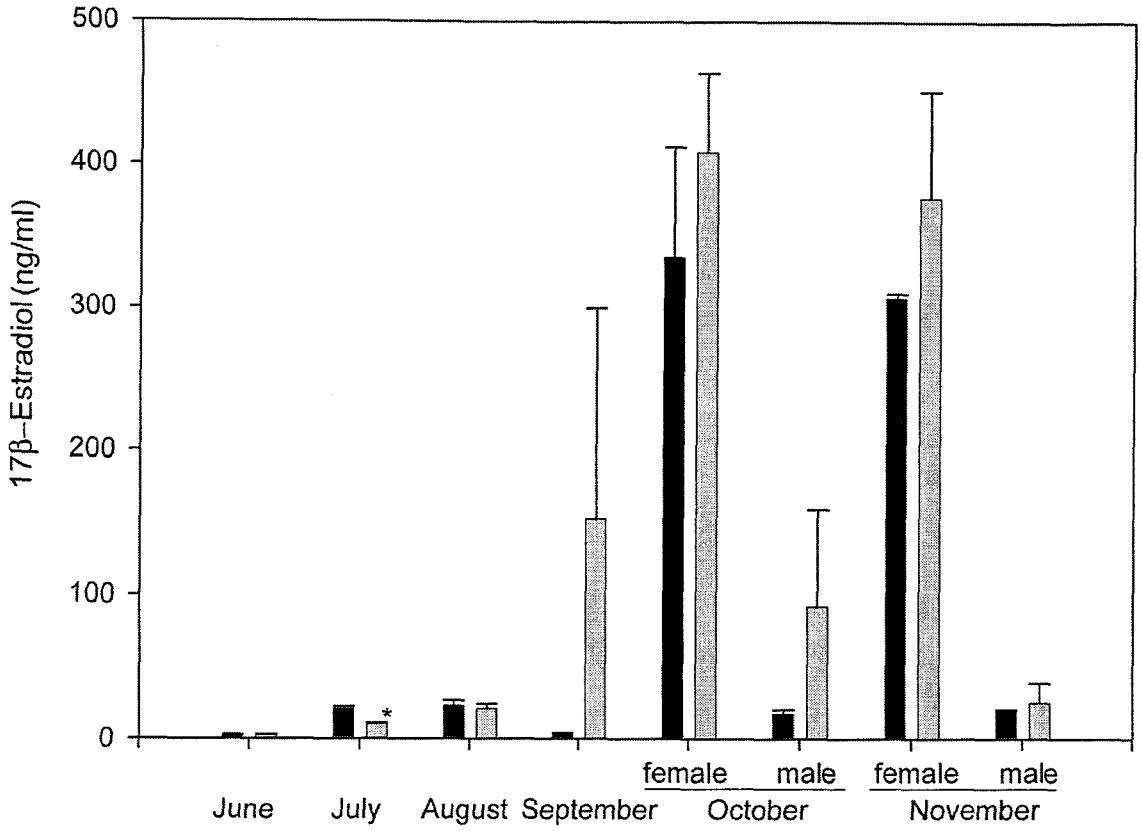

Figure IV-18. Plasma 17β-estradiol levels in control and CR ayu. The mean values were represented with standard deviation (n=3). The asterisk denotes that there are significant differences (*P<0.05) between the control (black column) and the CR (grey column).

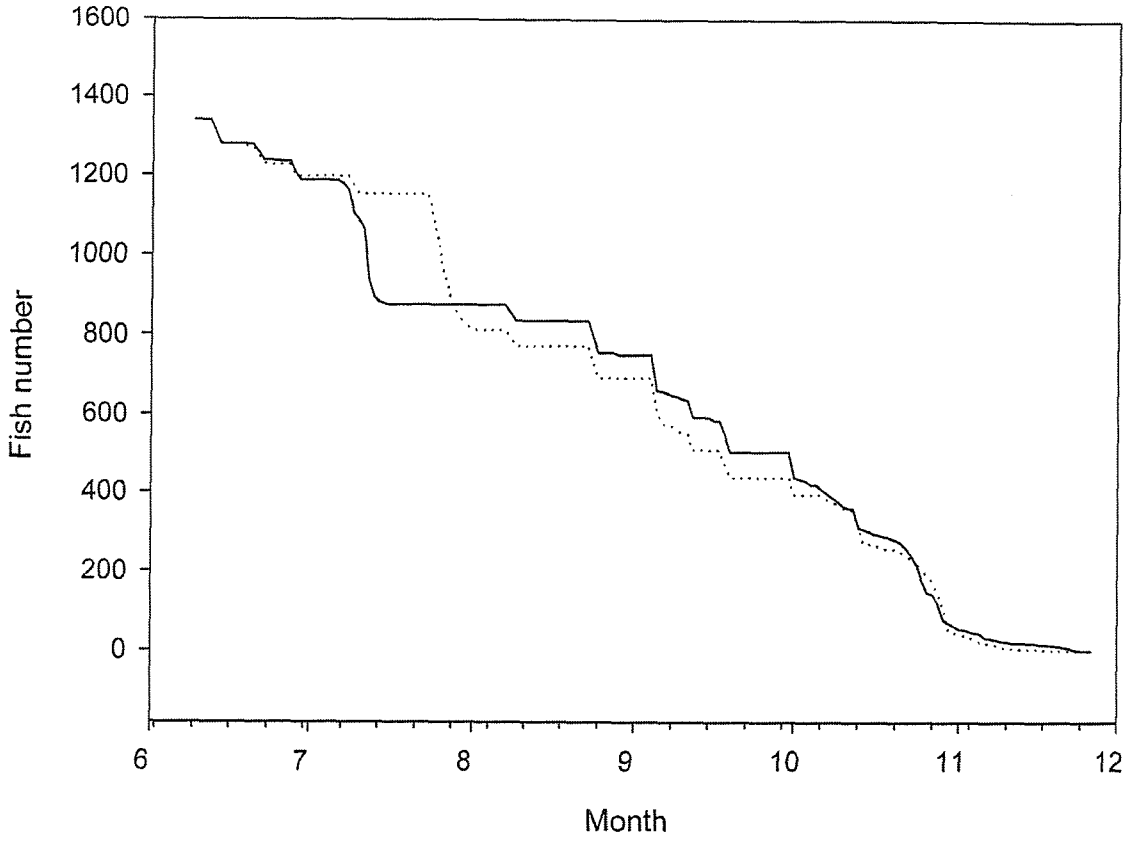

Figure IV-19. The number of survival in control (—) and CR (---) ayu. No significant difference was observed.

CHAPTER V

ROLES OF LEPTIN IN POST-SPAWNING DEATH OF AYU (*Plecoglossus altivelis*)

1. INTRODUCTION

In general fish have 5-7 years of life span, while the life span of some salmonids fish is only one year as well as ayu (*Plecoglossus altivelis*) a common freshwater fish in East Asia. The determinants for such a short life span are still ambiguous but probably involved in spawning and some accompanied changes in hormonal homeostasis. It is one of the accompanied changes that feeding activity of the ayu decreases during spawning and after spawning. The mechanisms for the decrease in appetite are also unclear and the decrease might be in part responsible for such a short life span of ayu.

Leptin is a hormone, the Lep^{ob} gene product, which is involved in the regulation of food intake and body weight homeostasis (Zhang et al., 1994) but also in very different function such as reproduction, immune response, and haematopoiesis (Cioffi et al., 1996; Loffreda et al., 1998). Leptin also plays important roles in neuroendocrine signaling and reproduction (Auwerx and Staels, 1998). Although leptin or leptin receptor has not been yet characterized in fish, heterologous Southern blotting (Zhang et al., 1994) and immunological screenings (Johnson et al., 2000; Yaghoubian et al., 2001) suggested fish would also express leptin-like proteins. Although some investigators, however, stated that mammalian leptin had no marked effect in immature coho salmon (Baker et al., 2000) or catfish (Silverstein and Plisetskaya, 2000), some leptin-administration studies suggest that leptin is able to modulate the fish food intake activity and other physiological responses. Carrillo and his colleagues demonstrated that leptin stimulated luteinizing hormone (Peyon et al., 2001) and somatolactin releases (Peyon et al., 2003) in European sea bass. Weil et al. (2003) have recently revealed that the high concentration of human leptin at the pituitary level directly stimulated FSH and LH releases in female rainbow trout. Volkoff et al. (2003) have recently demonstrated that murine leptin injection reduced food intake activity of goldfish and that the leptin function was antagonized by orexin A, a food intake enhancing hormone. It is, therefore, supposed that fish also have a functional leptin system for modulating food intake activity and some physiological signalings. Investigations in rodents indicate that sex hormones may be important in determining plasma leptin. Frederich et al. (1995) found that at any given body fat content, female rats had higher leptin levels compared to male rats. In woman of reproductive age, leptin and estradiol showed similar profiles throughout the menstrual cycle (Cella et al., 2000; Mannucci et al., 1998). The primary ovarian signal responsible for regulating body weight and adiposity has been suggested to be 17 β-estradiol (Czaja et al., 1983; Wade, 1975) and it has been shown that ovaries expressed leptin receptor messenger RNA (mRNA) (Cioffi et al., 1996; Karlsson et al., 1997). The administration of leptin also antagonized ovarian hormone secretion (Zachow et al., 1999). In mammals, 17 β-estradiol regulated leptin secretion (Kikuchi, et al., 2001). However, studies on the relationships between leptin and 17

β-estradiol are limited in fish.

Prolactin (PRL) is considered as a primary an osmoregulatory hormone in fish (Manzon, 2002). Some studies also suggest that PRL may be associated with production of steroid hormones in the gonads, the onset of gonadal development, and reproductive behavior (De Ruiter et al., 1986). The result that PRL stimulated leptin secretion in mammalian (Gualillo et al., 1999) bethinks us of a possible role for PRL in the regulation of food intake. On the other hand, 17 β-estradiol enhances PRL production by directly stimulating PRL gene transcription, leading to increased synthesis of PRL mRNA and PRL (Maurer, 1982). In teleosts, it is also suggested that 17 β-estradiol is involved in expression of PRL and PRL receptor mRNA of the gilthead seabream (Cavaco et al., 2003). It is, therefore, possible that PRL and 17 β-estradiol would also affect leptin regulation in ayu.

The actions of leptin are antagonized by another recently discovered peptide ghrelin (Shintani et al., 2001) secreted in the stomach and the hypothalamus of mammals (Date et al., 2000; Kojima et al., 1999). Ghrelin mRNA expression has been detected mainly in the stomach and shows low levels in the hypothalamus, pituitary, kidney, and placenta (Cowley, et al., 2003; Horvath et al., 2001; Kojima et al., 1999, 2001). Evidence in mammals suggests that, in addition to regulating growth hormone (GH) release, ghrelin produced in the stomach has a variety of regulatory actions in the brain and the periphery, which include energy balance (Cowley, et al., 2003; Horvath et al., 2001), regulation of gastrointestinal motility (Date et al., 2001), and feeding behavior (Nakazato et al., 2001; Unniappan et al., 2002; Wren et al., 2000). Only recently ghrelin has been identified in the bullfrog, chicken, and goldfish (Kaiya et al., 2001, 2002; Unniappan et al., 2002), hence, little is known about its role and regulatory mechanism in teleost.

The present study was carried out in order to reveal the relationships among leptin, ghrelin and appetite of ayu and to investigate whether plasma leptin was related to 17 β-estradiol and PRL. It was also investigated whether plasma ghrelin stimulated GH secretion in ayu.

2. MATERIALS AND METHODS

2.1. Fish

About 100 individuals of healthy ayu (*Plecoglossus altivelis*) were reared from hatch to death in the Tochigi Prefectural Fisheries Experimental Station, Japan. Until used in experiments, fish were stocked in outdoor ponds (14.8 m^2, 0.9 m in depth), where water was supplied from a natural stream. The fish were fed with commercial pellets for ayu (Oriental Yeast Co., Ltd., Tokyo, Japan) by an automatic feeding machine four times per

day. From our preliminary study, the hormones concerned in the present study, 17 β-estradiol, PRL and leptin, showed no significant change until July. Fish were, then, sacrificed before spawning in August (BS group), during spawning in October (DS group), and after spawning in November (AS group). In October to November, we determined sex of fish and further divided fish into two groups for each sex. The ovaries were excised out and the gonadosomatic index (GSI, gonadal weight/body weight × 100) of the fish were calculated.

2.2. Sampling

Blood was collected from caudal vessels of ayu with plastic syringes containing EDTA 2Na (50 mg/ml). Blood was centrifuged at 700 *g* for 5 min 4 °C to separate plasma from red blood cells. Plasma was frozen for subsequent assays of leptin and other hormone concentration.

2.3. Plasma leptin measurement

Leptin protein concentration in ayu plasma was measured by ELISA in 96-well microtiter plates (Costar, Corning, NY). After incubation for one hour at room temperature, wells were blocked with 150 μl of 5 % skimmed milk at room temperature for one hour. After washing the plate four times with Tris-buffered saline containing 0.5 % polyoxyethylene (20) sorbitan monolaurate, 100 μl rabbit anti-mouse leptin polyclonal antibody (1:500 dilution of stock, Chemicon international) was added to the wells and incubated for one hour. After washing, the wells were incubated with 100 μl peroxidase-conjugated goat anti-rabbit IgG (H+L) (Pierce, USA) at room temperature for one hour, followed by washing and development with 100 μl SIGMA FAST *o*-phenylenediamine dihydrochloride tablet sets (SIGMA, Japan). The reaction was stopped by adding 50 μl of 2 N H_2SO_4 and the absorbance at 490 nm was measured by a microplate reader. The rabbit anti-mouse leptin polyclonal antibody used in the present study successfully and dose-dependently detected the protein of about 15 k in molecular weight corresponding to the mouse leptin of 16 k in SDS-PAGE and subsequent Western blotting analysis as described by Johnson et al. (2000) (data not shown).

2.4. Plasma 17 β-estradiol and PRL measurement

Nine individuals of ayu for each group were separately used for measurements of plasma 17 β-estradiol and PRL. The Estradiol EIA Kit (Cayman Chemical Company, USA) was used to evaluate 17 β-estradiol concentrations. The plasma prolactin

concentrations were determined with the prolactin enzyme immunoassay kit (SPI-BIO, France).

2.5. Plasma ghrelin and GH measurement

Plasma sample for ghrelin assay earned from ayu immediately treated with 1/10 volume of 1 mol/L HCL. Samples were kept -80 °C until used. The Active Ghrelin ELISA Kit (Mitsubishi Kagaku Medical, Inc., Japan) was used to evaluate active ghrelin concentrations. The plasma GH concentrations were determined with the growth hormone enzyme immunoassay kit (SPI-BIO, France).

2.6. Statistical analyses

Data obtained were analyzed using one-way ANOVA.

3. RESULTS

3.1. Fish growth

Total length and body weight of the ayu, reared in the Tochigi Prefectural Fisheries Experimental Station, is shown in Table V-1. Fish were immature on the before-spawning stage and their mean GSI (Figure V-1) was low, 0.4 ± 0.2 %. In during spawning stages, the mean GSI increased and the value of female and male ayu reached the maximal level of 18.6 ± 3.3 %, 9.2 ± 1.5 % ($P<0.01$), respectively. In November, the body weight and gonadal weight slightly decreased and all individuals died off., Some female individuals died, though they still held eggs. There were significant differences between male and female in during- and after-spawning stages ($P<0.01$).

3.2. Plasma leptin levels at different sexual stages of ayu

Ayu plasma leptin levels in three different stages were shown in Figure V-2. There were significant differences between male and female in during- and after-spawning stages ($P<0.01$). In the DS and AS groups, leptin concentrations of female ayu were 11.8±4.2 ng/ml and 13.4±8.9 ng/ml, respectively. These levels were significantly higher ($P<0.01$) than that of the BS group. The leptin concentration in male of the DS group was 2.4±1.4 ng/ml, significantly higher than that of the BS group, 0.5±0.5 ($P<0.05$). In the AS group, the leptin level in male slightly decreased into 1.6±1.0 ng/ml, but significantly higher than

that of the BS group ($P<0.01$).

3.3. 17 β-Estradiol and prolactin in different stages

Figure V-3 shows 17 β-estradiol levels in three different stages of ayu. 17 β-estradiol concentrations in the DS and AS groups for female ayu were 320.8±94.6 pg/ml and 305.8±4.6 pg/ml, respectively. These levels were significantly higher compared with other groups ($P<0.01$). There were significant differences between male and female in both the DS and AS groups ($P<0.01$).

Plasma PRL levels were at the maximal level during spawning season (Figure V-4). In the DS groups, the level for female and male ayu were 9.7±2.3 ng/ml and 6.6±0.1 ng/ml, respectively. They were significantly higher ($P<0.01$) in the BS and AS groups. No significant difference was observed between male and female on each month.

3.4. Plasma ghrelin levels and GH concentrations at different sexual stages of ayu

Ayu plasma ghrelin levels in three different stages were shown in Figure V-5. There were significant differences between male and female in during-spawning stages ($P<0.01$). In the AS groups, ghrelin concentrations of two sexes of ayu were 2.7±0.1 fmol/ml and 2.8±0.1 fmol/ml, respectively. These levels were significantly lower ($P<0.01$) than that of the BS and female of DS group. The ghrelin concentration in female of the DS group was 3.8±0.2 fmol/ml, significantly higher than that of the BS group, 3.4±0.1 ($P<0.05$), and that of male of DS and AS group ($P<0.01$).

Figure V-6 shows plasma GH levels in three different stages of ayu. In the male of AS group, the level was 1.7±1.4 ng/ml. It was significantly lower ($P<0.05$) in the BS and female of DS. Significant difference ($P<0.05$) was observed between male and female on AS group.

4. DISCUSSION

The results of the present study provided the first description of the profiles of plasma leptin concentrations around spawning period of ayu and its relation to spontaneous seasonal changes in 17 β-estradiol and PRL.

Although reproductive processes of organisms are among the most energetically expensive, the feeding activity of ayu decreases during spawning. As shown in the Result section, the leptin levels of ayu were significantly high during and after spawning. If leptin has functions in fish similar to those in mammals, ayu would not recover the appetite for

maintaining their biological activities after spawning. Mustonen et al. (2002) reported that relatively long-life fish such as burbot had not increasing leptin levels in during spawning season of male. However, in the present study, leptin levels of male ayu increased significantly during spawning. Female burbot showed comparatively low leptin levels before and during spawning and increased significantly after the spawning period (approximately 3.0 ng/ml). On the other hand, comparing leptin levels between before- and after- spawning stages, the AS/BS leptin ratio was 24.4 for female ayu and 3.0 for male ayu, respectively. These values were much higher than 1.6 for burbot (calculated from the data of Mustonen et al., 2002). Thus, it is quite likely that both sexes of ayu are exposed to drastic increases in leptin levels around spawning period. Food intake in rats decreased with increasing leptin concentration, more than 3.5 ng/ml (Koopmans et al., 1998), while food intake of woodchucks decreased with increase in leptin more than 0.4 ng/ml (Concannon et al., 2001). Although the relationship between fish food intake and leptin is not well understood, leptin-induced reduction of appetite in these seasons might be one of reasons why almost ayu dye after the first spawning. An additional administration experiment with leptin for ayu will confirm this hypothesis in future.

It is well established that leptin is a hormone involved not only in regulation of body weight and metabolism, but in reproductive function as well in mammals. The mutant Lep^{ob}/Lep^{ob} female mouse, which does not produce an active form of leptin, has been shown to be acyclic and sterile (Chehab et al., 1996). Administration of recombinant leptin to these animals fully restores their fertility, indicating that leptin may be involved in regulating reproductive function. During the process of spermatogenesis and ovogenesis, human leptin stimulated FSH and LH release of rainbow trout pituitary cells (Weil et al., 2003). On the other hand, it has been suggested that 17 β-estradiol is the primary ovarian signal responsible for regulating body weight and adiposity in mammals (Pelleymounter et al., 1999; Schwarz et al., 1981). In this study, 17 β-estradiol showed profiles similar to leptin and significantly higher levels during spawning season as shown in Figure V-3. There were remarkable sexual differences in circulating leptin levels in accordance with 17 β-estradiol concentrations. Our data probably addresses that 17 β-estradiol could directly and/or indirectly affect leptin secretion also in ayu.

Okuzawa et al. (2003) reported that the 17 β-estradiol levels of female red seabream increased duirng vitellogenesis period, followed by rapid decrease after spawning. The plasma 17 β-estradiol levels of Lusitanian toadfish increased with gonadal growth, reached a maximum level a month before the start of spawning and then declined quickly (Modesto and Canário, 2003). On the other hand, as shown in this study, 17 β-estradiol in ayu was maintained at a high level in the after-spawning stage. This tonic phase in 17 β-estradiol level after spawning differentiates ayu from above other species able to spawn several times. The fish ayu would fail to recover appetite after spawning by force of the high leptin

secretion induced by the high level of 17 β-estradiol.

Despite the well-known roles of PRL in the reproductive physiology of higher vertebrates, most of the identified hormonal functions in fish are around the hydromineral homeostasis (Manzon, 2002). In female rats, it was reported that PRL boosted white adipose tissue leptin mRNA and plasma leptin levels *in vivo* (Guallilo et al., 1999). In the present study, plasma PRL levels increased at the maximal level during spawning season in both sexes ayu (shown in Figure V-4), and leptin levels also increased during spawning season. It is likely that PRL would also induce leptin secretion in fish.

A ghrelin-like ligand was detectable in the blood of a teleost as predicted by Shepherd et al. (2000). The concentrations of ghrelin in ayu plasma were quite low after spawning. The function of the ghrelin in the physiology of ayu remains an enigma. In rodents, exogenous ghrelin stimulates appetite and GH excretion (Kojima et al., 1999; Tschöp et al., 2000). A ghrelin-induced stimulation of GH secretion has also been observed in immature chicks (Ahmed and Harvey, 2002) and bullfrogs (Kaiya et al., 2001). In this study, GH concentration showed profiles similar to ghrelin and significantly lower levels after spawning season as shown in Figure V-6. If ghrelin is also able to increase appetite of teleost, ayu cannot increase the appetite after spawning, because of low quantities of gherlin levels at postspawing. Mustonen et al. (2002) reported that relatively long-life fish such as burbot had increasing ghrelin levels in after spawning season and the burbot consume great quantities of food after spawning (McCrimmon and Devitt, 1954). The high ghrelin levels could also function to increase the appetite of the spawned animals, a possible participation of ghrelin in teleost reproduction cannot be excluded. Therefore, it also considered that ayu would fail to recover appetite after spawning by low ghrelin secretion.

The present study suggests that the rising 17 β-estradiol levels and the increasing in PRL secretion with maturity would induce plasma leptin secretion in ayu. Moreover, it supposed that lower ghrelin levels at after spawning season decrease appetite of ayu. The inability to reduce the leptin level and to increase ghrelin level into the basal and to recover appetite after spawning would be in part responsible for a short life span of ayu.

This study attempted to disclose hematologically the relationships among the life span, appetite, leptin, 17 β-estradiol and PRL, ghrelin and GH homeostases in ayu and now investigate the 17 β-estradiol modulation manners of leptin secretion using estrogen receptor antagonists such as tamoxifen, in future revealing the governing mechanisms of life span in ayu.

ABSTRACT

It is well known that ayu (*Plecoglossus altivelis*) dye after spawning and the life span is only one year. The determinants for such a short life span are probably involved in spawning and some accompanied changes in hormonal homeostases. It is one of the accompanied changes that feeding activity of ayu decreases during spawning and after spawning. Then, it was investigated the relationships among leptin and ghrelin, they are regulators for food intake, and other major hormones, 17 β-estradiol and prolactin. Leptin levels were significantly higher during spawning, associated with decrease in appetite. Leptin levels were also synchronized with levels of 17 β-estradiol and prolactin. Ghrelin levels were no significant difference. Therefore, one possible explanation for decrease in appetite during ayu spawning is that the alteration of 17 β-estradiol homeostasis induced the secretion of leptin. The inability to recover the leptin level into the basal after spawning would be in part responsible for a short life span of ayu.

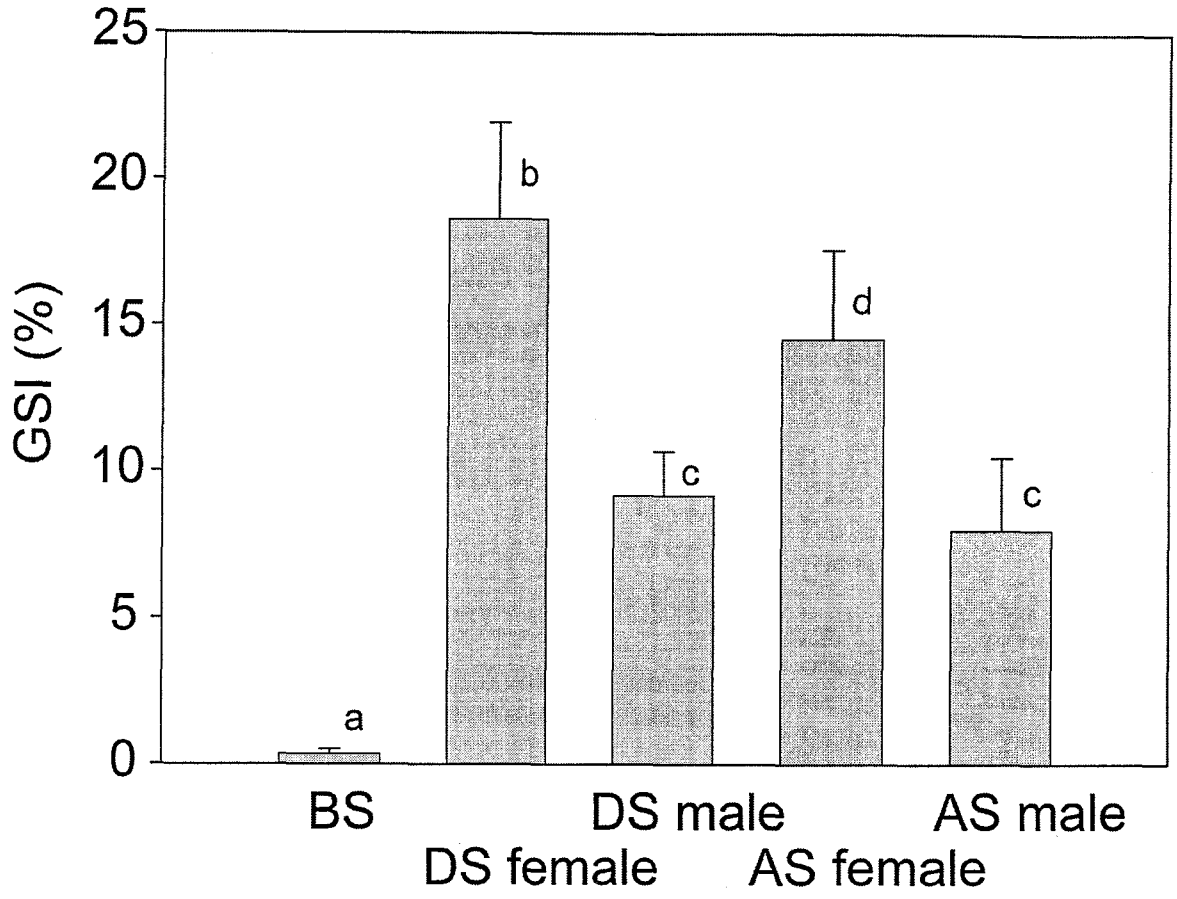

Figure V-1. Gonadosomatic index (GSI) in three maturation stages of ayu. The mean values were represented with standard deviation (n=20). The different characters represent significant differences ($P<0.01$).

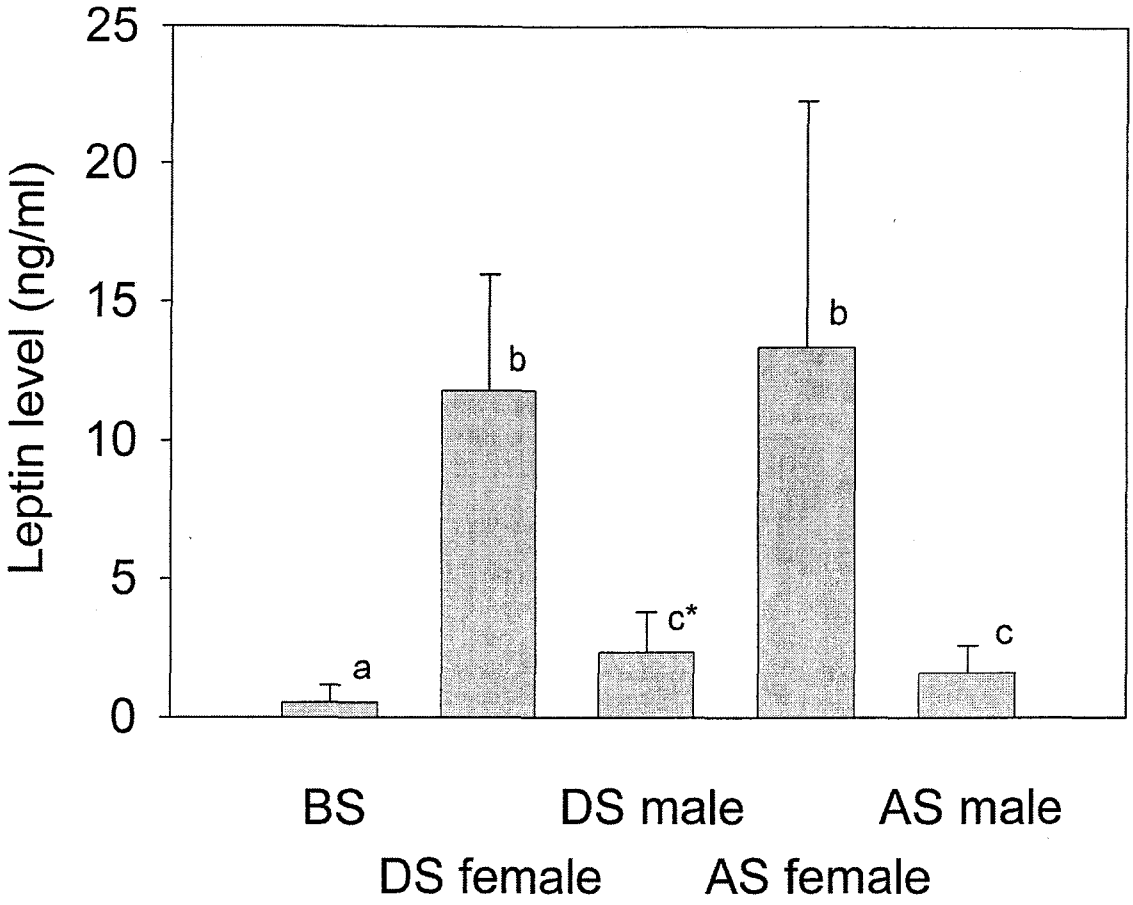

Figure V-2. Plasma leptin levels in three maturation stages of ayu. The mean values were represented with standard deviation (n=7). The different characters represent significant differences ($P<0.01$) except for the BS and DS male groups (*, $P<0.05$).

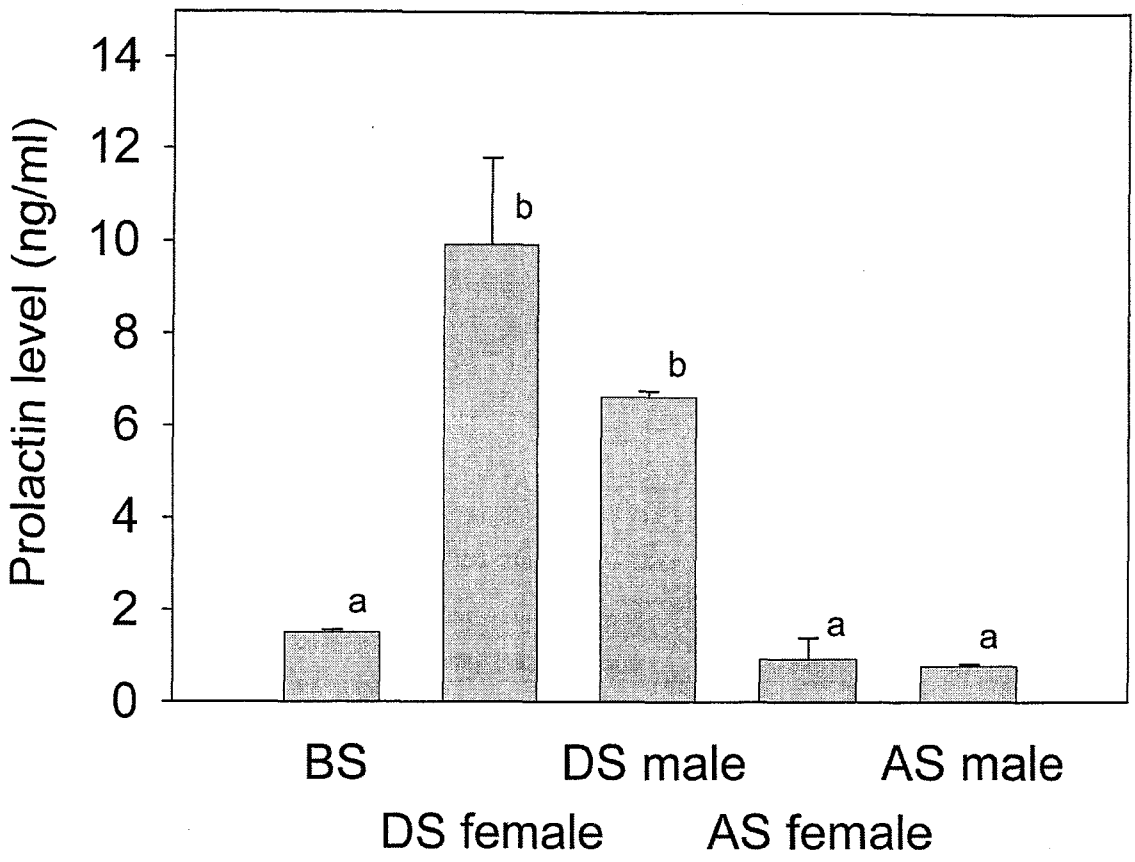

Figure V-4. Plasma prolactin levels in three maturation stages of ayu. The mean values were represented with standard deviation (n=9). The different characters represent significant differences ($P<0.01$).

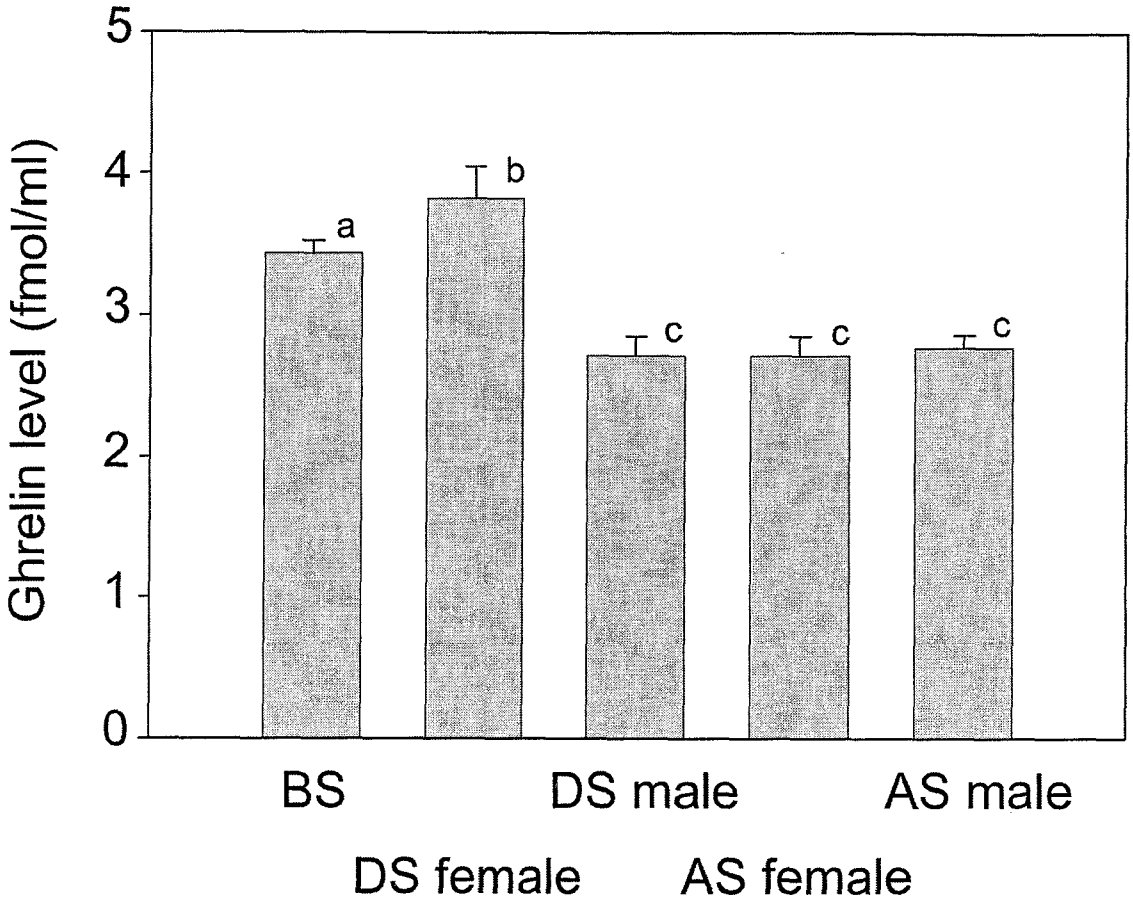

Figure V-5. Plasma ghrelin levels in three maturation stages of ayu. The mean values were represented with standard deviation (n=5). The different characters represent significant differences ($P<0.01$) except for the BS and DS female groups (a to b, $P<0.05$).

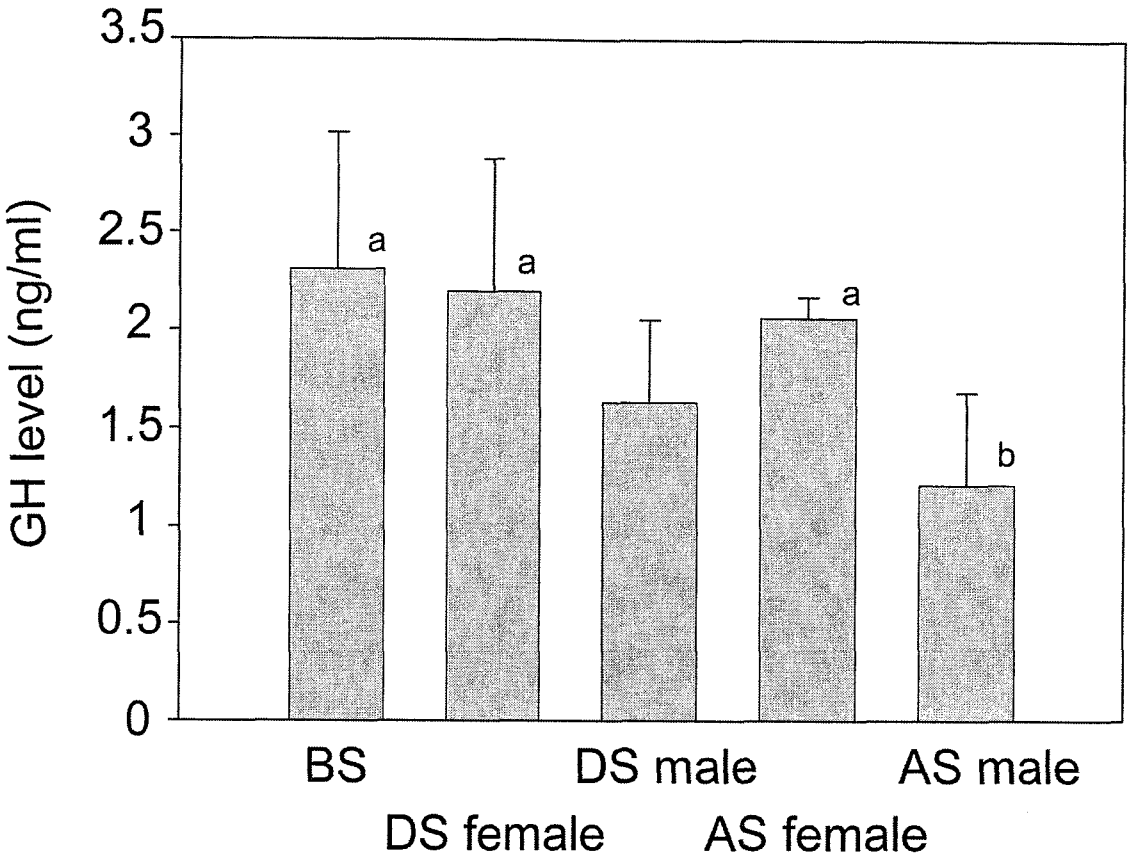

Figure V-6. Plasma growth hormone levels in three maturation stages of ayu. The mean values were represented with standard deviation (n=4). The different characters represent significant differences ($P<0.05$).

CHAPTER VI

GENERAL DISCUSSION

Ayu (*Plecoglossus altivelis*) is the most popular freshwater fish in Japan. The life span of ayu is only one year. They spawn in a river from late September to early November and die after spawning. Hatched larvae go down to the sea (catadromous migration) and winter there. The anadromous run of wild ayu juveniles begins from coast around early April and is over by early July. Soon after, they mature, spawn and then die after spawning (Figure 1).

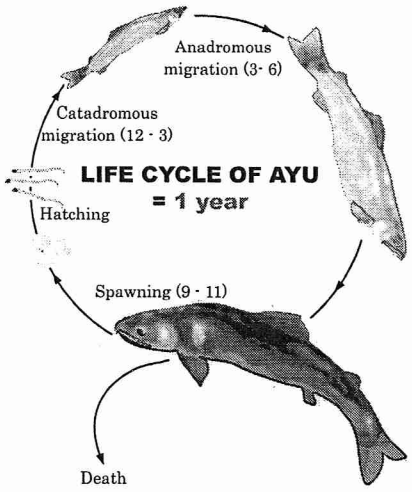

Figure 1. Life cycle of ayu

In recent decades, the study of aging has expanded rapidly both in depth and in breadth. Biological, epidemiologic, and demographic data have generated a number of theories that attempt to identify a cause or process to explain aging and its inevitable consequence, death. However, in recent years, the search for a single cause of aging, such as a single gene or the decline of key body system, has been replaced by the view of aging as an extremely complex, multifactorial processes (Kowald and Kirkwood, 1996). Several processes may interact simultaneously and may be operated at many levels of functional organization (Franceschi et al., 2000). Similarly, different theories of aging are not mutually exclusive and may adequately describe some or all features of the normal aging process alone or in combination with other theories. The definition of aging itself is open to various interpretations (Sacher, 1982). Aging is presented as an ontogenetic issue; the process of growing old and/or the sum of all changes, such as physiological, genetic, and molecular changes, that occur with the passage of time from fertilization to death. Because of aging is characterized by the declining ability to respond to stress and by increasing homeostatic imbalance through an incidence of pathology, death remains the ultimate consequence of aging. Theories to explain aging processes have been grouped into several categories, and some of the most widely used are the programmed and error theories of aging. According to the "programmed" theories, aging depends on biological clocks regulating the timetable of the life span through the stages of growth, development, maturity, and old age: this regulation would depend on genes sequentially switching on and

off signals to the nervous, endocrine, and immune systems responsible for maintenance of homeostasis and for activation of defense responses. The "error" theories is identified as environmental insults to living organisms that induce progressive damage at various levels (e.g., mitochondrial DNA damage, oxygen radicals accumulation, cross-linking).

On the other hand, the telomerase activities of cells of fish are very high, and it is hard to explain in a programmed theory for fish aging. Aging is an inevitable biological process and characterized by a general decline in physiological function. Aging may be defined as the increased probability of death with the accumulation of diverse adverse changes with aging, which belongs to the "error" theory group (Harman, 1998). This is counterbalanced by repair and maintenance factors that contribute to the longevity of the organism. Oxidative stress is associated with a disturbance in the balance between pro-oxidants (ROS) and antioxidants, in favor of the pro-oxidant (Sies, 1991). Oxidative damage to DNA, proteins, and lipids accumulates with age and contributes to degenerative diseases and the aging phenomenon by disrupting cellular homeostasis (Adelman et al., 1988; Ames and Shigenaga, 1992; Ames et al., 1993; Yu and Yang, 1996). It was, indeed, found that oxidative damage to DNA and lipids accumulates with age of fish as shown in Chapter II and III.

A salient question then is why these two modes of expenditure might exert "radically" different effects on life span. There is a considerable weight of evidence that this increased oxygen consumption leads to elevated rates of oxidative stress and stress-induced damage to both protein and DNA (McArdle and Jackson, 2000; McArdle et al., 2001). This effect is consistent with a negative effect of the elevations of such activity on life span. In contrast, the function of energy demands at rest remains obscure. It is, however, widely agreed that three components contribute mostly to the resting metabolic rate (RMR): the proton leak in mitochondria (Brand, 1990, 2000; Couture and Hulbert, 1995; Poehlman et al., 1993; Porter and Brand, 1993; Rolfe and Brand, 1996, 1997), the costs of sustaining ion gradients by sodium potassium pumping (Couture and Hulbert, 1995; Poehlman et al., 1993) and protein synthesis. Within this framework there are at least two mechanisms by which elevations in RMR might be associated with decreases in oxidative damage.

Animals can reduce the levels of proton motive force by increasing the extent of uncoupling in their mitochondria. Continuous generation of ATP requires elevated oxygen consumption, although the net production of free-radical species is diminished. The animals uncouple respiration to increase their survival (Brand, 2000). This effect is diametrically opposed to the prevailing notion that increasing uncoupling should lead to an increase in free-radical production because of the elevated oxygen consumption (Ramsey et al., 2000). Another link between oxidative stress and aging has focused on mitochondria, which consume ~ 85 % of the oxygen used by the cell *in vivo* and are the greatest source of oxidants. Mitochondria supply most of the ATP necessary for cell function and contain the

only DNA outside the nucleus in mammalian cells. If the permeability of ATP accumulates in mitochondria of fish with aging like erythrocyte as shown in Chapter II, cell function may be destroyed.

Peroxidized membranes and lipid oxidation products make threat to aerobic cells. It is now widely held that cells have also developed a variety of mechanisms for maintaining membrane integrity and homeostasis by repairing oxidatively damaged components in addition to preventing initiation of peroxidation (with compounds like vitamin E). Under normal conditions, the amounts of oxy-repair mechanism in older organisms would probably be sufficient to cope with the amounts of damage produced. However, under situations of oxidative stress, there may be some portions which cannot be restored, and if such things spill over, they may not be able to induce the oxy-repair mechanism necessary to maintain homeostasis. Chapter II presents the hypothesis that the pile of these small damages may contribute to the gradual accumulation of the oxidative damages with aging. Furthermore, the ability to mount an effective response to oxidative stress may decline with aging, thus predisposing older cells and organisms to death as shown typically in Chapter III.

It was reported that oxygen consumption of ayu was 325.99 ml/kg/hr, ranging from 208.76 to 390.12 ml/kg/hr (Aliah et al., 1991). Brain tissue represents 0.1 – 1 % of the body weight of vertebrates (excluding primates) but is responsible for 1.5 – 8.5 % of the total body energy consumption in endothermic vertebrates and a comparable range (2.7 – 3.4 %) are found for ectothermic vertebrates (Van Ginneken et al., 1996). Since fish is ectothermic animal, ayu brain requires 5.64 – 13.26 ml/kg/hr. Mass-specific energy expenditures of the brain of ectothermic vertebrates are similar to that of endothermic vertebrates, suggesting that heat production plays a minor role in the brain's energy expenditure. However, neural processing is metabolically expensive. These metabolic demands could be large enough to influence the design, function and evolution of brains and behavior. Most of the brain energy consumption is used to maintain ionic gradients across plasma membranes and to restore these gradients after depolarization. In fact, approximately 50 – 60 % of the ATP consumed by the brain is devoted to its electrical activity (Hylland et al., 1997; Purdon and Rapoport, 1998). The metabolic processes involved in ATP production are the same in the nervous system as in the rest of body. Only approximately 2 % of brain glucose flux or small amounts of blood-borne precursors such as ketone bodies have been estimate to support lipid synthesis in mammalian brain (Purdon and Rapoport, 1998). However, in terms of energy, lipid synthesis and phospholipids asymmetry across brain membranes may consume a significant part of the ATP used by brain cells. Therefore, in Chapter II, lipid membrane abnormalities induce exhaustion of ATP with aging, which must give damage to brain.

Life requires membranes. Their universal occurrence in living organisms suggests that

the earliest life-forms on the planet also possessed them. Indeed, just as DNA is described as an eternal molecule, membranes might be called eternal structures, since in modern organisms new membranes arise from pre-existing membranes. Biological membranes generally consist of bilayers of amphipathic molecules held together by non-covalent bonds. In eukaryotic cells, phospholipids are the predominant membrane lipids and consist of a hydrophilic head group to which are attached hydrophobic acyl chains. These acyl chains are either saturated, monounsaturated or polyunsaturated hydrocarbon chains that normally vary 12 to 22 carbons in length. Among cellular molecules, polyunsaturated fatty acids (PUFAs) exhibit the highest sensitivity to oxidative damage. It is generally accepted that their sensitivity increases as a power function of the number of double bonds per fatty acid molecule. As both oxygen consumption and oxygen free radical production occur in mitochondrial membranes, a low degree of fatty acid unsaturation in these membranes would be advantageous, because it would reduce the sensitivity to lipid peroxidation. This would also protect other molecules against lipid peroxidation-derived damage. The influence of fatty acid unsaturation on the transition temperature and hence on membrane fluidity have been extensively studied (Brenner, 1984). Whereas strong increases in lipid fluidity are observed after introduction of the first double bonds to a saturated fatty acid, progressively smaller effects are observed after the introduction of additional double bonds (Brenner, 1984). In Chapter II, it was revealed that membrane fluidity was decreased accompanied with decrease PUFA composition. This is because when a double bond is added near the center of the fatty acid chain (first double bond added) the impact on fluidity through the kink (or coiling) of the fatty acyl chain is much larger than when it is added nearer to its extremes (subsequent double bonds added). Many studies have shown that free radical damage and lipid peroxidation increases as a function of the degree of unsaturation of the fatty acid substrate present in the tissues *in vivo* (Bondy and Marwah, 1995; North et al., 1994). A modification of fatty acid unsaturation and oxidative damage in membrane occurs during aging can be prevented by food restriction (Laganiere and Yu, 1987, 1989a, 1993; Yu et al., 1992). Physiological treatments that extend lifespan can also give insight into the mechanisms underlying aging. Calorie restriction is the only physiological treatment known to extend life span in a wide range of animals (Sohal and Weindruch, 1996). During caloric restriction, metabolic rate is not reduced but there is a substantial decrease in lipid peroxidation in rats. This is not attributable to changes in membrane vitamin E content but is associated with changes in membrane acyl composition of both mitochondria and microsomes, resulting in a decreased susceptibility of these membrane bilayers to lipid peroxidation (Langaniere and Yu, 1987). Caloric restriction also modifies acyl composition of muscle membrane (Cefalu et al., 2000), as well as both phosphatidylcholine and phosphatidylethanolamine in liver (Leon et al., 2001), thus decreasing their ability to undergo lipid peroxidation. Although this thesis includes no

research for membrane in CR study, it is likely that there are something changes in membrane lipid environment by CR. Further studies along these lines are required.

One mechanism responsible for life span extension with caloric restriction (CR) would involve reduction in reactive oxygen species (ROS) production. CR has been shown to inhibit or delay age-related increases in oxidatively damaged proteins (Sohal et al., 1994), DNA (Kaneko et al., 1997), and lipids (Lass et al., 1998). The cellular changes were responses for this decrease in oxidative damages. In the present study, oxidative damage to nuclear DNA was investigated. In Chapter IV, DNA damage in brain and liver were elevated with ayu aging, but the value of 8-OHdG did not decrease by caloric restriction, inconsistent with the previous reports for mammals and insects. Since ayu are inherently exposed to the high level of endogenous ROS (Moritomo et al., 2003), even CR could not afford to reduce the DNA damage. Calorie-restricted feeding induces a change in the composition of the polyunsaturated fatty acid composition of mitochondrial and cellular membranes (Laganiere and Yu, 1987). Other reports may also explain the enhanced resistance to peroxidation damage with time (Laganiere and Yu, 1989a, b; 1993, Laganiere and Fernandes, 1991). It was also proposed that the changes in membrane structure were accompanied with decreased plasma concentrations of T_3 and insulin induced by a homeostatic response to a low energy diet (Herlihy et al., 1990; Wang et al., 1997). These two hormones are recognized to exert the expression of a number of lipid desaturase enzymes (Brenner, 1990; Wagner et al., 1994; Hulbert, 2000) and probably alter the physico-chemical properties of membrane. Chapter IV showed that CR reduced the testosterone level, which is well-consistent with the observation by Klibanski et al. (1981) that fasting decreased testosterone concentration in human. However, such changes in plasma hormone concentrations associated with CR in ayu would not affect the standard metabolic rate in ayu and endogenous ROS production and ROS accumlation.

Progesterone and 17β-estradiol levels in CR ayu were relatively higher compared to the control ayu as shown in Chapter IV. Estrogens have been shown to be powerful antioxidants, effectively preventing lipid peroxidation (Ayres et al., 1996; Maziere et al., 1991; Subbiah et al., 1993). Ayres et al. (1998) suggested that 17β-estradiol might prevent the oxidative DNA damage to some extent by inhibiting the formation of superoxides. The *in vivo* significance of this finding deserves some discussion in view of a previous report stating that 17β-estradiol decreases apoptosis of endothelial cells (Alvarez et al., 1997). In cellular apoptosis, the Bcl-2 gene plays a central role, and a variety of stimuli such as oxidants, toxins, oncogenes, and some growth factors can modulate expression of this gene (Thompson, 1995). 17β-Estradiol is known to modulate the transcription of a number of genes through their binding to cytosolic estrogen receptors, which translocate to nucleus. The receptor/estrogen complex binds to specific palidromin DNA targets (Brann et al., 1995). It is possible that, in this way, 17β-estradiol can directly or indirectly modulate

Bcl-2 expression. In amyotrophic lateral sclerosis, cell death is considered to be due to a mutation in SOD, causing inability to handle oxygen radicals (Rosen et al., 1993). *In vitro* superoxide-related cell death can be corrected by antioxidants (Vaca et al., 1988). Therefore, it is possible that the ability of estrogens to decrease might have some *in vivo* significance in term of apoptosis. In Chapter IV, every caspase activity of CR ayu was relatively low compared with control ayu. It is suggested that cellular caspase-induced apoptosis might be controllable by high secretion of 17β-estradiol by CR. As shown in Chapter V, ayu would, however, fail to recover appetite after spawning by force of the high leptin secretion induced by the high level of 17β-estradiol. Therefore, 17β-estradiol induced physical anorexia in ayu would offset the longevity by CR, although CR causes longevity in mammals. Leptin would appear to play a rple in relaying metabolic information to the reproductive axis, but the mechanisms by which this is accomplished remains unknown.

Physical activity in general declines through the life span and the decline is associated with a physiological anorexia. There are, however, minimal changes in extraction of energy from food with aging. After maturation, ingested resources are diverted from somatic growth to gonadal growth, resulting that growth increments are reduced after maturation. The author would like, if you allow me, to assume that fish lose weight, but never lose length and that fish with 20 % decrease of its maximum weight starve to death on the basis of a very conservative condition for starvation proposed by Mangel and Abrahams (2001). In November, control female ayu lost 5 % of their maximum weight, while control male, CR femail and mail ayu lost 18, 24, 36 % of their maximum weight, respectively as described in Chapter IV. Physiological anorexia in ayu after spawning may outstrip the reduction of physical activity, leading to weight loss and to death. Although data are limited, there are clear-cut directions in which future studies should be directed, studies of the role of reproductive hormones (17β-estradiol, testosterone and progesterone) on energy intake and metabolism with aging.

Leptin has been proposed as a physiological link between nutritional status and reproductive maturation and function and may be potentially served as a trigger or metabolic gate for sexual development (Campfield et al., 1995; Cunningham et al., 1999; Foster and Nagatani, 1999). It is well recognized that obesity in humans in associated with high blood pressure (Landsberg, 1986). It was reported that at least two pathways were involved in cardiovascular effects of caloric restriction: one dependent on leptin signaling and the other independent on the leptin axis (Swoap, 2001). In addition to its well-studied role in maintenance of body and fat mass, leptin may be important for the regulation of blood pressure via altering sympathetic nervous system (SNS) outflow. Fasting reduces the plasma leptin concentration and concomitantly suppresses gonadal, somatotropic, and thyroid hormones; however, fasting also increases plasma glucocorticoid levels.

Administration of exogenous leptin in fasting rats and mice reverses the fasting-induced hormonal state (Ahima et al., 1996). In rodents, secretion of leptin from adipocytes appears to be dually regulated (Schwartz et al., 2000). Leptin secretion is primarily related to body adipose levels; leptin gene expression and fasting plasma concentrations are positively correlated with the percentage of body fat. Although it was not measured leptin levels in Chapter IV, 17β-estradiol levels were relatively high in CR ayu. Therefore, leptin levels of during and after spawning ayu might be similar to those of ayu in Chapter V. This leads to one hypothesis that CR-induced increase in circulating leptin does not cause higher SNS outflow, then higher blood pressure in teleost, unlike mammals and that during and after spawning ayu RBC might hardly go through microcirculation and to perform satisfactory oxygen supply as partially oxidized RBC in Chapter II.

It is generally accepted that longevity can evolve only in situations in which background mortality rates are sufficiently low so that individuals can live to long ages without high probability of accidental death. Clearly, if the mortality rates are too high, then individuals simply do not have the opportunity to develop mechanisms for longevity. On the other hand, if the rate are too low, and competitors thus sufficiency abundant, individuals will lack the opportunity to grow into size large enough to aging. In the case of ayu the situation is more complicated than such a case. A window of background mortality rates exists and, even then, the ecological environment plays an important role. I would, thus, like to hypothesize that the ecological mechanisms described here provide the milieu in which a biological adaptation for short life occurred. Ayu can be understood by neither ecology/evolution alone nor cell biology alone. The interaction between the two leagues is essential to understand ayu. However, the result that a life was not prolonged even if calorie restrictions, it turns out that short-lived ayu might be important for an aquatic ecosystem. The switch to generations allows new individuals to take advantage of a new niche that is more energetically rewarding because of not continuing the niche occupied by parents. By focusing on the adult ayu, it is suggested that a significant cost is associated with this switch. Of particular interest is the cost associated with this switch. I proposed that reproduction is the beginning of death because maturity is often viewed as the onset of senescence. Of course, relatively short life means that even if ayu survive within a river, and they will be present for a relatively short time and therefore not obtain sufficient food.

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