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－Information－

**Hiroshima University has granted the Doctor's degree to the following researchers.
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Characteristics of the rate-pressure product during dynamic and static muscle contraction in normotensive men

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正常血圧者における動的および静的筋収縮時のRate-pressure productの特性

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Physiological meanings of the rate-pressure product (RPP), given by systolic blood pressure (SBP) times heart rate (HR), has become a useful and practical measurement to predict the oxygen consumption (VO_2) of the heart without trespass. RPP is also considered to be one of the best indices for heart muscle tension, since it is directly related to the VO_2 of heart muscle. RPP is used more and more for evaluation of heart function trouble and cardiovascular heart disease in medicine. Normal HR and SBP responses to static and dynamic exercise have been previously documented. Furthermore, the difference of these responses between the young and the elderly were also widely investigated. However, the normative responses of resting RPP, submaximal exercise RPP, and maximal exercise RPP have not been reported. In addition, there is little literature to investigate the relationship between RPP and physiological functions that effect the regulation of HR and SBP.
The first objective of my dissertation research was, therefore, to develop the normative RPP values during rest, static and dynamic exercises and to clarify the difference of RPP between the young and the elderly. The second objective was to examine the relationship between RPP and respiratory function and activity level of autonomic nervous system that effect the regulation of HR and SBP during static and dynamic exercise.

Change in the rate-pressure product during stepwise incremental exercise

The purpose of this investigation was to observe the change in SBP and RPP with increases in the intensity of exercise, and to determine if there is a relation between RPP and the pattern of increase in pulmonary ventilation (Ve). Eleven young male adults participated in this study. The subjects performed graded bicycling exercise increasing 20 watts every 2 min from 0 watts until the HR of 170 beats･min-1. During exercise SBP, HR and Ve were continuously measured. SBP gradually increased with the increase in workload, and then when the intensity of exercise became even stronger, the rate of increase became lower. On the other hand, the increase in HR was very small during the initial 5 min of exercise and when the intensity of exercise increased, the rate of increase in HR became higher. The polygonal regression analyses on the relation of RPP and Ve to elapsed time revealed clear break-points for both. On average, the break-point of RPP was 6.6 min (56 watts), whereas that of Ve was 10.8 min (100 watts); thus, a clear and significant difference was recognized. These results clearly showed that the break-point with an increase in workload appeared

even when the workload was relatively low, and did not relate to that of Ve during exercise.

The difference in the rate-pressure product between the elderly and the young in dynamic exercise

The purpose of this study was to investigate the difference in RPP between the young and the elderly during dynamic submaximal exercise. The subjects were six young and six older asymptomatic males. The subjects performed two bouts of exercise with workloads of 30 and 60 watts using a bicycle ergometer. During exercise, HR, BP, Ve, and VO_2 were continuously measured and RPP were calculated by HR and SBP. In addition, power spectrum analysis of R-R was performed to obtain the low frequency power (LF), the high frequency power (HF) and the ratio of LF to HF (LF/HF). The HRs of the elderly were significantly lower than those of the young during exercise at both 30 and 60 watts, whereas the elderly showed greater SBP than the young. As a result, there was no difference in RPP between the young and the elderly during exercise. These results suggested that the myocardial strain of the elderly was not different from the young when the workloads were relatively low.

Rate-pressure product and heart rate variability during maximal static exercise

The objective of this investigation was to monitor the change in RPP with time during maximal isometric exercise in normotensive young men. Furthermore, this chapter compared heart rate variability (HRV) using power spectral analysis to clarify the influence of contraction time on cardiac autonomic balance. Fourteen healthy normotensive students participated in this study. Each subject stood upright, contracting the biceps brachii against a load (4 kg) applied to the wrist downwards with the elbow at an angle of 90^{o} until exhaustion. SBP was measured at rest and every 2 min during exercise. The R-R intervals of the electrocardiogram (ECG) were measured and spectral analysis of HRV was performed by a fast Fourier transformation method. The standard deviation of R-R interval (SDNN), LF, HF and LF/HF were calculated. The RPP cuvilinearly increased with exercise, basically the same as responses of HR and SBP. The RPP at exhaustion increased 131 % from that at the start, whereas the HR and SBP increased only 111 % and 118 %, respectively. SDNN, HF and LF/HF remained unchanged during the maximal isometric exercise. These results suggested that the balance of the sympathetic nervous system and parasympathetic nervous system activity did not alter from the beginning to exhaustion during isometric exercise.

The difference in the rate-pressure product during static exercise in the elderly and the young

The purpose of this study was to clarify the difference in RPP, between the elderly (mean age of 66.4 years) and the young (mean age of 24.8 years) during isometric muscle contraction. Each subject maintained a 1, 3 or 5 kg weight applied to the wrist with the elbow flexed at about 90 degrees for two minutes in a sitting and standing positions, and the arterial pressure and HR were intermittently measured before, during and after the exercise. The elderly showed a lower HR during isometric exercise than the young. On the other hand, the SBP of the elderly was greater than that of the young and at a 5 kg workload a significant difference was observed ($P<0.05$). The young showed no significant increase in RPP between 1 and 3 kg, while for the elderly the RPP at 3 and 5 kg significantly increased compared to that for 1 kg ($P<0.05$). When comparing the RPP of the elderly to that of the young, there were no significant differences in RPP for any workload. These

results indicate that the similar RPP observed for the elderly and the young was due to the opposing response of HR and SBP associated with aging.

General Discussion

The pattern of increase in RPP during stepwise incremental dynamic exercise was not similar to that of respiratory function. Furthermore, the point of accelerating RPP during the exercise was not related to that of ventilatory threshold. These results suggest that the increase pattern had nothing to do with the anaerobic threshold. During maximal static exercise, RPP remained unchanged while SBP was between 120 - 170 mmHg, increasing exponentially until exhaustion with the increase in SBP. During both dynamic and static exercise, there were no differences in RPP between the young and the elderly. This was attributable to the opposite response patterns of HR and SBP in the two groups. Namely, the young showed a higher HR and lower SBP during dynamic exercises. In contrast to the young, the elderly demonstrated a more marked increase in SBP with lower HR. These physiological characteristics of the elderly were considered to be due to functional changes associated with aging.

Key words: rate-pressure product, heart rate, systolic blood pressure, static and dynamic exercise, elderly people

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Studies on the structure and expression of group IB phospholipase A_2 isoforms in the red sea bream

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マダイのIB型ホスホリパーゼA2アイソフォームの構造と発現に関する研究

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Phospholipase A_2 (phosphatide 2-acyl hydrolase, EC 3.1.1.4) (PLA_2) hydrolyzes the fatty-acyl ester bond at the sn-2 position of glycerophospholipids. PLA_2 has now become a large superfamily of distinct enzymes that play a central role in diverse cellular processes including phospholipid digestion and metabolism, host defense and signal transduction. Secretory PLA_2 ($sPLA_2$) have been comprehensively investigated in land animals, and many novel sequences encoding $sPLA_2$ have been determined according to recent advances in genomic information and molecular biological techniques. $sPLA_2$ are now found to consist of twelve molecular species. On the other hand, there is little of information about the enzymatic property and the structure of fish $sPLA_2$. Five low molecular weight Ca^{2+}-dependent PLA_2 have been purified from the hepatopancreas and gill of the red sea bream, and classified the hepatopancreas DE-1 and DE-2 PLA_2 and gill G-1, G-2 and G-3 PLA_2 as group I PLA_2, based on the analysis of their N-terminal amino acid sequences and enzymatic properties. Although the cDNA encoding DE-2 PLA_2 had been isolated, the primary structures of DE-1 and Gill PLA_2 remain to be determined. In this study, the cDNA cloning of DE-1 and Gill PLA_2 were firstly attempted to determine their primary structures. Isolation of a cDNA clone encoding a novel PLA_2 in the red sea bream were also tried. In addition to the cDNA cloning, bacterial expression systems for DE-1, DE-2 and G-3 PLA_2 were constructed, and the enzymatic properties of their purified recombinant mature $sPLA_2$ were characterized. Furthermore, the localization of protein and gene expression of group IB $sPLA_2$ were investigated in red sea bream tissues.

Cloning of the group IB phospholipase A_2 isoforms in the red sea bream

Two cDNA encoding the red sea bream hepatopancreas DE-1 and gill G-3 PLA_2 were cloned by RT-PCR and RACE methods. In addition, a cDNA clone encoding a novel $sPLA_2$ was also isolated from the intestine of the red sea bream, and it was named as IN PLA_2. The cDNA of the red sea bream DE-1, G-3, and IN PLA_2 encoded a mature protein of 125, 124, and 127 amino acid residues, respectively, with an apparent signal peptide and propeptide. Comparison of the amino acid sequences for the mature DE-1, DE-2, G-3 and IN PLA_2 showed that their proteins contain 14 cysteines, Ca^{2+} binding region, and pancreatic loop conserved in the group IB $sPLA_2$. A previous report concerning the cDNA cloning of the red sea bream DE-2 PLA2 and the present results represent the first cloning and sequencing of four distinct isoforms of the group IB PLA_2 in a single

fish species, red sea bream. A phylogenetic tree shows that the red sea bream $sPLA_2$ were divided into two subgroups, DE-1, G-3 and IN PLA_2 and DE-2 PLA_2 more distantly related to the mammalian group IB sPLA2, respectively.

Recombinant expression of the group IB phospholipase A_2 isoforms in the red sea bream

Bacterial expression systems for DE-1, DE-2 and G-3 PLA_2 were constructed. Their cDNA encoding for the mature $sPLA_2$ were amplified by polymerase chain reaction and subcloned in-frame with a glutathione S-transferase (GST) encoded by the vector pGEX-4T-1. The resulting plasmids were used to transform *Escherichia coli* BL21 cells. Three recombinant $sPLA_2$ were expressed as a fusion protein with GST in *E. coli* cells by induction of isopropyl-1-thio-β-D-galactopyranoside. The bacterial cells were lyzed with strong alkaline solution and the fusion proteins were recovered as a soluble form. The fusion proteins were purified with affinity chromatography and cleaved by trypsin. Then, the recombinant DE-1 and G-3 PLA_2 were purified to near homogeneity by reversed-phase high-performance liquid chromatography (HPLC), and the recombinant DE-2 PLA_2 was by ion exchange chromatography and reversed-phase HPLC. The enzymatic and structural properties of the purified recombinant DE-1, DE-2 and G-3 PLA_2 were found to be essentially the same as those of native $sPLA_2$.

Expression of the group IB phospholipase A_2 isoforms in the red sea bream

The mRNA expression of group IB $sPLA_2$ isoforms was firstly investigated in the red sea bream larvae/juveniles. The mRNA expression of DE-1 and G-3 PLA_2 were detected in eggs, and those of DE-2 and IN PLA_2 were from the larvae of 1 day and 7 days post-hatching, respectively. In immature red sea bream, mRNA and protein of DE-1 and DE-2 PLA_2 were detected in the hepatopancreas of the red sea bream. Both $sPLA_2$ were synthesized as $preproPLA_2$ and were processed with signal peptidase in endoplasmic reticulum of pancreatic acinar cells. After being secreted in the intestine, both $sPLA_2$ were activated by tryptic proteolysis and digest dietary phospholipids. DE-2 PLA_2 was detected not only in the digestive tissues, but also in the non-digestive tissues, such as the spleen and heart. This means that DE-2 PLA_2 would play a physiological role via the specific receptor for group IB PLA_2, in addition to the digestion of the dietary phospholipids. G-3 PLA_2 mRNA was expressed in the gill and ovary of the red sea bream, and IN PLA_2 mRNA was mainly in the pyrolic caeca and intestine. In addition, IN PLA_2 mRNA was detected in the goblet cells of the intestine by *in situ* hybridization. Effect of LPS stimulus on the mRNA expression of the red sea bream group IB $sPLA_2$ was further investigated. The mRNA level of DE-2 and IN PLA_2 increased, while that of DE-1 PLA_2 decreased. It has been known that mouse group IIA $sPLA_2$, which has a bactericidal activity, was expressed in the epithelial cells and the paneth cells of the intestine, and the mRNA was induced by bacterial infection. These aspects would indicate that IN PLA_2 also has a function of non-specific defense against bacteria, in addition to the digestion of the dietary phospholipids in the gastrointestinal tract.

Key words: fish, red sea bream, group IB phospholipase A_2, gill, hepatopancreas, intestine, cloning, recombinant

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Genomic organization of the chicken T-cell receptor β chain

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ニワトリT細胞抗原受容体β鎖遺伝子の塩基配列の解析

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Chapter 1 describes the background and purpose of the present work. In chapter 2, I examined overlapping genomic clones containing the chicken T cell receptor (TCR) Dβ-Jβ-Cβ complex, which contains a single diversity segment, four joining segments and four exons that encode the constant region. This sequence comprised 18.3 kb. All four Jβ sequences possessed typical recombination signal sequences (RSS) with intervening 12-bp spacers at their 5'-ends and splice sites at their 3'-ends. No Jβ-pseudogenes were identified. TGTG sequences in the RSS heptamer sequences were well conserved, as is the case in mammals. As germline sequences revealed complete Jβ sequences, the CDR3 (complementarity-determining region) sequences of TCR β from non-immunized splenocytes were analyzed. Non-coding (N) and palindromic (P) nucleotides were frequently observed at the Dβ-Jβ recombination sites. There were differences in length of deletion at the 5'-end of each Jβ. Deletion of the 5'-end of Jβ-1280 was particularly short when compared with that of Jβ-1336, but there were no changes in the length of the CDR3 using any of the four Jb sequences.

In chapter 3, chromosomal organization of the chicken TCR Vβ1 genes was investigated. Southern blotting analysis indicates that Vβ1 genes have many members and spanned about long length. Then TCR Vβ1 segment positive clones were screened form chicken genomic library constructed in lambda FIX II vector. Four genomic clones were isolated and sequenced. Sequenced lengths were about 14.8 kb, 13.5 kb, 12.4 kb, 10.8 kb, respectively. All four clones contain only one Vβ1 segment each other. In addition, except for retroposon sequences, there are very high sequence similarities over the length of clone sequence.

In chapter 4, diversity of TCR Vβ1 genes was investigated. 56 PCR clone sequences obtain from chicken genome were compared to each other. 50 pattern of distinct Vβ1 were described. And that 50 kinds of Vβ1 have exactly similar RSS sequences not only 7-mer and 9-mer but also intervening 23-bp spacers.

Although there appears to be limited diversity in the germ line elements that encode the chicken TCR β chain, this thesis indicates the valuable information about the chicken TCR β gene genomic organization and non-rearranged TCR β gene sequences.

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Physiological study on the cytoarchitecture of the locomotor region in the fish midbrain

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魚類の中枢内遊泳運動領域の細胞構築に関する組織化学的研究

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The objective of the study is clarification of cytoarchitecture of the nucleus of medial longitudinal fasciculus (Nflm) and spinal neurons concerning with fish swimming. I investigated first the distribution of inhibitory interneurons in spinal cord, and subsequently relationships namely connections between Nflm neurons and spinal cord inhibitory interneurons in this research. Since Nflm and the oculomotor neuron were located extremely close, then I tried to investigate the boundary of the nuclei. To grasp a distribution and connections of neurons of the brain and the spinal cord in these analyses. Then, I investigated shapes of Nflm neurons by using technique of retrograde and immunohistochemical methods.

1. Distribution of spinal interneurons in the carp

The distribution of spinal inhibitory interneurons of the carp was investigated immunohistochemically. Some GABAergic and glycinergic neurons distributed in the spinal cord and extended axons to an opposite side. The sizes of GABAergic neurons were 200 μm^2 or less, glycinergic neurons were of 100-300 μm^2 mostly. They made cell clusters corresponding to spinal segments usually along with each ventral root. The distribution of labeled GABAergic and glycinergic neurons on cross -sections of spinal cord was observed, then demonstrated that neurons of small and a medium size were distributed mostly entire the ventral horn, and the larger neurons were near the central canal.

2. Intraspinal projection of Nflm neurons and contacts with spinal interneurons

To determined connctions of axon collaterals of the carp Nflm neurons and spinal inhibitory interneurons, both the neurons were anterograde labeling and IHC, respectively. It was observed that collaterals of Nflm axons running on cell bodys of GABAergic and glycinergic spinal interneurons. As mentioned above, Nflm neurons were considered to carry out excitately signals onto not only motor neurons but GABAergic and glycinergic interneurons. Thereby, it was suggested strongly that Nflm neurons activates central pattern generator of the spinal cord.

3. Discernment of the cholinergic neurons in a central nervous system with ChAT mRNA expression

A specific mRNA probe produced based on cloned goldfish choline asetyltransferase (ChAT) cDNA (*Carassius auratus*). ChAT is a enzyme synthesizing acetylcholine in neurons. Specificity of the probe was determined in the goldfish spinal cord by *in situ* hybridization (ISH) method and retrograde labeling of motor neurons with a neural tracer True Blue in the same individual. In the brain of the carp (*Cyprinus carpio*) which is an animal used as the subject of this research, ChAT mRNA positive neurons were detected by ISH. The oculomotor neuron was a motor neuron and accordingly contain ChAT. By this research, it was clearly demonstrated that there are in the rostrocaudal order Nflm, the noculomotor nucleus, and the trochlear nucleus in the midbrain of the carp. The caudal part of Nflm is overlaps about 180-200 μ m with the rostral part of the oculomotor nucleus. Nflm is distributed over the caudal part of the midbrain tegmentum, and oculomotor neurons were distributed along with central line mainly.

4. Cytoarchitecture of Nflm neurons

By injection with a tracer in the spinal cord, shape of retrogradely labeled Nflm neuron was observed in serial cross sections of the brain. Two types of the neuron were distinguished. The one has dendrites extending to the contralateral side, and cell bodies assumed to be spindles. The neurons were distributed mainly near the mid-line of the midbrain. Of the other types, their dendrites were thicker than the former and expanded only in the ipsilateral side. The round cell body of the latter neuron was distributed over the whole Nflm.

Key words: carp, brain, swimming, spinal cord, acetylcholine, GABA, glycine, *in situ* hybridization, immunohistochemistry

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Detection of Betanodaviruses, the causative agent of viral nervous necrosis, from cultured and wild marine fish

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海産魚からのベータノダウイルスの検出

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Viral nervous necrosis (VNN) caused by piscine nodavirus (*Nodaviridae, Betanodavirus*) has emerged as major constraints on aquaculture of marine fish worldwide. The spreading of VNN might be attributable to either vertical or horizontal transmission of the causal agent. There was strong evidence for vertical transmission of infection in VNNs of some fish species like striped jack *Pseudocaranx dentex*. However, the mode of horizontal transmission has not yet been verified thoroughly, although the importance of subclinically infected fish as a source of nodavirus was suggested.

The present study has demonstrated that large populations of wild marine fish in aquaculture areas are inaparently or sometimes apparently infected with a genetically closely related, pathogenic betanodavirus. These wild fish could be a natural host or reservoir of betanodavirus and pose a serious risk for the spread of VNN to the cultured fish.

Chapter 1. New primer design for the sensitive polymerase chain reaction amplification of betanodaviruses

A sensitive polymerase chain reaction (PCR) method to detect betanodaviruses in vivo and in vitro was developed. The degenerate primers were designed on highly conserved regions of the known betanodavirus RNA2 (coat protein gene, 1.4kb) sequences available in database. The first set of primers amplified a fragment of 570 bp by reverse-transcription (RT)-PCR, while the second set amplified an internal segment of 420 bp by nested PCR. The present PCR with degenerate primers was superior to the previously reported PCR procedure, particularly in the selectivity and sensitivity against RGNNV (redspotted grouper nervous necrosis virus), which is most frequently isolated from cultured warm-water marine fish affected with VNN.

Chapter 2. Detection of betanodaviruses from wild redspotted grouper with clinical signs

Wild redspotted grouper *Epinephelus akaara* were collected in the Seto Inland Sea in August, 2002. Fish showed erratic swimming behavior and inflation of swim bladder. The brains of the fish were positive for nodavirus in both RT-PCR and nested PCR. The sequence of the RT-PCR products (300 nt) was highly homologous (similarity 99-100%) and closely related to that of a known betanodavirus (RGNNV). When young sevenband grouper *E. septemfasciatus* were challenged intravitreously with the isolated virus, abnormal swimming behavior and high mortality were

produced. These results indicate that the wild redspotted grouper were severely affected by RGNNV infection. This is the first report on VNN in wild population of redspotted grouper.

Chapter 3. Detection of betanodaviruses from cultured and wild marine fish with no clinical signs

Apparently healthy 539 fish consisting of 66 species were collected in four geographically remote aquaculture areas in Japan. The brains of fish were used to detect the betanodavirus by the PCR and to isolate the virus using E-11 cells. In Yashima (Kagawa Pref.), 2 and 13 of 20 cultured fish were positive for nodavirus in RT-PCR and nested PCR, respectively, and 4 of 5 wild fish were positive only in nested PCR. In Goto Island (Nagasaki Pref.), 28 and 99 of 106 wild fish were positive for the virus in RT-PCR and nested PCR, respectively. In Kakeroma Island (Kagoshima Pref.), 30 and 139 of 295 wild fish were positive for the virus in RT-PCR and nested PCR, respectively. In Kamiura (Oita Pref.), 106 of 113 wild fish were positive for the virus in nested PCR only. When sevenband grouper were challenged intravitreously or intramuscularly with the virus isolates from fish, all the isolates induced behavioral abnormalities in fish and mortalities ranged from 50 to 100%. The sequences of the nested PCR products (177 nt) from 39 fish species were highly homologous to each other (similarity: 98-100%) and these were closely related to that of RGNNV.

Chapter 4. Detection of betanodaviruses from fresh fish and mollusk for feeding cultured fish

A total of 420 (5 species) fresh fish for feeding cultured fish were collected from 4 stations of Fisheries Research Agency, Japan. The brain of the samples was examined by RT-PCR and nested PCR to detect the betanodavirus. In Miyako Station, only 1 of 60 samples (2 species) was positive for nodavirus in both RT-PCR and nested PCR. In Hakatajima St., 12 and 16 of 90 samples (2 species) were positive in RT-PCR and nested PCR, respectively. In Amami St., 18 and 33 of 150 samples (4 species) were positive for nodavirus in RT-PCR and nested PCR, respectively. While in Kamiura St., 32 and 59 of 120 samples (3 species) were positive for nodavirus in RT-PCR and nested PCR, respectively. When sevenband grouper challenged intramuscularly with the virus isolates, the fish displayed behavioral abnormalities and mortalities ranged from 90 to 100%. The sequences of the nested PCR products (177 nt) from 2 species were highly homologous to each other (similarity 98-100%) and these were closely related to that of RGNNV. These results suggest that fresh fish used for feeding cultured fish might be another potential source of nodavirus.

Chapter 5. Mortality of wild golden grey mullet associated with VNN in Iranian Caspian Sea

Unknown acute mortality occurred in wild golden grey mullet *Lisa auratus* in Iranian Caspian Sea (brakishwater) in February, 2004. Clinical signs of the moribund fish were erratic swimming behavior such as spiral and belly-up at rest and high distention of swimming bladder. In virological examinations, whole brain homogenates were positive in the RT-PCR test for betanodavirus. The sequence of the PCR amplicons is closely related with that of the betanodavirus RGNNV. Although I have not succeeded to isolate the virus, experimental infection with the brain homogenates of affected golden grey mullet in sevenband grouper, produced neurological abnormality followed by mortality. These results suggest that the present mortality of golden grey mullet in Caspian Sea is associated with VNN.

Key words: betanodavirus, viral nervous necrosis, wild marine fish, virus carrier

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Studies on viral epidermal hyperplasia of flounder larvae

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ヒラメのウイルス性表皮増生症に関する研究

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ヒラメは漁業資源として重要な位置を占めており，全国各地で天然資源量増大を目指して積極的な放流事業が展開されている。しかし，種苗生産においては疾病による大量減耗が頻発しており，その原因究明と対策法の確立が急務である。本研究は、種苗生産期のヒラメに発生する「ウイルス性表皮増生症」について，その防除対策の確立に資するため、主にウイルス学的および病態生理学的視点から検討を行ったものである。

第一章では，本研究全般に関係するため，まず正常なヒラメの発育段階を再整理した。また，ヒラメの成長に伴う表皮細胞タンパク質組成を分析した結果，仔魚期または稚魚期に特有のタンパク質の存在が明らかになった。

第二章においては，本病の原因を明らかにするため，主としてウイルス学的検討を行った。本病罹患魚は鰭の白濁を主徴として，多くの場合2週間以内に全滅状態となった。病魚の磨砕濾液を0.45 μmフィルター濾過した液を接種源として健康なヒラメ仔魚に対して攻撃試験を行ったところ，症状が再現された。しかし，稚魚期のヒラメあるいはその他の魚種の仔魚は発病しなかった。原因体の分離培養には成功しなかったため，感染因子の理化学的性状を実験感染により調べたところ，感染因子は大きさ100?220 nmで，エーテル，酸および熱（50℃・30分間）に感受性が認められた。また、病魚の表皮細胞にはその形態的特徴からヘルペスウイルスと思われるエンベロープを有する粒子が確認された。そこで，本症をヒラメ仔魚のウイルス性表皮増生症，原因ウイルスをヒラメヘルペスウイルス（flounder herpesvirus; FHV）と称することを提案した。

第三章においては，FHVの免疫学的検出法として酵素抗体法を開発し，ウイルス抗原の局在性等感染病理の研究への有用性を示した。また，迅速診断法としてPCR法を開発した。本PCR法は，感染細胞数にして100細胞が存在すればFHVの検出が可能なことから，高感度かつ迅速診断法として今後本病の診断および感染機構の研究に有用であると考えられた。

第四章では，病魚の死因について病態生理学的に検討した。様々な酸素分圧下でヒラメ仔魚を飼育したところ，病魚が高い生残性を示すのに必要な分圧は正常魚のそれの約２倍の280 Torr以上であった。病魚の表皮細胞層は正常魚の平均２倍の厚さがあることから，表皮増生症病魚の主たる死因は表皮細胞増生に伴う皮膚呼吸機能の障害と結論された。一方，体表の塩類細胞を免疫学的に検出したところ，病魚は正常魚に比べてその数が減少していた。病魚は全海水で飼育するよりも希釈海水で飼育した方が高い生残性を示したことから，表皮増生に伴い仔魚期には体表が担う塩類調節機能が失調していることも死因と考えられた。

第五章では，仔魚と稚魚におけるFHV感受性の違いを明らかにするため，ヒラメの変態とFHVによる発症との関係を調べた。ヒラメ仔魚に甲状腺ホルモン（チロキシン：T4）処理を施して変態促進させた魚，すなわち小型稚魚にFHVで攻撃したところ発症しなかった。一方，抗甲状腺剤（チオウレア）で変態を抑制した浮遊大型仔魚はFHV攻撃後，発症した。このことから，仔魚と稚魚の表皮細胞の質的な違いが発症に影響をしていると考えられた。

第六章において，種苗生産場での防除に向けた技術開発を行った。原因ウイルスの紫外線感受性を調べた結果，4,000 μW cm^{-2} sec^{-1}で不活化出来ることがわかった。野外試験の結果，流水式紫外線殺菌装置は本症の水平感染の防除に有効と考えられた。ポビドンヨード製剤50 ppm・5分間あるいはグルタールアルデヒド製剤125 ppm・10分間の卵消毒は，本症の垂直感染の防除に有効と考えられた。

Key words: ヘルペスウイルス，ヒラメ，ウイルス性表皮増生症，病態生理，迅速診断法，防除法

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The effect of group context and individuals' internal process on self-categorization

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集団文脈と個人内過程が自己カテゴリー化に及ぼす影響

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When would in-group members reject a superior in-group member who can enhance their group evaluation, and when would they praise and accept the superior member? This dissertation aimed to reexamine self-categorization process through exploring some answers to the previous questions.

In resent years, social psychological theorizing and research have emphasized the distinction between personal identity and social identity. The former means the self as a unique individual relative to other individual, and underlying interpersonal behavior. The latter means the self as an interchangeable exemplar of some social category, and underlying intergroup behavior. The distinction seems to involve the assumption that these identities are antagonistic. Self-categorization theory (Turner, 1987) explains in what contexts social identity becomes salient, and in what contexts individual identity becomes salient. And this theory emphasizes the role of intergroup context making social identity salient, and argues that this process leads individuals to engage in the intergroup and intragroup behavior. However, as Endo (1999) and Spears (2001) noted, contextual factors alone are unlikely to be able to explain all processes of self-categorization. Individuals would actively work the self and contexts to maintain and enhance both personal identity and social identity.

Thus, in this dissertation, self-categorization process was reexamined from a point of view of individuals' motive for self-evaluation. Specifically, the effect of interaction between intergroup contexts and individuals' internal processes on self-categorization process was investigated. At the same time, this dissertation attempted to explain the interpersonal relationships surrounding a superior in-group member mentioned above.

The constitution of this dissertation is as follows;

Chapter1: At first, the necessity of reexamination of self-categorization process was argued considering individuals' motive for self-evaluation. It was also stated that focusing on interpersonal comparison with an in-group member would be meaningful for this reexamination. Finally, basic hypotheses were described.

Chapter2: The effect of intergroup context on self-categorization process was examined. Taking into account individuals' motive for self-evaluation, it was hypothesized that the direction of salient intergroup comparison would have different effects on self-categorization process. Individuals would

regard themselves in terms of social identity only in the intergroup upward comparison condition, in which they were motivated to enhance their in-group evaluation. In order to examine this hypothesis, using a company as in-group and using employees of the company as participants, Study 1 was conducted. Study 2 also examined the effect of trait self-esteem added to intergroup context, using the faculty for female university students as in-group. Results of these studies showed that the direction of intergroup context determined individuals concern for self-evaluation, and this affected on self-categorization process as hypothesis.

Chapter3: In this chapter, the effects of perceived changeability of intergroup relationship on self-categorization process were examined. Based on Blanton, Christie, & Dye (2002), it would be predicted that if individuals could perceive such changeability, they would enhance their concern for social identity in the intergroup context. On the other hand, if not, they would enhance their concern for personal identity. In order to examine these hypotheses, in Study 3, laboratory experiment was conducted. In this experiment, participants who appraised their sex category highly or lowly were recruited from university students, and the direction of intergroup comparison was manipulated using their sex category as in-group. It was found that participants who gave their in-group high appraisal in the intergroup downward comparison condition, as well as those who gave their in-group low appraisal in the intergroup upward comparison condition could endorse the context as confirming their appraisal, so they became concerned for personal identity. On the other hand, It was found that participants who gave their in-group high appraisal in the intergroup upward comparison condition, as well as those who gave their in-group low appraisal in the intergroup downward comparison condition, regarded this context as an opportunity to loss or gain their status and in-group evaluation, so they became concerned for social identity. Furthermore, study 4 investigated the effect of the level of perceived changeability of intergroup relationships on self-categorization process, using the sex category for university as in-group category. Results of these studies, as hypothesized, illustrated that not only intergroup context, but also internal factors would affect self-categorization process.

Chapter4: This chapter examined the effect of individuals' internal process as the strategy of deflecting the threat to personal identity on self-categorization process. Based on Mussweiler, Gabriel, & Bodenhausen (2000), it was hypothesized that after engaging interpersonal upward comparison, individuals would maintain their self-evaluation by emphasizing an unshared social identity with the comparison target. In order to examine this hypothesis, study 5 manipulated another category sharing added to the factors in study 2. Furthermore, study 6 conducted an experiment using university students as participants, in order to examine whether the level of trait self-esteem has different effect on the former process. Results of these studies showed presence of the voluntary self-categorization process. Especially, study 5 implied that, if possible, individuals attempt to protect both personal and social identity at the same.

Chapter5: First, this chapter generalized the results mentioned above and insisted on importance of considering the multiple motives for self-evaluation for understanding more precisely self-categorization process. Second, it was proposed that further examination will be needed to identify other important factors and interactions between contextual factors and individuals' factors. Finally, practical contributions concerning interpersonal relationships in in-group were also suggested.

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The study of relationship between reminiscences cued music in mental health of elderly people

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音楽を手がかりとした回想と高齢者の精神的健康に関する研究

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第１章　音楽を用いた回想法における問題点と本研究の目的

近年，高齢者の精神的健康の維持・向上をめざした方法の一つとして，音楽療法が広くおこなわれるようになってきた。高齢者を対象とした音楽療法では，高齢者にとって親しみ深い曲を用い，聴取や歌唱を通して回想を促し会話を進めていくという，音楽を手がかりとした回想法が実施されている。しかし，音楽と回想及び高齢者の精神的健康との関係についてのメカニズムはいまだ明らかにされていないため，経験則による音楽の選択や手続きがとられているのが現状である。呈示された音楽や回想内容によっては，高齢者が心理的に不安定になる場合もあることから，音楽と回想される量や内容との関係や音楽による回想が精神的健康に与える影響について明らかにすることは大きな課題であるといえる。

本研究では，音楽を手がかりとした回想法の有効性を検討することを目的とし，音楽と回想との関係及び高齢者の精神的健康との関係について明らかにするために，実証的な検討をおこなった。

第２章　音楽と回想内容との関係及び音楽を用いた回想法の短期的な効果との関係に関する研究

第２章の目的は，懐かしさを生起させる音楽と回想内容との関係を明らかにすることと，精神的健康の短期的な側面である主観的気分との関係について明らかにすることであった。

高齢者16名に対して，懐かしさを生起させる曲又は統制曲を聴かせ，聴取中に思い浮かんだことについて自由に発言させた。分析の結果，音楽に対する関心が高く，発言した内容が自己と関連が強いときに自伝的な回想が多く引き出されていたことが示された。また，懐かしさを生起させる曲では自伝的な内容が，統制曲では音楽の印象や気分・歌手に関する内容が，最も多く引き出されていた。さらに，音楽により自伝的な回想が引き出されたときには，ポジティブ気分が向上することが示された。

第３章　音楽と回想内容に対する評価との関係及び音楽を用いた回想法の短期的効果に関する研究

第３章の目的は，懐かしさを感じる音楽が，自伝的な回想内容に対する再評価及び気分に与える影響について明らかにすることであった。

高齢者15名に対して，懐かしさを感じる曲と懐かしさを感じない曲それぞれ２曲ずつを聴取させ，音楽聴取後に思い浮かんだことについて自由に発言させた。音楽聴取前と回想後で主観的気分の測定を行った。分析の結果，懐かしさを生起させる音楽では，自伝的な回想が多く引き出されていること，さらに，懐かしさを感じる曲の中でもゆったりした静かな印象の音楽では，回想した内容に対して再評価が多くなされることが示された。さらに，懐かしさを生起させる音楽を用いた場合，回想前後でポジティブ感情が高まることが示された。

第４章　音楽の主観的特徴と回想内容の評価との関係及び音楽を用いた回想法の短期的効果に関する研究

第4章の目的は，「懐かしさ」を感じる音楽を手がかりとした回想を経時的におこなうことによって，回想内容の評価と主観的気分との関係について明らかにすること，さらに，音楽の特徴と回想内容に対する評価との関係について明らかにすることであった。
高齢者13名に対して，1週又は２週に１日のペースで１日２回セッション全11～12セッション（２ヶ月～３ヶ月）を実施した。１回のセッションで，1曲を呈示し，その後半構造化面接をおこなった。音楽は幼少期から成人期（昭和50年代）に聴いた曲の中から本人が「懐かしい」音楽として選曲した曲を用いた．回想後に主観的な気分を測定した。分析の結果，懐かしさを生起させる曲の中でも，曲に対して「重い」という印象を強く感じるときには，ポジティブに再評価された内容が多く引き出されていた。このことから，音楽の印象や特徴が，回想内容に対するポジティブな再評価を促している可能性が示された。

第５章　音楽を用いた回想法の長期的な効果に関する研究

第５章では，懐かしさの生起を伴う音楽を用いた回想における，回想内容に対する評価と精神的健康との対応関係，及び懐かしさの生起を伴う音楽を用いた回想における短期的な効果と長期的な効果の関連を明らかにすることを目的として検討をおこなった。
手続きは第４章と同様であった。測定指標のうち，精神的健康の長期的側面を示す因子として，現在に対する満足感と活動意欲，人生に対する満足感の３因子が抽出されたため，各因子の変化の違いによって回想評価や気分の比較を検討した。

その結果，人生に対する満足感が改善した者は悪化した者に比べて，過去よりも現在の方がポジティブに再評価された内容を多く回想していたことや，過去も現在もポジティブである内容が少なかったことが分かった。

また，人生に対する満足感が改善した者は悪化した者に比べて，過去よりもポジティブに再評価された内容や過去も現在もポジティブに評価している内容を回想したときに，憂うつ感が低かったことが分かった。現在に対する満足感が改善した者は悪化した者に比べて，回想後にポジティブ気分が高まっていたことが分かった。

本章の結果から，回想による精神的健康の変化と回想内容の回想内容の評価との間には関連性があり，また，短期的な効果である気分は長期的な効果の予測につながることが示された。

第６章　総合考察

第６章では，第２章から第５章までをまとめ，音楽と回想及び高齢者の精神的健康の関係に関するメカニズムと臨床への応用可能性について考察した。

第２章から第４章の結果から，音楽の印象や音楽によって引き起こされる気分が，回想量や回想内容，回想内容に対する評価に影響していることが明らかにされた。第２章から第５章の結果からは，音楽によって影響を受けた回想内容が気分に影響することや，個人の回想内容に対する評価の特徴が，人生に対する満足感の変化に関連していることが明らかとなった。これらの結果から，音楽が回想の量的･質的側面を決定する要因となり，さらに音楽によって影響を受けた回想が精神的健康へ影響するという一連のメカニズムが示され，音楽を用いた回想が高齢者の精神的健康の向上に有効であることが実証されたといえる。

本研究により明らかにされた回想を引き出す音楽の特徴や精神的健康の維持や向上につながる回想の特徴は，音楽を用いた回想を行なう際の音楽の選択方法や回想の促し方へ応用することができると考えられる。

Key words: music, reminiscence, elderly people, mental health

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Fruits trade in Asia and export production center in Thailand
-Dynamism for export in "Conventional production center of fruits"-

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アジア果実貿易の拡大とタイの輸出産地
－在来型果樹産地にみる輸出対応のダイナミズム－

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目的と課題

本論文の目的は，タイの農産物輸出産地の発展に対する「在来型農産物輸出」の役割を明らかにすることである。アジア域内貿易が深化する中，タイの果樹産地では輸出の促進をいかに受け止め，いかなる対応をとっているのか，という分析視角から，タイの輸出向け果樹産地の対応を実証的に分析し，「在来型農産物輸出」の存在形態を明らかにする。本研究の課題は，次の３つである。 第１に，「在来型農産物輸出」の概念を明示し，タイにみる「在来型果実輸出」を支える要因を明らかにする。「在来型農産物輸出」は，大規模な多国籍アグリビジネス企業による産地の包摂過程では，説明できない現象である。第２に，タイの「在来型果実輸出」の対応を実態調査に基づいて明らかにする。特に，①産地流通の対応，②輸出先市場別にみた産地，及び生産者の対応，③消費者主導型の産地形成としての対応，という３つの視点をもって，生鮮ドリアン，生鮮マンゴスチン，生鮮ホムトンバナナ輸出という３種類の農産物について，「在来型果実輸出」にみる産地の対応と産地の再編過程について分析する。第３に，「在来型農産物輸出」の性格を明らかにしながら，アジア域内貿易が深化する中での，今後の役割を考察する。

第１章　　多国籍アグリビジネスの動向とタイの「在来型農産物輸出産地」

第１章，第２章においては，先行研究の成果を踏まえ，「在来型農産物輸出」の展開と存在形態について独自の果実輸出論の展開を試みた。

第１章では，世界の多国籍アグリビジネス企業の動向を整理し，タイのアグリビジネス企業の特徴を抽出した。アメリカに拠点を構えるアグリビジネス企業は，産地の農業経営を，アグロ・フード・システムの原料供給部門として位置づけ，垂直的統合を図っている。しかし，タイのアグリビジネス企業と農産物産地との関係では，従来のアグリビジネス企業の産地包摂過程では説明できない農産物輸出の現象がみられる。そこで，タイの生鮮果実輸出にみる産地とアグリビジネス企業との関係の実態を論拠に，新しい輸出の捉え方を「在来型農産物輸出」と定義した。「在来型農産物輸出」とは，①農産物が輸出用に開発されたのではない在来品種である，②高度な加工を必要としない，③流通システムは，国内市場向け流通形態の発展系である，④輸出ルートは，伝統的な周辺諸国への移出ルートの延長，及び既存のネットワークの活用形態である，⑤中小企業が個別に分散した零細農家を，小規模に組織化し輸出へ結びつけている，である。

第２章　食料貿易のグローバル化とタイの果実輸出

第２章では，タイの輸出指向型の経済発展と，タイ農業がもつ比較優位の関係について述べている。経済発展の経緯を時系列的に整理し，農業の生産・流通構造が輸出指向型の発展を支える要因であることを指摘

した。また，生鮮果実輸出の動向を明らかにし，1990年代から顕著になったアジア各国との競争関係の中で，在来型果実輸出の展開を支える要因を整理した。

在来型農産物輸出では，中小規模のアグリビジネス企業が，産地を海外市場へ結びつけている。中小企業が在来型農産物輸出の展開を支える要因の一つは，開発政策の核となるNAIC（Newly Agro-Industrializing Country：新興農業関連工業国）戦略であった。農村には，労働集約的生産が可能な豊富な低賃金労働力が滞留し，転換能力が高い生産者や，アジア各国に広がる人的・流通ネットワークが備わっていた。こうしたタイ農業の生産・流通構造の特徴は，タイ農業がもつ比較優位であり，輸出指向型戦略の展開を図る条件となった。果実の輸出産地では労働集約的栽培を行う農家も出現している。特に1990年代以降，農村の低賃金労働力の維持や，果実輸出に新規参入する専門的業者の出現によって，輸出向けの果樹生産が展開されてきた。

第３章　生鮮ドリアン輸出にみる産地流通の再編

第３章，第４章，第５章では，タイ南部の果樹産地であるチュンポン県を中心とした実態調査に基づき，３種類の果実輸出の対応を実証的に考察した。

第３章では，生鮮ドリアン輸出がさかんなチュンポン県ムアン郡を事例とし，産地流通の再編過程について考察した。従来から存在する小産地流通が輸出に接合され，物流システムが形成されていく過程と，輸出の専門的流通業者の登場による産地間流通ネットワークの解明を行った。産地では，国内向け出荷を行っていた流通業者が，輸出請負業者となり，専門的に輸出流通に取り組む動きがみられる。同時に，輸出会社も新規参入が容易な産地の流通構造を活かし，生産者や流通業者を系列化してきた。既存の流通業者が海外市場に連なる「産地ネットワーカー」として育つことで，輸出ルートが確立される過程を明らかにした。

第４章　生鮮マンゴスチン産地の輸出市場の多様化

第4章では，チュンポン県ランスワン郡を中心とした生鮮マンゴスチン輸出を事例として取りあげ，輸出先市場別にみる産地，及び生産者の対応の違いを検討している。マンゴスチンは，東・東南アジア諸国への輸出と同時に，日本向け輸出という高品質市場向けの輸出が特徴的である。東アジア向けについては，ドリアンと同様，輸出請負業者を中心とした流通システムであり，排他的流通システムを形成しない。それに対し日本市場向けは，農民グループを単位に，契約栽培を実施している。その契約栽培は，多国籍企業経営とは異なり，輸出会社がグループを直接，系列化するのではなく，選別や集荷方法などは農家が自由に対応できる仕組みである。品質の良いものを安定的に確保するため，輸出会社は高い買取価格を設定し，農家へのインセンティブとして位置づけている。小規模な個別農家を，海外市場へ結びつけようとする在来型輸出対応の輸出会社の戦略といえる。

第５章　消費者主導型アプローチによるフードシステム形成と輸出産地
ー日本向け無農薬バナナ産地の展開ー

第５章では，３章，４章とは分析視角を変え，在来型果実の消費者側からのアプローチによって形成されたフードシステムと，その中の産地対応について分析している。チュンポン県ラメー郡の無農薬ホムトンバナナ輸出を事例として取りあげる。バナナ輸出を担うラメー農民グループは，無農薬バナナの供給先である大阪よどがわ市民生協からの働きかけによって組織化された。無農薬バナナ輸出事業は，消費者の食品の安全性が脅かされ，かつ生産者が輸出によって健全な農業環境が奪われる貿易に対峙し，消費者側から提案された。また，それは賦存の資源や農法を活かし，在来型を意識した消費者主導の輸出事業であり，フェアトレードの要素が含まれた輸出展開であった。消費者の需要に応えながら，自立的な発展も遂げる農民組織が誕生した。

第６章　アジア食料貿易の拡大と在来型果樹産地の輸出対応

最終章である第６章では，自然発生的にみえる在来型果樹産地の輸出対応に潜む，既存産地が輸出へ結びつく動的メカニズムを明らかにした。さらに，農産物輸出産地の発展に対する在来型農産物輸出の役割を考

察した。

３つの事例分析から，既存の国内向け産地が輸出産地として成長する要因は，第1に，「産地ネットワーカー」の存在，及び産地の既存の特徴を活かす産地形成の仕組みである。これらは，既存の流通形態や産地との柔軟な関係を維持する，という共通の性格をもつ。第２に，輸出に積極的な農家の出現である。農家自らが輸出を目指す姿勢や，組織化による輸出出荷の可能性を高めようとする取り組みが３つの事例に共通する。

在来型農産物輸出の展開には，アジアという膨大な消費市場圏の存在が不可欠であった。アジア域内のボーダレス化と消費市場の拡大が，在来型輸出対応の果樹産地の成長を支えている。在来型農産物輸出産地は，アジア域内貿易の拡大と強く相乗して，発展を遂げていく可能性を有している。

在来型農産物輸出は，大きな産地だけでなく，既存の零細な産地を輸出へリンクさせる役割をもつ。今後，アジア域内のグローバル化の深化とともに，産地が輸出へ連結する度合いは高まり，零細農家が在来型農産物輸出を契機に発展する可能性がある。

ただし，在来型農産物輸出の消費市場は今後，変化する可能性は十分にある。また，在来型農産物産地の規模は小さく，比較優位に基づく産地間移動を特徴とする。それは，国内の産地間はもとより国を超えての産地間競争の激化を引き起こし，在来型農産物産地の消滅の事態を招くと考えられる。さらに，生産者は，流通業者や輸出会社と柔軟な関係を保っている。それは，取引の変更や休止の可能性が高い関係を意味し，生産者のリスクが高い取引である。在来型農産物輸出は，過渡的な性格を持ち合わせている。

この過渡的性格を踏まえ，在来型農産物輸出の政策的課題を展望する。積極的農家を中心に農家のレベルアップを図り，新たな市場対応が可能な産地育成を目指すことが，産地が生き残る一つの方法である。生産者への輸出関連情報の提供や，農業普及局をはじめとする関係機関の連係が求められる。また，

市場価格低下の状況が既にある。生産コストの削減，価格支持政策の実施なども，優良産地の育成に重要な施策であろう。

キーワード：在来型農産物輸出，タイ，果実輸出，多国籍アグリビジネス，産地ネットワーカー，輸出産地，アジア消費市場

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A study on population structure and virulence evolution of pinewood nematode *Bursaphelenchus xylophilus*

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マツ材線虫病の病原線虫の個体群構造と毒性の進化に関する研究

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序　論：マツノザイセンチュウは材線虫病の病原生物であり，マツノマダラカミキリによって媒介される。日本ではアカマツやクロマツに急激な枯死を引き起こす。近年，病原体の毒性進化の理論的研究は，遺伝的に異なる毒性（宿主の適応度を低下させる病原体の形質）を持つ複数の病原体が宿主の１個体に感染する（重複感染）と，遺伝的に単一の病原体が感染する場合より毒性の高い病原体が個体群内に存続し，また毒性の変異が大きくなることを示した。マツノザイセンチュウの毒性には大きな変異が知られているが，毒性の時間的変化，毒性に変異が生じる機構とその変異が維持される仕組みは明らかでない。本研究は病原体の毒性の進化理論に基づいて材線虫病の病原線虫の毒性進化を理解するために行った。

毒性の進化理論の仮定の検証（第３章）：重複感染下の理論では，病原体は半数体であり，宿主体内で強毒性の系統が弱毒性の系統より高い増殖力を持つことを仮定している。マツノザイセンチュウは二倍体の生物である。そこで本種もこの仮定を満たすかどうかを明らかにするために，毒性の異なる線虫アイソレイトをクロマツに単独あるいは混合して接種した。枯死後クロマツから線虫を分離し，マイクロサテライトマーカーを用いて個体群の遺伝的組成を調べた。強毒性アイソレイトの対立遺伝子は弱毒性アイソレイトの対立遺伝子より増加率が高く，病原線虫は重複感染下の毒性進化の理論の仮定を満たした。

材線虫病の流行終息期のアカマツ林分におけるマツノザイセンチュウの遺伝的構造と毒性（第４章）：病原線虫の毒性の遺伝子は不明であるので，マイクロサテライトマーカーを用いて野外線虫個体群の遺伝的な構造を調べた。遺伝的変異の大部分は枯死木内や媒介昆虫内の線虫個体群に存在した。また，枯死木内の線虫個体群に構造化が生じる場合があった。野外線虫個体群からアイソレイトを確立して，アカマツ苗に接種した結果，毒性（苗の枯死率）はアイソレイト間で大きく異なった。媒介昆虫内より枯死木内の線虫個体群が平均的に高い毒性を持っていた。

マツノザイセンチュウの毒性とクロマツ樹体内における長期生存（第５章）：個体群の一部が次世代に繰り越されることは変動環境下の個体群の維持に有利である。病原線虫の毒性と宿主内における個体群の存続期間の関係を明らかにするために，毒性の異なる線虫アイソレイトを単独あるいは混合してクロマツに接種した。接種の２，３年後に線虫は健全木から検出されなかった。しかし部分的に枯死した木から線虫が検出された。それらは強毒性のアイソレイトの対立遺伝子を持っていた。

種間交雑と病原線虫の遺伝的多様性（第６章）：分化した個体群の二次的な接触は新しい遺伝子型の創出を通じて動物の系統進化に重要な役割を持つと考えられている。病原線虫とニセマツノザイセンチュウの種間交雑を明らかにするために，２種を識別する種特異的PCRプライマーを作成した。広島県と山口県のアカマ

ツ３林分の各４～６本の枯死木から線虫を分離して種を同定すると，１個体が２種の種特異的なバンドパターンを示した。また，実験室で作成した２種の雑種個体は両種のバンドパターンを持つことを示した。この結果，野外で２種線虫の種間交雑が起こっていることを初めて明らかにした。近縁種との交雑が毒性の進化に及ぼす影響について議論した。

宿主内における病原線虫の個体群構造に関わる要因（第７章）：樹体に侵入後，線虫は宿主と相互作用をしながら樹体全体に広がる。そのため，樹体内の線虫の移動は宿主内における線虫個体群の構造化に大きく関与すると考えられる。

線虫の分散に対するアカマツの阻害能の季節的変化を明らかにするために，６週おきにアカマツの２年生枝を採取して，５cmの切り枝にしてバイアルに垂直に立てた。この上端に200頭の線虫を接種し，24時間の間に切り枝を通過した線虫の数を調べた。実験にはマツノザイセンチュウの強毒性の２アイソレイトと弱毒性の２アイソレイト，ニセマツノザイセンチュウの１アイソレイトを用いた。枝内における分散能力は卵と増殖型第２期幼虫で低く，その他の発育段階で高かった。分散能力はアイソレイト間で変異があり，分散能力と毒性は関係がなかった。線虫の分散に対する阻害能は８月と12月から２月の間に低下した。

上述の５アイソレイトの線虫を長さ2.5cmと5.0cmのアカマツ切り枝の上端に接種した。切り枝の長さが長くなるとアイソレイトに関係なく通過線虫数は80％減少した。さらにアカマツによる分散阻害に対する線虫の感受性を調べるために，長さ5.0cmの生きた切り枝と煮た切り枝の上端に線虫を接種した。その結果，線虫の感受性にアイソレイト間で変異が見られた。そのため，アカマツ樹体内における線虫の分散は線虫自身が本来持つ分散能力，マツの阻害能の程度および阻害能に対する線虫の感受性によって決まると考えられた。

線虫の樹体内分散に対する阻害能についてアカマツ個体間に変異があることを示すために，野外の６本の健全木から４方位に伸びる枝を２つの高さで採取した。切り枝の通過線虫数は樹木個体間で異なったが，枝の高さや方位による違いはなかった。別の６本の健全木から４時間ごとに枝を採取し，アカマツの阻害能の日変化を調べた。その結果，通過線虫数の周期的な日変化は急峻な斜面上部に生える１本においてのみ観察された。

アカマツの材線虫病抵抗性と線虫分散阻害能の関係を明らかにするために，抵抗性の異なるアカマツの17クローンから枝を採取した。長さ５cmの生きた切り枝と煮た切り枝の上端に線虫を接種した。線虫通過数は生きた切り枝より煮た切り枝で多く，枝の阻害反応はクローン間で変異があった。通過線虫数と抵抗性の間には相関がなかった。このことから，リギダマツのような抵抗性樹種の抵抗性機構と感受性樹種の抵抗性クローンのそれは異なることが示唆された。

総合考察（第８章）：本研究の結果から材線虫病の病原線虫の毒性の時間的変化は重複感染下の毒性進化の理論に基づいて理解できることが明らかになった。そして，染色体の組み換えや種間交雑による遺伝的変異の増加，宿主個体内における病原線虫の分散および構造化がマツノザイセンチュウの毒性進化に影響することが示唆された。

キーワード：個体群構造，重複感染，毒性の進化，マツ材線虫病，マツノザイセンチュウ

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Identification and function of neurosteroids in vertebrate brains

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脊椎動物の脳におけるニューロステロイドの同定と作用

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The brain has traditionally been considered to be a target site of peripheral steroid hormones. In contrast to this classical concept, new findings over the past decade have shown that the brain itself also has the capability of forming steroids *de novo*, the so-called "neurosteroids". Studies on mammals and nonmammals indicate that *de novo* neurosteroidogenesis in the brain from cholesterol is a conserved property of vertebrates. Since their discovery, diverse functions of neurosteroids have been identified, mediated by their genomic and nongenomic actions.

The production of steroids requires the coordinate action of several steroidogenic enzymes that start with cholesterol. Tsutsui and his colleagues have recently demonstrated that the avian brain possesses the key steroidogenic enzyme P450scc and 3β-HSD, and produces pregnenolone and progesterone using biochemical and immunochemical techniques. The amphibian brain also possesses P450scc and produces pregnenolone. Despite these observations, however, our knowledge of the biosynthetic pathway of neurosteroids from cholesterol in the vertebrate brain is still incomplete. Therefore, in Chapter 1, I attempted to reveal the biosynthetic pathway of neurosteroids from cholesterol in the vertebrate brain using quails. The present study revealed that progesterone is further metabolized to 5β-DHP and 3β,5β-THP in the avian brain. The present study also demonstrated that the avian brain possesses not only P450scc and 3β-HSD, but also other key steroidogenic enzymes, $P450_{17\alpha,lyase}$ and 17β-HSD, and produces androstenedione and testosterone from progesterone. These observations indicate that the vertebrate brain has a capability of forming various neurosteroids *de novo* from cholesterol.

Interestingly, preliminary studies suggested that the vertebrate brain may produce an unknown neurosteroid, which is different from any neurosteroids identified in Chapter 1, from pregnenolone. Furthermore, it also is suggested that an unknown neurosteroid was actively metabolized from pregnenolone in the brain of the newt, a seasonal breeding wild amphibian, rather than in the brain of the quail and rat. Because the production of the unknown neurosteroid was greater than that of any known neurosteroid, I propose that this neurosteroid might well be involved in important aspects of brain function in the newt. Therefore, in Chapter 2, I attempted to identify this unknown amphibian neurosteroid. Employing biochemical techniques combined with HPLC, TLC, and GC-MS analyses, I could identify this unknown neurosteroid as 7α-hydroxypregnenolone. The formation of 7α-hydroxylated neurosteroids, such as 7α-hydroxypregnenolone and 7α-hydroxydehydroepiandrosterone, has been observed only in the brain of mammals, such as

laboratory rodents and humans. In mammals, 7 α -hydroxylation of dehydroepiandrosterone may be part of a metabolic pathway to more potent derivatives, and 7a-hydroxydehydroepiandrosterone is more active than dehydroepiandrosterone in preventing hypoxic cell death of neurons *in vitro*. In contrast to these mammalian studies, no investigation has been published on the formation of 7 α -hydroxypregnenolone in nonmammalian vertebrates, to my knowledge. Therefore, this study is the first observation showing that 7 α -hydroxypregnenolone is actively produced in the nonmammalian brain. In Chapter 3, I demonstrated the physiological change, function, and mode of action of 7 α -hydroxypregnenolone in the brain. 7 α -Hydroxypregnenolone synthesis in the brain showed marked changes during the annual breeding cycle, with a maximal level in the spring breeding period when locomotor activity of the wild populations of the newts increases. Interestingly, exogenous 7 α -hydroxypregnenolone acutely activated locomotor activity of newts in the nonbreeding period when endogenous 7 α -hydroxypregnenolone synthesis in the brain was low. *In vitro* analysis further revealed that 7 α -hydroxypregnenolone treatment resulted in a dose-dependent increase in the release of dopamine from cultured brain tissue of nonbreeding newts. The effect of this neurosteroid on locomotion was also abolished by dopamine D_2-like receptor antagonists. These results indicate that 7 α -hydroxypregnenolone acts as a neuronal activator to stimulate locomotor activity of breeding newts by means of the dopaminergic system. The increase in locomotor activity of newts in the spring breeding period may be due to an increase in the production of 7 α -hydroxypregnenolone. This study shows a previously undescribed physiological function of 7 α -hydroxypregnenolone in the brain of a vertebrate.

In conclusion, the present study first revealed that the vertebrate brain has a capability of forming various neurosteroids *de novo* from cholesterol, and clarified the biosynthetic pathway of neurosteroids in the vertebrate brain. Subsequently, this study revealed that 7 α -hydroxypregnenolone is actively produced in the vertebrate brain, and demonstrated the physiological change, function and mode of action of 7 α -hydroxypregnenolone that has not been described previously in any vertebrate class. This study is the first observation showing the physiological function of 7 α -hydroxy-pregnenolone in the brain. This study also provides findings on the regulatory mechanism of locomotor activity from a unique standpoint.

Key words: Neurosteroids; Dopamine release; Seasonal changes; Quail Brain; Newt Brain

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Electrophysiological study on neurophysiological basis of time perception

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時間知覚の神経生理学的基盤に関する電気生理学的研究

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Time is an important source of information that influences behavior of human and animal. Recent studies have revealed that frontal cortex, basal ganglia, cerebellum, and hippocampus play important roles in the temporal processing (reviewed in Onoda, 2004). Matell & Meck (2000) proposed striatum beet frequency model that cortico-striatal network encode specific durations as components of a neural circuit used to represent duration. However, this model does not describe the relationships between cortico-striatal circuits and other regions. Finch (1996) reported existence of inputs from the frontal cortex, thalamus, and hippocampal formation to the basal ganglia. This suggests the basal ganglia might do integration of the temporal information from the other regions. The activities of brain must be measured with high time resolution for evaluating the relationships between these regions. Animal EEG was adopted for the measurement in this study, because it directly evaluated the brain activity with high time resolution. The purpose of this study is to examine the relationship among the regions relevant to the time perception by using the animal EEG.

EEG recordings of rats in this experiment were performed in a timing task and a control task. In the timing task, the subjects were trained to discriminate between two stimulus intervals (500 and 2000 ms). The stimulus was a pure tone signal. It was 1000 Hz, 80 dB and 100 ms duration. When a trial started, the tone stimulus (S1) was presented at first. The second tone (S2) was presented 500 or 2000 ms after the S1 onset. The subjects were required to judge the S1-S2 interval, the temporal processes were essential to solve the task. The control task was the same as the interval discrimination task except the S1-S2 interval. The S1 was presented when a trial start, the S2 was always presented 500 ms after the S1 onset. The subjects were required to response the lever after the S2, so the temporal processes were not essential to perform the task because the S1-S2 interval was fixed for 500 ms.

First, the concurrence of the brain activity related with temporal processes was examined by event-related potential (ERP). Onoda et al. (2003) reported that animal ERP waves in a task required temporal processes differ from ones in a control task. This means that the difference of the ERP waves can detect the activities that reflect the temporal processes. If the plural regions concurrently show the difference of ERP waves in the temporal task and control task, the result suggests that these regions cooperate to process the time with each other. The ERP waves for the S1 in both tasks consisted of P2, N2, P3. T-tests were performed in each time point and each region. Difference of ERP waveforms between the tasks was defined as the periods that the significant values ($p<.05$)

successively lasted at least 20 ms. The cerebellum, striatum, and thalamus concurrently showed the ERP differences in 100-200 ms latency. Further, the frontal cortex, striatum, and thalamus also showed the ERP differences in 400 ms latency at the same time. These results suggested that the front-striatum circuit and projections from the cerebellum to the striatum via the thalamus play important roles in the temporal processing.

Second, the brain activity related with temporal processes was examined by event-related spectral perturbation (ERSP). The ERSP are increasingly used in the EEG literature to visualize mean event-related changes in spectral power over time in a broad frequency range. The event-related changes in the power spectrum of each region were compared between the timing task and the control task. Power of frequency bands (6-12 Hz) correspond to a hippocampal theta in the S1-S2 interval was larger in the timing task than in the control task. This result suggests that the hippocampal function in time perception are related to working memory, because the change of the hippocampus theta wave was connected with working memory (Givens, 1996).

Third, directions of information transmission between the regions were examined by event-related phase coherence (ERPCOH). ERPCOH was calculated to evaluate a delay of the phase between the two activity measures. The phase of the frontal cortex, the cerebellum, and the hippocampus advanced in comparison with the striatum, these suggest that the information transmissions from the frontal cortex, the cerebellum, and the hippocampus to the striatum exist.

The striatum integration model based on these findings was proposed in general discussion. This model emphasizes that the striatum forms loop circuits respectively with the cortex, cerebellum, and hippocampus, the striatum encodes the temporal information from not only the cortex but also the cerebellum and hippocampus. Although a large number of studies have been made on time perception, there is little agreement as to form of the fundamental temporal information. It is necessary to examine the fundamental form of pace-maker.

Key words: time perception, striatum, event-related potential, event-related spectral perturbation, event-related phase coherence

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Individual's adaptation as a function of exclusivity of romantic relationships

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親密な関係における排他性が個人の適応に及ぼす影響

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The purpose of this study was to investigate how exclusivity of romantic relationships influences romantic partners' adaptation.

Previous studies about exclusivity of interpersonal relations have shown that exclusivity enhances the availability of interpersonal resource inside the relationship (Yamagishi, 1998) and eventually promotes the member's adaptation. However, research of social support and interpersonal conflict in multiple relationships has suggested that individuals who have exclusively supportive relationship with a specific partner can less effectively cope with conflict happened within the relationship (e.g., Lepore, 1992). In such a case, it is likely that individuals who cannot effectively cope with conflict suffer from violence at the hands of their partners (Walker, 1979). Therefore, this study examined whether or not exclusivity of romantic relationship affects romantic partners' cooperative and uncooperative orientation inside the relationship and lastly their orientations affect violence from their partner.

The framework of this study is as follows. In the chapter 1, the author reviewed previous studies on exclusivity of relationship and pointed out that they have devote attention to positive function of exclusivity, but pay little attention to negative function. Taking this fact into the consideration, from the chapter 2 to the chapter 4, the author carried out eight studies about functions of exclusivity. Finally in the chapter 5, the author discussed how exclusivity can influence individuals' adaptation from the results of the eight studies.

In the chapter 2, the author examined whether closeness of relationship affects the degree of the exclusivity. The study 1-1 showed that members of romantic relationship are reluctant to seek social support outside their relationship. In the study 1-2, it was shown that members belonging to romantic relationship perceive stronger distinctiveness about their relationship than members belonging to friendship. Furthermore, this study found that the stronger distinctiveness the members in a relationship perceive, the more they feel reluctance to seek support outside their relationship.

In the chapter 3, the author examined whether exclusivity of romantic relationship affects how a romantic partner acts for their partner. In the study 2, it was found that individuals who have the exclusive support relationship with a romantic partner can less effectively cope with conflict happened within the relationship than counterparts. In the study 3, it was found that members in romantic relationship have higher cooperative orientation and lower uncooperative orientation in

their relationship than members in friendship. Additionally, it was found that the strength of distinctiveness that romantic members perceive in their relationships mediates the relation between closeness of relationship and those orientations. In the study 4 it was shown that the strength of the perceived distinctiveness bolstered cooperative orientation, but on the other hand, inhibited uncooperative orientation romantic partners have. The results of this study additionally showed that the influence process of the perceived distinctiveness to the orientations is independent of the influence process of interdependence variables (comparison for alternatives, satisfaction, investment and commitment; Rusbult, 1983) to the orientations.

In the chapter 4, the author examined how exclusivity of romantic relationship affects the degree of violence offered by their partner. In the study 5, it was found that the less availability of social support romantic partners can perceive outside their relationship, the less uncooperative orientation they have, and that partners with neither cooperative nor uncooperative orientations suffer from violence at the hands of their partners. In the study 6-1, it was shown that the perceived distinctiveness romantic partners have affects violence at the hands of their partners through two processes. One process is that the stronger distinctiveness romantic partners perceive, the more cooperative orientation they can have and the less they suffer violence at the hands of their partner. The other process is that the stronger distinctiveness romantic partners perceive, the less uncooperative orientation they can have, and the more they suffer violence at the hands of their partner. In the study 6-2, it was found that individuals who can maintain highly both cooperative and uncooperative orientation will less suffer from violence at the hands of their partner than the others.

In fifth chapter, based on the results of the eight studies, the author summed up about the influence processes of exclusivity in romantic relationship to participants' adaptation. Furthermore, the author pointed out two originalities of this study. One is that this study found that easiness of interpersonal exchange varies depending on relationship status. The other is that this study found that exclusivity of a relationship may cause the member maladaptation. Further, the author indicated prevention policy about domestic violence from results shown in this study. Finally, the author suggested the need to examine in what relationship other than romantic relationship exclusivity negatively functions the members' adaptation.

Key words: exclusivity, romantic relationship, domestic violence, cooperative and uncooperative orientation

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Biosynthesis and action of gonadotropin-inhibitory hormone

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生殖腺刺激ホルモン放出抑制ホルモンの生合成制御機構と作用機構

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脊椎動物において脳下垂体からの生殖腺刺激ホルモンの放出は視床下部ペプチドである生殖腺刺激ホルモン放出ホルモン(GnRH)が制御すると考えられている。一方，生殖腺から分泌される性ステロイドやインヒビン等による生殖腺刺激ホルモン放出の修飾作用は知られているが，生殖腺刺激ホルモンの放出を抑制する視床下部ペプチドの存在は知られていなかった。最近Tsutsuiらの研究により，鳥類のウズラにおいて脳下垂体からの生殖腺刺激ホルモンの放出を抑制する新規視床下部ペプチドが発見され生殖腺刺激ホルモン放出抑制ホルモン(GnIH)と名づけられた。GnIHはアミノ酸12残基からなるペプチド(SIKPSAYLPLRF-NH_2)である。GnIH含有ニューロンの細胞体は室傍核に局在し，その神経繊維を正中隆起に投射する。GnIH前駆体のクローニングにより，GnIH前駆体はGnIHの他に2つの遺伝子関連ペプチド(GnIH-RP-1, -RP-2)をコードすることが明らかになっている。そこで本研究では，ウズラを用いてGnIHの生合成制御機構および作用機構を明らかにした。

第１章では，GnIHの生合成制御機構および作用機構を解析する基礎的知見を得るため，GnIH発現の発達過程における変動を，競合PCR法，競合ELISA法，免疫組織化学法を用いて解析した。その結果，ウズラの孵化前後にGnIH mRNA及びGnIHの発現が高まることが見出された。また，この時期GnIHニューロンは室傍核に局在しており，その神経繊維を正中隆起に投射することが形態的に確認された。従って，GnIHは脳下垂体制御をウズラの孵化前後に開始することが示された。GnIHの発現は孵化後1週間で一旦減少し，ウズラの成熟後再び発現が高まった。GnIHが減少する時期において生殖腺刺激ホルモンの放出が高まることから，ウズラの発達過程においてGnIHは生殖腺刺激ホルモンの放出を抑制的に制御することが示唆された。

GnIHの生合成制御機構を明らかにするために，第２章ではGnIHの発現を誘導する分子の同定を試みた。ウズラは長日期に生殖腺を発達させ短日期に退化させる季節繁殖動物であり，メラトニンによる生殖腺活動と生殖腺刺激ホルモン放出の抑制作用の報告がある。そこで，メラトニンはGnIHの発現を誘導するのではないかと考え，メラトニンのGnIH発現誘導作用を解析した。その結果，GnIH mRNAおよびGnIHの発現はメラトニン合成器官である松果体および眼球の除去により減少し，松果体および眼球除去個体にメラトニンを投与するとGnIH mRNAおよびGnIHの発現が濃度依存的に高まることが分かった。またGnIHの発現は，メラトニンの合成・分泌時間の長い短日期に高まることが分かった。次に，GnIH の発現を誘導するメラトニンの作用機構を明らかにするために，メラトニン受容体の局在を*in situ hybridization*法を用いて解析した。その結果，GnIHニューロンにはメラトニン受容体 (Mel_{1c}) mRNAが発現していることが明らかになった。これらの結果から，松果体および眼球から分泌されるメラトニンはGnIHニューロンに局在するメラトニン受容体(Mel_{1c})を介してGnIHの発現を誘導することが分かった。

第３章では，GnIHの作用機構を解析するために，まずGnIH受容体の同定を行った。クローニングされたGnIH受容体は７つの膜貫通領域を持つ新規のGタンパク共役型受容体であった。GnIH受容体をコードするcDNAを発現させたCOS-７細胞の膜画分はGnIHおよびGnIH遺伝子関連ペプチド(GnIH-RP-1, -RP-2)と特異的に結合した。Scatchard plot解析により，同定されたGnIH受容体は高親和性の結合部位を持つことが明らかに

なった。次に，GnIHの作用機構を明らかにするために，GnIH受容体の発現する部位を調べた。サザンブロット解析により，GnIH受容体mRNAは脳下垂体および間脳を含む脳領域に発現していた。これらの結果から，GnIHは脳下垂体に存在するGnIH受容体を介して生殖腺刺激ホルモンの放出を抑制することが分かった。また，GnIH受容体はGnRHニューロンの存在部位にも発現していることから，GnRHニューロンの働きを抑制する間接作用によっても生殖腺刺激ホルモンの放出を抑制することが示唆された。

GnIHの生殖腺刺激ホルモン放出抑制作用は培養脳下垂体を用いたin vitroの研究により明らかにされたが，個体レベルでの研究はなされていない。そこで第4章では，GnIHの生理作用をin vivoで詳細に解析した。成熟ウズラにGnIHを2週間投与し，脳下垂体の生殖腺刺激ホルモンサブユニット(common α，LH β，FSH β) mRNAを競合PCR法により，生殖腺刺激ホルモン放出量をRIA法により定量したところ，生殖腺刺激ホルモンの発現と放出が，投与したGnIHの濃度に依存して抑制された。またGnIHを投与すると血中テストステロン濃度もGnIHの濃度に依存して減少した。さらに，未成熟ウズラにGnIHを投与すると生殖腺の発達や血中テストステロン濃度の上昇が抑制された。これらの結果より，GnIHは生殖腺刺激ホルモンの合成と放出を抑制することにより生殖腺の発達や機能維持を抑制することが明らかになった。

本研究により，GnIHの生合成制御機構が明らかになった。GnIHの発現は，松果体および眼球から分泌されるメラトニンがGnIHニューロンに局在するメラトニン受容体(Mel_{1c})を介して誘導することが分かった。また本研究により，GnIHの作用機構が明らかになった。GnIHは脳下垂体に存在するGnIH受容体を介して生殖腺刺激ホルモンの放出を抑制することが分かった。さらに本研究により，GnIHは生殖腺刺激ホルモンの合成と放出を抑制することにより生殖腺の発達や機能維持を抑制することが明らかになった。

Key words : 生殖腺刺激ホルモン放出抑制ホルモン，メラトニン，Gタンパク共役型受容体，生殖腺刺激ホルモンの合成と放出，生殖腺の発達と機能維持

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Molecular characterization of multidrug resistance in food-borne pathogenic bacteria

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食中毒起因菌における多剤耐性の分子機構の解析

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Food-borne pathogenic bacteria are the most common causes of food-borne illnesses. The illnesses range from a simple upset stomach to more serious epidemic outbreaks. The antimicrobial resistance associated with food-borne pathogenic bacteria is an issue of great significance for public health at the global level. However, it is of particular concern if the bacteria are multidrug-resistant types. Levels of resistance are increasing and much is yet unknown about this problem. In this study, I characterized the molecular bases of this resistance in some important strains of food-borne pathogenic bacteria.

The first part of this study is the characterization of the genetic bases of resistance in multidrug-resistant strains of pathogenic *Escherichia coli*. A plasmid-encoded class 1 integron carrying three gene cassettes was characterized in an enterotoxigenic *E. coli* (ETEC) O159. This strain was isolated from a diarrheic patient in Tokushima prefecture. It showed resistance to streptomycin, kanamycin, gentamicin, chloramphenicol and ampicillin. The first gene cassette is the streptothricin acetyltransferase gene, *sat*, which confers resistance to streptothricin. The second one is an open reading frame (ORF) whose product is a putative phosphoserine phosphatase (PSP), and the last one is an aminoglycoside adenyltransferase gene, *aadA2*, which confers resistance to streptomycin and spectinomycin.

I also analyzed an enteroinvasive *E. coli* (EIEC) O164 which was isolated from a patient suffering from diarrhea at Osaka airport. This strain showed multidrug resistance (MDR) phenotype against streptomycin, spectinomycin, co-trimoxazole (trimethoprim/sulfamethoxazole) and ampicillin, and reduced susceptibility to ciprofloxacin. The molecular characterization of this strain revealed the presence of class 1 integron that contains three genes, dihydrofolate reductase gene type 12, *dfrA12*, which confers resistance to trimethoprim, aminoglycoside adenyltransferase gene, *aadA2*, which confers resistance to streptomycin and spectinomycin and an ORF of unknown function. Southern blot hybridization and conjugation experiments showed that the class 1 integron is located on a transferable plasmid of less than 90 kb in size. The resistance of EIEC O164 to ampicillin was found to be due to the presence of TEM-1 β-lactamase. On the other hand, a single mutation which has not been described before, proline (P)158→ serine (S), was detected outside the quinolone resistance-determining region (QRDR) of *parC* of topoisomerase IV which may be responsible for the reduced susceptibility to ciprofloxacin in this strain.

Finally, the genome of a multidrug-resistant strain of enterohemorrhagic *E. coli* (EHEC) O157:H7 was analyzed. This strain was responsible for a family outbreak in Hiroshima prefecture. It showed a MDR phenotype against streptomycin, spectinomycin, co-trimoxazole (trimethoprim/sulfamethoxazole), ampicillin and tetracycline. DNA fingerprinting profiles obtained by pulsed-field gel electrophoresis (PFGE) showed that this isolate had unique *Xba*I *and Bln*I profiles. Also, plasmid analysis results revealed that this strain contains an extra plasmid that is larger than the classic large plasmid of EHEC O157, pO157 (93.6 kb). This new plasmid was named pMDR157. Molecular analysis of the MDR phenotype in this unique strain revealed the presence of a class 1 integron containing two gene cassettes: a dihydrofolate reductase type 1 gene, *dfrA1*, which confers resistance to trimethoprim and an aminoglycoside adenyltransferase gene, *aadA1*, which confers resistance to streptomycin and spectinomycin. The ampicillin resistance was found to be due to the presence of the TEM-1-type β-lactamase gene.

The second part of this study is related to *Salmonella* serovars. The first extended-spectrum β-lactamase (ESBL) producing *Salmonella* in Japan was characterized. This strain is a non-clinical isolate of *S. enterica* serovar Senftenberg which harbored CTX-M-3. It was more resistant to cefotaxime, ceftriaxone and cefpodoxime than to ceftazidime by MIC testing results and gave a positive cephalosporin/co-amoxiclav synergy test. Southern hybridization showed that the CTX-M-3 gene was located on a plasmid of < 38 kb in size.

On the other hand, class 1 and class 2 integrons were characterized among non-typhoid *Salmonella enterica* serovars isolated in Hiroshima prefecture. PCR sequencing analysis revealed the presence of seven profiles of class 1 integrons in addition to a new type of class 2 integron. The identified gene cassettes within class 1 integrons were as follows; *aadA1*, *aadA2* and *aadA5* which confer resistance to streptomycin and spectinomycin; *aadB*, which confers resistance to gentamicin, kanamycin and tobramycin; *dfrA1* and *dfrA17*, which confer resistance to trimethoprim; *pse-1*, which confers resistance to ampicillin; *catB3*, which confers resistance to chloramphenicol and *sat1*, which confers resistance to streptothricin. Two strains of the multidrug-resistant *S.* Typhimurium DT 104 were characterized in this study. DNA sequencing of the class 2 integrons identified one with an unusual array of gene cassettes, *sat, sat1* and *aadA1*.

Finally, a multidrug-resistant strain of *Salmonella* Paratyphi B was characterized as the first report in Japan. This strain was recently isolated from an infant (one year old) suffering from acute gastroenteritis in 2002 in Hiroshima prefecture. MIC phenotypes, PCR and DNA sequencing results revealed that *S.* Paratyphi B contains the entire *Salmonella* genomic island 1 (SGI1) of resistant *S.* Typhimurium DT 104.

The third part of this study characterized several mechanisms of antibiotic resistance in *Vibrio* species. A new aminoglycoside acetyltransferase gene, *aac(3)-Id*, has been identified in a multidrug-resistant strain of *Vibrio fluvialis*. This gene confers resistance to aminoglycosides, mainly gentamicin. This strain was isolated from a hospitalized infant aged 6 months suffering from cholera-like diarrhea in India in 2002. It showed resistance to chloramphenicol, streptomycin, spectinomycin, co-trimoxazole, ampicillin, furazolidone, nalidixic acid, and gentamicin. It was found that this novel gene, *aac(3)-Id*, is located in a class 1 integron with another gene, aminoglycoside adenyltransferase gene, *aadA7*, which confers resistance to streptomycin and spectinomycin. Both *aac(3)-Id* and *aadA7* genes were cloned and expressed in *E. coli*. Phylogenetic analysis suggested that the *aac(3)-Id* represents a fourth evolutionary lineage in the aminoglycoside acetyltransferase genes of the

aac(3)-I types. Southern hybridization showed that this integron is located in the chromosome.

Furthermore, a variant type of *Vibrio cholerae* SXT element was characterized in *V. fluvialis*. The SXT element is a *V. cholerae*-derived integrative and conjugative element (ICE) that has also been referred to as a conjugative transposon.

Finally, class 2 integrons were identified for the first time in *V. cholerae*. *V. cholerae* is the causative agent of cholera, a potentially epidemic and life-threatening disease. Class 2 integrons were detected in two strains of *V. cholerae* non-O1, non-O139. One is a clinical strain (RC121: O27 serotype) isolated in October 2001 in India and the other is an environmental strain (B0320: O39 serotype) isolated in July 2003 in Bangladesh. MIC results showed that both strains had multidrug resistance (MDR) phenotype against different types of antibiotics including trimethoprim, streptomycin and spectinomycin whose resistances were encoded by the class 2 integron gene cassettes.

In conclusion, in this study, many types of antibiotic resistances genes, integrons and a transposon were found to be responsible for the multidrug resistance phenotypes in many strains of food-borne pathogenic bacteria.

Key words: antibiotic resistance, food-borne pathogenic bacteria, integrons, β-latamases

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Growth inhibition and inactivation of microorganisms by hydrostatic pressure

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加圧条件下での微生物の発育抑制と不活性化に関する研究

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Microorganisms naturally contaminate almost all the food ingredients, and some of them, especially bacterial spores can grow in the foods exposed to heat because of their heat resistance. One of the most effective methods to kill the spores is moist heating at temperatures higher than 100 ºC in food industries. However, the heat treatment at such temperatures affects the qualities of foods. Therefore, an alternative way of killing bacterial spores has been needed in food industries instead of the moist heating.

Increase in consumer demand for high quality product has prompted the exploration of physical treatments other than traditional heat treatments as potential alternatives. These alternatives include thermal methods, such as ohmic heating or microwave heating, and non-thermal methods, such as irradiation, high electric fields and high hydrostatic pressure (HHP).

HHP can inactivate microorganisms without altering the flavor and nutrient contents of foods owing to non-thermal methods. In addition, when exposed to pressure, objects such as foods and their ingredients are uniformly and immediately pressurized regardless of its dimension, differently from thermal methods. Many studies about inactivation of microorganisms by HHP have been carried out all over the world, since reported by Roger in 1895. It has, on the contrary, come to be known that only HHP cannot be killed bacterial spore even at 1,000 MPa. In addition, many researchers have suggested a combination of HHP and temperature to kill the spores. Even in these cases, pressures at higher than 100 MPa was nevertheless needed, so reduction of pressure for application becomes an important key in practice.

Over this thesis, the effects of the pressure below 100MPa on two physiological phenomena of microorganisms, growth inhibition and germination of spore were investigated, and the results were applied to practical uses.

A pressure range that prevents growth of microorganisms (two yeasts, three lactic acid bacteria, *E. coli*, three bacilli, one clostridium and two microorganisms isolated from spoiled anchovies) was investigated in order to apply it to food processing. The growth rate of all microorganisms used in the present study was delayed under a pressurized condition compared to the atmospheric pressure. The growth of the microorganisms could be restrained in a pressure range of 40-70 MPa depending on the species of microorganism. That is, growth of two kinds of yeasts was inhibited at 40 MPa. In lactic acid bacteria, *L. plantarum* was inhibited at 70 MPa, *T. halophilus* at 60 MPa and *L. lactice* at

50 MPa. Growth of *E.coli* and vegetative cells of *B. subtilis* was inhibited at 50 MPa. In bacterial spores, growth of *Bacillus* and *Clostridium* spores was inhibited at 50 MPa. In the microorganisms isolated form spoiled anchovies, they were inhibited at 50 MPa. Practical usefulness of these results was verified in the autolysis process of fish meat without decomposition. Growth inhibition and inactivation of spores by treatment at pressures less than 100 MPa can be utilized as a new technique for killing microorganisms and for inhibiting their growth.

The death behavior of *B. subtilis* spore during pressurization was shown in detail. *B. subtilis* spore was pressurized at 60 MPa at 40℃ for 0-24 hour in phosphate buffer and in GAM broth, respectively. After 24 hours the viable and spore counts decreased in the extent of about 5 log-cycles in the broth, and only 1.6 log-cycles in phosphate buffer. And most of spores in the broth changed in darkness from brightness during 4 hours and afterward some of them became budding. These results showed that the spores germinated in the presence of the pressure and germination-inducing components in the broth, and afterward the geminated spores were inactivated before changing into vegetative cells. Thus, it is possible to inactivate *B. subtilis* spores by pressure-holding in non-thermal condition. The same phenomenon was observed in other spore-forming bacteria, *B. licheniformis, B. cereus*, and *B. coagulans*, but their viable counts did not decrease as much as that of *B. subtilis*.

The combined effect of mild heating and pressurization on germination and inactivation of *B. subtilis* spores was investigated. The spore counts remarkably decreased at 60℃ + 80 MPa in glucose broth rather than in phosphate buffer. This result suggested that the spores germinated with the pressure and germination-inducing components in the broth, and that the geminated spores were inactivated by mild heating. The induction of germination remarkably started at 10 MPa at 40℃ or 20 MPa at 60℃ (the spore population decreased in the extent of two log-cycles for one hour). In phosphate buffer, the relationships between logarithm of spores and pressure became linear in the pressure range of 0.1-300 MPa. The germination ratio was 1.7 log-cycles / 100 MPa at 40℃ or 1.4 log-cycles / 100 MPa at 60℃ for one half-hour. In glucose broth, the relationships between logarithm of spores and pressure became linear in the pressure range of 50-300 MPa except for the data of 0.1 MPa. The germination ratio was 0.95 log-cycles / 100 MPa at 40℃ or 0.82 log-cycles / 100 MPa at 60℃ for one half-hour. The technique of the spore germination and inactivation owing to pressure and mild heating is efficient to reduce the spore in processed foods, especially, as a pre-preparation of food material.

Key words: pressurization, growth inhibition, pressure-holding, bacterial spore, germination, inactivation

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Studies on a novel inhibitor of xanthine oxidoreductase, Y-700; its inhibition mechanism and application

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キサンチン酸化還元酵素の新規阻害剤Y-700に関する研究
－ その阻害機構と応用 －

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Xanthine oxidoreductase (XOR) is a member of the molybdenum-containing hydroxylase family. The mammalian enzyme has two interconvertible forms, xanthine dehydrogenase (XDH) and xanthine oxidase (XO). In the catalytic cycle, XDH can reduce either oxygen or NAD^+ but has greater affinity for the latter, and generates NADH. On the other hand, XO only reduce molecular oxygen, and generates superoxide anion as well as hydrogen peroxide. Both forms catalyze the conversion of hypoxanthine to xanthine and xanthine to urate, the last two steps of urate biosynthesis. Thus, XOR is a target of drugs against gout and hyperuricemia. Moreover, as XOR generates superoxide anion and hydrogen peroxide, which are reactive oxygen species (ROS), the enzyme has been hypothetically proposed to be responsible for several oxidative stress-related diseases.

In clinical situation, allopurinol is the only available inhibitor of XOR for the treatment of gout and hyperuricemia. However, rare but severe side effects of allopurinol, including rash and hepatotoxicity etc., have been well known. Although, the exact mechanism of allopurinol's toxicity has not been identified, a rise in the blood concentration of the actual metabolite, oxipurinol, which has primarily renal route of excretion, has been pointed out, especially in patients with renal insufficiency. There have been efforts to develop the potential inhibitors with less toxicity; however, so far none has been made clinically available. More recently, a series of 1-phenylpyrazoles have demonstrated XOR inhibitory activity in vitro. Of the compounds prepared, 1-[3-cyano-4-(2,2-dimethylpropoxy)phenyl]- 1*H*-pyrazole-4-carboxylic acid, Y-700, had the most potent enzyme inhibition and displayed longer-lasting hypouricemic action than allopurinol. However, the mechanism of inhibition of XOR by Y-700 has not been precisely clarified.

The purpose of this study was to characterize the mechanism of action of Y-700 on XOR inhibition. A second purpose was to estimate the potential advantage of Y-700 for the practical or experimental application to pathophysiology in which XOR may be involved.

Mode of Action of Y-700 on XOR Inhibition

Steady-state kinetics with highly purified XO from bovine milk showed that the inhibition by Y-700 is a mixed type with K_i and K_i' values of 0.6 nM and 3.2 nM, respectively. The enzyme

titration experiment showed that Y-700 binds tightly to both the active sulfo-form and to the inactive desulfo-form of XO with K_d values of 0.9 nmol/L and 2.8 nmol/L, respectively. The crystal structure of the XDH/Y-700 complex showed no covalent bond between Y-700 and molybdenum. Instead, Y-700 was bound in a narrow channel leading to the molybdenum center of the enzyme, through a variety of hydrogen bonds and hydrophobic interactions and some of them were seem to contribute strong binding similar way of substrate recognition.

Suppressive Effects of Y-700 on Urate Biosynthesis in Rats and Humans

In rats, oral Y-700 dose-dependently decreased amounts of urinary allantoin, the end-product of purines in most animals, increasing the amounts of oxypurines (hypoxanthine and xanthine), the substrates of XOR. In terms of the observed effect, Y-700 appeared to be at least 10-fold more potent than allopurinol. Y-700 showed no uricosurics action. In hyperuricemic rats, established by repeated treatment with a uricase inhibitor, potassium oxonate, orally administered Y-700 dose-dependently reduced plasma urate levels. At a dose of 10 mg/kg, the hypouricemic action of Y-700 was more potent and of longer duration than that of allopurinol. Furthermore, single oral administrations of Y-700 (5, 20 or 80 mg) to Japanese male volunteers dose-dependently reduced serum urate levels. Urinary excretion of xanthine and hypoxanthine were markedly increased after dosing of Y-700 compared with placebo group. The hypouricemic action of Y-700 in rats and humans is presumably due to its long-lasting suppressive effect on urate biosynthesis by its potent XOR inhibitory action.

Pharmacokinetic Properties of Y-700 in Rats and Humans

In rats, orally administered Y-700 was absorbed rapidly with high bioavailability (84.1%) at a dose of 1 mg/kg. After oral administration of ^{14}C-Y-700 (1 mg/kg) to rats, unchanged Y-700 was hardly excreted in urine (1.1%) but mainly excreted in feces (45.3%). Also in the human subjects, single orally administered Y-700 was absorbed rapidly, and C_{max} and AUC of the compound were increased dose-dependently (5, 20 and 80 mg). Urinary excreted Y-700 was only 1.5% of dosing in volunteers. In both species, only unchanged Y-700 was detected in the plasma, and there was no evidence of any major circulating metabolites. No notable adverse event was observed throughout these study periods. These data elucidate that Y-700 is an orally effective inhibitor of XOR in rats and humans with hepatic route of excretion.

Suppressive Effect of Y-700 on Chemically-induced Colon Carcinogenesis in Mice

In 1,2-dimethylhydrazine (DMH)-treated mice, feeding of Y-700 (10 - 20 mg/kg diet) caused a significant suppression in the development of aberrant crypt foci (ACF) in the colon mucosa, accompanied by decrease in serum urate levels. The mucosal labeling index of bromodeoxyuridine was also significantly suppressed by Y-700. These results suggest that Y-700 suppresses DMH-induced carcinogenesis, and that ROS generated by XOR may act as an important mediator of mitogenic signaling on cancer cells.

In the present study, I clarified that Y-700 is a novel inhibitor of XOR with a distinctively different mechanism of inhibition from allopurinol. Since Y-700 is mainly eliminated via the liver but not kidneys, the compound is expected to provide safe and effective treatment of gout and

hyperuricemia, even in patients with renal insufficiency. Moreover, I elucidated the suppressive effect of Y-700 on DMF-induced colon tumorigenesis in mice. This finding suggested that Y-700 might be a useful probe to investigate pathogenesis of oxidative stress-related diseases in which XOR may be involved.

Key words: xanthine oxidoreductase, urate, Y-700, oxidative stress, colon carcinogenesis

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Species diversity and distribution patterns of lotic Chironomidae (Diptera)

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河川産ユスリカ類の種多様性と分布様式に関する研究

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Chironomidae is a dipteran family including as many as about 10,000 worldwide species, of which about 1,200 are recorded from Japan. The larvae predominate both in species richness and density of freshwater benthic ecosystems, and play an important role to connect primary producers with consumers in the food webs.

Variations in macrodistribution among lotic chironomid species have so far been attributed to within-river environmental factors, e.g., water quality, topographic type of streams. As for without-river factors, riparian forest provides the leaf litter for the larvae and supports the detrial food web, which is the majority of lotic community. Since the leaf litter is captured by the interstices of the riverbed at riffles, microhabitat of chironomid larvae should be different between exposed and interstitial surfaces of cobbles and/or boulders.

The aim of the present study was to clarify the relationships between chironomid assemblage composition and river-environmental factors. Multiple regressions and direct ordination techniques were used to determine which environmental variables mainly cause the variations in abundance, species richness, diversity and composition of the assemblages. Guidelines for conservation of species diversity in lotic ecosystems were proposed from the above results.

Chapter 1. Species diversity of lotic Chironomidae

Chironomid fauna were investigated at the main stream and tributaries of the Ohta River basin in Hiroshima, Japan. A total of 216 species belonging to four subfamilies was recorded, of which 137 and 161 were collected by larval samplings from riverbed and by imaginal samplings (light trap) from ambient air, respectively. Descriptions of new species were given in this study, i.e., *Polypedilum akisplendens, Polypedilum fuscovittatum, Polypedilum paranigrum, Polypedilum bingoparadoxum* and *Stempellinella coronata*.

Seventy-nine species, including terrestrial species, e.g., *Smittia aterrima*, and nuisance species emerging from paddy fields, e.g., *Polypedilum kyotoense*, were collected only by imaginal samplings, however, of which 49 were considered to dwell in the riverbed. Thus, imaginal samplings can collect species occurring in particular habitats difficult to investigate, e.g., hyporheic zone, so that be useful in evaluating lotic chironomid fauna.

Excluding 30 species that would occur outside of the riverbed, the subfamilies Chironominae and Orthocladiinae, of which many species were recorded from warm and cold regions, were 93 and

68 species, which accounted for 50.0 and 36.6 % of all the lotic species, respectively. This means that chironomid fauna of the Ohta River basin reflects warm-temperate river environment. The larval species only from tributaries accounted for 38.7 % of the 137 species, indicating that the tributaries contribute to raise species diversity at basin level.

Chapter 2. Relationship between within-river environment and microdistribution patterns of chironomid larvae

Chironomid microdistribution patterns on the surfaces of artificial substrates, concrete blocks, placed at riffles of streams with and without riparian forest were investigated. The block surfaces were classified into three types, i.e., 'exposure', 'hollow' and 'interstice', based on the conditions against water flow and accumulation of leaf litter. Larvae attached to the blocks were collected separately by the surface type.

At the streams with riparian forest, species richness and density on 'interstice' surface were about five and ten times higher than that on 'exposure' one, respectively, whereas no significant differences were found among the surface types at the streams without riparian forest. Assemblage ordination by partial redundancy analysis indicated that 20 out of 34 species collected at streams with riparian forest tended to be abundant at 'interstice' one, and 9 species, e.g., four *Rheotanytarsus* species, were significantly abundant.

These results indicate the importance of interstices as a microhabitat of chironomid larvae, which is due to accumulation of the leaf litter provided by riparian forest.

Chapter 3. Relationship between macro-environmental factors and distribution patterns of chironomid larvae

The relative importance of natural and anthropogenic factors, e.g., topographic type, riparian canopy, altitude, temperature and bank protection, on larval chironomid assemblage, was investigated in the Ohta River basin. A concrete block was used as an artificial substrate for chironomid collection in order to sampling regime should be identical among the sites.

Partial canonical correspondence analysis revealed that topographic type, riparian canopy coverage, water temperature and altitude were the main factors influencing the species distribution. *Stempellinella tamaseptima, Polypedilum tamanigrum* and five *Rheotanytarsus* species showed positive association with canopy coverage, while five *Cricotopus* species showed negative associations. Stepwise multiple regressions of the assemblage indices on the environmental variables were applied. Bank protection and depth showed negative correlations with Shannon diversity H'. Both topographic type and depth showed negative correlations with Pielou equitability J. Topographic type (lower reach) and specific conductance showed positive, while bank protection showed a negative correlation with abundance.

As a whole, topographic type was the most directly related factor to larval chironomid assemblages, but anthropogenic modification of riparian terrain was shown to be an important factor disturbing the natural composition.

These results suggest that the following endeavors should be needed to conserve lotic ecosystems: (1) grasping species composition of various taxa as much as possible, (2) clarifying the mechanism how species diversity is sustained with giving attention to habitat scale and (3)

comprehensive preservation and/or management of river environment including riparian terrain.

Key words : lotic Chironomidae, species diversity, distribution patterns, riparian environment, artificial substrates, multivariate analyses

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Nutritional studies on anti-tumor effect of vitamin B_6

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ビタミンB_6の抗腫瘍作用に関する栄養学的研究

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There is growing evidence that several dietary factors affect colon carcinogenesis. For example, high fat diet and high calorie diet are risk factors of colon carcinogenesis, and dietary fibers, DHA, folate, polyphenols, vitamin D are protective factors of the carcinogenesis. Recent epidemiological studies in USA and seven countries of Europe suggested an inverse relationship between consumption of vitamin B_6 and the incidence of colon cancer. Recent studies have indicated that consumption of vitamin B_6 by Japanese and Americans is generally not enough for its requirement. This study was conducted to test the possibility that dietary vitamin B_6 suppresses colon tumorigenesis.

Mice were fed diets containing 1, 7, 14, 35 or 70 mg vitamin B_6(pyridoxine HCl)/kg for 5 weeks and treated with a colon carcinogen, azoxymethane (AOM). Supplementation of vitamin B_6 to 1 mg vitamin B_6/kg diet caused a reduction in colon aberrant crypt foci (AOM), a precursor of colon cancer. In the long-term experiment of 22 weeks, AOM-treated mice were fed 1~35 mg vitamin B_6/kg. Dietary supplementation of vitamin B_6 lowered development of colon tumor in a dose-dependent manner among 1~14 mg vitamin B_6/kg. This finding is the first *in vivo* evidence for the anti-tumor activity of dietary vitamin B_6, and consistent with epidemiological studies.

Further study was performed to investigate the mechanisms of the anti-tumor effect of dietary B_6. Supplementation of vitamin B_6 lowered colon BrdU-labeling index (an index of cell proliferation) and expression of c-myc and c-fos. Colon oxidative stress markers including 8-OHdG and 4-HNE and expression of iNOS were also reduced by higher vitamin B_6 intake. The results imply that the anti-tumor effect of vitamin B_6 is ascribed to reduced cell proliferation, oxidative stress and NO production.

Experiment was conducted to examine the combined effect of dietary level of fat and vitamin B_6 on colon cell proliferation. Mice were fed low (50g corn oil/kg)- or high-fat (200g corn oil/kg) diets containing 1~35 mg vitamin B_6/kg for 5 weeks. The mice were treated with or without AOM injection. The results indicated that the inhibitory effect of dietary vitamin B_6 was markedly enhanced by high-fat diet, but slightly affected by AOM treatment. This finding suggested that combination of high-fat diet and lower vitamin B_6 intake is an important risk factor of colon cancer. In addition, the results raised the possibility that the molecular mechanism of higher colon tumor by high-fat diet appeared to be closely associated with that of the higher tumor by lower vitamin B_6 consumption.

This study elucidated the physiological function of dietary vitamin B_6 as a protective factor of colon cancer, and its potential mechanisms. This finding suggest that higher intake of vitamin B_6 is necessary for the prevention of colon cancer in Japan and USA.

Key words: vitamin B_6, high-fat diet, colon cancer, cell proliferation, oxidative stress

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Molecular biological studies on the bovine ovarian follicular cysts

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ウシ卵胞嚢腫の分子生物学的研究

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ウシの卵胞嚢腫は非常に高率に発生する障害であり，分娩後の空胎期間を延長させるため畜産経営に重大な被害を及ぼす。これまでウシ卵胞嚢腫の研究は視床下部―脳下垂体―卵巣系における内分泌系の機能障害に注目した内分泌学的検討や，顆粒層細胞や卵胞膜細胞における内分泌関連分子の検討を中心として行われており，嚢腫卵胞中の卵胞液に関する報告は少ない。特に卵胞液中のタンパク質の変動に注目した研究は殆どない。本研究は，ウシ卵胞嚢腫のさらなる病態解明の一助とすべく，プロテオミクスの手法を用い，嚢腫卵胞中のタンパク質の変動について検討した。

第２章では，ウシ卵胞液タンパク質の適切な二次元電気泳動像を得るため，卵胞液中タンパク質の電気泳動法を検討した。卵胞液タンパク質から等電点電気泳動を阻害するといわれている塩，脂質や核酸等，さらに多量に存在するアルブミン及びIgGの除去，並びに銀染色の適用により，タンパク質のスポットの染色性が増し，可視化されたスポット数は明らかに増加した。従って，適切に処理した卵胞液タンパク質を用いた二次元電気泳動は卵巣生理の研究の非常に有効な手段に成ると考えられた。

第３章では，食肉処理場で採取したウシ嚢腫卵胞の性状を明確にする目的で，種々の発育段階にある卵胞の形態，及び卵胞液のタンパク質及びステロイドホルモン濃度を測定し，正常卵胞と嚢腫卵胞を比較・検討した。卵胞は，直径が5~8㎜の正常小型卵胞（YF），９~12 mmの正常大型卵胞（DF），15~25 mmの準嚢腫卵胞（YC），25 mm以上の嚢腫卵胞（DC）に分けて採取した。その結果，正常卵胞は膜は正常な重層状の構造を呈していたが，準嚢腫卵胞（YC卵胞）では顆粒層細胞の規則的な配列が消失するとともに細胞の遊離が軽度にみられた。嚢腫卵胞（DC卵胞）では顆粒層細胞が完全に消失し，内卵胞膜の薄層化が認められた。嚢腫の形成に拘わらずタンパク質濃度に変化は無かった。ステロイドホルモンの測定では嚢腫形成に伴いテストステロン（T）及びエストラジオール-17β（E2）濃度は低下，プロジェステロン（P）濃度は上昇し，E2/P4濃度比は減少した。

第４章では，ディファレンシャルプロテオミクスの概念を導入し，正常及び嚢腫卵胞液中のタンパク質を第２章で示した方法を用いて二次元電気泳動を行い，嚢腫卵胞中で増加するタンパク質を検索し，次いでMALDI-TOF MSによる質量分析を行い増加するタンパク質の同定を試みた。その結果，嚢腫卵胞液のゲル上で特異的に増加する８つのタンパク質スポットが確認された。ついで，それらのスポットをMALDI-TOF MSで質量分析し，Peptide Mass Finger Print法によりNCBIデータベースを検索した。その結果，それらのタンパク質は，それぞれbovine mitochondrial f1-atpase，erythroid associated factor，methionine synthase（MeS），vascular endothelial growth factor-receptor (Fragment)，glyceraldehydes 3-phosphate dehydrogenase (Fragment)，heat-shock cognate 70kd protein 44kd atpase N-terminal mutant with Cys 17 replaced by Lys（Heat chock proteinn70, HSP70），β-lactoglobulin及びsuccinate dehydrogenae Ip subunitと同定された。これらのタンパク質，特にHSP70やVEGF受容体の過剰発現が卵胞の閉鎖遅延，卵胞液の蓄積あるいは卵胞の異常成長に関与し，卵胞嚢腫の形成に重要な役割を果たしていることが示唆された。

第５章では第４章において二次元電気泳動による検討で増加が確認された８つのタンパク質の中で，アポ

トーシスを抑制する機能を持つことから排卵や卵胞閉鎖に深く関与している可能性のあるタンパク質としてHSP70を，栄養や代謝に関連するタンパク質としてMeSを選択し，リアルタイムPCR法により，嚢腫卵胞と正常卵胞の各組織における遺伝子発現を比較し，嚢腫卵胞での発現増加組織を検討した。嚢腫卵胞では顆粒層細胞及び卵胞膜細胞でHSP70遺伝子の発現コピー数が増加していた。正常卵胞と嚢腫卵胞の比較では，嚢腫卵胞の顆粒層細胞及び卵胞膜細胞でのHSP70遺伝子の発現コピー数が，正常卵胞のそれぞれの細胞での発現コピー数より有意に高い値を示した。嚢腫卵胞の顆粒層細胞及び卵胞膜細胞で認められたHSP70遺伝子発現の増加は，嚢腫卵胞の顆粒層細胞及び卵胞膜細胞でのアポトーシス制御メカニズムに関連する非常に重要な変化であると考えられた。MeS遺伝子の発現コピー数の正常卵胞と嚢腫卵胞における比較では，いずれの細胞においても有意差は認められなかった。

本研究では，卵胞嚢腫の病態解明にプロテオミクスという新たな手法を導入し，卵胞液生理の研究に二次元電気泳動法が有効であることを示した。また卵胞液中のタンパク質を網羅的に観察することにより，嚢腫卵胞の卵胞液中で８つのタンパク質が増加することを確認した。さらにリアルタイムPCR法による検討で，嚢腫卵胞の顆粒層細胞及び卵胞膜細胞においてHSP70遺伝子の発現の増加が確認された。HSP70はアポトーシスを阻害することから，HSP70の過剰発現が内卵胞膜でのアポトーシスを抑制し，卵胞の閉鎖を遅延させ嚢腫卵胞が形成される可能性が強く示唆された。これまでに卵胞嚢腫とHSP70の関係について検討した報告は全くなく，本研究の成果は卵胞嚢腫のさらなる病態解明の大きな足がかりとなると考えられる。さらに，嚢腫卵胞中で増加が確認された８つのタンパク質を卵胞嚢腫のバイオマーカーとして使用した触診やホルモン濃度測定に因らない新たな卵胞嚢腫診断法の確立が期待できる。

キーワード：ウシ卵胞嚢腫，二次元電気泳動，リアルタイムPCR，Heat shock protein 70

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Basic and applied studies of recombinant chicken interleukin-6

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組み換えニワトリIL-6 の基礎並びに応用的研究

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IL-6 は，T 細胞，B 細胞, マクロファージおよび肝細胞など種々の細胞の活性化および分化に関与する重要な多機能性サイトカインである。IL-6 は，IL-6 特異的受容体IL-6R と，シグナル伝達受容体gp130 を介して細胞内にシグナルを伝達する。IL-6 のシグナルは，主にJAK/STAT シグナル経路により伝達されるが，このシグナルは，ネガティブフィードバック因子SOCS3 に代表されるような阻害因子により「負の制御」を受け必要以上のシグナル伝達を自身で抑制する機構を持っている。

近年，ニワトリの卵黄抗体が臨床診断や検査薬の分野で注目されているが，その要因の一つは，ニワトリが進化の点で哺乳類と大きく異なる動物であり，哺乳類で作出困難な哺乳類間高度保存分子に対する抗体を作出するのに有用な免疫動物だからである。そのため，将来はニワトリ型モノクローナル抗体 (mAb) 活用の期待も高まっている。当研究室で確立されたニワトリハイブリドーマ作製技術は，プリオンタンパク質 (PrP) 等の哺乳類間で高度保存された分子に対して特異性が高く，バックグランドの低い優れたニワトリ mAb の作製を可能にした。しかし，樹立されたニワトリハイブリドーマの抗体産生能は，マウスハイブリドーマの抗体産生能と比較して低いという問題点を抱えている。この問題点を解決することは，ニワトリ型 mAb の大量調製を可能にし，同抗体の汎用性拡大に繋がるものと考えられる。マウスでは， IL-6 がマウスハイブリドーマの細胞増殖や抗体産生の促進に作用することが知られており，ハイブリドーマの培養系に有用な因子として利用されている。

そこで本研究では，ニワトリハイブリドーマ培養系にニワトリIL-6 (chIL-6) を活用することを目的として，はじめに組み換えchIL-6 (rchIL-6) を作製し，ニワトリハイブリドーマHUC2-13 に対する生物活性試験を実施し，同細胞株に対する有効性を検討した。続いて，chIL-6R をクローニングし，特にHUC2-13 に注目してニワトリ組織および細胞株における発現を解析した。最後に，chIL-6R のニワトリハイブリドーマ培養系への活用法として，chIL-6RとchIL-6 の融合タンパク質Hyper-chIL-6 (H-chIL-6) を作製し，HUC2-13 培養系に対してchIL-6 添加条件とのSTAT3 のリン酸化および抗体産生効果を比較すると共に，chIL-6 またはH-chIL-6 添加時におけるHUC2-13抗体遺伝子および抗体遺伝子発現の関連遺伝子の発現を解析した。

[ニワトリハイブリドーマに対する組み換えニワトリIL-6 の有効性の検討]

rchIL-6 は大腸菌および動物細胞発現型の 2 種類を作製し，IL-6 依存性マウスハイブリドーマMH60 および抗ヒトPrP mAb 産生ニワトリハイブリドーマHUC2-13 に対して細胞増殖促進効果を試験した。その結果，両rchIL-6 は，濃度依存的にMH60 の細胞増殖を促進したが，HUC2-13 に対しては効果を示さなかった。しかし，rchIL-6 添加により，HUC2-13 の抗体産生量が増加していた。また，rchIL-6 添加によりSTAT3 のリン酸化がマウス，ニワトリ両ハイブリドーマで観察されたことから，chIL-6 添加によるSTAT3 のリン酸化がHUC2-13 抗体産生能に影響を与えている可能性が示唆された。しかし，rchIL-6 同濃度添加条件下のHUC2-13 とMH60 を比較した場合，HUC2-13 のリン酸化STAT3 量はMH60 と比べて少なかったことから，このリン酸化の違いは両ハイブリドーマにおけるIL-6R の発現量に依存している可能性が考えられた。よってニワ

トリハイブリドーマ培養系におけるrchIL-6 の活用を展開していくには，chIL-6R の解析をする必要があると考えられた。

[ニワトリIL-6 レセプターの解析]

chIL-6R 完全長cDNA は，ニワトリ肝細胞株LMH cDNA を鋳型としてRACE 法によりクローニングした。クローニングしたchIL-6R cDNA の全長はポリA 配列を含めて1857 bp であり，そこから予想されるアミノ酸は445 アミノ酸であった。chIL-6R と哺乳類IL-6R のアミノ酸レベルでの相同性は40% であったが，FN III ドメインのN 末端側に4 つのシステイン残基が，C 末端側にWSXWS モチーフが完全に保存されていたこと，ヒトIL-6R においてIL-6 との結合に重要なアミノ酸がchIL-6R でも保存されていた。ニワトリ細胞株由来cDNA を用いて，chIL-6R およびchgp130 mRNA の発現を解析したところ，ニワトリマクロファージ様細胞株HD11, ニワトリ単球性白血病細胞株IN24 およびLMH ではchIL-6R が強く発現していた。一方，HUC2-13 ではgp130 の高い発現は認められたものの，chIL-6R の発現は低いことがわかった。LMH とHUC2-13 にrchIL-6 を添加して，刺激培養後にリン酸化STAT3 を検出したところ，LMH におけるリン酸化STAT3 量は，rchIL-6 同濃度添加条件下のHUC2-13 よりも有意に多かったことから，rchIL-6 の標的細胞に対するシグナル量は，chIL-6R の発現量に依存していることが強く示唆された。

[ニワトリIL-6 リセプターのHUC2-13 培養系への活用並びにIL-6 添加によるHUC2-13抗体産生機構の解析]

近年哺乳類では，IL-6 と可溶型IL-6Rの融合タンパク質H-IL-6 が作製され，gp130 陽性細胞に対してシグナルを伝達することが報告されている．そこでchIL-6R のニワトリハイブリドーマ培養系への活用法として，chIL-6RとchIL-6 の融合タンパク質H-chIL-6 を作製し，rchIL-6 添加群とのリン酸化STAT3量を比較した。その結果，H-chIL-6 添加群はchIL-6 添加群に比べてリン酸化STAT3 量が大きく増加していた。しかし，H-chIL-6 添加条件では，予想に反して抗体量の増加は認められなかった。哺乳類では，IL-6 による抗体産生促進効果は，転写因子を介して転写レベルで制御されている。そこでrchIL-6 またはH-chIL-6 添加条件のHUC2-13 cDNA を用いて，HUC2-13 抗体遺伝子およびその発現制御に関与すると思われる遺伝子群の発現をRT-PCR により解析した。その結果，rchIL-6 添加群ではSTAT3 のリン酸化に伴い，転写因子C/EBPβ，ネガティブフィードバック因子SOCS3 および抗体重鎖 (VH) 遺伝子の発現量が増加していた。また，EMSA 試験から，リン酸化STAT3 がC/EBPβ およびSOCS3の発現を制御し，C/EBPβ がVH の発現を制御していることが示唆された。それに対して，H-chIL-6 添加条件は，rchIL-6 添加条件のようにC/EBPβ およびVH の発現上昇は認められなかった。その一方で，SOCS3 の発現は経時的に増加しており，rchIL-6 添加時よりも「負の制御」が強く引き起こされた可能性が考えられた。

最後に，chIL-6 を培養24 時間毎に添加して，初日のみに添加した培養条件と抗体産生量を比較したところ，継続添加群で抗体量が増加しており，chIL-6 の継続添加がニワトリハイブリドーマの抗体産生能を増強させるのに有効であることが示された。

本研究は，rchIL-6 がSTAT3 をリン酸化，C/EBPβ の発現上昇を経てVH の発現量を上昇させ，HUC2-13 抗体産生量の上昇を誘導することを示した。また，継続添加が有効な活用法であることを示した。今後，chIL-6 およびH-chIL-6 を有効活用することに加えて，C/EBPβ 以外の抗体遺伝子の発現を制御する転写因子を同定することでニワトリハイブリドーマ抗体遺伝子の転写機構の解析を進めることで，ニワトリmAb の大量調整にさらに寄与できると考えられる。

Key words: rchIL-6, ニワトリハイブリドーマ，mAb, STAT3, C/EBPβ, SOCS3

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Studies on effect of dietary fats on the gene expression in brain

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食餌脂肪の脳・中枢遺伝子発現に及ぼす影響に関する研究

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There is growing evidence that brain diseases and functions are affected by several dietary factors, including dietary types and level of fats. Epidemiological studies have indicated that high consumption of saturated fatty acids elevates the risk of brain diseases such as Alzheimer disease (AD) and Parkinson disease (PD), as well as diabetes and obesity. Experimental studies have also suggested that higher intake of saturated fatty acid lowers learning ability. On the other hand, epidemiological studies have suggested that consumption of n-3 polyunsaturated fatty acids including docosahexanoic acid (DHA) reduce the risk of Alzheimer disease. Further, DHA intake is known to improve brain functions.

My studies were performed to investigate the influence of dietary fats on the gene expression in mouse brain by the methods of differential display and suppression subtractive hybridization. In chapter 1, experiments were conducted to examine the influence of animal fats on gene expression in mouse brain. In chapter 2, experiments were conducted to examine the influence of fish oil on gene expression in mouse brain.

Identification of brain factor affected by high beef tallow diet

High-fat diet is well known to be a risk factor of obesity, heart disease, diabetes and cancer. Recent epidemiological and clinical studies suggest that higher intake of saturated fatty acids lead to development of AD and PD. In this study, I postulated that high intake of saturated fatty acids might enhance development of several brain diseases. To examine this hypothesis, experiment was conducted to identify the factors in mice brain affected by high beef-tallow diet. Mice were fed either 20% beef tallow diet or 5% corn oil diet for 4 weeks. The responses of several gene expressions to the beef tallow diet were examined by differential display method. As a result, expression of ZPR1 gene was found to be up-regulated by the beef tallow diet. ZPR1 is known to be involved in the signal transduction by signals of cell proliferation, and interact with Elongation factor 1α and Survival motor neuron protein to regulate cell cycle and RNA processing. Higher expression of ZPR1 was observed in the cerebellum and hippocampus of mice fed high-fat diet and obese KKAy mice. The results suggest that higher expression ZPR1 is associated with high-fat intake and obesity.

To analyze the physiological significance of higher expression of ZPR1 in the brain, stable ZPR1-transfected clones from Nuro-2A cells were obtained, and exposed to oxidative stress (H_2O_2). The results showed that cell survival of transfectans was significantly reduced compare to mock

clones. This finding implies that higher expression of ZPR1 exaggerates the damage caused by oxidative stress. Taken together, high-fat diet appears to cause the development of brain diseases by a mechanism involving higher expression of brain ZPR1.

Identification of brain factor affected by fish oil diet

Previous studies have suggested that DHA, contained in fish oil, prevents brain diseases including AD. In this study, the effect of fish oil feeding on gene expression in the brain was investigated by suppression subtractive hybridization. Mice were fed either 20% sardine oil diet or 20% corn oil diet for 4 weeks. The results showed that pyruvate dehydrogenase E1 alpha (PDHE1 α) mRNA expression is down-regulated by fish oil feeding. Further, I examined whether the expression of PDHE1 α mRNA is altered by DHA treatment in differentiated PC12 cells. PDHE1 α mRNA was reduced by supplementation of DHA with a significant decrease in cellular ATP level. These results indicate that fish oil feeding might modulate energy metabolism in the brain.

Key words : high-fat diet, DHA, brain disease, gene expression, ZPR1, pyruvate dehydrogenase E1 alpha

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Cryo-cutting of frozen fish

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凍結魚肉の低温切断法に関する研究

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At very low temperatures under their freezing point, many materials show a great decrease in fracture stress or fracture energy, called “low temperature brittleness”. This behavior of materials in association with vitrification at temperatures is applied to “frozen crushing”, and this characteristic behavior of the materials is able to cut when an external load added is using. For instance, ceramics that are in a glass state at room temperature can be cut into arbitrary sizes by applying external loads (bending or compression) without causing large quantities of cutting dust. This idea can be applied to food, in particular as a good processing system for fish that is presently mainly cut with a band saw.

We have developed a new method for cutting frozen fish called “Cryo-cutting” in which a bending force is applied to the frozen fish at an appropriate low temperature. The influence of the direction of the distribution of the muscle fiber to the direction of the bend load has been examined. However, it is necessary to undertake actual bending tests at low temperatures to establish suitable temperature conditions for cryo-cutting for individual fish species.

This study researched the determination of an optimum cryo-cutting temperature using DSC (Differential Scanning Calorimeter) analysis equipment as that is able to measure small amounts of sample fish. First, the change in the start temperature for cutting and the bending energy were examined by material testing using model fish material with different moistures and lipid amounts, and the correspondences of the results of the material testing with the DSC measurements of the model fish were examined. Further, whether a similar examination using an actual fish (mackerel) with a known moisture and the amount of lipid, could be forecast by DSC was examined.

Chapter 1

An introduction to this thesis; the background, meaning, and purpose of the research are described.

Chapter 2

Frozen surimi (ground fish-meat) gel samples with different moisture contents were subjected to measurements of their fracture stress (bending fracture energy) in a low temperature range. The optimum conditions for low-temperature cutting (“cryo-cutting”) were estimated from the values of enthalpy change measured by a differential scanning calorimeter (DSC).

Frozen surimi gel of 90% moisture could not be cut by bending at -40℃, although it could be cut this way at -60℃. The temperature, A, at which surimi gel starts to be cut by bending fell in conjunction with a reduction in moisture content. Bending energy was observed to fall around -70℃ and was nearly constant below a temperature, B, which corresponded to the glass transition start point temperature (Tig) obtained by DSC measurement while the glass transition end point temperature (Teg) corresponded to temperature A. As a result, an appropriate cutting temperature as calculated by DSC measurement was below -80℃, but was dependent on moisture content.

Chapter 3

Frozen surimi samples having different moisture and cod oil contents were subjected to measurements of their fracture stress in a low temperature range below 0℃. The optimum conditions for low- temperature cutting, "cryo-cutting," were estimated from the results of enthalpy change measured by a DSC.

Frozen surimi of 60~90% moisture and of 1~20% cod oil, had frozen at -40℃, but was not cut by bending, though it could be cut at -80℃ (temperature A). The melting temperature of cod oil by DSC measurement was above -80℃. Thus, we could cut the surimi below the melting temperature of cod oil detected by DSC.

No great decrease of bending fracture energy of surimi sample was measured below temperature A, and glass transition was not observed on the DSC chart. It was considered that cod oil restrained the appearance of glass transition in the surimi sample because the melting temperature of cod oil overlapped with the temperature range of glass transition of surimi without oil.

The result showed that the optimum cutting temperature was considered below -80℃, and that it could be estimated by DSC measurement.

Chapter 4

Frozen mackerel meat samples were subjected to measurements of their fracture stress in a low temperature range. The optimum conditions for low temperature cutting, "cryo-cutting," were estimated from the results of enthalpy change measured by a DSC.

There were two enthalpy changes showing grass transition on the DSC chart of mackerel, one was at -63 ~ -77℃ and another was at －96 ~ -112℃. Thus, we estimated that mackerel was able to be cut by bending below -63℃ and that a great decrease of bending energy would occur around -77 ℃ and -112℃.

Test pieces of mackerel froze at -40℃, but it could be cut this way at -70℃ by bending. There were two great decreases of bending energy, one at -70 ~ -90℃ and the other at -100 ~ -120℃.

As a result, DSC measurement of mackerel meat provides a good estimation for the appropriate cutting temperature of mackerel, and it is applicable to this estimation method for other actual fish species.

Chapter 5

It became clear in this study that an appropriate freezing temperature for cryo-cutting is greatly influenced by the glass transition temperature of the material. Glass transition temperature of an individual fish can be ascertained by cryo-DSC using a refrigerative such as liquid nitrogen with a small amount of sample. If the glass transition temperature of the fish can be measured by electric

capacity measurement, it becomes possible to determine an appropriate cryo-cutting temperature of an individual fish by nondestructive means. It is possible to cut fish in the direction almost appropriate for cutting by considering the direction of the distribution of the muscle fiber. By using the stress concentration effect, frozen fish can be cryo-cut in a straight line by making an incision in the part where cutting should begin, and as the incision is deepened, a decrease in the cutting load becomes possible.

As noted above, the reality of the cryo-cutting of frozen fish has become a reality because of accumulated results of reviews of work done to date. A useful result showing the practical application of this cutting method was obtained for individual fish in this study.

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Genetic Evaluation of Japanese Native Chicken Populations Based on Microsatellite DNA Polymorphisms

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マイクロサテライトDNA多型に基づいた日本鶏集団の遺伝学的評価

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In the present study, by using 20 microsatellite DNA markers, genetic analyses were carried out to quantify the variability in Japanese native chicken breeds sampled in different Japanese locations and to investigate the relationship between Japanese native chickens with other imported commercial breeds present in Japan. The results were summarized as follows.

1. Genetic variability and relationships of Oh-Shamo and its related breeds

In this experiment, Shamo-group breeds (Oh-Shamo, Ko-Shamo, Yakido, and Kinpa), Oh-Shamo-related breeds (Koeyoshi, Minohiki-dori, Satsuma-dori, and Hinai-dori), Shoukoku, and one European breed (White Leghorn) were used. A total of 167 alleles were detected across the 10 breeds, and the mean number of alleles per locus was 8.35. In each breed, the number of alleles per locus (*MNA*), proportion of polymorphic loci (P_{poly}), and mean expected heterozygosity (H_e) ranged from 1.750 (Koeyoshi) to 4.70 (Satsuma-dori), 0.50 (Koeyoshi) to 1.00 (Oh-Shamo, Satsuma-dori, Hinai-dori, and Shoukoku), and 0.212 (Koeyoshi) to 0.671 (Satsuma-dori), respectively. All the breeds studied had one or more private allele(s). In the neighbor-joining tree reconstructed based on the genetic distance D_A showed that there was clear separation between the Japanese breeds and White Leghorn. Within Japanese native breeds, three groups were determined as follows: (1) Oh-Shamo and Ko-Shamo, (2) Yakido, Kinpa, and Koeyoshi, and (3) Minohiki-dori and Shoukoku. However, the Hinai-dori and Satsuma-dori breeds were located far from these three groups.

2. Genetic variability and relationships of the breeds established in Kochi Prefecture

In this study, seven Kochi Prefecture native breeds (Miyadi-dori, Ohiki, Onaga-dori, Tosa-Jidori, Tosa-Kukin, Toutenkou, and Uzurao), Shoukoku, and two foreign breeds (White Leghorn and Rhode Island Red) were investigated. A total of 155 alleles were detected across the breeds, with the mean number of alleles per locus, 7.75. Genetic variability of 20 microsatellites examined varied depending on the breeds, as *MNA,* P_{poly}, and H_e ranged from 2.05 (Miyadi-dori) to 3.90 (Rhode Island Red), 0.75 (Miyadi-dori) to 1.00 (Rhode Island Red, Shoukoku and Uzurao), and 0.330 (Miyadi-dori) to 0.607 (Rhode Island Red), respectively. All breeds had one or more private microsatellite allele(s). According to the neighbor-joining tree reconstructed based on D_A genetic distance, among the breeds established in Kochi Prefecture, fancy and utility breeds belonged to different clusters.

Among the fancy breeds, those having thick and long feathers in the tail and saddle showed a close relationship to the Shoukoku breed, which also has thick and long feathers in the tail and saddle.

3. Genetic variability and relationships of the breeds designated as Natural Monuments of Japan

In this experiment, 22 breeds of Japanese native chickens that have been designated as Natural Monuments of Japan (Chabo, Gifu-Jidori, Hinai-dori, Jitokko, Kinpa, Koeyoshi, Kuro-Kashiwa, Ko-Shamo, Kawachi-Yakko, Minohiki-dori, Mie-Jidori, Ohiki, Onaga-dori, Oh-Shamo, Satsuma-dori, Shoukoku, Tosa-Jidori, Toumaru, Toutenkou, Ukokkei, Uzurao and Yakido) coupled with two foreign breeds (White Leghorn and Rhode Island Red) were investigated. A total of 206 alleles were detected across all studied breeds and the mean number of alleles per locus was 10.30. In each breed, *MNA,* P_{poly}, and H_e ranged from 1.750 (Koeyoshi) to 4.70 (Satsuma-dori), 0.55 (Koeyoshi), to 1.00 (Chabo, Gifu-Jidori, Hinai-dori, Jitokko, Oh-Shamo, Rhode Island Red, Satsuma-dori, Shoukoku, White Ukokkei and Uzurao), and 0.212 (Koeyoshi) to 0.671 (Satsuma-dori), respectively. Microsatellite alleles being unique to a particular breed were detected in some breeds. According to the neighbor-joining tree based on the D_A genetic distance, the chicken breeds were divided into three major groups. The first group consisted of Koeyoshi, Kinpa, Mie-Jidori, Yakido, Kuro-Kashiwa, Toumaru, Rhode Island Red, White Leghorn, Hinai-dori, Ukokkei, Jitokko and Satsuma-dori; the second group comprised Ohiki, Onaga-dori, Toutenkou, Shoukoku, Gifu-Jidori, Kawachi-Yakko, Tosa-Jidori, Chabo and Uzurao; and the third group included Ko-Shamo, Oh-Shamo and Minohiki-dori.

4. Genetic variability and relationships of Japanese native chickens and some foreign breeds

In this survey, 28 breeds of Japanese native chickens (Aidu-Jidori, Chabo, Ehime-Jidori, Gifu-Jidori, Hinai-dori, Jitokko, Kinpa, Koeyoshi, Kuro-Kashiwa, Ko-Shamo, Kumamoto, Kawachi-Yakko, Minohiki-dori, Mie-Jidori, Miyadi-dori, Nagoya, Ohiki, Onaga-dori, Oh-Shamo, Satsuma-dori, Shoukoku, Ukokkei, Tosa-Jidori, Tosa-Kukin, Toumaru, Toutenkou, Uzurao, and Yakido) and seven foreign breeds (Barred Plymouth Rock, New Hampshire Red, Red Cornish, Rhode Island Red, White Cornish, White Leghorn, and White Polymath Rock) were examined. A total of 217 alleles were detected across all breeds, and the mean number of alleles per locus was 10.85. In each breed, *MNA,* P_{poly}, and H_e ranged from 1.75 (Koeyoshi) to 4.70 (Satsuma-dori), 0.55 (Koeyoshi) to 1.00 (Barred Plymouth Rock, Chabo, Ehime-Jidori, Gifu-Jidori, Hinai-dori, Jitokko, New Hampshire Red, Oh-Shamo, Red Cornish, Rhode Island Red, Satsuma-dori, Shoukoku, Ukokkei, Uzurao and White Plymouth Rock), and 0.212 (Koeyoshi) to 0.671 (Satsuma-dori), respectively. Private microsatellite alleles were detected in some breeds. According to the neighbor-joining tree reconstructed based on D_A genetic distance, with a few exceptions, Japanese native chicken breeds and foreign breeds were clearly separated from each other. Furthermore, the Japanese native chickens were divided into 4 main classes by their body morphology: (1) Cochin type, (2) Malay type, (3) intermediate type between Malay and layer types, and (4) layer type, although there were some exceptions. This is the first discovery in Japanese chicken breeds.

This is the first study in which microsatellite profiling approach was applied to a large number of Japanese chicken breeds to reveal their genetic variability and relationships. The results of the

present study will be useful in order to support the decision on conservation and further use of Japanese native breeds. Furthermore, breed specific alleles could offer an efficient means to trace origins of meat in the commercial circulations.

Key words: Japanese native chickens, microsatellite DNA polymorphism, genetic variability, genetic relationship

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Physical analyses of lipid nanospheres for materials design of delivery systems of sparingly soluble drug materials

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リピッドナノスフィアを利用した難水溶性薬物の製剤設計と物性解析に関する研究

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With the aim of developing new drugs, new technologies such as genomic research, combinatory chemistry are useful for the screening and synthesizing of new compounds. The tendency of the new compounds shows sparingly water-soluble character. So it is necessary to use new device or high technology for the formulation using such compounds. The technology of lipids sphere is used for the improvement of bioavailability as controlled release and targeting of sparingly water-soluble drugs. However, lipids sphere has many problems such as physical stability, amounts of enclosed drug, handling and so on.

In this study, we examined the properties of lipid spheres, and our new studies about (1) crystallization and polymorphic behavior of μm and nano-meter sized emulsions (nm-sized emulsion), (2) stability of nm-sized emulsion in heating process, (3) lording capacity of drug in nm-sized emulsion.

In the first chapter, we explained about our purpose using lipids spheres for pharmaceutical, and problems of lipids spheres. In the second chapter, we described crystallization and polymorphic behavior of palm stearin (PS) in a bulk state and oil-in-water (O/W) emulsion droplets (average diameter, 1.7 ± 0.3 μm). We used PS for oil phase in emulsion, because of the high stability of PS in air condition. Differential scanning calorimetry (DSC), *in-situ* X-ray diffraction with synchrotron radiation (SR-XRD), and polarized microscope were employed in the experiments. As for the bulk sample, the DSC measurements showed three main exothermic peaks at 31 ℃, 21 ℃ and 3 ℃ on cooling, and broad endothermic peaks around -3 ℃, 8 ℃, 15～25 ℃, 37 ℃ and 53 ℃ on heating. The SR-XRD patterns taken during cooling from 60 ℃ to -5 ℃ clarified that the DSC exothermic peaks at 31 ℃ and 3 ℃ corresponded to crystallization of α form of high-melting and low-melting fractions, respectively, and that the occurrence of β' form corresponded to the small endothermic peak of 21℃. Then, the SR-XRD patterns taken during heating from -5 ℃ to 60 ℃ showed that the DSC endothermic peaks corresponded to the following transformation: melting of α of the low-melting fraction (-3 ℃), melt-mediation transformation from α form to β' form (15～25 ℃), melting of β' form (36 ℃) and melting of β form (53 ℃) of the high-melting fraction. As for the O/W emulsion sample, the DSC and SR-XRD measurement during the cooling and heating processes showed basically the same behavior as that of PS in the bulk state, except for such manners that the crystallization of β' form during the cooling process did not occur, and that the

crystallization of α form, melt-mediated $\alpha \rightarrow \beta' \rightarrow \beta$ transformation, and melting of β form occurred at lower temperature in the emulsion droplets.

In the third chapter, we discussed about crystallization and polymorphic behavior of PS dispersed in nm-sized emulsion (average diameter, 120 ± 30 nm). We employed the same methods as those used for the bulk and μm-meter sized emulsion. As for the Decaglycerin -mono-laurate (10G1L) having a small hydrophobic group for emulsifier, the crystallization temperature (Tc) of emulsion was 15 ℃ which was decreased from that of the bulk PS (31 ℃). On the other hand, Tc was increased as the fatty acid moiety was varied from lauric to myristic, palmitic and stearic acids. In particular, Tc was 49 ℃ when decaglycerin -mono-stearate (10G1S). This result suggests that using the emulsifiers with long fatty ester with high melting point may cause the crystallization of its fatty acid chains that may induce the crystallization of PS in the nm-sized emulsion. We assume that the crystallized emulsifier may act as template for the nucleation of PS crystals, whose effects are more remarkable than the PS crystallization in μm-sized emulsion droplets. This effect might be ascribbed to tight packing of the hydrophobic region of the interfacial emulsifier membrane that comprises the nm-sized emulsion.

In the fourth chapter, we explained the stability of the nm-sized emulsion in heating process. As for the 10G1S which has large hydrophobic group, it indicated highest stability in three types emulsions (10G1S, Decaglycerin-mono-myristrate: 10G1M and 10G1L). But as for the 10G1L, it indicated coalescence of nm-sized-emulsion. This is because that there is a tight hydrophobic interaction between 10G1S and oil phase. So this result indicates that we can use nm-sized emulsion for injections and infusions.

In the fifth chapter, we described solublization by nm-sized emulsions. In this study, we used soybean oil for oil phase in emulsion and prepared several sized emulsions (100 nm, 200 nm and 400 nm). Then we added several types of model compounds as sparingly water-soluble character to nm-sized emulsions. The result shows that as smaller as the size of emulsion become or as longer as fatty ester of emulsifier become, the amount of model compound soluble in nm-sized emulsion is increased. The last chapter summarized the results obtained in this work.

In these studies, we indicated physical problems about nm-sized emulsion revealing new several functions. We also showed several physical date about lipid spheres. These date support us for using lipid spheres as career of sparingly water-soluble drugs, and this career will be able to has several properties such as high stability in heating process or in storing low temperature, high lording capacity of drug, controlled release or targeting.

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Study on bio-molecule which participates in the reaction of the damaged skin

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傷害刺激時における皮膚の反応に関与する生体分子の研究

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The skin which is the largest organ in the body serves as a protective cover, and is also a major producer of various functional proteins like growth factors and cytokines. Since the outermost layer epidermis, which is supported by basement membrane and overlies the dermis, is exposed to the atmosphere, it has a role as an important barrier against infection by external microbes or physical/chemical wounds. Furthermore, the epidermis has an ability to regenerate the impaired tissue by itself. Cutaneus wounds elicit a series of cellular responses including clotting, inflammatory cells infiltration, re-epithelialization, the formation of granulation tissue comprising of fibroblasts and new blood vessels, and then matrix deposition and wound contraction at later stage. During the recovering process, the dermal keratinocytes, fibroblasts, and other cells should interact, co-operate, proliferate and differentiate properly. Furthermore, growth factors and cytokines produced in response to injury are also involved in regulating the processes. Some of those functional proteins are known to be produced by the dermal keratinocytes. Thus, keratinocytes are crucial to keep skin turnover under normal physiological conditions and in the maintenance of skin. In this study, we paid attention to the epidermal cells and investigated the bio-molecule that participates in the reaction of the damaged skin.

Establishment of a series of monoclonal antibodies that specifically recognize hamster keratinocytes

First, we took a forward genetics approach to find novel functional molecules that are involved in the functions of skin keratinocytes. By immunizing mice with hamster keratinocytes, we established a series of monoclonal antibodies (mAbs) and obtained five mAb clones that specifically recognized hamster skin keratinocytes. The characteristics of each mAb clone were examined using hamster skin sections or skin ketatinocytes. We found that the K114 mAb, one of the established mAbs, recognized cell-surface protein that is expressed restrictedly in the dermal sheath cells near bulge area of the hair follicle and in the differentiated sebocytes of normal adult hamster skin.

Identification of the AgK114 recognized by a mAb K114

The cDNA of AgK114 was obtained from 1 day cultured hamster keratinocytes by expression cloning using mAb K114. Sequence analysis revealed that it had 242 amino acid residues with a

signal peptide at the N terminus, six potential N-glycosylation sites, a characteristic repetitive threonine rich domain, and a possible glycosylphosphatidylinositol (GPI) anchoring site near the C terminus. Based on the amino acid sequence of the hamster AgK114, we searched for homologous molecule in both GenBank and the Swiss-Protein databases. Mouse AgK114 was found to be approximately 60% homologous to the hamster AgK114. However, neither functional motifs nor biological functions have been reported for any AgK114 molecules of any species.

Study on expression pattern of AgK114

To elucidate biological significance of AgK114, we examined the expression of AgK114 under various conditions in hamster, such as the UV irradiation, wounding, and inflammation. Interestingly, AgK114 molecule was expressed accompanying tissue damages of the skin. It was transiently induced in the basal epidermal keratinocytes after UV exposure. In addition, AgK114 was also induced in elongating edged epidermal keratinocytes during tissue regeneration after an excised wounding. The location and expression patterns of AgK114 on the skin suggest that AgK114 is involved in the wound healing response.

The function of AgK114 on wound healing process

To analyze the biological functions of AgK114, we did an experiment in the murine excisional wound model by using the recombinant mouse AgK114FL variant protein, in which the FLAG epitope was attached to the C-terminal. Exogenous mouse AgK114FL promoted wound closer of impaired skin and formation of blood vessels in granulation tissue. In Addition, exogenous mouse AgK114 up-regulated pro-matrix methalloprotease-9 (MMP-9), vascular endotherial growth factor, transforming growth factor-beta 1, IL-6, and IL-1 β production during early stage of wound healing in impaired tissue. Interestingly, mouse AgK114 induced the MMP-9 activity of wound fibroblasts prepared from impaired skin, in the presence of proinflammatory cytokines. These results suggest that mouse AgK114 mediates the wound response during the healing process, and promotes wound repair.

In this study, we have identified the hamster AgK114 as a novel molecule from hamster keratinocytes, and we have clarified that its protein is induced promptly by replying to the wound reaction. Moreover, we made it clear that AgK114 promotes the production of wound response proteins/factors. These findings are useful in that AgK114 becomes one of the key molecules in the wound healing treatment and in the advanced medical field such as the organogenesis and the tissue engineering.

Key words : skin, keratinocytes, monoclonal antibody, AgK114, wound healing

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Transcriptional regulation for the plant specific transcription factor, Dof protein.

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植物特異的な転写因子Dofタンパク質による転写調節機構

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Ascorbate oxidase (AAO; EC 1.10.3.3) catalyzes the oxidation of ascorbic acid to monodehydroascorbic acid. AAO is highly expressed in cucurbitaceous plants such as pumpkin and cucumber. Its definitive biological function remains unclear, although the enzyme is considered to be localized in the cell wall. AAO is markedly induced by auxin, which causes cells to elongate. In tobacco, the AAO gene is highly expressed in young tissues where rapid elongation is required. Furthermore, the transgenic tobacco protoplasts over-expressing pumpkin AAO expanded more rapidly than wild-type protoplasts. These results suggest that AAO plays an important role in cell growth. We have studied the transcriptional regulatory mechanism of the AAO gene.

The unique element AGTA repeat (AAAAAGTAAAAAGTAAAAAGTA AAAAG), which has three AGTA sequences surrounded by A tracts, is found in the silencer region of the pumpkin AAO gene. The AOBP (ascorbate oxidase gene binding protein) has isolated as a protein bind to this unique sequence, AGTA repeat. AOBP has a zinc finger DNA-binding domain named the Dof domain, which is conserved only in higher plants. Although the Dof proteins are considered as the plant specific transcriptional factors, it is little known the physiological functions and how the Dof proteins regulate the expression of the target genes as well as AOBP.[1] In this study, I focused on one of the Dof proteins, AOBP, and analyse its DNA-binding function, the molecular mechanisms for the transcriptional regulation and the physiological functions in plant.

At first, in order to investigate the functions of the AOBP, I isolated the cDNA of AOBP homologue from tobacco by RT-PCR method. Tobacco (*Nicotiana tabacum*) is one of the model plants for research and the experimental methods using tobacco such as transformation, cultivation of cells or protoplasts, are well-established. The Dof domain region in NtAOBP (Gln106 - His178) was expressed as a GST-fused protein in *E.Coli*, then analyzed the DNA-binding activities of this fused protein by the gel mobility shift assay. As the results, the Dof domain in NtAOBP was able to bind to the AGTA repeat as well as the pumpkin AOBP. Furthermore, I investigated whether the DNA-binding activity of Dof domain is inhibited by various metals. As a result, the Dof domain was shown to be more sensitive to toxic metals because more metal ions that inhibited the DNA binding in the Dof domain than in other zinc fingers. Furthermore, we investigated the possibility of coordination of metals other than zinc in the Dof domain. The results show Manganese ion as well as zinc ion were coordinated by the Dof domain *in vitro*. On the other hand, the analysis using

inductively coupled argon plasma mass spectrometry (ICP-MS) showed that the Dof domain contained zinc ion but not manganese ion. Thus, the Dof domain was proved to function as a Cys2/Cys2 zinc finger domain.

It is suggested that AAO plays an important role in cell growth, because the AAO, a target gene for NtAOBP, is markedly induced by auxin, which causes cells to elongate and is highly expressed in young tissues where rapid elongation is required. Furthermore, the transgenic tobacco protoplasts over-expressing pumpkin AAO expanded more rapidly than wild-type protoplasts. Therefore, it is thought that NtAOBP was related to the transcriptionaln regulation for the cell growth and elongation. However, it is little known how NtAOBP regulate the transcription of the target genes and the physiological functions of NtAOBP in plants. In order to clarify the functions of NtAOBP, I investigated the cellular localization and the transcriptional activity of NtAOBP. Although the NtAOBP protein has localized in nucleus, NtAOBP don┐t had the activation nor repression activities. However, I analyzed the phenotype of the transgenic plants and cultured cells overexpressing the NtAOBP mRNA. These transgenic plants were suppressed growth and became shorter than the wild type plant. Furthermore, the cell size of the transgenic cells was smaller than the wild type cells. These results suggest that NtAOBP has important role to regulate the growth for plants.

I have isolated a cDNA for the protein interacted with NtAOBP by the yeast two-hybrid method to reveal the molecular mechanisms of the transcriptional regulation for the AAO gene. The isolated cDNA has encoded one of the bHLH (basic helix-loop-helix) proteins containing ZIP (leucine zipper) domain. This bHLH has localized in nucleus, therefore it was thought that this bHLH protein function as a transcription factor. Then, I investigated the transcriptional activity of this bHLH protein. The result showed that this bHLH protein function as a transactivatior while the NtAOBP had no transcriptional activity. On the other hand, the expression pattern of bHLH mRNA was not similar to that of NtAOBP but that of AAO. These results suggested that NtAOBP and bHLH are competitively involved in the transcriptional regulation for the AAO gene. It is not reported that the Dof proteins interact with bHLH proteins. Further analysis will elucidate the molecular mechanisms of the transcriptional regulation only in plants.

Key words : Dof domain, Transcriptional regulation, Transcription factor, Ascorbate oxidase

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Studies on photosynthetic oxygen evolving complex by means of light-induced Fourier transform infrared (FTIR) difference spectroscopy

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フーリエ変換赤外分光法を用いた光合成酸素発生系の研究

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Photosynthetic water oxidation occurs within an oxygen-evolving complex (OEC), the catalytic center of which is a tetranuclear Mn cluster that resides on the lumenal side of the D1 protein in photosystem II (PS II). Two water molecules are oxidized to an oxygen molecule through a light-driven reaction cycle via five intermediate states designated as S_0-S_4. When PS II is illuminated with a series of short flashes, the S_1-state OEC, which is thermally stable and is predominant in the dark, is oxidized to the higher S-states in a stepwise fashion by absorbing a photon at each step. After the third flash, the OEC reaches the highest oxidation state, S_4, and then subsequently decays to the lowest oxidation state, S_0, concurrent with the release of an oxygen molecule.

Essential information to understand the reaction mechanism of the photosynthetic oxygen evolution, can be obtained by detecting chemical changes within the OEC, including the protein matrices, amino acid ligands for Mn cluster, substrate water, and hydrogen-bonding networks among these components, during the reaction process (S-state cycling) of the water oxidation. Vibrational spectroscopy can answer this directly, therefore, light-induced Fourier transform infrared (FTIR) difference spectroscopy has been extensively and successfully applied to the studies of the photosynthetic oxygen evolution in these ten years. Studies in the mid-frequency region (1800 - 1200 cm^{-1}) indicated characteristic structural changes of protein matrices and amino acid side groups during S-state transitions. In addition, high-frequency (3800 - 2150 cm^{-1}) spectra showed S-state-dependent changes in the OH vibrational mode of water molecules. These may include modes arising from amino acid ligand of the Mn cluster and from the substrate water molecules bound to the cluster, but in an indirect manner. In contrast, metal-ligand vibrations generally appear in a low-frequency (< 1000 cm^{-1}) region, and therefore chemical bonding between Mn cluster and its ligands, including the substrate water molecules, can be directly detected by means of low-frequency vibrational spectroscopy. However, because of the technical difficulties for the low-frequency measurements, very few studies were reported.

In the present study, in order to obtain direct information on the coordination sphere of the Mn cluster, the methods for measuring for light-induced FTIR difference spectra during S-state cycling in the mid- to low-frequency (1200 - 350 cm^{-1}) as well as mid-frequency (1800 - 1200 cm^{-1}) region were developed. For assignment of the observed bands, the spectra were also measured in samples that were labeled with isotope, such as ^{13}C, ^{15}N, and ^{18}O-water ($H_2{}^{18}O$).

In chapter 3, the entire mid- to low-frequency (1800 - 350 cm^{-1}) FTIR difference spectra for the S-state cycling of PS II core particles from *Thermosynechococcus elongatus* were reported. This is the first time report on a complete set of S-state cycling spectra in the 1800 - 350 cm^{-1} region. Interesting bands, that changed their signs and intensities as the S-state advanced, were observed.

In chapter 4, the mid-frequency (1800 - 1000 cm^{-1}) FTIR difference spectra for S-state cycling were further investigated. S-state cycling spectra in the PS II core particles from thermophilic cyanobacterium (*Thermosynechococcus elongatus*), mesophilic cyanobacterium (*Synechocystis* sp. PCC 6803), and higher plant (*Spinacia oleracea*) were very similar, suggesting that largely identical processes take place during the oxygen evolution in prokaryotic and eukaryotic OEC. Effects of global ^{15}N- and ^{13}C-isotpe labeling on the spectra were examined to assign the observed bands in the core particle from *Synechocystis*. With respect to the frequency region below 1200 cm^{-1}, several bands in 1200 - 1140 cm^{-1} range were attributable to the nitrogen- and/or carbon-containing group(s) that are closely related to the oxygen evolution process. Especially, the putative histidine ligand exhibited a band at 1113 cm^{-1} which was affected by both ^{15}N- and ^{13}C-labeling and showed distinct S-state dependency. Effects of ^{18}O-water substitution were also studied using the core particles from *Thermosynechococcus*. The 1800 - 1000 cm^{-1} spectra were scarcely affected by the ^{18}O-water substitution, indicating that few vibrations related to the substrate water or these from groups including oxygen atom exchangeable with oxygen from water are included in this frequency region.

In chapter 5, the mid- to low-frequency (1000 - 800 cm^{-1}) FTIR difference spectra during S-state cycling were further investigated systematically by the use of ^{15}N- and ^{13}C-isotope labeling and ^{18}O-water substitution. Possible assignments of the bands are discussed. Notably, the 1000 - 800 cm^{-1} spectra were scarcely affected by the ^{18}O-water substitution, suggesting that the vibrational modes from the Mn-water interactions in the OEC are undetectable or entirely absent in this frequency region.

In chapter 6, the low-frequency (650 - 350 cm^{-1}) FTIR difference spectrum corresponding to S_1- to S_2-state transition in PS II core particles from *Synechocystis* and each transition during S-state cycling in the core particles from *Thermosynechococcus* were analyzed in detail by using ^{15}N- and ^{13}C-isocope labeling and ^{18}O-water substitution. The ^{18}O-water sensitive modes showed characteristic changes during the S-state cycling, suggesting the changes in chemical interactions between the Mn cluster and oxygen ligands derived from the substrate water molecules during the process of the water oxidation. Further assignment of the low-frequency bands using ^{17}O-water and $^{2}H_2O$ will bring deeper insight into the chemistry of photosynthetic water oxidation.

Key words : photosystem II, oxygen evolving complex, Fourier transform infrared

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Production and decomposition mechanisms of reactive oxygen species by red-tide causing phytoplankton - Case study for hydrogen peroxide

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赤潮藻類による活性酸素種の生成機構と分解機構の解明―過酸化水素を中心に―

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In this doctor's thesis, the distribution and the behavior of Reactive Oxygen Species: ROS (mainly hydrogen peroxide (H_2O_2)) in the ocean was studied. I have studied the biological generation of H_2O_2 as well as the production by photochemical processes. Especially the phytoplankton that may cause harmful algal bloom with the mortality of cultured fish and bivalves, in late spring to early summer in the Seto Inland Sea and other coastal seas in Japan were investigated for their ability of ROS production and decomposition by analyzing of natural red tide seawater and cultured samples.

In chapter 1, previous studies of production, distribution and decomposition of H_2O_2 in the environment mainly in the atmosphere and the ocean was summarized and on the basis of previous studies, the aim and significance of this study were described.

In chapter 2, the concentration and the behavior of H_2O_2 in the Hiroshima Bay seawater was investigated during 8 cruises in 1996 to 2002 (except 2000). H_2O_2 was characterized as higher concentrations at the surface water with decreasing trend with depth. The H_2O_2 concentration showed higher during the daytime (140-450 nmol L^{-1} at 5:00-19:00) than during the nighttime (85-260 nmol L^{-1} at 20:00-4:00) and suggested that H_2O_2 at the surface seawater was generated by photochemical reaction and also partly by biological production on the process of photosynthesis by phytoplankton. The correlation of H_2O_2 with environmental factors such as salinity, water temperature, solar radiation, concentration of dissolved organic matter was examined by statistical analysis and H_2O_2 concentration was found to be controlled by mainly salinity and water temperature in Hiroshima Bay probably due to the influence of river waters running into the bay. H_2O_2 photo-production rate was estimated to be 8.0-16 nmol h^{-1} by solar irradiation experiment and indicated faster production rate than those in another sea areas reported previously. Estimated H_2O_2 half-life time under the dark condition was 12-14h and seemed to be faster decomposition rate compared with those in other sea areas. Decomposition of H_2O_2 was prevented by filtration of seawater before the incubation, suggesting that the decomposition was taken place by microorganisms including phytoplankton in seawater.

In chapter 3, biological production of H_2O_2 in Hiroshima prefecture coast seawater was observed. Concentration of H_2O_2 in natural red tide seawater was compared with that in natural seawater (containing no red tide dominant species). Natural red tide seawater was taken from 3

harbors in Hiroshima prefecture. I have observed a phytoplankton *Chattonella ovata* (Raphidophyte) that had never occurred red tide until now in Japan has caused big blooms and generated high concentration of H_2O_2 (1,700-5,600 nmol L^{-1}). After the separation of *C. ovata* from the red tide seawater, the production ability of H_2O_2 under the artificial culturing condition (21℃, 12h light: 12h dark, 42-62 μ photons) was observed and confirmed that *C. ovata* produces H_2O_2, with the increase of cell number, as the same phenomenon has been observed in other Raphidophyte species such as *Chattonella antiqua* and *Chattonella marina* in previously reports.

In chapter 4, production mechanism of H_2O_2 by *C. antiqua* was observed. I have measured the activity of Super Oxide Dismutase (SOD) to estimate an enzymatic formation of H_2O_2 from O_2^- in algal cells. High SOD activity was found in the cell of *C. antique*, indicating large production of H_2O_2 while low SOD activity was detected in the cell of *H. circularisquama*, indicating little or no production of H_2O_2. The result of Native-PAGE active staining analysis for cultured samples suggested that both *C. antiqua* and *H. circularisquama* contain Mn-SOD in the cell as a kind of antioxidant system.

In chapter 5, the mechanism of H_2O_2 decomposition by *C. antiqua* and *H. circularisquama* was observed. Decomposition ability of H_2O_2 was completely diminished by filtration of the culture solution containing the phytoplankton studied, which suggested that cell itself (probably cell surface) is involved in the decomposition process of H_2O_2. *H. circularisquama* has shown strong decomposition ability compared with that of *C. antiqua*. I found that *C. antiqua* has a high catalase activity (5.2 units^{-1} 10^3 cells mL^{-1}) but *H. circularisquama* indicated the low activity (1.3 units^{-1} 10^3 cells mL^{-1}). The confirmatory test was done by using 3-amino-1,2,4-triazole (3AT) which inhibits the activity of catalase. Since higher concentration of 3AT added to the cell suspension resulted in higher H_2O_2 concentration, the involvement of catalase against the decomposition of H_2O_2 was highly likely. Ascorbate peroxidase (APX) activity was found to be strong with *H. circularisquama* (1.73 μmol mg Chl^{-1}min^{-1}) and low with *C. antiqua* (0.55 μmol mg Chl^{-1} min^{-1}). To decompose the harmful H_2O_2, *C.antiqua* will use mainly catalase to keep the balance of H_2O_2 concentration both inter-cellular and outer-cellular environments. In other hand, *H. circularisquama* will use both catalase and APX to rapidly decompose H_2O_2.

In chapter 6, I have summarized and discussed on all the experiments mentioned above. From my results, H_2O_2 in Hiroshima Bay seawater is produced by both photochemical reaction and biological process. The photochemical reaction is probably the dominant pathway of generation of H_2O_2 during no blooms of phytoplankton in Hiroshima Bay while during some phytoplankton bloom period biological process may be the dominant for H_2O_2 generation. Now it is clear that some Raphidophyte species that cause red tide in Japan and other countries have the specific mechanism of H_2O_2 production and decomposition. Considering significant fishery damage reported by this species, further clarification of production and decomposition processes of ROS is needed.

Key words : H_2O_2, *Chattonella antiqua, Heterocapsa circularisquama*, photochemical reaction, red tide, H_2O_2 production and decomposition

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Consideration for several factors of the weakening and withering of pine trees

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マツ類樹勢弱体化及び枯損諸要因考察について

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現在，全国各地でマツ，モミ，シラビソなどの針葉樹をはじめコナラ，ブナなどの広葉樹を含めた森林衰退が進行している（中根，2000; 佐久川，2000; 井川，2000）。その中でなぜ主として針葉樹であるマツに集中して被害が見られるのであろうか。若齢の衰退林でマツノマダラカミキリによる後食跡が殆ど見つからないにもかかわらず，松枯れが確実に進行している事実があるのは見逃せない(Nakane & Kimura，1992；中根，1992; 中根･戎，1998；岡馬ら，1999; Kume *et al*.，2000a,b)。以上のマツ枯れにおいては，従来の「松枯れ」の直接的な原因とされる「松くい虫」のみではこの被害の拡大の説明が困難であり，他に決定付ける要因として「大気汚染」によるマツの弱体に起因している可能性が大きいと思われる（Kume *et al*.，2001）。今まで，葉上の酸性物質やOHラジカル物質がマツ葉の生理・生態機能を著しく傷つけていることが明らかにされている（Nakane，1992; Kume *et al*.，2000; Naemura *et al*.，2000）が多環芳香族化合物について注目した研究例は無い。そこで，マツ葉（特に気孔中）上の多環芳香族化合物などの汚染物質に注目し調査を行った（第２章）。

さらにマツ林内におけるマツノザイセンチュウの伝播形態（様式）について（第３章）の新しいプロセスの解明についても調査した。

調査地は静岡県沼津市および裾野市の10箇所の道路沿い，および海岸沿いのクロマツ林である。静岡県東部に位置する沼津市は東海道の要所として，交通網が発達し，新幹線･国道1号･246号･414号･旧国道１号などの基幹をなす流通網が集中している。特に，交通量を考慮して選定した。

本研究ではSEM（走査型電子顕微鏡）像，マツ葉表面の反射電子組成像，SEM-EDS測定によるマツの気孔の閉塞状況，付着物の測定および，マツ葉（特に気孔部分）に付着した多環芳香族炭化水素の解析，エネルギー分散型X線分析装置（EDS）により付着物の元素分析を行った。測定条件で，コーティングをAuスパッタ膜にすることにより，２次電子像を観察した。また，無コーティングで，反射電子組成像観察およびEDS元素分析した。また，多環芳香族炭化水素は蛍光性物質であることにより，蛍光量の測定もした。さらに，車の排気ガスに含まれる多環芳香族炭化水素のうち，３種類 anthracene，fluoranthene，pyreneの標準試料を選択し，マーカーとしてそれぞれの量をマツ葉に付着している多環芳香族炭化水素と交通量との相関関係を調査した。

その結果，国道246号沿いのマツ葉が最も車排気ガス・粉塵によって汚染されており，交通量の多さが原因と思われた。また，この排気ガス中の多環芳香族炭化水素のうち３種をマーカーとして調査した結果，国道246号沿いのマツ葉が最も多環芳香族炭化水素の付着量が多く，その量は交通量，特にトラック通行量と相関関係があるものと思われた。また，一般に鉱物質の粉塵が観察されたが，有機成分は極微量であった。これらの結果より，付着物量でグループ分けすると，内陸部の国道246号沿い，海岸沿い，山間部の裾野と大別され，順に付着物量が減少した。

松枯死率は，マツ葉上のトラック排気ガスに含まれる芳香族炭化水素量との相関が高く，必ずしも，気孔

中の閉塞率とは有意な相関は見られなかった。これら気孔閉塞は国道246号，千本松公園は車排気ガスにより，海岸近くの旧国道1号，我入道は砂埃による可能性があると思われた。

総合的には交通量の最多の246号沿いのマツ葉が最も汚染され，損傷をうけていることが判明した。マツ枯れの背景には，マツの気孔が汚染物質（ディーゼルガソリン排ガス成分）によって閉塞・損傷をきたし，マツが衰弱したことがあると推察された。このように衰弱したマツは殺虫作用のある物質（スチルベン）の生産が減少し，２次的にマツノザイセンチュウがマツに侵入し増殖しやすくなり，マツが枯死する可能性があるとの指摘がある（遠藤1995，2003；佐久川，2002）。

次に，「松くい虫」（ザイセンチュウ）を運ぶとされているマツノマダラカミキリが，各地で薬剤散布後，その死骸がほとんど見当たらないという報告が多くあり，松枯れの一つの原因と言われているマツノザイセンチュウは如何にして，伝播拡散していくのか。風に乗ったり，水に流されたり，土壌中を移動したりしないといわれているが，果たしてマダラカミキリ以外に媒体となるものは無いものか検討した。すなわち，花粉が空中を風に乗って，飛散するように枯死したマツに生息している0.6～1.0㎜程の微小な線虫が空中に飛散する可能性を検討した。

ザイセンチュウの飛散調査地はJR常磐線 内原駅（茨城県西茨城郡内原町）より南西1km先の旧操車場跡地（JR用地内）で殆ど管理されていないマツ林である。薬剤散布は施行されていない。方法としては，スライドガラスに白色ワセリンを塗布し，これをビニール製の金網を貼った木枠（600×1200㎜）をマツ林内に主風向き（東依り）にほぼ，直角，30度，平行に3基（1基60枚）セットし，合計180枚となる。

主風向に対し，垂直ないし30度ぐらいの角度で捕集板をセットしたもの方が風向きに平行にセットしたものより大量のセンチュウが付着していた。光学顕微鏡で検鏡し，確認した。180枚中，約120枚にセンチュウが付着していた。そのプレパラートのワセリンを除去し，ザイセンチュウの同定の試料とした。

分離されたセンチュウがマツノザイセンチュウかどうか，茨城県林業技術センターより提供して頂いたマツノマダラカミキリと比較した。その結果，プレパラート上のザイセンチュウは分類形態学上より，非常に高い確率でマツノザイセンチュウと同定された。また，このマツ林内でマダラカミキリの生息を確認できなかったが，マツ林中で，マツノザイセンチュウが飛散していることが実証された。

樹木枯れ現象は大気汚染，とりわけ酸性雨・霧･露，土壌の酸性化，オキシダント，オゾン，OHラジカル物質等による樹勢の弱体化が考えられ，高い相関関係にあると考えられている（Izuta *et al.*，1996; Kobayashi *et al.*，2002）。そこで，気孔（マツ葉）の形態・閉塞状況・その閉塞物質と松枯れの関係を究明し，車排気ガス中の多環芳香族炭化水素によるマツ樹勢への直接的影響は無視できないものと考え，大気汚染と松枯れとの因果関係があるのではないか調査した結果，松枯れの直接の原因はマツ特有な構造，マツ葉1本あたりの気孔（陥没構造）が多く存在し，これに比例して大気汚染物質の蓄積量も多く，かつ，常緑樹（3年間で落葉）のため長時間にわたり影響を受け，マツ葉の生理活性が衰退し，生産力が低下することが指摘されている（中根，1992；Kume *et al.*，2000, 2001）。また，若齢アカマツ自然林での調査で，マダラカミキリの後食を受けたマツと後食を受けなかったマツの枯死率に有意な差異が無かったことはマダラカミキリが松枯れを有意に促進していないということも示唆されている（中根，2000）。

ザイセンチュウの媒介が〝マダラカミキリ〟以外に風（大気）による飛散は形態学的に確認されたが，ザイセンチュウがその後，マツ木に感染し，増殖するかの過程については今後の課題となる。

今後の「松枯れ対策」は原因を〝松くい虫〟に固執するのでは無く，大気汚染（特に，ディーゼル排気ガス；自動車，船舶）など環境要因を含めた対策に積極的に転じるべきと思われる。

キーワード：気孔閉塞，芳香族炭化水素，交通量，マツノザイセンチュウ媒介，風向

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Assessment of the bioresource sustainability in marine coastal waters: An attempt based on retrospective studies in Hiroshima Bay, Osaka Bay, Ise Bay and Tokyo Bay

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遡及的アプローチによる本邦代表的内湾域（広島湾，大阪湾，伊勢湾，東京湾）の
生物資源持続性評価と相互比較

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本研究は，内湾域の生物資源持続性管理手法の確立を目指すために，①生物資源持続性評価手法の提案を行い，本邦の代表的富栄養内湾である広島湾，大阪湾，伊勢湾，東京湾にその手法を適用し，②生物資源持続性が失われた原因項目について追求し，③今後の対策について検討した。

１．生物資源持続性を評価するために，生物資源の生産性（PI），効率性（EI），安定性（SI）の３基準指標を包括する総合的な生物資源持続性指標（BSI）を提案した。なお，PIとして漁獲量，SIとして漁獲物食性別多様性，EIとして窒素回収効率を用いた。

２．調査対象期間は，本邦内湾域が大きな環境変化に晒された1950年から2000年までとし，その間の人間活動と内湾の環境を把握できる多くのデータを収集し，生物持続性指標の変化とその変化をもたらした要因の追求を行った。

３．広島湾の生物資源持続性は1967－1975年の間に急激に失われ，現在も緩やかに失われつつあると判断された。生物資源持続性が急激に失われた1967－1975年の時期は，広島湾の浅海域が３－12％に減少し，同時に急激に富栄養化した時期と一致した。

４．大阪湾の生物資源持続性は1962－1972年の間に急激に失われ，現在も失われつつあると判断された。1962－1972年の時期は，大阪湾の浅海域の減少が著しく，この時期11－29％の浅海域が失われた。また富栄養化が最も顕著に進行した時期とも一致した。

５．伊勢湾の生物資源持続性は1961－1975年と1990－1998年の2回の期間に顕著に失われたと判断された。最初に持続性が失われた1961－1975年の時期は，富栄養化が進行し，また浅海域の０－９％が消失した時期と一致した。1990－1998年の場合は，浅海域が14％消失した時期に相当したが，富栄養化は停止した状態下で起こったことから，主として浅海域の減少の要因が効いていると推定された。

６．東京湾の生物資源持続性は1966－1972年の間に急激に失われ，現在も失われつつあると判断された。持続性が失われた時期は，浅海域が急激に11－20％まで減少した時期と一致し，また最も富栄養化が進み水質が悪化した時期と一致した。

７．広島湾，大阪湾，伊勢湾，東京湾において生物資源持続性の変遷パターンで共通していることは，1960年代から1970年代前半にかけての生物資源持続性の顕著な低下であり，その期間は我が国の高度経済成長期に相当し，埋立による浅海域の減少が著しく，富栄養化が急速に進んだ時期であった。

８．東京湾と伊勢湾は共に比較的浅い内湾であるが，この50年間に東京湾の方が相対的に生物資源持続性が失われた。伊勢湾は相対的に生物資源持続性が比較的今日まで高く維持された湾であった。この違いをもたらしたのは両湾の浅海域減少率の違いにあるものと考えられた。

９．広島湾と大阪湾は比較的深い湾であるが，この50年間に大阪湾の方が広島湾より相対的に生物資源持続

性が失われた。大阪湾の浅海域の減少率が大きいこと，また広島湾ではマガキ養殖が行われていることが，このような相違をもたらしたと考えられた。

10．本研究で提案した生物資源持続性指標は，本邦の代表的 4 内湾でほぼ同時に起こった大きな環境変化（浅海域の減少と富栄養化）に対して低下した。現在では他の環境要因との対応をより詳しく追求することは困難であるが，本指標は大まかには内湾の環境の良し悪しと生物資源状態の良し悪しを表していた。今後もさらなる修正や検討を行い，より正しく生物資源持続性が評価されるよう改良する必要がある。

11．本研究を通して内湾の生物資源持続性に最も大きな影響を及ぼすのは，浅海域の喪失であることが改めて示された。生物資源持続性の回復のためには，埋立の禁止と浅海域の造成などが必要である。

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Phylogenetic analysis of ultramicro-organisms that pass membrane filters with a pore size of 0.2 μm

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孔径 0.2 μmの濾過膜を通過する極微小生物の分子系統学的解析

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微生物研究者の多くは孔径0.2μm濾過膜（フィルター）を通過する微生物（0.2μm通過微生物）の存在は無視できると考えてきた。その理由は，「生存に必要な生体高分子から微生物の最小サイズを見積もった場合，その直径は0.2μm未満にはなりえない」という理論的な仮説があるためである。しかし，実際に0.2μm通過微生物の報告例がある。そのため，0.2μm通過微生物が無視できる存在なのかを微生物生態学の観点から調べる必要がある。

まず，第一章では，既報の仮説や既知の事実を概説する。0.2μm通過微生物の研究例は1970年代から存在し，全菌数に対する割合が求められた。1980年代初期では，孔径0.2μmフィルターを通過する生涯極微小サイズのウルトラミクロバクテリアが海水から単離され，現在まで水圏環境中から複数の単離例が報告されている。対して，環境中の0.2μm通過微生物の群集構造解析を行った例はほとんどない。特に極限環境中の0.2μm通過微生物の群集構造解析は皆無である。そこで本論文では極限環境に生息する0.2μm通過微生物の群集構造を解明し，それらの微生物生態学的な位置付けを明らかにすることを目的とした。

次に，第二章では深部地下水中の極微小生物について述べる。地下生物圏は陸上生物圏および海洋生物圏に匹敵する巨大な生物圏であると考えられてきた。しかし，微生物群集構造に関する研究例は少ない。そのため，本研究が地下水中の0.2μm捕集および通過微生物を網羅した最初の研究例である。本研究では，岐阜県土岐市の東濃鉱山内の試錐孔KNA6号孔から堆積岩地下水および花崗岩地下水を採取した。0.2μm捕集および通過微生物は，それぞれ，KNA6号孔に孔径0.2μmステリベクスフィルター（ステリベクス）を直列で接続し濾過，およびKNA6号孔に孔径0.2μmコンパクトカートリッジフィルターおよび0.1μmステリベクスを直列に接続し濾過して回収した。濾過後のステリベクスはDNA抽出，アーキアおよびバクテリアの16S rRNA遺伝子のPCR，クローニングを行った。クローンのグループ分けにはRFLPを行った。得られたphylotypeから代表クローンを選び，シーケンスを行った。その結果を，近隣接合方，最大節約法および最尤法のアルゴリズムを用いて系統樹作成およびbootstrap分析を行った。本研究の結果，KNA6号孔堆積岩および花崗岩地下水のから，0.2μm捕集および通過バクテリアそれぞれ14および23 phylotypes得られた。また，アーキアのPCR産物は得られなかった。これらの中で0.2μm通過バクテリア中の15 phylotypesは他系統群とは進化距離が大きく異なるDeep-branchingを示し，環境から得られたクローンのみで形成されるcandidate divisionに属していた。これら以外の0.2μm通過バクテリアはBeta proteobacteriaおよびFirmicutesに属していた。対して0.2μm捕集バクテリアでは，Beta-，Delta-proteobacteria，Acidobacteria，Nitrospira，Green nonsulfurおよびcandidate division OP3に属していた。

さらに，第三章では熱水噴出域の極微小生物について述べる。熱水噴出孔は地下生物圏と海洋生物圏をつなぐ「窓」であり，その熱水には地下生物圏に生息する微生物が存在している。しかし，その微生物研究例は非常に少なく，さらに0.2μm通過微生物の研究例は皆無である。そのため，本研究は熱水中の0.2 μm捕集および通過微生物を網羅した最初の群集構造解析例である。0.2μm捕集および通過微生物を回収するため，

マリアナトラフおよび水曜海山で得た熱水を孔径0.2μmステリベクスで濾過をした。続いて濾液を孔径0.1μmステリベクスで濾過し，0.2μm捕集および通過微生物を回収した。濾過後，第二章の方法と同様に行い，phylotype分類は全シーケンス解析後，97%以上の相同性のクローンをphylotypeとしてグループ分けした。そして得られたphylotypeから代表シーケンスを選択し，近隣接合方，最大節約法および最尤法のアルゴリズムを用いて系統樹作成およびbootstrap分析を行った。また，微生物の多様性を評価するためにrarefaction分析も行った。本研究の結果，マリアナトラフおよび水曜海山にて6種類の熱水から全11種類のクローンライブラリーを得た。マリアナトラフの第797潜航のアーキアのみPCR産物が得られなかった。11種類のクローンライブラリーからアーキアおよびバクテリアそれぞれ27および62 phylotypesが得られた。これらphylotypeの中でMa-NA02，Sd-NA，Sd-EA01およびSc-EA05は新規系統群を作るDeep-branching phylotypeであった。これら以外は過去に報告例のあるMGⅠおよびMGⅡに属していた。一方，本研究で得られたバクテリア62 phylotypesの中で9 phylotypesがcandidate divisionに属すDeep-branching phylotypeであった。逆に機知phylotypeとしてAlpha-，Gamma-，Epsilon-，Beta-およびDelta-proteobacteria，Actinobacteria，Firmicutes，およびGreen nonsulfurが得られた。

これらを踏まえたまとめとして，本研究では孔径0.2μmフィルターを通過する微生物を選択的に解析した結果，Deep-branchingなphylotypeが多数得られた。この結果から新規微生物は環境中で極微小サイズの微生物として存在しているという可能性を提案する。本研究は，孔径0.2μmフィルター通過画分にも微生物生態学的に重要な知見が含まれていることを明瞭に示すものである。

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Dynamics, characteristics and photochemical processes of fluorescent dissolved organic matter and peroxides in river water

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河川水中蛍光性溶存有機物および過酸化物の動態、特性、光化学過程に関する研究

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Dissolved organic matter (DOM) in river waters is originated from various natural and man-made activities occurring at water catchment's areas. DOM plays a significant role in controlling the water quality, biogeochemical carbon cycle and energy sources for microorganisms in natural water. The major parts in DOM in river waters are the humic-like substances (50-90%), composed of fulvic acid and humic acid. Fluorescent whitening agents (FWAs), such as distyryl biphenyl (DSBP) and diaminostilbene type (DAS1) are most commonly used whiteners in household detergent productions and papers, which are significantly detected in major rivers in Japan, U. S. A and Europe. As the fulvic acid and FWAs are specific organic compounds in natural water, characterization of those substances is an important step to elucidate the chemical nature of DOM and its decomposition processes in natural water. Fluorescent dissolved organic matter (FDOM), such as fulvic acid and FWAs in waters are easy to characterize by the fluorescence properties. As the superiority of the three-dimensional (3-D) fluorescence spectroscopy for the characterization of FDOM in water is well known, this technique is widely used for investigation of sources and chemical nature of FDOM in natural water.

Hydrogen peroxide (H_2O_2) and organic peroxides (ROOHs) are frequently present in all natural waters and they are involved in the red-oxi reactions of various chemical species dissolved in natural water. H_2O_2 is considered to be a final product through a chain reaction among DOM, dissolved oxygen and natural sunlight. Due to characteristic differences of DOM composition and their sources in the upstream and downstream river waters, it is interesting to examine the peroxides concentration, their sources, and the causes of the variations in the upstream and downstream river waters. Moreover, investigation of ROOH as well as H_2O_2 may be a crucial for better understanding the photochemical or biological processes in river.

In Chapter 1 it described about the overview of the chemistry of DOM, FDOM and peroxides in river water. The major objectives of this study are:

1. To understand the concentration variations and transport of DOM from upstream to downstream areas in Kurose and Ohta River waters, Hiroshima prefecture, Japan.
2. To characterize the optical nature of FDOM in the river waters with the measurement of fluorescence properties by comparing with that of the standards of the Suwannee River Fulvic Acid (SRFA) and Humic Acid (SRHA), tryptophan, and FWAs, such as DAS1 and

DSBP.

3. To investigate the spatial-temporal variations of H_2O_2 and ROOHs in Kurose and Ohta River waters and then to discuss about the factors controlling their concentration.
4. To examine the photochemical production mechanisms of peroxides in river waters by conducting photoirradiation experiment on various standard FDOM using solar simulator.

In Chapter 2 dynamics and optical nature of FDOM were investigated in two rivers (Kurose and Ohta) in Hiroshima prefecture, Japan during 2002-2003, by measuring dissolved organic carbon (DOC) and three-dimensional excitation emission matrix fluorescence (3-D EEM). In monthly collected samples, DOC varied from 43 to 146 μM C at upstream sites in both rivers, and from 130 to 349 μM C and from 45 to 164 μM C in Kurose and Ohta downstream, respectively. The 3-D EEM of FDOM in the river waters identified three characteristic peaks, indicating the occurrence of fulvic acids (peak F), FWAs (peak W), and protein-like substances (peak T). The upstream FDOM in the both river waters and the downstream FDOM in the Ohta river contained the peaks F and T, identified by comparing with that of the SRFA and tryptophan and their photo-irradiated standards. The 3-D EEM of FDOM in Kurose downstream waters, however, contained the peaks W and T. The ratio of fluorescence intensity (FI) for peak F or W to DOC (FI/DOC-index) was estimated to be high (2.00 ± 0.51 to 2.09 ± 0.38 QSU (quinine sulphate unit)/μM C) at Kurose downstream compared to Ohta rivers (0.73 ± 0.35 to 0.74 ± 0.38 QSU/μM C) and to the Kurose River upstream (0.55 ± 0.22 to 0.65 ± 0.21 QSU/μM C), and the absolute FI values were several times higher in Kurose downstream waters than Kurose upstream and Ohta river waters. Moreover, studies on the photo irradiation experiments of various standard FDOM indicated that the peak W is easily photo-decomposed while the peak F appears to be photo-resistant. These results indicate that the chemical properties of FDOM in the Kurose downstream waters are different from those in the Kurose upstream and Ohta river waters.

In Chapter 3 H_2O_2 and ROOHs were investigated in two river waters (Kurose and Ohta), Hiroshima prefecture, Japan, during the period of 2002 - 2003. H_2O_2 monthly varied from 6 to 213 nM (mean 52 ± 48 nM) and from 33 to 188 nM (84 ± 51 nM) at Kurose River and Ohta River, respectively. ROOHs varied from 0 to 73 nM (29 ± 18 nM), and from 1 to 80 nM (30 ± 21 nM) in both rivers, respectively. Concentrations of peroxides were higher during summer than winter. H_2O_2 concentrations were well correlated with DOC contents and FI of FDOM in Kurose River, but in Ohta River H_2O_2 was only correlated with FI, suggesting DOM and FDOM to be major H_2O_2 sources. Photo-irradiation of various standard FDOM using solar simulator implied that fulvic acid is the dominant DOM for the production of peroxides in both rivers. A negative correlation was observed between the number of bacterial cells and the peroxides from the Kurose downstream waters only where a large number of bacteria exist. From these results the peroxides concentration in the river water is regulated mostly by photochemical production through the degradation process of fulvic acid, except for the downstream waters of Kurose River, in where it is also controlled by bacterial decomposition process.

In Chapter 4 it discusses and summarizes on the most important results obtained in this study.

Key words : Fulvic acid, protein-like substances, hydrogen peroxide, organic peroxides, photochemical processes in river water

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Physiological responses and adaptations of plants to water stress and analysis of drought tolerance in leguminous plants

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マメ科植物の水ストレスに対する応答および水ストレス耐性に関する研究

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西日本では降水量のほとんどが６月の梅雨時期に集中し，続く７，８月には高温寡雨のため干ばつが発生し，農業生産に甚大な影響を及ぼしている。とくに，ダイズでは開花期から子実肥大期がこの時期にあたり，水ストレスはダイズの収量低下の最も大きな原因となっている。このことから，水ストレスによる被害を軽減し作物の安定多収を図るためには，作物の水ストレス耐性機構を明らかにし，水ストレスの影響を軽減する栽培技術などを確立することが重要である。本研究は，水ストレス耐性の高いマメ科植物を選抜し，耐性の異なる植物種間で水ストレスに対する応答の違いを生理生態学的に解析するとともに，生育ステージが異なるダイズの水ストレスに対する生理生態的応答を解析した。

ダイズ(*Glycine max* (L.) Merill cv.クロセンゴク)，ピジョンピー（*Cajanus cajan* (L.) Millsp），セントロ（*Centrosema pubescens*），ディスモディウム(*Desmodium intortum* DC.)，サイラトロ(*Macroptilium atropurpureum* DC.Urb)およびスタイロ（*Stylosanthes guianensis*）を用いて，土耕ポット試験と圃場試験において水ストレスによる地上部乾物重の減少程度から水ストレス耐性を比較した。ポット試験において各植物の生育は水ストレスにより抑制されたが，その程度はサイラトロが最も小さかった。圃場試験でもサイラトロの地上部乾物重の減少程度は，最も小さく，また，サイラトロでは，茎および葉柄への乾物分配率が高かった。葉の水ポテンシャルは，ピジョンピーとサイラトロで減少程度が小さかった。水ストレスにより各植物種とも窒素固定能は低下したが，その低下程度は，サイラトロが最も小さかった。また，水ストレスによる窒素集積量の減少程度もサイラトロが最も小さかった。以上の結果から，水ストレス耐性はサイラトロが最も高くことが明らかとなった。

サイラトロの水ストレス耐性機構を耐性の低いダイズと比較して解析した。サイラトロでは，水ストレスによる茎と根の乾物重の減少程度が小さかった。また，サイラトロでは，水ストレス下でも茎の伸長が見られたが，ダイズでは茎の伸長が著しく阻害された。サイラトロは，葉の水ポテンシャルがより低い条件下でも気孔伝導度と光合成速度を高く維持した。光合成産物の根粒と根への転流率はダイズに比べてサイラトロで低かったが，茎への転流率は逆にサイラトロで高かった。以上の結果，サイラトロでは茎の生育を維持することにより，水ストレス耐性を獲得しているものと考えられた。

次に，ダイズが水ストレスに弱い要因と収量低下要因をさらに明らかにするために，品質が高く，近年需要が増加している丹波黒大豆を用いて開花期の水ストレスの影響を解析した。実験には京都府奨励品種の新丹波黒を用いた。水ストレスにより新丹波黒の地上部および根部の乾物重とも，その増加程度が著しく抑制された。光合成速度，気孔伝導度および蒸発速度は，水ストレスにより減少したが，葉内CO_2濃度は，わずかな減少にとどまった。光化学系Ⅱにおける最大量子収率と電子伝達能は，水ストレスにより変化しなかった。水ストレスは，昼間の茎の収縮を促進させ，逆に夜間の拡張を減少させた。このことから歪みゲージ式変位計による茎径の変動は，ダイズ体内の水分状態を把握するための重要なパラメーターになることが明らかとなった。開花期の水ストレスは，個体当たりの莢数や百粒重には影響を及ぼさなかったが，個体当たり

の子実数を減少させ，子実収量は対照区の75％に止まった。以上の結果，開花期の水ストレスは，その後の補償作用もあるため，落花や落莢に与える影響は小さいが，光合成能の低下や茎の収縮の増大などを通じて，子実の受精や肥大に影響を与えている可能性があった。

さらに，ダイズ子実肥大期の水ストレスに対する応答を品種，新丹波黒を用いて解析した。水ストレスにより新丹波黒の葉と莢の乾物重は減少したが，茎の生育には影響は認められなかった。歪みゲージ式変位計で測定した莢の厚さは，日中に縮み，夜間に膨張する日変化が認められ，水ストレスにより莢の肥大が著しく抑制された。一方，茎径は水ストレスによりわずかながら増大した。葉から茎への光合成産物の転流率は，水ストレスにより増加した。また，水ストレスにより莢のデンプン含有率が減少し，逆に可溶性糖は増加した。茎の総炭水化物含量は，水ストレスにより増加し，水ストレス下では炭水化物の利用形態が変化し，収量へ影響を及ぼしていることが示唆された。以上の結果，子実肥大期の水ストレスは，子実の肥大や葉の乾物重を減少させるものの，逆に茎の生育をわずかに増大させ，光合成産物や窒素を優先的に茎へ供給することによって生育を維持していることが明らかとなった。水ストレスによって個体当たりの莢数，子実数および百粒重ともに強く抑制され，子実収量が著しく減収したことから，子実肥大期の水ストレスが収量により大きな影響を及ぼすことが明らかとなった。

本研究では，数種マメ科作物を水ストレス下で栽培し，作物生産量から耐性種の選抜を行い，耐性の高い種の特性を生理生態的に解析した。また，干ばつのため収量が著しく減少し，深刻な問題を抱えているダイズの水ストレス耐性と生育阻害機構を水分生理，光合成，光合成産物の転流などから明らかにした。以上の結果から，ダイズ，特に丹波黒大豆の安定生産と多収を図るためには，梅雨明け以降の夏季においては早い時期から土壌水分の低下を防ぎ，一定の土壌水分を保つことが重要であり，特に子実肥大期においては土壌水分の急激な変動を抑制し，子実の肥大を促進することが重要であると考えられた。

キーワード：光合成，サイラトロ，耐乾燥性，ダイズ，丹波黒，水ストレス

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Estimation of the contribution of riparian vegetation to the matter flow in a river basin ecosystem

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流域物質循環における河畔植生の役割の定量的評価

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第１章　序論

近年，流域を通じた物質の輸送に大きな関心が寄せられている。しかし，物質の起源やその供給プロセスを含む，流域全体の物質循環のメカニズムについては，未知の部分が多く残されており，更なる情報の蓄積が必要である。流域における物質供給源の一つとして，河畔植生が挙げられる。河畔植生は，一次生産の過程で炭素や栄養塩を獲得して有機物を生産し，それらの一部を落葉として還元するという働きを通して，河畔域の物質の流れに深く関わっている。また落葉として供給された有機物の一部は，河川を通じて下流へと輸送されると予想されるが，河畔植生の流域の物質循環に対する影響については，その寄与の大きさやメカニズムを含めてほとんど分かっていない。そこで本研究では，河畔植生の役割を定量的に評価する目的で，広島県を流れる一級河川太田川とそこでの優占種であるネコヤナギ（*Salix gracilistyla*）を対象とし，群落における一次生産から有機物分解にかけての物質の動態を群落レベルで定量した。その結果をもとに，流域全体の河畔植生から供給される物質量を示すことを通して，流域の物質循環における役割について評価した。

第２章　河畔ネコヤナギ群落のバイオマスと一次生産量

まずネコヤナギ群落が年間につくりだす有機物量を明らかにするために，地上部バイオマスと一次生産量について調べた。太田川中流域の砂州に調査地を設け，森林の調査で広く用いられる相対成長関係を応用して推定を行った。その結果，地上部バイオマスは9月に最大（2.2 kg m^{-2}）となった。９月における群落1 m^2 あたりの葉のバイオマスは0.6 kgであった。５月から12月にかけての旧年枝・幹のバイオマスの増加と，９月における葉，当年枝，当年生シュートのバイオマスから，ネコヤナギ群落の地上部純一次生産量は1.3 kg m^{-2} yr^{-1}と推定された。この値は他の先駆性木本や温帯林の生産量に匹敵する値であり，河畔ネコヤナギ群落が潜在的に高い生産力を持つことが明らかになった。

第３章　ネコヤナギの栄養塩経済

3-1　群落内の窒素・リン動態

第３章では，季節を通じた河畔ネコヤナギ群落内の窒素（N），リン（P）動態を明らかにした。また，秋に落葉として還元される炭素（C）量の推定も行った。太田川中流域の調査地でサンプリングを行い，植物体器官中のC, N, P含有量の季節変化を調べた。採取したサンプルはそれぞれ器官別（葉，当年枝，２年枝，冬芽，花芽）に分け，炭素と窒素の含有量は微量元素分析システムで，リン含有量はサンプルを灰化後モリブデン酸アンモニウムによる比色定量によって求めた。さらに群落面積あたりのN，P量とその器官別の分布を，第２章で求めた群落の地上部バイオマスをもとに推定した。その結果，群落が毎年新たに多量の栄養塩を獲得し（10 g N, 0.94 g P m^{-2} yr^{-1}），その5割に相当する量を落葉として環境中へ戻していることが示された。同時に，落葉を通した炭素のリリース量も非常に大きい（280 g C m^{-2} yr^{-1}）ことが明らかになった。

3-2　栄養塩吸収に対するVA菌根の影響

３章の第２節では，ネコヤナギの栄養塩吸収に対するVA菌根の影響に着目し，野外調査と実生を用いた栽培実験を行った。太田川中流域の調査地で，野外での菌根の形成状況を調べた結果，成木，実生に関わらず，VA菌根の形成が認められた。栽培実験ではVA菌根がリン吸収を促進している可能性が示唆されたものの，実生の生育に対する直接的な効果は認められなかった。また窒素施肥による大幅な生育促進とshoot：root比の増加が認められた。以上のことから，ネコヤナギ実生の成長に対するVA菌根の影響は小さく，むしろ土壌中の窒素が大きく影響していることが示唆された。

第４章　河畔域における落葉分解

第２章，第３章の結果から，ネコヤナギ群落が高い生産力を持ち，特に落葉の供給を通して流域の物質循環に影響を与えている可能性が示唆された。そこで第4章では，供給された落葉の，陸上と河川水中における分解過程について調べた。

一次生産と栄養塩経済について調べた調査地で，落葉（リター）の重量減少をリターバッグ法によって調べた。その結果，実験開始から一年を経ても陸上で65%，河川水中では約40%の落葉が粗粒有機物として残っていた。有機物の分解に伴う重量減少は，無機化と溶存態・細粒有機物の流出によって起きる。そこで次に，調査地に一定期間設置した落葉の無機化速度の温度依存性と，調査地の気象データをもとに，年間の落葉無機化量を見積もった。陸上での無機化速度は赤外線ガス分析装置を用いたopen-flow法で，水中での無機化速度は，サンプルを入れた密栓容器内の溶存酸素消費量を溶存酸素計で測定して求めた。その結果，陸上での推定無機化量は，野外での重量減少の結果を大きく上回った。陸上での無機化速度の測定は，サンプルに水分を充分に与えた状態で行ったことから，野外では主に乾燥によってリターの無機化が抑制されている可能性が示された。一方河川水中の推定無機化量は重量減少の結果とほぼ一致し，河川水中に入ったリターは，一年を経ても約4割が粗粒有機物として残ることが示唆された。

第５章　流域における有機物の動態

前章までの結果から，河畔ネコヤナギ群落が多くの落葉を供給し，その約４割以上が一年を経ても粗粒有機物として残ることが示唆された。これらの粗粒有機物は河川の増水に伴って下流へ流出すると予想される。第５章では，増水の規模と頻度を考慮した群落からの粗粒有機物流出モデルを作成し，長期間にわたる有機物動態のシミュレーションを行った。モデルでは，増水とそれに伴う粗粒有機物の移動のタイプとして，毎年起きる小規模な増水（transport）と，数年に一度起きる大規模増水（wash out）を想定した。そして，transportの規模とwash outが起きる頻度を変えてシミュレーションを行い，ネコヤナギ群落から供給される潜在的な粗粒有機物量を推定した。さらにこのプロセスを流域全体に拡張し，太田川での植生分布データをもとに流域全体のネコヤナギ群落から流出しうる粗粒有機物量を推定した。その結果，ネコヤナギ群落から供給される粗粒有機物の総量は数十tから百tを越すと見積もられた。

以上のことから，ネコヤナギ群落に代表される河畔植生が，群落周辺だけではなく，河川の下流域や河口域に多量の有機物をもたらしている可能性が示され，河畔植生が流域における有機物供給源の一つとして重要な役割を果たしていることが示唆された。

キーワード：河川流域　河畔植生　シミュレーションモデル　物質循環　有機物分解

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Ecological study of the effect of hydroxyl-radical on root system of the Japanese appricot (*Prunus mume*)

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ウメの根系にヒドロキシルラジカルが及ぼす影響の生態学的研究

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田辺市とその近傍で発生したウメの生育障害を畑作障害でなく森林衰退とみなした。わが国では1960年代末に制定された公害対策基本法や大気汚染防止法によって，二酸化硫黄の濃度は劇的に低下し，今日では1970年代からおおよそ１桁減少している。二酸化硫黄の濃度の減少によって，植物の可視障害は減少したが，二酸化窒素の濃度は減少せず，地域によってはむしろ増加傾向にある。同様に増加傾向にあるオキシダントは工場などから排出される１次汚染物質の窒素酸化物と炭化水素などが大気中で光を受けて光化学反応を起こし，２次的に発生する酸化性の物質で，主成分はオゾンである。梅生育障害対策研究会の調査（梅生育障害対策研究会，2000.）でも田辺市のオキシダント濃度の年平均値は0.036～0.046ppmと和歌山県内の監視局の年平均値（0.021～0.039ppm）に比べ比較的高濃度であったという。オゾンの曝露実験で葉面に可視障害の見られない状態で，個体当りの乾重特に根の乾重が著しく低下した（Izuta *et. al*., 1996）。オキシダントと同様に窒素酸化物を起源とする硝酸や亜硝酸，過酸化水素などの2次物質が朝露や霧などの水滴中で凝縮され光を受け，ヒドロキシルラジカル（OHラジカル）が発生することが明らかになった（新垣ほか，1998，1999）。ヒドロキシルラジカルは活性酸素種の中でも極めて反応性が高く強力な酸化作用をもつラジカルであるため，オゾンと同様に樹木に対する影響が考えられる。ヒドロキシルラジカル生成物質を含む溶液をアカマツに曝露する実験から，ヒドロキシルラジカルの生成が植物の光合成や気孔コンダクタンスに大きな影響を与えることが明らかになった（Arakaki *et. al*., 2000; Kobayashi *et. al*., 2000）。一方，中根ら（2003a）は2000年と2001年，田辺市のウメの生育障害が多発している地域において採取された，大気に由来する葉上沈着物から平均でそれぞれ4.68 μ mol^{-1}h^{-1}と4.00 μ moll^{-1}h^{-1}のヒドロキシルラジカルの生成を検出している。さらに，ウメの葉面へヒドロキシルラジカルの生成物質を含む溶液を散布する実験において，最大光合成速度と気孔コンダクタンスが処理区間で，曝露溶液の濃度に応じた有意な低下傾向を認めている。しかし，地上部では可視障害ならびに処理区間の生長の差異は認められてない。いくつかの植物では，酸性雨やオゾンなどの影響で光合成能が低下すると，葉茎は光合成産物の利用に関して根に比べ優先権をもっているため，葉茎への光合成産物の分配量が相対的に多くなる（根系への光合成産物の分配量が少なくなる）（Irving，1985）。地上部の葉の色や成長量に変化がみられない場合でも地下部の細根量に影響がみられる （Vogt *et al*., 1993）と云われているように地上部に可視障害が認められる以前に地下部では根系の生長に影響がではじめているものと思われる。

以上の知見から，梅の生育障害においても，ヒドロキシルラジカルが関与する可能性があること。根系の観察によって地上部の観察より早く，高感度にヒドロキシルラジカルの影響が検出できるのではないかと考え，ヒドロキシルラジカル生成溶液をウメの葉に散布し，実証を試みた。実証に当って，２つの仮説を設けた。

仮説１：ヒドロキシルラジカルの曝露によって根系の生長が阻害される。
仮説２：根系の生長阻害は地上部に可視障害が認められるより以前に発生する。

この２つの仮説を実証するために，ウメの木に現地で測定されたヒドロキシルラジカル発生量の1/3倍，１倍，３倍の量を発生するヒドロキシルラジカル発生溶液を散布する処理区と対照区を設け，着葉期の間２シーズンに亘り散布した。地下部の観察の方法は生育の結果を示す現存量を測定する静的な方法「掘上げ法」と生育中の総生産を測定するための動的な方法「Minirhizotron法（内視鏡法）」によって行った。

その結果，ポット栽培の現存量の測定では細根率に対照区と3倍区の間に統計的に有意な差が生じヒドロキシルラジカルの影響が認められた。地植え栽培の測定では細根率について対照区と３倍区の間に有意な差が生じヒドロキシルラジカルの影響が認められた。また，「Minirhizotron法（内視鏡法）」による細根の発生量は，対照区と３倍区の間に有意な差が生じた。

以上の事実から，ヒドロキシルラジカルの発生がウメの根系に影響を与え，仮説１の「ヒドロキシルラジカルの曝露によって根系の生長が阻害される」は実証されるものと考えられた。論文第３章において，地上部での生長量や相対成長率に対照区と処理区の間に有意差が発生しなかったこと，第４章においても同様に地上部での生長量や相対成長率に対照区と処理区の間に有意差が発生しなかったこと，さらに可視障害が見られなかったにもかかわらず，細根，根系細根率。個体細根率に対照区と３倍区で有意な差が生じたことから，仮説２の「根系の生長阻害は地上部に可視障害が認められるより以前に発生する。」は実証されたと考えられた。

地上部では短期間（１年～２年）の曝露では可視障害がみられことがあるが，ユンら（2005）の研究によってヒドロキシルラジカルの曝露によって光合成能が低下することが知られており,また,光合成の低下による光合成産物配分減少は根系が最も受けやすいこともわかっている.さらこのような生育環境が長年継続すれば,生育障害の発症をもたらすと考えている。また，中根らの研究でも葉上降下物と1/3倍区のヒドロキシルラジカル発生液を３年間曝露した実験で，この曝露環境が５～６年継続されたら地上部現存量は対照区の半分になると推定している（中根ら，2003)。細根の発生量の観察から同様な推定をすると

$Wt=0.87Wc$　となる。

この式は「１倍区の細根の量（W_t）は１年間で対照区（W_c）の87%になる」ことを示し,この環境が継続するならば約５年で１倍区の細根の量は対照区の約半分になることを示し,中根らの結果とよく一致した.

キーワード:　ウメ，hydroxyl-radical，細根，生育障害，光合成

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The effect of hydroxyl radical in the aqueous phase on the decline of *Prunus mume* trees in Tanabe City, western Japan

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和歌山県田辺市における液相中で生成されたヒドロキシルラジカルの梅木の衰退への影響

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The decline of Japanese apricot trees (*Prunus mume*) has been observed in some orchards in Tanabe City and its surrounding areas, Wakayama Prefecture, western Japan since the mid 1980's. Furthermore the decline spread throughout most orchards in the northwestern part (declined area) of the Tanabe City in the 1990's.

In these declined areas, air pollutants (wet and dry deposition) derived from the anthropogenic activities were considered one of the primary causes of the decline. The amount of dry deposition in washoff solutions of leaves and stemflow of trees in the declined areas were higher than those on the southwestern areas of the City (non-declined area). In the exposure experiment conducted in order to evaluate the effects of dry deposition on *P. mume* in experimental greenhouses, the maximum CO_2 assimilation rate, stomatal conductance and RGR (Relative growth rate) of the trees decreased with increasing concentrations of pollutants in the solutions.

Photochemical formation of oxidants such as hydrogen peroxide and the hydroxyl (OH) radical in the aqueous phase have significant effects on the composition and chemistry of the troposphere. Such oxidants cause oxidative stresses in plants, and have been suggested to cause forest decline due to their high reactivity. Recent researches reported the harmful effect of the hydroxyl radical on the ecophysiological traits of Japanese red pine trees (*Pinus densiflora*). They indicated that the liquid-phase free radicals (OH radicals) were formed on the leaf surface by photochemical reactions in dew droplet, and conclude that the stress of polluted dew induced decreases in leaf photosynthetic rate and stomatal conductance. Thus, dew polluted with dry deposited on *P. mume* leaves, were focused on as a factor affecting the physiology and growth of Japanese apricot trees. In the present thesis, effects of mists which simulated the polluted dew and authentic dew of leaf surface on the ecophysiological characteristics and growth of *P. mume* were examined at Tanabe City.

In Chapter 2, the effects of the hydroxyl radical on the growth, gas exchange rates and chlorophyll fluorescence of 3-year-old seedlings of *P. mume* were evaluated. In the declined area, hydroxyl-radical-generating solutions simulating the polluted dew were sprayed on the leaf surfaces of seedlings grown in experimental greenhouses three times a week from May to November 2001. Four hydroxyl radical generating solutions, which were formulated utilizing the photo-Fenton reaction (HOOH with Fe(Ⅲ) and an oxalate ion), were used in the exposure experiment. Three contained 6, 18 and 54 μM of H_2O_2 (HOOH-6, -18, -54) and one was distilled water as a control

solutions (HOOH-0).

After five months from the beginning of treatment, the leaves exposed to the hydroxyl-radical-generating mist with 54 μM H_2O_2 (HOOH-54) showed a significantly smaller maximum CO_2 assimilation rate (A_{max}) and stomatal conductance (g_s) as compared to the leaves exposed to the mists with 0 μM H_2O_2 (HOOH-0). The hydroxyl-radical-generating mist exposure on *P. mume* seedlings caused a reduction in the dry weight and relative growth rate (RGR) of the above-ground parts (stem + branch) in the end of growing season. Positive significant correlation was shown between RGR and A_{max}. Thus, the effects of oxidants in the liquid phase generating in polluted dew on the leaf surface are supposed to be a cause of decrease in the leaf photosynthesis and growth of *P. mume*.

In Chapter 3, the growth reduction as a result of long-term exposure (for three years) of *P. mume* to low level of hydroxyl-radical-generating mist was quantified. Four-year-old *P. mume* trees planted on ground in the experimental greenhouses were exposed to hydroxyl-radical-generating solutions (simulated polluted morning dew) for three years from 2000 to 2002.

Two solutions (HOOH-0 and HOOH-6) were used in the exposure experiment. During the experiments, maximum CO_2 assimilation rate (A_{max}) and stomatal conductance (g_s) of HOOH-0 exceeded that of HOOH-6 since the ends of each growing seasons. For three years (2000-2002), exposure of *P. mume* leaves to low concentration of hydroxyl-radical-generating mist (HOOH-6) decreased A_{max} and g_s and reduced dry weights and RGR of the above-ground parts. Furthermore, this decrease of RGR was maintained and expanded for three years. On the other hand, there were no significant decreases of GR and RGR in below-ground parts between them. Nevertheless, the GR and RGR of HOOH-6 significantly decreased comparing to those of HOOH-0 at the whole plant level.

In order to verify the effect of hydroxyl radical generated from polluted dew at the declined area, two equipments were installed. Transparent roof and fans to prevent frost falling and the dew formation were installed at declined orchards. In this experiment, the growth of *P. mume* trees was measured and the comparisons between control (in the declined field conditions) and treatment were made. Throughout the transparent roof experiments for three years from 2001 to 2003, each parameters(A_{max}, g_s and RGR) of inside were higher than that of outside and the differences between inside and outside became greater. Also, in the fan-installation experiment, although the RGR remarkably declined trees in non-installed area decreased dramatically for two years, that of fan-installed was maintained with the almost same level. In these experiments, by avoiding the dew formation to block the effect of hydroxyl radical and acid deposition, the recovery of physiological activity and growth in declined trees was possible.

The results presented in this thesis demonstrate that hydroxyl-radical-generating solutions, intended to simulate polluted dew, induced an ecophysiological disorder in *P. mume* trees. Although significant differences were observed in the gas exchange parameters, the chlorophyll fluorescence parameter showed no significant differences for the different concentrations of solution throughout the experiment. These results suggest that hydroxyl radical generating solutions had little effect on the potential maximal quantum yield of PSⅡ. Also, A_{max} tended to increase with an increase in g_s. Similar trends were also reported for Japanese red pine trees. Therefore, the decrease in the A_{max} of *P. mume* leaves induced by hydroxyl-radical-generating solutions was thought to be due to stomatal disorder rather than an effect of PSⅡ. In this thesis, furthermore, it became clear that the above-ground growth of *P. mume* trees decreased after exposure to hydroxyl-radical-generating solutions.

The concentration gradient of hydroxyl-radical-generating solutions showed a significant negative correlation to the RGR of seedlings. Thus, hydroxyl-radical-generating solutions are appear to be responsible for the changes in physiological functions and the reduced the growth of *P. mume* trees.

By control of micro-climatic conditions using dew formation block roof and fans, the recovery of growth in declined trees was possible. Therefore, the effects of oxidants in the liquid phase, especially free radicals in polluted dew on the leaf surface, are an important cause of the decline in *P. mume* trees in Tanabe City and its surrounding areas.

Key words : *Prunus mume*, Hydroxyl radical, CO_2 assimilation rate, Stomatal conductance, Relative growth rate

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Isolation and characterization of novel bioactive peptides from the central nervous system of the cephalopod, octopus

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頭足類タコの中枢神経系から単離した生理活性ペプチドの構造と生物活性に関する研究

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序論

頭足類タコは，軟体動物の他の網に比べ体制構造が複雑に発達し，その生態的・生理的機能が著しく特化しており，海棲無脊椎動物の中で進化の最高峰に達したものとされている。中でも特記すべき点は，高度に発達した神経系と循環系である。他の軟体動物の神経系は，体を縦走する２対の神経幹とそれらに分散する６対の神経節からなっているのに対し，頭足類では，頭部においていくつかの神経節が融合していわゆる“脳”を形成している。また，タコの循環系は，高血圧の閉鎖血管系からなり，大静脈神経分泌系による制御と外套内臓葉による神経支配により複雑な制御を受けている。これらのことから，タコは，解剖学や行動学の分野で注目を集め，中枢神経系の組織化学，知覚・学習能力，生殖や保育行動に関する研究が知られているが，神経ペプチドやホルモンなどの物質・遺伝子レベルでの研究はほとんどなされていない。そこで，タコの体心臓に対する活性を指標として，中枢神経系に存在する新奇のペプチドの探索を開始した。

第１章：D-アミノ酸を含む４残基の心臓作動性ペプチド（octopus cardioactive peptides）の構造と生物活性

テナガダコ中枢神経系より，体心臓に対する活性を指標として４種類のoctopus cardioactive peptides（ocp-1,-2,-3,-4）を純化した。Ocp-1とocp-2はGly-Phe-Gly-Asp，ocp-3とocp-4はGly-Ser-Trp-Aspのアミノ酸配列を示したが，ocp-1とocp-4では，N末端から２番目のアミノ酸残基がD-異性体になっていることがわかった。さらに，ocp-1（Gly-DPhe-Gly-Asp）とocp-3（Gly-Ser-Trp-Asp）は，体心臓に対して興奮性作用を示したが，それぞれの異性体であるocp-2とocp-4は活性を示さなかった。TOF-MSとtandem MSにより，体心臓にGly-Phe-Gly-AspとGly-Ser-Trp-Aspの存在を確認した。このことから，ocp-1とocp-3は中枢神経系で合成され，体心臓を標的組織として興奮性作用を示すペプチドであることが示唆された。これまでに，軟体動物に由来する幾つかのD-アミノ酸を含むペプチドが知られている。中でも，アフリカマイマイから単離されたachatin-I（Gly-DPhe-Ala-Asp）の構造はocp-1（Gly-DPhe-Gly-Asp）と非常に似ており，achatin-I前駆体タンパク質にはGly-Phe-Gly-Asp配列も存在することから，ocp-1とachatin-Iは同族関係にあると考えられる。また，ocp-4のようにD-アミノ酸を含むペプチドに活性が認められない例も報告されている。エスカルゴ（*Helix pomacia*）から，N末端から２番目がL-Pheである*Helix* CCAP-RP-IIとD-Pheである*Helix* CCAP-RP-IIIが単離された。前者は，ムラサキイガイ足糸前牽引筋に収縮活性を示したが，後者は，活性を示さなかった。D-アミノ酸の存在意義や役割については，①「活性コンフォメーション」を形成することができる：②生物的多様性を生み出す：③ペプチドの生物的半減期を延長させるなどが考えられ，L-アミノ酸からD-アミノ酸への変換は生物的に有利な条件をもたらすと考えられてきた。Ocp-4や*Helix* CCAP-RP-IIIがその動物の他の生理作用においてL-アミノ酸ペプチドより強い活性を示す可能性は残されているが，これらのペプチドの存在は，生物的多様性の一部を示しているものかもしれない。

第２章：生殖腺刺激ホルモン放出ホルモン様ペプチド（octopus gonadotropin- releasing hormone）の構造と生物活性

GnRHは，脊椎動物に広く存在し，視床下部-脳下垂体-生殖腺軸の中心となるペプチドホルモンである。無脊椎動物においても，GnRH様物質の存在が免疫組織化学的実験により示唆されていたが，脊索動物ホヤを除いてその構造は全く明らかにされていなかった。今回，マダコの中枢神経系より体心臓に興奮活性を示す12残基のペプチド（<Glu-Asn-Tyr-His-Phe-Ser-Asn-Gly-Trp-His-Pro-Gly-NH_2）を純化した。その構造がN末端から２，３番目の残基を除いて，GnRHファミリーペプチドの構造上の共通点（N末端ピログルタミン酸，His^2，Ser^4，Pro^9，Gly^{10}の保存，C末端アミド化）を満たしていたことから，octopus GnRH（oct-GnRH）と名付けた。また，脊椎動物のGnRH前駆体タンパク質の基本構造（シグナルペプチド，GnRH配列および-Gly-Lys-Arg-の切断部位，さらに50残基前後のGnRH関連ペプチド（GAP））とoct-GnRHの前駆体タンパク質の基本構造が一致していたことからも，oct-GnRHは脊椎動物GnRHの同族体と考えられる。タコのgonadotropinの構造が明らかにされていないため，タコを用いた分泌アッセイ系が存在しない。そこでoct-GnRHをウズラの脳下垂体細胞に作用させると，濃度依存的に黄体形成ホルモンを放出させた。このことから，少なくともoct-GnRHはgonadotropin放出活性を持っていることが示唆された。また，mammalian GnRHはモルモットの心臓に対して増強効果を示すことが知られているが，10^{-9}M のoct-GnRHは，マダコ体心臓に対して収縮頻度および収縮幅ともに増加させた。Oct-GnRH からAsn^2-Tyr^3配列を削除したペプチドおよびchicken GnRH-IIは，10^{-5}Mでもマダコ体心臓に対して活性を示さないが，chicken GnRH-IIにAsn^2-Tyr^3配列を挿入すると増強活性を示すようになった。このことより，oct-GnRHのAsn^2-Tyr^3配列は，マダコ体心臓活性に必須であると思われる。

第３章：生殖腺刺激ホルモン放出ホルモン様ペプチド（octopus gonadotropin- releasing hormone）の中枢神経系および末梢組織における発現分布と機能の多様性

In situ hybridizationと免疫組織化学的手法を用いたoct-GnRHの発現と分布から，oct-GnRHの生物学的機能の推測を試みた。タコの生殖腺は，視索上にある視柄腺から分泌される因子によって成熟し，視柄腺の活動は，脳下脚葉を源とする視柄腺神経によって抑制的に支配されている。脳下脚葉にoct-GnRH mRNA発現細胞体と免疫陽性細胞体および線維があり，視索に免疫陽性線維，後嗅葉に免疫陽性細胞体および線維が存在した。さらに，視柄腺内の視柄腺神経と主細胞である星状細胞に免疫陽性反応を確認した。末梢では，免疫陽性線維が卵管および卵管球に存在し，oct-GnRHにより卵管の自動収縮が増大した。In situ hybridizationによるシグナルが観察されなかったことから，視柄腺においてoct-GnRHが合成されているのかどうか確証は得られなかったが，星状細胞にoct-GnRHが含まれていることを初めて確認した。これらの結果により，oct-GnRHの生殖に対する機能として，①脳下脚葉-視柄腺?生殖腺軸および後嗅葉-視柄腺-生殖腺軸（視床下部-下垂体-生殖腺軸に相当する）において性成熟を誘導する：②視柄腺の星状細胞から分泌され，生殖腺に作用する：③末梢生殖器を支配する神経修飾物質であることが示唆された。

脊椎動物の非視床下部GnRH系，すなわち終神経および中脳GnRH系は，脳全体に広く投射する神経ネットワークを構成し，行動，代謝，免疫などさまざまな生体機能の制御にかかわっていると考えられている。Oct-GnRHは，脳の食道上部および下部をあわせて10の領域の細胞体で合成され，脳全体に神経ネットワークを構築している。たとえば，oct-GnRHを含む細胞体と神経線維は，摂食行動の制御を行う上位口球葉，接触記憶の中枢である下位前頭葉，視覚と視覚による記憶の中枢である視葉，心臓の神経支配を含む内臓制御の中枢である外套内臓葉などに存在する。このことは，oct-GnRHが非視床下部GnRH系と同様に，タコの行動，知覚，記憶・学習などに関与する多機能な神経伝達物質もしくは神経修飾物質であることを示唆している。

まとめ

タコの体心臓は，摘出した実験系でも長時間拍動し，様々な物質に対して反応することから，ペプチドの探索において優れたアッセイ系である。本論文のペプチドに加えて，プロクトリン様ペプチド，バソプレッ

シン／オキシトシンスーパーファミリーペプチドなど，複数の新奇なペプチドをマダコより純化している。これらは，従来，大静脈神経分泌系で免疫陽性物質として報告されていたものである。この結果は，タコの中枢神経系には新奇の生理活性ペプチドが豊富に存在していることを示し，in situ hybridizationと免疫組織科学的手法を用いた実験により，これらのペプチドが神経ペプチドとして，あるいはペプチドホルモンとしてどのように機能しているのか推測することができた。つまり，中枢神経系における分布と解剖学的知見から標的組織を推測し，収縮活性などの生物活性を調べることにより，末梢での作用を解明することが可能である。タコの中枢神経系は無脊椎動物において著しく発達しているが，脊椎動物の脳と比較すると，その構造にはあまりにも違いがある。しかし，同族と推測できる生理活性ペプチドが存在することから，それらペプチドの生理的役割を比較することにより，脊椎動物での脳の進化と軟体動物での神経節から脳への進化がどのような過程をたどったのかについて重要な知見を得ることができるかもしれない。

キーワード：頭足類，タコ，生理活性ペプチド，心臓増強活性

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Biogeography of large-sized ectoparasites on mammals and birds with a special reference to their fauna of Chugoku District, Japan

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哺乳類・鳥類の大型外部寄生虫に関する生物地理学的研究
～特に中国地方の外部寄生虫相について～

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哺乳類・鳥類の大型外部寄生虫，すなわち，昆虫綱のシラミ目，ノミ目，ハエ目蛹生類，およびダニ綱のマダニ目は，人獣共通の感染症を媒介することによって，宿主の健康や生命に影響を与えるものが少なくなく，医学，獣医学，畜産学，そして保全生物学分野にとって重要な存在である。しかしながら，中国地方において，外部寄生虫が体系的に研究されたことはなく，分布や宿主―寄生者関係といった基礎的な情報が不足していた。そこで，中国地方の大型外部寄生虫に関する分布・宿主調査を行ない，それらの知見を包括した。また，マダニ科の分布拡大に深く関連する寄生生態として，「雌生殖器内における精子貯蔵」に関する研究を行った。さらに，新興・再興感染症の発生を考慮に入れ，外部寄生虫の分布が今後どのように変化するかを予測するため，外部寄生虫の生物地理について考察を行った。

中国地方のシラミ類を調査し，哺乳類９種と鳥類26種からシラミ目10科33属32種14未同定種を確認した。これらのうち，中国地方初記録は９種：ハヤブサハジラミ，*Ciconiphilus decimfasciatus*，ヒヨドリハジラミ，カモハジラミ，*Craspedorrhynchus nisi*，*Degeeriella rufa*，ヒツジハジラミ，シカハジラミ，イノシシジラミであった。

中国地方のノミ類を調査し，哺乳類13種と鳥類３種からノミ目５科12属16種を確認した。これらのうち，中国地方初記録は５種：ミカドケナガノミ，タヌキナガノミ，ムササビナガノミ，*Ceratophyllus farreni chaoi*，*Ceratophyllus gallinae dilatus*であった。なお，新宿主記録としてチョウセンイタチよりネコノミとミカドケナガノミを報告した。また，ツバメの巣から発生した*C. farreni chaoi*による初の人体刺咬例を確認し，本種の衛生害虫としての重要性を指摘した。

中国地方のハエ目蛹生類を調査し，哺乳類４種と鳥類８種からハエ目蛹生類３科８属14種を確認した。これらのうち，中国地方初記録は３種：*Ornithomya fuscipennis*，ヒメシカシラミバエ，クロシカシラミバエであった。なお，新宿主記録としてハイタカより*O. fuscipennis*を報告した。さらに，ダニ類によるシラミバエ科への便乗例を日本から初めて発見した。便乗ダニはトリハダダニ科の１種*Myialges* sp.で，便乗されていたのはハトシラミバエであった。この発見により，日本において，飛翔能力を持たないダニ類がシラミバエ科を移動分散に利用していることが明らかとなった。

中国地方のマダニ類を調査し，哺乳類20種と鳥類19種からマダニ目２科８属25種１未同定種を確認した。これらのうち，中国地方初記録は４種：サワイカズキダニ，ツノチマダニ，タヌキマダニ，コウモリアシナガマダニであった。なお，新宿主記録として，ムササビよりキチマダニ，ヌートリアよりタカサゴキララマダニとキチマダニとフタトゲチマダニ，ハイタカよりキチマダニ，ジョウビタキよりキチマダニ，トラツグミよりアカコッコマダニ，ツグミよりアカコッコマダニ，コヨシキリよりキチマダニ，ムクドリよりアカコッコマダニを報告した。また，重要な畜産害虫であるフタトゲチマダニが野生哺乳類からきわめて多く採集されている点は，中国地方におけるマダニ相の特徴であると考えられた。さらに，陸鳥類とコウモリ類はマ

ダニ目の宿主となりうるが，陸鳥類とコウモリ類がマダニ目の分布域拡大に大きな役割を担っているとは考えにくいことが示された。

マダニ属の移動分散に関連して，マダニ科Prostriata群（*Ixodes*属のみを含む）に属するヤマトマダニを用いて，交尾から吸血へと続く一連の生殖行動の中で雄性生殖細胞（精子）の動態を記載し，雌体内において精子貯蔵が行われることを明らかにした。また，雌体内における精子の形態変化を記載した。交尾前，雄貯精嚢内の精子はprospermiaの状態であり，また，交尾後，精子にspermateleosisが起きる時期，及びそれに伴う微細構造の変化も他のマダニ類と基本的に同様であった。しかしながら，Metastriata群のダニにおいて受精嚢でみられる内精包の破壊が，本種においては膣頸部で確認された。内精包の破壊後，未吸血の雌では，精子が総卵管まで移動し，決して卵管まで上走することはなかった。そして，総卵管において，少なくとも30日間は精子の貯蔵が可能であることが明らかとなった。一方，交尾後吸血し飽血離脱した雌では，離脱後４日目（交尾後９日目）には精子が卵管まで達していることが確認され，精子の上走が吸血によって促進されることが明らかとなった。なお，総卵管で貯蔵中の精子の微細構造は，交尾後30日が経過しても交尾後２日目のそれと同じであった。マダニ科Prostriata群においては，雌体内における精子貯蔵が可能であるため，宿主の移動に伴う分布拡大の可能性が高いと考えられる。

中国地方から記録された外部寄生虫を，それらの分布パターンに基づいて８つの分布型に分類した。これにより，中国地方の外部寄生虫には，北方系要素（シベリア型とウスリー型）が強いが，南方系要素（マレー型と汎熱帯型）も少し含まれており，外部寄生虫の分布から，中国地方が旧北区と東洋区の移行帯に位置することが示された。さらに，中国地方の日本海沿岸域にはマレー型の外部寄生虫が遺存的に分布することも明らかとなった。中国地方内にみられる，「阿哲要素」，「カワトンボ線」，および「環瀬戸内型分布」といった生物地理学的な要素は，本論文で扱ったいずれの外部寄生虫からも確認されなかった。これは，植生や河川レベルの地理的障壁が哺乳類・鳥類の分布を制限する要因となりにくいため，それらの寄生虫にも分布の制限が生じないことを示すものと考えられた。以上の結果，哺乳類・鳥類の外部寄生虫は，宿主の移動分散能力が高いため植生や河川レベルの地理的障壁に関連する生物地理学への適用は困難であるが，より広域を対象とした生物地理学に適用可能であることが示された。

キーワード：マダニ目，シラミ目，ノミ目，ハエ目蛹生類，便乗，移動分散，分布