検出された試料について各種培養細胞を用いたウイルス分離を試みる。本研究により分離または検出された RPV 及び APPV については、臨床現場で実際に用いられる豚熱ウイルス検査（RT-PCR 及びリアルタイム RT-PCR）を行い、豚熱診断への影響を検討する。

4. 研究の成果

2012 年に豚から分離された BDV FNK2012-1 株は、羊由来細胞２種及び牛腎由来細胞４種に高い感受性を示したが、豚由来培養細胞５種においては細胞株種によってその感受性に顕著な差を示した。FNK2012-1 株を構成する準種を調べた結果、自然免疫を抑制するウイルスが主たる構成ウイルスであるが、ウイルス株全体の 0.1 ～ 1% の割合で自然免疫を誘導するウイルスが混在していることが判明した。さらに、両ウイルスの自然免疫制御能の相違にウイルス蛋白質 N^{pro} の 34 番目のアミノ酸が関与していることが明らかになった。これらの結果は、CSFV や BVDV と共通の生物性状又は特徴を持つことを示している（論文準備中）。また、両ウイルスともに豚熱ウイルス遺伝子検査で弱陽性又は陽性を示し、豚やイノシシの豚熱診断に影響する恐れがあることが判明した。以上のことから、BDV は豚由来培養細胞にも感受性を示し、CSFV や BVDV と共通の生物性状を有し、豚熱遺伝子検査で検出されうることが明らかとなり、すなわち豚熱の検査において RPV の存在にも留意し、必要に応じて鑑別する必要があることが明らかとなった。今後、臨床現場で実践可能な、高感度で、迅速かつ簡便な識別法を確立する必要がある。

ダンス病が疑われる豚の臓器又は血液から APPV の遺伝子を検出し、APPV Anna/2020 株として日本で初めて全塩基配列を解読し、ウイルス RNA 全長が 11,567 塩基であることを明らかにした（Accession No.LC596433）。また、分子疫学解析の結果、世界的にも新しい系統である Genotype 3 であることが判明した（論文投稿中）。本ウイルス試料を用いて豚熱ウイルス遺伝子検査を行ったところ陰性であり、また、解読した APPV の遺伝子配列を基に検討したところ APPV の主要抗原と豚熱の主要抗原との交差性は低い可能性があることから、APPV が豚熱の診断に影響する可能性は低いものと考えられた。次に、世界的にも APPV の分離に適した培養細胞が見つかっておらず、ウイルス分離は困難とされるが、APPV の分離を試みることとした。豚由来細胞４種、牛由来細胞３種、サル由来細胞、猫及び犬由来細胞を用いてウイルス分離（３代継代）を行っている。現在までに豚由来細胞１種で APPV が維持されている可能性があり、詳細に解析している。また、今後、ウイルス分離系やウイルス人工合成系を確立して活性のある APPV を入手する計画であり、病原学的及び血清学的な研究を継続し、APPV の獣医学的知見及び豚熱防疫に貢献する予定である。

2020年度大学間連携等による
共同研究に係る研究成果報告書（慶應義塾大学）

1　ゲノム編集技術を駆使した肺高血圧関連遺伝子改変マウスの作製と解析

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2．研究の目的

肺動脈性肺高血圧症（PAH）は女性に偏り発症する難治性循環器疾患であり、低浸透率だが原因遺伝子としてBMPR2が発見され10年以上が経過した。PAH国際コンソーシアムは2000症例以上、我々も300症例以上の臨床と遺伝子変異の情報を蓄積してきた。PAHにおいてはBMPR2を介する系が発症に関わる主要な原因と想定されている。しかし､BMPR2変異が発症･表現型に繋がる過程、性差や妊娠等の生理的因子が発症に深く関与する機構は未解明のままで、自然発症疾患モデルも確立されていない。我々は次世代シークエンサによる全エキソーム解析を250症例に実施し超長寿者と患者集団の比較という独自の戦略を用い、血管疾患に共通する可能性の高い感受性遺伝子RNF213のHot Spot変異をPAH患者に高頻度に見出した（Suzuki, Kataoka, et al. Circ Genom Precis Med. 2018）。また、新しいPAH原因遺伝子としてSox17を報告した（Hiraide, Kataoka, et al. Am J Respir Crit Care Med. 2018）。そこで、BMPR2遺伝子、RNF213遺伝子、Sox17遺伝子のそれぞれのHot Spot変異を保有するマウスを作製し、発症疾患モデルの確立と発症メカニズムの解明を目的とした。

3．研究の計画・方法

BMPR2遺伝子、RNF213遺伝子、Sox17遺伝子のそれぞれのHot Spot変異をCRISPR-Cas9システムを用いてノックインしたマウスを作製した。作製したマウスを正常酸素ならびに低酸素刺激による肺高血圧症発症の有無を評価した。刺激したマウスをイソフルランで吸入麻酔をした後､右内頸静脈よりカテーテルを挿入し右室圧を評価した。安楽死させた後、肺高血圧症による右心不全の程度、および肺病変を評価した。また肺動脈内皮細胞や平滑筋細胞を分離し、mRNAや蛋白の発現解析を実施した。

4．研究の成果

BMPR2 R899X変異マウスは、低酸素刺激によってWTマウスと比較して著明に肺高血圧症が増悪し、予後が悪化することを確認した。肺組織を用いたsingle cell RNA-seq解析によって、BMPR2 R899X変異マウスにおける肺高血圧症発症に重要な遺伝子発現変化を解析中である。

また、RNF213遺伝子p.Arg4810Lys変異マウスにおいては、低酸素飼育刺激によってWTマウスと比較して、右室収縮期圧の上昇・右室肥大を認めることを確認した。これらのマウスの肺組織から抽出したRNAを用いた発現解析によって、RNF213シグナル経路で重要ないくつかの細胞周期調整・細胞増殖を制御する因子を同定することに成功した。それら重要な因子の阻害薬等を用いた研究によって創薬の可能性についても検証中である。

さらに、SOX17変異マウスにおいては、低酸素刺激がなくとも通常飼育下で左室・右室ともにWTマウスに比べて有意な心拡大を認めることを確認した。心拡大をもたらすメカニズムについての詳細な解析を実施中である。

5．研究発表（2020年度の研究成果）

〔雑誌論文〕

1）Shinya Y, Hiraide T, Momoi M, Goto S, Suzuki H, Katsumata Y, Kurebayashi Y, Endo J, Sano M, Fukuda K, Kosaki K, Kataoka M. TNFRSF13B c.226G>A (p.Gly76Ser) as a Novel Causative Mutation for Pulmonary Arterial Hypertension. J Am Heart Assoc. 10. 2021. e019245. doi: 10.1161/JAHA.120.019245.

〔学会発表〕

1）片岡雅晴. 肺動脈性肺高血圧症における日本人患者での併用療法の実態と今後求められる治療戦略. 第84回日本循環器学会学術集会･ランチョンセミナー . 2020年7月31日. Web講演.
2）片岡雅晴. 肺高血圧症の遺伝的素因. 第60回日本呼吸器学会学術講演会･シンポジウム. 2020年9月21日. Web講演.

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Immunological study on age-dependent susceptibility to *Ochroconis* infection in marbled rockfish, *Sebastiscus marmoratus*

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Ochroconis infection is a fungal disease caused by *O. humicola*. This disease has been reported in some marine fish species including marbled rockfish. Because these case reports suggest that *O. humicola* consistently infects juvenile fish, young age fish seem to be a target of the infection. The purpose of this thesis was to study the effect of age on the susceptibility and pathogenesis of the infectious disease, and to investigate the difference of immune response in marbled rockfish of different age categories.

Effect of age on susceptibility of marbled rockfish to *O. humicola* was examined by experimental challenge against juvenile fish of three age categories that showed different body length: small (29 ± 2 mm), medium (55 ± 3 mm), and large (74 ± 6 mm). The cumulative mortalities for small, medium and large fish were 100 %, 20 % and 0 %, respectively. These results demonstrated that the younger fish among juveniles were more sensitive to *O. humicola*.

For observation of inflammatory response in the different age categories, younger fish (52 ± 1 mm) and older fish (76 ± 4 mm) were intraperitoneally injected with *O. humicola*. Both fish group showed pathological symptoms in adipose tissues. In younger fish, infiltration of mononuclear leukocytes appeared at an early phage, increased the number of cells with time, and then these cells became necrotic at a late phase. The fungi spread the hyphae in the tissue with progress of pathological symptoms. In older fish, remarkable pathological symptoms were not observed, but granulomatous inflammatory response was found in some fish at a late phase. The fungal growth was limited and a few hyphae were present only in granulomatous inflammatory area. These results indicated that the quick and intense inflammation were characterized in the younger fish.

Cloning of immune relevant genes of adaptive cellular immunity that has been known to protect fungal infection was conducted for analysis of the immune activity in marbled rockfish. Four T cell marker genes and 2 cytokine genes could be identified based on the primary structure and phylogenetic analysis. The gene expression of CD4 and IFNγ was upregulated in the spleen of younger fish (52 ± 1 mm) and older fish (77 ± 2 mm) after the intraperitoneal injection with *O. humicola*. The results showed a unique profile that the younger fish characterized quick and intense responses, but the older fish characterized slow and moderate response.

The quick and intense response of helper T-cell function (CD4 and IFN-γ gene expression) in the younger fish probably initiates the inflammatory reaction in the adipose tissues after the infection. The immune response could be crucial for the recruitment for inflammatory cells to eliminate the fungal hyphae but this response in younger fish failed in elimination of the pathogen. The younger marbled rockfish could be characterized by operation of ineffective immune response which provokes intense inflammation resulting in tissue damage. It may be associated with the susceptibility of marbled rockfish against *O. humicola* infection.

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Studies on the contamination of retailed chicken by Campylobacter spp.and Listeria spp And the sanitation measures with HACCP

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The situation of *Campylobacter* species (C spp.) and *Listeria* species (L spp.) contamination in retail chicken meat was clarified, and its relationship with hazard analysis and critical control points (HACCP) certification was examined in this study.

In Chapter 1, 90 samples of chicken meat (categories: general broiler (' General'), Meigara-brand (Meigara), and Jidori Japanese local (Jidori) chicken) were purchased from 16 stores in Tokyo from 2017 to 2018. In total, 232 strains of C spp. and 175 strains of L spp. were isolated from the meats. Among the C spp., *C. jejuni* (Cj) accounted for the majority of the strains, and among the L spp. contaminated rate was high of *L. innocua* (Li) and *L. gray*. Contamination by only L spp. was three times more frequent than that by only C spp. The contamination rates were high in Jidori and Meigara, and low in ' General'. Bacteria of both genera were isolated from 43% of the samples, and the combination of Cj and Li was the most common combined contamination. *L. monocytogenes* (Lm) was found to contaminate meat only together with either Cj or *C. coli*. The current situation of contamination by bacteria of both genera was clarified from these results.

In Chapter 2, 219 strains were randomly selected from among preserved Lm strains and examined for benzalkonium chloride (BC) resistance. BC resistance was found in the 633 C3, 772C2, 868 C4, and 824 C3 strains, and the minimal inhibitory concentration s were 32 μ g/ ml in 633C 3, 772 C2, and 868C 4, and 8 μ g/ ml in 824C 3. Furthermore, it was shown that BC-resistant Lm strains have been in Japan since at least 2004, and their frequency has increased since 2017.

In Chapter 3, contamination by both bacteria was analyzed in 17 species of Jidori and Meigara in relation to the distribution channels and HACCP certification. Contamination by L spp. was confirmed in 13 routes, and in seven of the routes, the contaminatio n rate was more than 80%. Contamination by C spp. was confirmed in 17 routes, and the isolation rate of L spp. was high in six routes that had a contamination rate of more than 70%. Different processing plants had different contamination rates and bacteria l species even though the same meat shop, indicating route- dependent contamination. High contamination rates were revealed, i. e., the contamination rate of L spp. was 80% in eight categories of meats, and that of C spp. was 100% in nine categories of meats. Regarding HACCP certification, stores that acquired the certification tended to have lower isolation rates of C spp. than stores that did not. Regardless of certification acquisition, the isolation rate of C. spp. was significantly higher in stores handl ing Jidori and Meigara, and a lower contamination rate was found in chicken produced from windowless rearing. The high contamination rates in both Jidori and Meigara were therefore considered to reflect these factors.

In this study, the current situation of C spp. and L spp. contamination was examined in retail chicken, which were classified into ' General', Meigara, and Jidori categories, and the occurrence of combined contamination was clarified. It was also shown that BC- resistant Lm strains have been in Japan since at least 2004, and their frequency has been increasing in recent years. Furthermore, it was clarified that the contamination depended on the distribution channels, and in particular, delays in the implementation of sanitation measures in distribution channel and breeding system appear to be responsible for the higher contamination rates in Meigara and Jidori.

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Study of the Protective Effect of Tibial Plateau Leveling Osteotomy on Ligament Degeneration in Canine Cranial Cruciate Ligament Disease

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Canine cranial cruciate ligament rupture (CrCLR) is a common cause of hind limb lameness in small animal orthopedics. Tibial plateau leveling osteotomy (TPLO) is a useful treatment for CrCLR. Recently, particular attention has been paid to early intervention of TPLO to inhibit the progression of postoperative osteoarthritis (OA) and reduce mechanical stress in the cranial cruciate ligament (CrCL). However, it is unclear whether CrCL degeneration has been histologically suppressed. This study aimed to evaluate the usefulness of the early intervention of TPLO for CrCLR by analyzing whether TPLO has a protective effect on CrCL degeneration.

The present study showed that TPLO could improve weight-bearing function from as early as 3 months postoperatively and maintain it for as long as 36 months postoperatively. Although OA progressed over time after TPLO, its progression was more gradual in stifles with partial tears than in those with complete rupture. This result suggests that the adaptation of TPLO has a favorable outcome. Using a robotic system, TPLO promoted instability under the craniocaudal and internal–external rotation tests without compressive force conditions following CrCL transection. This may be one of the factors for the significant progression of OA in cases of complete CrCLR.

Based on previous studies, we created a model of CrCL degeneration by increasing the tibial plateau angle (TPA) and subsequently adopted TPLO to evaluate its effect on CrCL degeneration histologically. The results showed the maintenance of collagen 1 and inhibition of the production of proteoglycans and elastic fibers, which suggested that TPLO inhibited the progression of CrCL degeneration. This suggests that, by reducing the mechanical load on the CrCL with TPLO in the presence of CrCL, the CrCL degeneration process induced by excessive TPA can be suppressed.

Therefore, the present study suggests that early intervention with TPLO may decrease the biomechanical stress of CrCL, inhibit CrCL degeneration, and preserve CrCL function. The preservation of CrCL function is expected to minimize the instability associated with TPLO and inhibit the progression of postoperative OA.

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Study on the usefulness of tear components in respiratory disease of Japanese Black Calves

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This study conducted a series of experiments to investigate whether changes in tear properties are useful as an index for detecting the acute inflammatory stage of respiratory diseases, which have a large economic loss for Japanese Black breeding farmers.

In Chapter 2, the Schirmer Tear Test I method of clinically healthy calves is used to measure tear volume (STT I value) and total protein concentration in tear fluid, and the factors affecting the tear volume were examined. The STT I value of clinically healthy calves was 18.9 ± 2.9 mm/min (n=263). The total protein concentration in tears of clinically healthy Japanese Black calves from 15 to 90 days of age was 1.18 ± 0.30 mg/ml (n=38). It was suggested that age and ammonia concentration are related to fluctuation of tear volume.

In Chapter 3, the STT I value (22.2 ± 3.0 mm/min) and total protein concentration in tears (1.85 ± 0.47 mg/ml) of calves (n=63), which are in the acute inflammatory stage of respiratory diseases, are described. It was measured and significantly higher than that of clinically healthy calves. Therefore, the protein components in tears were subjected to qualitative analysis by two-dimensional electrophoresis and Liquid Chromatography-Mass spectrometry (LC-MS/MS). 1,329 spots of protein in tears of clinically healthy calves were obtained, and spots showing a significant increase in calves in the acute inflammatory stage of respiratory disease were identified using LC-MS/MS. Although it was not identified, the amino acid sequence suggested that it may be an immunoglobulin L chain. Then, when the immunoglobulin concentration in the tear fluid was evaluated including digestive diseases typical of calves, the IgA concentration in tears of respiratory disease calves was significantly increased compared with clinically healthy calves (0.31 ± 0.32 mg/ml, n=32) and gastrointestinal disease calves (0.07 ± 0.04 mg/ml, n=6). The increase in tear fluid and IgA in tear fluid observed in calves with respiratory diseases was considered to be caused by irritation to the conjunctiva and cornea, which are the upper respiratory tract and ocular mucosa, caused by respiratory diseases. Furthermore, when tears were collected over time in two calves evaluated as clinically healthy calves, high IgA levels in tears were observed before the onset of external symptoms of respiratory disease.

If it is possible to observe the area around the eyes and treat in the early stages of respiratory disease during feeding management, reduce the number of treatment days, prevent the spread of infection, and reduce the number of injuries and injuries of livestock mutual aid. It is thought that it can contribute to connection, improvement of productivity and stabilization of farm management.

*Supervisor : Prof. Hidekazu KOYAMA

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Characterization of novel diabetic rat model (Diabetes with Enlarged kidney: DEK) and the role of kidney in the development and the progression of diabetes

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The number of patients with diabetes continues to increase and has been estimated to reach 700 million persons worldwide by 2045. About 90～95% of patients with diabetes are estimated to have Type 2 diabetes mellitus (DM), which is thought to be caused by insulin resistance and/or relative insulin insufficiency. Because the pathogenesis and pathology of DM are complex and involve many genetic and/or environmental factors, there are limitations to providing a single guideline of medical treatment to all diabetic patients. This limitation has been addressed by developing several classes of anti-diabetic drugs that differ in their mechanisms of action, as well as by generating several animal models of diabetes, which differ in phenotypes. The treatment of patients with diabetes requires strict control of blood glucose concentrations, as failure of blood glucose control could seriously affect patient quality of life. Conventional major antidiabetic medicines act by stimulating endogenous insulin secretion and/or by reducing insulin resistance. However, despite treatment, some patients may develop diabetes as well as diabetic complications, such as diabetic nephropathy. Sodium glucose cotransporter 2 (SGLT2) inhibitors are a novel class of antidiabetic drugs. Determination of their therapeutic effects has suggested that the kidneys play roles in glycemic control. The present study describes the phenotype of diabetes with enlarged kidney (DEK) rats established in our laboratory, assesses the effects of the SGLT2 inhibitor empagliflozin on this phenotype, and analyzes the association between increased renal parenchyma and hyperglycemia in these animals. This study found that only male DEK rates developed diabetes. These rats were deficient in insulin secretion with decreases in β cell populations and enlarged kidneys, but without renal dysfunction. Examination of the enlarged kidneys of DEK rats showed that increases in the renal parenchyma were associated with increased renal tubules with luminal dilation. Because the inheritance of the phenotype of DEK rats is polygenic, as yet unidentified genetic factors responsible for these renal alterations may contribute to renal reserve capacity and resistance to the progression of diabetic nephropathy. In addition, long term empagliflozin administration eliminated chronic hyperglycemia and improved systemic metabolism as well as renal tubule function. However, these renal tubules remained dilated, with no histological improvement in response to empagliflozin. The enlarged kidneys of DEK rats had a congenitally increased number of nephrons, with the progression of renal tubular dilation and renal overgrowth being dependent on the duration of hyperglycemia. Uninephrectomy (1/2Nx) reduced blood glucose concentrations to normal levels, suggesting that increased parenchyma in enlarged kidneys may be responsible for hyperglycemia in DEK rats. However, renal overgrowth was not prevented under normoglycemic conditions induced by empagliflozin and 1/2Nx, indicating that renal growth was independent of hyperglycemia. Taken together, these studies have shown that DEK rats are useful as a novel animal model of diabetes and suggested the potential existence of a novel pathological mechanism, in which high renal reserve capacity might contribute to the development and deterioration of diabetes.

*Supervisor：Prof. Hiroetsu SUZUKI

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Examination of fecal microbiota transplantation as a new treatment for canine Lymphocytic-plasmacytic enteritis

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Inflammatory bowel disease (IBD) is a common cause of idiopathic, chronic, and relapsing gastrointestinal (GI) disease in dogs. The most typical histological change associated with IBD is lymphocytic-plasmacytic enteritis (LPE). As for the treatment of the dogs with symptomatic LPE, a diet, an antimicrobial agent, a corticosteroid agent and an immunosuppressive drug are used frequently. However, some of the dogs with symptomatic LPE which is resistant to those treatments is present. Therefore, the establishment of the new treatment of dogs with symptomatic LPE is urgent business.

In human medicine, fecal microbiota transplantation (FMT) has been reported as an effective treatment for disease with dysbiosis. In FMT, fecal matter is collected from a tested healthy donor, mixed with saline or other solutions, strained, and administered to a patient by colonoscopy, endoscopy, sigmoidoscopy, or enema.

A purpose of this study is to consider being the treatment that FMT for the dogs with symptomatic LPE is safe and effective. Therefore, we pushed forward this study by the following contents by this thesis.

In the symptomatic LPE (sLPE) group, α diversity of the microbiome significantly decreased as compared with asymptomatic LPE (aLPE) group. The predominant bacterial phylum was Proteobacteria of total bacterial population in the sLPE group, and it was significantly higher than aLPE group. While Fusobacteria comprised < 0.1 % in the sLPE group, it was significantly lower than aLPE group. Moreover, it was found that acetic acid and propionate concentrations were raised by the increase of the bacterial count of *Bacteroides* and *Fusobacterium*. Hence, it was found to have to give SCFA production these high bacterial strains of the specificity to raise the SCFA concentrations in the intestinal tract for symptomatic LPE with dogs. Therefore we conducted clinical application of FMT for the symptomatic LPE dogs to be resistant to treatment with drugs. After FMT fecal microbiome, the bacterial counts of *Enterobacteriaceae* was significantly decreased, while the bacterial counts of *Fusobacterium* was significantly increased. Based upon the foregoing, the sLPE dogs were a state of dysbiosis, and the possibility that this state is involved in the clinical status mainly on the digestive symptom is suggested. Furthermore, FMT treatment improved these sLPE dog's symptoms of diarrhea and vomiting. In conclusion, we show the safety and efficacy of FMT in the sLPE dogs. We conclude that FMT should be considered as a treatment option for canine symptomatic LPE cases in the future.

*Supervisor : Prof. Hidekazu KOYAMA

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Studies on changes in diagnostic markers for metabolic disorders in healthy cats with aging

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In our country, super-aging society is expanding at a speed that is so fast that we don't see it in the world. Non-communicable diseases (NCDs) such as obesity, diabetes and cancer have increased with progress of super-aging society. In recent years, the life span of dogs and cats has significantly extended and the number of age-related diseases of animals as well as human is increasing, and it is necessary to respond from a different viewpoint from conventional veterinary medicine. Therefore, the new animal medical systems that the preventive medical care for animals for individuals by early diagnosis and treatment is marked for NCDs. It is necessary to develop appropriate diagnostic markers for the early diagnosis with aging. It is necessary to investigate changes in diagnostic markers in healthy cats with aging because these markers change with aging. The subject were 67 clinically healthy cats (10 months to 16 years and 9 months old, 25 females, 42 males) that were divided into 5 groups with age. It was clarified that the weight and BCS increased with aging, and glucose (GLU), triglyceride (TG), albumin (ALB), malate dehydrogenase/lactate dehydrogenase (M/L) ratio, serum amyloid A (SAA), AMP activated protein kinase (AMPK) in serum changed with aging. It is suggested that healthy cats decrease energy metabolism on lipid metabolism with aging. From this result, GLU, TG, ALB, M/L, AMPK, SAA, and ADN were useful markers for detecting early small changes in metabolic diseases. It is necessary to be changed standard value of the marker with aging because energy metabolism changes with aging. We attempted to establish standard value with aging for TG, ADN, SAA, and M/L ratio related to lipid metabolism by study so far. As a result, it was suggested that early diagnosis of hyperlipidemia, obesity, and associated diseases may be possible. In addition, AMPK activity is also one of the markers to attend in the future that decreases with aging and lead to an extension of lifespan. In the future, it will be necessary to set more accurate standard values by accumulating various data and to attend other markers such as immunity. It is possible to apply this initiative to human with a declining birthrate and an aging population.

*Supervisor : Prof.Toshiro ARAI

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Study on the Association of Proximodistal Patellar Position and Canine Gait

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Motion analysis is used to analyse the mechanical function of humans or animals in motion. However, it has not yet been widely applied in clinical practice in the veterinary field. One of the most common orthopaedic diseases in small animals is medial patellar luxation (MPL). Patella alta, a condition in which the patella is displaced proximal to the trochlea, has been suggested to be associated with MPL. There has been no motion analysis of MPL or patella alta performed in dogs to date, and it is unclear whether dogs display crouch gait associated with these conditions, as in humans. This study aimed to evaluate the effect of the proximodistal patellar position on the gait of dogs.

In the first study using radiographic images, it was shown that the maximum stifle joint extension angle was greater in small dogs with MPL than in healthy dogs, with consequent proximal displacement of the patella. The effects of the hip and stifle joint angles on the quadriceps muscle length/femoral length ratio (QML/FL) and patellar ligament length/patellar length ratio (PLL/PL) were then examined. It was shown that the PLL/PL did not change with joint angles, whereas the QML/FL decreased with hip joint flexion and stifle joint extension. These results indicate the possibility of functional patella alta, a condition in which the patella displaces more proximally than normal over the femoral trochlea due to stifle joint hyperextension, even with the proximodistal position of the patella relative to the stifle joint angle being normal. Such conditions might be associated with MPL in small dogs.

An inverse dynamics analysis was performed on healthy dogs during trotting to calculate the angular alterations in the stance phase as well as the associated joint moments and joint power. Based on these angular alterations in healthy dogs and dogs with MPL, we examined the association between the movement of the stifle joint during trotting and image examination values related to patella alta. As the PLL/PL increased or the patellar position during hyperextension became more proximal, both the maximum angle of flexion and extension of the stifle joint in the stance phase significantly decreased. It was suggested that the crouch gait, which is equivalent to the one reported for patella alta in humans, is also present in dogs. Finally, gait analysis was performed in dogs wearing an orthosis that limits the range of motion of the stifle joint to investigate how the degree of restriction affects the ground reaction force. The alteration in the ground reaction force varied with the degree of restriction on the range of motion of the stifle joint. These alterations could increase the load on some joints.

In this study, it was concluded that functional patella alta may be present in small dogs with MPL, possibly in association with crouch gait and these gait changes could result in load changes other than stifle joint.

*Supervisor : Prof.Yasushi HARA

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Studies on effects of anti-oxidant astaxanthin supplementation in healthy and obese dogs

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In recent years, overweight and obesity are frequently observed also in dogs and cats. Obesity and its related disorders such as diabetes mellitus and Cushing's syndrome become social problem in veterinary medicine. In this thesis, pathophysiology and onset mechanism of obesity is reviewed, and astaxanthin (ASX) supplementation effects were investigated in healthy, obese, diabetes and Cushing' s syndrome dogs.

Obesity is a risk factor to induce various metabolic disorders in both human and animals. Excess lipid as accumulated visceral fat induces proinflammatory reactions in various tissues. The most important factor for obesity to induce various metabolic disorders is remodeling of fat cells in accumulated visceral adipose tissues. Enlarged fat cells secret various inflammatory cytokines (adipokines) such as non-esterified fatty acids (NEFA), M1 macrophages, which induce activation of NADPH oxidase and oxidative stress leading acceleration of obesity domino reaction consequently metabolic syndrome. ASX is generally known as vigorous anti-oxidant substance. As ASX shows suppressive lipid peroxidation function and singlet oxygen elimination function, ASX is used as a functional food in various fields, food industry and fish culture, etc. Effect of ASX supplementation was investigated in healthy dogs with BCS3 (ideal body weight). ASX was orally supplemented at a dose of 0.3 mg/kg BW/day for 8 weeks, plasma TG and MDA concentrations and LDH activities decreased significantly. These results seem to clarify that ASX supplementation ameliorates liver function leading to improve lipid metabolism in dogs.

ASX was supplemented to 5 obese dogs with BCS 4 and 5 at a dose of 0.3mg/kg/day for 8 weeks. After the supplementation, plasma TG concentrations and alanine aminotransferase (AST) activities were decreased in all 5 dogs. Plasma LDH activities decreased in 4 of 5 dogs, and MDH concentrations decreased in 3 of 5 dogs. BW and BCS were not changed after the ASX supplementation.

ASX supplementation effect was investigated in dogs with diabetes mellitus and Cushing's syndrome. On 60 days after the supplementation, plasma ALT, γ-GTP, NEFA and MDA concentrations decreased significantly. Miniature Dachshund (spayed, 11 years old) with Cushing's syndrome showed overweight (BCS4). ASX was supplemented orally with this dog at a dose of 0.28mg/kg/day. On 30 days after the supplementation, plasma total cholesterol (TC), TG, ALT and NEFA values were not changed, however, on 120 days plasma TG and NEFA concentrations decreased.

As the above results, ASX supplementation may suppress lipid peroxidation in tissues, in particular in liver, and may improve liver function, which is effective to treat metabolic disorders such as diabetes mellitus and Cushing's syndrome in dogs. It is very important to ameliorate liver function by suppression of lipid peroxidation for prolongation of lifespan in obese dogs. Therefore, supplementation of anti-oxidant substance like ASX is very effective to prevent obesity and its related metabolic syndrome.

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Usefulness of advanced MR imaging in the canine and feline intracranial tumors

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The purpose of this study is to evaluate the usefulness of advanced MR imaging in canine and feline intracranial tumors. In the present study, the MRI data of canine and feline with intracranial tumors obtained each definitive histopathological diagnosis were used. The obtained advanced MR imaging sequences included diffusion weighted imaging, diffusion tensor imaging, MR spectroscopy, and perfusion weighted imaging.

Diffusion weighted imaging (DWI) was performed in 35 cases and evaluated using apparent diffusion coefficient (ADC) and the ADC ratio, which is a relative value obtained by dividing the tumor ADC value by those of the contralateral normal-appearing white matter. As a result, in intratumoral regions, feline meningiomas and canine histiocytic sarcomas showed significantly lower ADC ratios compared to canine meningiomas. This finding is compatible with the actual clinical picture of histiocytic sarcomas and feline meningiomas. The ADC ratio for gliomas in this study was similar to the previous canine reports, and the ADC ratio for bone tumor was also identical to that of humans. In peritumoral areas, the lowest ADC ratio was observed in feline meningiomas, that was likely to be resulted from the strong displacement of the surrounding normal brain tissue by the tumor. However, the peritumoral ADC ratio in this study was not helpful to differentiate invasion in peritumor, and between vasogenic edema and cytotoxic edema.

Diffusion tensor imaging (DTI) was performed on 36 cases and evaluated using fractional anisotropy (FA) and FA ratio, as with ADC. The intratumoral FA ratio suggests that feline meningioma and bone tumor are relatively solid tumors, and that canine and feline gliomas have variable directions of diffusion. In peritumoral areas, FA ratios of histiocytic sarcomas and gliomas were lower than those of canine and feline meningiomas. It is suspected that histiocytic sarcomas and gliomas would be infiltrating and/or destroying the surrounding brain parenchyma while meningiomas compress surround tissues without nerve damage. In addition, the vasogenic edema was suggested in the peritumoral region with T2-weighted hyperintense for histiocytic sarcomas.

Magnetic resonance spectroscopy (MRS) of thalamus in normal dogs and cats were measured at first, and then those results were used as the reference values for the following comparative MRS study of 15 cases with intracranial tumor. Creatine and N-acetyl-L-aspartate were reduced in all tumors. Choline was elevated in association with active cell proliferation in histiocytic sarcoma and glioma. However, the marked choline peak seen in human meningiomas was not observed in feline meningiomas in this study. As with human gliomas, elevated lipid and lactate were observed in canine gliomas in this study, this finding suggests the possibility that MRS is useful for grading of glioma.

Perfusion weighted imaging (PWI) is a sequence to assess cerebral blood volume, cerebral blood flow, and mean transit time. The comparison of infusion rate (IR) 1 ml/sec and 4 ml/sec was investigated using normal dogs and cats at first. In some brain regions, there were some significant differences among the difference of IR. Then, each parameter of PWI in 10 cases with intracranial tumors was evaluated. Those results suggested that the lesions may be underestimated at low IR as known in human medicine. Also, it was challenging to assess tumor invasion or edema in the peritumoral region with PWI.

As mentioned above, this study suggests the combination of these advanced MR imaging techniques

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has the possibility to differentiate intracranial tumors in veterinary patients as well as human medicine. Further studies with more case accumulation would establish the noninvasive diagnosis of intracranial tumors using advanced MR imaging.

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Studies on assisted reproductive technology in Amur leopard cats

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Small wild cats of the Felidae family indigenous to Japan, Tsushima leopard cats and Iriomote wild cats, are on the verge of extinction, as are many wild cats of the Felidae family. To protect these wild animal species, it is necessary to introduce various artificial reproductive technologies (ART) as in-situ conservation, in addition to ex-situ conservation of habitats, but these techniques for small wild cats of the Felidae family, such as Tsushima leopard cats, have not yet been established.

Thus, using a subspecies of leopard cats belonging to the same species, Amur leopard cats, to which Tsushima leopard cats belong, as a model of Tsushima leopard cats, we performed a series of study to establish ART techniques.

The conditions of a method collecting semen from male Amur leopard cats were investigated using the transrectal electric stimulation method and it was clarified that semen with favorable characteristics capable of being used for artificial insemination (AI) can be collected by inserting a rectal probe assembled with electrodes into a single bundle on the ventral side parallel to the rectum at a site about 6.5 cm from the anus and applying stimulation at 1-4 V in September-April.

When semen storage was investigated, it was possible to store semen retaining favorable characteristics by either cryopreservation or low- temperature storage. The characteristics of sperm collected from the cauda epididymis excised after the animal died and those after freezing and thawing were also investigated. The sperm characteristics were favorable and these types of semen may be sufficiently applicable for AI.

Female Amur leopard cats showed no marked estrus sign in captive breeding and it was difficult to identify the optimum time of mating. Thus, methods to induce follicular growth and ovulation by eCG and hCG administrations to female Amur leopard cats in the non-breeding season were investigated. Although it has not been investigated in this animal species, each condition could be set. When surgical intrauterine injection of semen (AI) collected from a male Amur leopard cat was performed 20-22 hours after hCG administration in 2 female Amur leopard cats with grown follicles, both animals became pregnant. Early embryonic death occurred in one, but pregnancy was maintained in the other and a normal newborn could be acquired. Birth of a newborn by artificial reproduction in this animal species has not previously been reported and this was the first success in the world.

These techniques may be useful for the reproduction of not only Amur leopard cats but also small wild cats of the Felidae family on the verge of extinction and help increasing the currently reduced population.

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Studies on *Sarcocystis* detected Japanese sika deer (*Cervus nippon centralis*) in Hyogo Prefecture

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Sarcocystis form sarcocysts, which are large cysts that can be seen with the naked eye, in the muscles of the intermediate host. Where the popularization of meat and livestock was inferior to that of the West, these wild animal meat has been supplied as a vital protein source since ancient times, but Japanese sika deer were caught only a little until more than ten years ago and were not as common as wild boar as food. However, the report on the present state of *Sarcocystis*, belong to the phylum Apicomlexa, in Japanese sika deer is not sufficient yet.

From the 64 Japanese deer (*Cervus nippon centralis*) caught in the central mountainous area of Shimomikata district, Fukuchi, Shiso City, Hyogo Prefecture for hunting and harmful control, materials were collected by age (under 1 year old to 5 years old) and by site (myocardium, diaphragm muscle, biceps femoris, and longissimus dorsi) to investigate the parasitism rate of sarcocysts observed there. The parasitism rate in the investigated Japanese deer was as high as 81.3%. By site, a significantly higher parasitic density was observed in the myocardium.

When we examined sarcocysts in muscle to investigate the parasitism status of *Sarcocystis* species of Japanese deer, differences in their size and morphology were observed, which suggested that they might be undescribed species different from any species examined. The taxonomic position of this *Sarcocystis* was studied using *18 S rDNA* and *cox1* genes. As a result of the classification of *Sarcocystis* protozoa originated from deer by the *cox1* gene, this *Sarcocystis* formed a clade independent of those previously reported. This indicated the possibility of this species being the single species which has not been reported until now from the genetic analysis. We had an opportunity of performing an experimental infection by giving myocardium of Japanese deer containing sarcocysts detected from the muscle of Japanese deer to two dogs. This proved that the dog becomes the definitive host, and the prepatent period of this species is 5-6 days.

These results suggested that *Sarcocystis* detected in Hyogo Prefecture is a new species different from the known species based on morphological, genetic, and prepatent period differences, and revealed that the life cycle of this species is maintained by canids as the definitive host and deer as the intermediate host.

*Supervisor : Prof. Kazunori IKE

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Study of DNA Polymorphisms of the *CMAH* gene in Dogs and Cats Abstract of Doctoral Thesis

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In the feline blood group system, N-glycolylneuraminic acid (Neu5Gc) and N- acetylneuraminic acid (Neu5Ac) are the type A and type B antigens, respectively, and the cytidine monophospho-N-acetylneuraminic acid hydroxylase (Cmah) enzyme is involved in the synthesis of Neu5Gc from Neu5Ac. Each type of antigen in this blood group system results from mutations in the *CMAH* gene that affect the production of Neu5Gc and Neu5Ac. A DNA screening scheme involving *CMAH* variants that can accurately determine blood types within the feline AB blood group system would be of great use and is highly desirable to complement phenotypic tests. Even though the *CMAH* gene has been well characterized in cats, not much is known about it in dogs. Furthermore, it was recently reported that canine and feline parvoviruses preferentially recognize Neu5Gc. Therefore, we characterized the dog *CMAH* gene for the first time. Additionally, we further studied the association between mutations and diplotypes of the cat *CMAH* gene and compared the results to those of the dog *CMAH* gene.

cDNA cloning showed that the dog *CMAH* gene contained a 1737-bp open reading frame that encodes a polypeptide of 578 amino acids; it was predicted to have high similarity to the *CMAH* gene of other mammals, including cats. The dog *CMAH* gene was expressed in many tissues, but not all tissues (n=28); the cat *CMAH* gene has been reported to be expressed in almost all tissues. We also identified 15 single-nucleotide polymorphisms and an indel in *CMAH*. Focusing on c.554A>G (p.Lys185Arg), the G allele was widely distributed in western breeds (n=229), although the Shiba dog was identified to be the most polymorphic at this locus among the breeds used in the study. To investigate the c.554A>G in dogs with the presence or absence of Neu5Ac expression, the phenotype of the binding of lectin to Neu5Ac (positive or negative) was determined in Shiba dogs and Labrador Retrievers. Thereafter, we genotyped the dogs at c.554A>G. However, the results did not clarify whether this mutation influences the expression of Neu5Ac.

We also investigated mutations and diplotypes of the cat *CMAH* gene in type B (n=21) and AB cats (n=6), and compared the results to those of a previous study (Omi et al., 2016). Results showed that the diplotype distribution in type B cats was not so discordant to that of the previous report, although some novel diplotypes were found. However, in type AB cats, some of the genotypes were discordant to those of the previous report as some showed diplotypes that were reported to have at least one intact allele (A type). Therefore, we need to analyze other exons in which other variants might be discovered.

Compared to the cat *CMAH* gene, the results on the dog *CMAH* gene suggest that variants with an amino acid substitution at the *CMAH* locus are not associated with the expression of Neu5Ac or Neu5Gc. Analysis of the regulation mechanism of mRNA expression may enable the cause of this difference to be identified.

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Properties of p53 Mutant in Canine Mammary Tumor-derived Cell Line CTB-m2

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The tumor suppressor protein, p53, plays a central role in cell cycle and maintains the genomic integrity. The p53-encoding gene is frequently mutated in human cancers. Such germline mutations are the underlying cause of Li-Fraumeni syndrome. The human p53 protein comprises an N-terminal transactivation domain, a proline-rich region, a structured DNA-binding domain connected to a tetramerization domain (TD) via a flexible linker, and a C-terminal regulatory domain. Active p53 protein is a homotetramer, which adopts a dimer-of-dimer topology. TD in human p53 (amino acid residues 326–356) exhibits a dihedral symmetry of dimers. Two monomers interact with the β-strands (Glu326-Arg333) to form a dimer, two dimers then interact as a α-helix bundle (Arg335-Gly356) to form the tetramer. Nine residues in the TD of human p53 (Phe328, Leu330, Ile332, Arg337, Phe338, Met340, Phe341, Leu344, and Leu348) are critical determinants in stabilizing the p53 tetramer. Leu344 mutants lacking the ability to dimerize (L344P) or tetramerize (L344R) have been previously reported, and notably, L344P germline mutation was observed in a family with Li-Fraumeni syndrome. Leu344 is located in the α-helix, which forms the hydrophobic core of the tetramer interface.

Although numerous cell lines containing mutated p53-encoding genes have been established from cancer tissues, there are few cell lines that contain a mutated p53 TD. Uyama *et a*l. established and characterized four pairs of canine mammary tumor cell lines of either primary or metastatic origin. One of these cell lines, CTB-m, was passaged 50 times in our laboratory to obtain a cell line containing spontaneous L332Q mutation in p53 (corresponding to human L344); the new cell line was designated as CTB-m2. In a previous study, glutaraldehyde cross-linking assays and a functional reporter assay revealed lack of tetramerization abilities of L332Q mutant of canine p53. In this study, we attempted to clone the canine p21 homolog, transcription of which is promoted by p53, and conducted expression analysis of p21-ecoding gene in canine p53-forced-expressed cells.

Chapter 1: We novel cloned canine p21 homolog, which is transcribed by tetramerized p53. RT-PCR of cloned canine p21 formed a 495 bp open reading frame and 165 amino acid residues (GenBank accession: LC586433). The cross-reactivity between human antibodies against p21 and canine p21 homologs was validated. Antibodies against human p21 could specifically detect the Halo-tagged canine p21 homolog transfectants in HeLa cells.

Chapter 2: We analyzed the changes in morphological properties of CTB-mVP and CTB-m2 by doxorubicin stimulation. The change in expression levels of canine p21 and related molecules following 1, 2, 5, and 10 μM doxorubicin stimulation was also validated. After 24 h of stimulation with 10 μM doxorubicin, CTB-m2 showed remarkable cell death. CTB-m2 also showed DNA fragmentation with >2 μM doxorubicin stimulation. In contrast, cell death and DNA fragmentation were not induced in CTB-mVP at <10 μM doxorubicin stimulation. However, CTB-mVP showed acetylation of Lys382 in p53-encoding gene and expression of p21-encoding gene on stimulation with 1 μM doxorubicin; CTB-m2 showed acetylation of Lys382 in p53-encoding gene with and without doxorubicin stimulation and no expression of p21-encoding gene. These results suggest that CTB-mVP with normal p53-encoding gene induces tetramerization of p53 following doxorubicin stimulation and thereby mediates expression of p21-encoding gene, cell cycle arrest, and progression of the DNA repair process. However, CTB-m2 with L332Q mutant of p53-

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encoding gene lacked the tetramerization ability and hence failed to induce expression of p21-encoding gene post doxorubicin stimulation. Subsequent cell cycle progression with genomic instability results in cellular apoptosis. Furthermore, mRNA expression of CTB-mVP and CTB-m2 on stimulation with 1 μM doxorubicin was also observed. We reported a reduced expression of p21-encoding mRNA in CTB-m2 compared to that observed in CTB-mVP and thereby confirmed the increased expression of mRNA coding DNA synthesis and DNA recombination repair proteins in CTB-m2. These phenomena support our hypothesis that impaired p53 tetramerization promotes abnormal DNA synthesis and cell division with DNA damage, leading to cell death.

In this study, we analyzed the characteristics of CTB-m2, which expresses L332Q mutant of p53 in the TD. CTB-m2 lacked the ability to induce expression of p21-encoding gene on stimulation with DNA damaging doxorubicin, resulting in abnormal cell division. CTB-m2 cells can be used to investigate p53 pathogenesis and evaluate new strategies for restoration of p53 function.

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Effect of inhalation of hydrogen gas on blood pressure-lowering in a rat model of renal hypertension

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Hypertension in humans is defined as systolic/diastolic blood pressure higher than 140/90 mmHg. In patients with chronic kidney disease and hypertension, one goal is to maintain blood pressure lower than 130/80 mmHg. Hypertension is classified as either essential hypertension, whose cause is unclear, or secondary hypertension, which is induced by specific cause. Essential hypertension is thought to be caused by genetic factors and environmental factors such as lifestyle habits, whereas secondary hypertension is induced by abnormal hormone secretion, heart disease, and kidney disease. Because chronic hypertension accelerates decline in renal function and the development of cardiovascular disease, blood pressure must be managed in patients with chronic kidney disease. The kidneys play an important role in blood pressure regulation. When the kidneys are under stress, the renal afferent nerves increase and the sympathetic nerve from the brain activates, which result in hypertension. In the treatment of patients with resistant hypertension, renal denervation, and baroreflex activation therapy exerts an antihypertensive effect.

Many antihypertensive agents have been developed and their effects have been reported. In 2010, Nakayama et al. reported that in comparison with standard dialysate, dialysate containing hydrogen improved hypertension after dialysis in patients with chronic kidney disease. In 2007, Ohsawa et al. reported the therapeutic effects of hydrogen gas on cerebral ischemia–reperfusion injury in rats; since then, therapeutic effects of molecular hydrogen on various animal models of disease have been reported. Molecular hydrogen is thought to exert therapeutic effects by its antioxidant activity against reactive oxygen species (ROS) produced by ischemia–reperfusion injury. However, the effect of molecular hydrogen on blood pressure regulation remains unclear.

In this study, on the basis Nakayama et al.'s reports, we investigated the blood pressure–lowering effect of hydrogen gas inhalation in a rat model of renal hypertension. To assess the antihypertensive effect of hydrogen gas in the acute phase, a rat model of acute kidney disease was used. A rat model of chronic kidney disease was used to evaluate the effect of hydrogen gas in the chronic phase. In addition, we examined the method by which telemetry systems can be operated over a long term to measure vital signs (e.g., blood pressure and heart rate) in conscious animals.

Nine-week-old male Lewis rats were used in the study. To induce renal hypertension in the rats, the branches of the renal artery were ligated in 5/6 nephrectomy. The rats used immediately after 5/6 nephrectomy served as the model of acute kidney disease, and those used 3 weeks after nephrectomy served as the model of chronic kidney disease. Inhalation of hydrogen gas or control gas was performed for 1 h per day for 4 weeks. In the acute kidney disease model, renal function was measured, and a telemetry system was used for measuring blood pressure, heart rate, and autonomic nerve activity. Furthermore, for implantation of the telemetry system, we evaluated the effectiveness of the introducer we developed . In the model of chronic kidney disease, blood pressure and heart rate were measured by means of tail-cuff manometry. The data of the two groups of rats were compared and statistically evaluated.

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The introducer we developed shortened the time needed to insert a telemetry catheter into the rat femoral artery and reduce complications before and after catheter insertion.

In the model of acute kidney disease, inhalation of hydrogen gas significantly decreased blood pressure without affecting the level of renal function ($P < 0.05$). The heart rate also tended to decrease. Hydrogen gas significantly suppressed the 5/6 nephrectomy–related increase in sympathetic nerve activity and decrease in parasympathetic nerve activity ($P < 0.05$).

Hypertension is partly caused by activation of the sympathetic nervous system, which increases ROS production in the rostral ventrolateral medulla (RVLM). The antihypertensive effect of hydrogen gas in this study was to suppress central sympathetic nerve activity by scavenging ROS in the RVLM through the antioxidative effect, which is a known function of hydrogen, thereby decreasing blood pressure and heart rate. Recently, hydrogen dynamics in the blood of pigs after a single inhalation of hydrogen gas was measured. Hydrogen gas inhalation increased the concentration of hydrogen to a higher level in the carotid bloodstream than anywhere else in the body. This result indicated that hydrogen lowered blood pressure by acting directly on the brain.

In the model of chronic kidney disease, blood pressure was significantly decreased ($P < 0.05$) and heart rate tended to decrease in the rats that inhaled hydrogen. Therapeutic effects of hydrogen have been investigated mainly during the acute phase, when inflammation is common immediately after ischemia–reperfusion injury; in this study, however, the efficacy in the chronic phase, when inflammation has subsided, was examined, and hydrogen exerted the antihypertensive effect regardless of inflammatory status. Although the increase in ROS production in the RVLM during the chronic phase was not examined in this study, the mechanism by which blood pressure was reduced in the model of chronic kidney disease is thought to be the regulation of autonomic nerve activity, as in the model of acute kidney disease.

This is the first study in which pharmacological effects of hydrogen on renal hypertension in rats were experimentally demonstrated. Although further studies are necessary to elucidate whether molecular hydrogen improves sympathetic activation by scavenging ROS at the cellular and tissue levels, this study showed that inhalation of hydrogen gas is a potential new therapy for hypertension.

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Pathological analysis of captive Japanese rock ptarmigan (*Lagopus muta japonica*)

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The Japanese rock ptarmigan (*Lagopus muta japonica*) is the order of Galliformes, family of Phasianidae, genus of Lagopus and grouse subfamily which inhabits only the alpine zone of the Japanese Alps. The population of wild Japanese rock ptarmigan has been declining in recent years, and the Ministry of the Environment revised the Red List, which is a list of endangered species, making Japanese rock ptarmigan endangered from the previous endangered species II in 2012. It was raised to class 1B, and in the same year, a conservation and breeding business plan was formulated. One of the projects is consideration of breeding in captivity and reintroduction to wild habitats, and as part of the agreement between the Ministry of the Environment and the Japanese Association of Zoos and Aquariums, an attempt to raise Japanese rock ptarmigan in the zoo as ex situ conservation has been attempted since 2015. In this study, the cause of death of Japanese rock ptarmigan in captivity was analyzed histopathologically with the aim of obtaining findings that can be utilized for ex situ conservation of them and reintroduction in habitats.

In chapter1, we analyzed histopathologically 42 Japanese rock ptarmigan. of the 46 that died in captivity between August 2015 and September 2020, and summarized the characteristics of the lesions by microscopically observation with HE tissue-stained specimens. In 2015, when the breeding started, eggs derived from wild individuals were artificially hatched, but five eggs kept in the same zoo died one after another around two months after birth. Anemia was observed in the dead ptarmigan, a decrease in the proportion of hematopoietic cells in the bone marrow, and immaturity of lymphoid follicles in the spleen were confirmed, suggesting poor development of the immune system. In addition, oxalate pyelonephritis was observed, and although it could not be confirmed by autolysis, septic bacterial infection was also suspected. In 2016, eggs derived from wild individuals were hatched as in 2015, and all birds grew to adult. Since 2017, eggs derived from artificial breeding have been used for hatching, but many bird dead suspected of having bacterial infectious diseases such as Salmonella, Pseudomonas aeruginosa and Escherichia coli were observed. When the age of death and the cause of death were summarized for each hatching year, it was confirmed that the proportion of individuals dying within one week after 2017 is as high as about 1/4. Individuals hatched from wild fertilized eggs in 2015 and 2016 may have stronger properties due to the effects of natural mating in the wild and the nutritional status of their parents. In some cases, it was thought to be a bacterial infection from the feces of the mother bird, indicating that hygiene management is an important issue in transferring hatching and raising birds from by human to the mother bird. In addition, as characteristic lesions of Japanese rock ptarmigan, deposition of oxalate in the kidney and accumulation of macrophages containing crystalline in the bronchi of the lung were observed. Although these have rarely been associated with causes of death so far, they may affect age-related changes in the future, and these are lesions to be noted.

In chapter 2, for individuals suspected of histologically infectious disease, we attempted to identify the bacteria histopathologically by adding Gram stain and

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immunohistochemical staining for bacteria, and analysis using an electron microscope.

We tried immunohistochemical staining with antibodies against three types of bacteria suspected to be pathogenic bacteria: Salmonella, Pseudomonas aeruginosa, and Escherichia coli, and bacteria in lesions were positive in some cases. The bacteria were gram-negative, and it was revealed that they were salmonellosis, pseudomonas aeruginosa, and Escherichia coli disease, respectively.

Even if bacteria were detected in the many parts of the body by the microorganism test, the inflammatory reaction by the bacteria could not be confirmed by the histopathological examination in some cases. So, it is important that performing both the bacterial test and the histopathological examination for confirming the influence of bacteria for cause of death.

An electron microscopic search of a suspected case of Salmonella confirmed phagosomes in macrophages, and bacteria in it had wall construction of gram-negative bacteria, they were capture the characteristics of Salmonella. In a case suspected of Escherichia coli infection, immunohistochemical staining suggested that the cells adhered to the microvilli, and that it was intestinal-adherent microvilli-disappearing Escherichia coli (AEEC). But it was not possible to observe the disappearance image of the microvilli by electron microscope observation.

In this study, we have established immunohistological analysis methods for some pathogenic bacteria, and revealed that control infectious diseases, especially within the first week of life, is important in the breeding of Japanese rock ptarmigan. In order to preserve this valuable species, it is necessary to further enhance the pathological diagnosis techniques with such as immunohistochemical staining and to accumulate the pathological knowledge of Japanese rock ptarmigan, in the future.

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Relationship between spatial distribution of hard mast production in the beech family and home range size and shape in Asiatic black bears

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Home range is referred as the place to use for the forage, the mate, the parental care, and the territories. Home range is determined depending on various factors. In particular, it seems to be strongly influenced by the amount and quantity of food sources. The amount of food sources varies widely be¬tween years or between seasons, and even temporal variation has an effect on the size, shape, and location of the home range for wildlife animals. Hard mast in the beech family is one of the major food resources in the fall for Asiatic black bear (*Ursus thibetanus*). Previous studies reported in bears that the mast production varies with time and affects the home range. Since mast production varies with not only a temporal scale but also a spatial scale, the spatial distribution of the mast production may also affects characteristics of the home range. For example, local mast production sometimes becomes rich, even if the mast production of the whole region is poor. This means that the distribution of mast production would be not uniform. Nevertheless, only a few studies have quantitatively demonstrated such prediction. In addition, it is not clear how these distribution patterns of mast production affect the home range of Asiatic black bears. In this study, I attempted two subjects: first, I estimated the distribution pattern of mast production by the Kriging method, one of the spatial completion methods. Second, I tested the relationship between spatio-temporal resource variation of hard mast production and characteristics of home range (size, shape and location) in Asiatic black bears.

This study was conducted in and around Karuizawa town, Nagano Prefecture, Japan, from 2014 to 2018. For the survey of the mast production, I created 72 survey sites and counted mast production using binoculars on Mongolian oak (*Quercus crispula*, 409 trees), Japanese chestnut (*Castanea crenata*, 352 trees), and Konara oak (*Quercus serrata*, 272 trees). For the distribution pattern of the mast production, I determined the synchro¬nized distances using the data of the mast production, and estimated distribution pattern using the Kriging method. For the estimation of the home range in female Asiatic black bears, I used the telemetry data that was obtained to determine the locations of each individual during the autumn season (Septem¬ber 1 to November 30), and esti-mated the 50% and 95% home range of 29 individuals by the fixed kernel method and calculated size and shape complexity of home range for each individual.

To investigate the relationship between the home range of Asiatic black bears and the distribution of mast production, I conducted generalized linear mixed models (GLMMs). The objective variables were 50% and 95% home range size and shape. The explanatory variables were related to the mast production for each species (mean, maximum, minimum, and standard deviation). Then, I developed three candidate models to be used for each of the objective variables. The three candidate models were composed of the following explanatory variables: i) only temporal variables in the mast production, ii) only variables related to spatial variables in the mast production, and iii) spatio-temporal variables in the mast production. I compared each model using Aikaike Information Criteria (AIC). Next, I developed resource selection function (RSF) to clarify whether the location of each bear' s home range affects the distribution pattern of the mast production. For RSF, I compared the ratio of the mast production in the study area to the mast production of each oak in the home range of Asiatic black bears using the chi-square test.

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The results of the spatio-temporal resource variation showed that mast production of all tree species in 2017 was highest in the investigated years, while the mast production of Mongolian oak in 2016 was lowest among all tree species and years. The synchronized distances of mast production were 988 ± 385 m for Mongolian oak, 1364 ± 875 m for Konara oak, and 1213 ± 404 m for Japanese chestnut. Furthermore, I found that the distribution pattern of mast production was spatial heterogeneity in all years. Home range size and shape of Asiatic black bears in 2016 tended to be the smallest and most com¬plex. Resulting from GLMM, 50% and 95% home range size is affected by the standard deviation of Konara oak, the mean value and the maximum value of Mongolian oak and the minimum value of Konara oak. Moreover, shape of 50% and 95% home range was affected by only the standard deviation of Konara oak. Previous studies showed that home range size is strongly influenced by the mast production of Mongolian oak, but this study showed that home range size and shape complexity is strongly influenced by the mast production of Konara oak. One of the reasons for the inconsistent results is that Konara oak is an alternative food resource to Mongolian oak in the study site. Therefore, it was revealed that when the mast production of Mongolian oak was low and spatial variation in the mast production of Konara oak was high, the home range of Asiatic black bear may have large size and complex shape.

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Detection of catecholamine using saliva sample in adult male mouse

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Introduction

Blood is one of the most commonly used samples for measuring bioactive substances, however, blood collection is highly invasive for small animals such as mice, and it is difficult to collect blood samples repeatedly from the same individual. By contrast, saliva samples can be collected with less invasiveness compared to blood samples and be obtained repeatedly, suggesting that might contribute to Refinement and Reduction in the principles of 3Rs. Our laboratory has reported that salivary corticosterone is available to evaluate stress response and salivary testosterone is available to evaluate testicular function in mice. Since these two hormones are steroids, it is assumed that they are rapidly transferred from blood to saliva, but it is not clear how much the water-soluble bioactive substances can be detected in saliva of mice.

Catecholamine (CA), low molecular weight, is one of water-soluble bioactive substances and is important as a neurotransmitter such as noradrenaline (NA) or adrenaline (Ad). These NA and Ad secreted from adrenal glands are available to evaluate acute stress response. The purpose of this study is first, we examined how much isoproterenol (ISO), an exogenous CA, could be detected in blood and saliva of mice treated with continuous intravenous ISO infusion (Experiment 1) and second, to detect endogenous CA in mouse saliva, we examined blood and saliva concentrations of NA and Ad in response to restrain stress. (Experiment 2).

Materials and method

Animals and Agents

Adult male mice 8-10weeks of age were used in this study. Three types of mixed anesthetic agents (medetomidine / midazolam / butorphanol = 0.3 / 6.0 /7.5 mg/kg; i.p.) was selected for anesthesia. Saliva was collected by placing a cotton ball in the mouth of mice under anesthesia. This study was approved by Nippon Veterinary and Life Science University (2019K-15, 2020K-42).

Experiment1

Ten minutes after anesthetic injection, mice were continuously injected ISO (50, 100, 200 μg/ml) through the jugular vein using a pump (200 μl/40 min). Ten minutes after the onset of ISO administration, mice were injected pilocarpine hydrochloride (1.0 mg/kg; i.p.) that enhanced saliva secretion, and saliva was collected for 30 minutes. Blood was drawn from the posterior vena cava at the end of saliva collection. Both blood and saliva samples were extracted using alumina, the concentration of ISO in both samples was detected by high-pressure liquid chromatography (HPLC) with electrochemical detection (ECD).

Experiment2

Male mice were acclimated to the environment of experimental room at 1 hour before the onset of this experiment. To examine blood concentrations of CA in response to stress, blood samples were taken by decapitation at just before the onset of stress (0 min) and at 7.5, 15, 30, and 60 min after the onset of stress. To detect endogenous CA in saliva, mice were restrained at 10 minutes after the injection of pilocarpine hydrochloride (3 mg/kg; s.c.) dissolved in 10 % polyvinylpyrrolidone, and then saliva samples were collected for 15 minutes with or without anesthesia. In the control group, saliva was collected from mice without restraint stress under anesthesia. Blood was collected by decapitation at 25 minutes (the end of restraint stress) after the injection of pilocarpine hydrochloride. Samples were extracted using alumina

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and the concentration of CA in both blood and saliva samples was detected by HPLC with ECD.

Result

Experimnet1

Both salivary and plasma ISO levels increased in a dose-dependent manner (plasma: R^2= 0.86, p <0.05, saliva: R^2= 0.82, p <0.05), and it was confirmed that ISO transferred from blood to saliva was about 11%.

Experiment2

The plasma NA and Ad levels, endogenous CA, were significantly increased at 7.5 min after the onset of restraint stress compared to the 0 min-control (p <0.05). By contrast, there was no significant difference in the plasma CA levels at 15, 30, and 60 min after the onset of restraint stress compared to the control group. The salivary NA and Ad were not detected in mice treated without restraint stress, whereas the salivary CA could be detected in both anesthetized and conscious mice treated with stress. In addition, it was confirmed that the ratio of CA transferred from blood to saliva was 3.9% for NA and 1.6% for Ad under conscious condition, and the ratio of CA transferred from blood to saliva was 15.6% for NA and 2.5% for Ad under anesthetized condition.

Discussions and Conclusions

The endogenous CA transferred from blood to saliva was low compared with the transfer of exogenous ISO except for NA under anesthetized conditions (15.6%). Since an endogenous CA is easily affected by general anesthesia and the decreased level of blood NA may be due to the effects of anesthesia, the mechanism of the increased level of salivary NA is not clear. The exogenous ISO is metabolized by catechol-*O*-methyltransferase (COMT), but is less affected by monoamine oxidase (MAO). On the other hand, the endogenous CA, NA, and Ad are metabolized by both COMT and MAO.

In conclusion, it is suggested that CA, water-soluble bioactive substances, can be detected in the saliva of mice. The level of salivary endogenous CA is very low and can be detected in the saliva of mice treated with restraint stress but it can be not detected in it of mice treated without stress. Further studies are needed in order to detect endogenous CA in saliva samples of mice treated without stress. Saliva samples can be collected with less invasiveness and be obtained repeatedly, and the technique of detection of water-soluble bioactive substances in saliva samples of mice may contribute to Refinement and Reduction in the principles of 3Rs.

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Analysis of IgE antibody production and antigen processing in mice administered with *C. elegans*.

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[Introduction]

In recent years, the cause of the increase in allergic disease patients is explained by the hygiene hypothesis in which contact with microorganisms and parasites in early childhood has decreased, the natural development of the immune system has become insufficient, and then susceptibility to allergic diseases has increased. Allergies develop in response to hypersensitivity to originally harmless antigens (allergens), because IgE antibodies to them are produced and mast cells are activated. In order to select the production of IgE isotype antibody, T cells are presented with antigen by dendritic cells, which are antigen-presenting cells, and differentiate into Th2 cells after activation. Then, the cytokine IL-4 is secreted from Th2 cells and binds to a receptor on B cells to activate a class switch to IgE isotype, which finally induces IgE antibody production.

On the other hand, the immune response to parasites differs depending on various things such as the type of parasites, their developmental stage and secretory molecules. Among parasites, when helminth nematodes are infected, IgE antibody is produced as in allergies, and worm exclusion is promoted resultantly. There are many unclear points about how nematodes promote IgE antibody production. *C. elegans* is a non-parasitic nematode and is widely used as an experimental material because its entire genome sequence has been clarified. Previous studies in our laboratory have shown that when *C. elegans* was subcutaneously administered to mice, IgE antibodies are produced in the same manner as parasitic nematodes. Therefore, in order to investigate the mechanism by which *C. elegans* produces IgE antibody, experiments such as confirmation of immune cells that bind to the worm body after administration of the worm to mice, administration of the stained worm for follow-up its fate, and search for target molecules of blood antibodies, were carried.

[Materials and methods]

(1) Administration of *C. elegans* and blood sampling: *C. elegans* killed at 37 ℃ was subcutaneously administered to mice. Blood was collected from the tail of the mouse before the first administration, and after confirming that the IgE concentration in the serum was low by the ELISA method, the worm administration was carried out 3 times every 7 days. Blood was collected from the tail of the mouse 7 days after the final administration, and after confirming that the concentration of IgE antibody in the serum was high by the ELISA method, cardiac blood was collected to obtain antiserum. Conversely, if the IgE antibody concentration was low in the blood from the tail, the nematode *C. elegans* was administered again, and 7 days later, a cardiac blood sample was taken. (2) Staining and administration of nematodes: *C. elegans* that has been killed is stained with 0.2% trypan blue, 2.0% Evans blue, Coomassie brilliant blue (undiluted solution), and ink, and the dye that can stain *C. elegans* is determined by comparison. The nematodes stained overnight with 2.0% Evans blue were administered subcutaneously and intraperitoneally to BALB/c mice, and the mice were dissected 1 day and 5 days later, respectively, and observed with a stereomicroscope. (3) Western blot analysis: TNE Buffer was added to the pellet of *C. elegans* nematode, then ground, and the lysate was collected after centrifugation. Subsequently, SDS-PAGE was performed, and after transfer to a membrane, antiserum was used as a primary antibody for reaction, and then visualized with anti-IgG or anti-IgE antibody. (4) Immunoprecipitation and silver staining: In order to analyze the molecules

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of the worm recognized by IgG and IgE antibodies in the blood, antiserum was applied to the lysate of nematode C. elegans prepared above, then followed by immunoprecipitation with anti-IgG and anti-IgE antibodies, and silver staining was performed after electrophoresis.

[Results and discussion]

(1) After subcutaneous administration of *C. elegans* 3 or 4 times, the amount of IgE in the blood was compared with that before administration, and it indeed increased 3 to 11 times, reconfirming that the nematode *C. elegans* promotes the production of IgE antibody. (2) As a result of trying to stain the dead *C. elegans* with 0.2% trypan blue, 2.0% Evans blue, Coomassie brilliant blue, and ink overnight, it was found that 2.0% Evans blue stained the worms at any stage of development. Next, mice were subcutaneously administered with Evans blue-stained worms for the follow-up. One day later, it was confirmed that many worms remained at the administration site while maintaining their shape under a film-like tissue. After 5 days, the worms still remained at the administration site, but their shape were almost lost and turned to paste. When administered intraperitoneally, many worms remained in the omentum near the pancreas one day later and were covered with a film-like tissue while maintaining its shape. After 5 days, the number of sites where worms were observed decreased, and worms were sparsely present, covered with a film-like tissue in the mesentery, and their shapes were not well maintained. Therefore, when the nematode was administered subcutaneously, it remained at the administration site even after 5 days, suggesting that the antigen processing rate may be slow under the skin.

(3) Western blot with IgG antibody in antiserum of nematode *C. elegans*-administered mice detected bands over 250 kDa, near 250 kDa and 140 kDa, while IgE antibody in antiserum detected bands in the vicinity of 75 kDa and 140 kDa, suggesting the possibility of a target molecule. (4) As a result of trying to immunoprecipitate the target molecules of IgG antibody and IgE antibody, followed by silver staining, intensive bands were confirmed around 35 and 43 kDa by the IgG antibody. By the IgE antibody, intensive bands were confirmed around 50 and 140 kDa, and a faint band was confirmed around 75 kDa. Therefore, the bands from Western blot and immunoprecipitation did not necessarily match, but the bands around 75 and 140 kDa detected by the IgE antibody appeared to be same, suggesting that these may be target molecules. In the future, a sufficient amount of these bands needs to recover from the gel for analysis to identify the molecule, and finally verify the possibility that these are molecules that induce the production of IgE antibody.

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Stress induced by animal care and experimental procedures in mouse - assessment of changes in body temperature -

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Introduction

In rodents, acute stress such as encountering predators activates the sympathetic nervous system that increases body temperature, which is stress-induced hyperthermia (SIH). The SIH is caused by β_3-adrenoceptor (β_3R) on brown adipose tissue localized around axillae, kidneys, and between shoulder blades in endothermic animals. In laboratory mice, SIH is mainly observed by intentional experimental stress models including restraint and social defeat stress. However, there are few studies have reported that the transition of body temperature was used as an indicator for measuring stress induced by daily animal care, such as cage-change and cage transfer, and experimentally manipulates including treatments of manual restraint and injection. Stress is known to affect some physiologic parameters (e.g. blood hormone levels, heart rate, blood pressure, and body temperature). Therefore, it is extremely important for researchers or technicians to understand how much stress mice might be received.

In the present study, to assess the degree of stress induced by daily animal care and experimental procedures, the transition of core body temperature was measured in mice. Furthermore, to investigate the mechanism of hyperthermia after cage-change, mice were pretreated with SR59230A (A/β_3R), a selective β_3R antagonist and confirmed the effects of A/β_3R on the hyperthermia. In addition, we focused on two factors might account for hyperthermia after cage-change, 1) odors in bedding such as urine scent or pheromones used as social identification and 2) acclimation to handling which reported to reduce the sensitivity of stress in mice.

Materials and Methods

We used adult male ICR mice kept under group-rearing conditions in this study, and they were intraperitoneally implanted the device under anesthesia. The device can measure and record the core body temperature and the activity levels in mice without touching them. The mice were allowed recovery periods over 2 weeks after surgery for implantation and the body temperature and the activity levels were measured from 11 weeks of age. Firstly, the mice were exposed some treatment, which is assumed the daily husbandry (viz. entering the breeding room, peeking into the rearing cage, cage transfer, and cage-change at light phase), and experimental procedures namely manual restraint and injection processes (viz. i.p., s.c., i.v., p.o.). The baseline was defined as the temperature at the time of just before entering the room for each experimental procedure. Then, we calculated the body temperature variations(ΔT) and the activity levels. In the experiment using A/β_3R, we performed a cage change in both light and dark phases. The light phase is assumed to perform a general cagechange in a breeder's or experimental facility, and the dark phase is done to increase the body temperature because of the circadian rhythm. In order to examine the effects of odor factors on hyperthermia-induced by cagechange, two groups were set up; the "no odor group" in which bedding was replaced with sterilized ones, and the "odor group" in which bedding was reused with replacement. In both groups, the rearing cage was replaced with sterilized ones. Additionally, to investigate the effects of acclimatization, mice purchased at the age of 3 weeks were subjected to the handling treatment once a day, 5 days a week (Monday-Friday), and the measurement device was implanted at 9 weeks. After a week of the

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recovery period, the handling treatment was resumed at the age of 10 weeks. At the age of 11 weeks, the body temperature and the activity level were measured when the mice were subjected to the following stimuli: entering the breeding room, peeking into the rearing cage, cage transfer, cagechange at light phase, and manual restraint. The present study was taken in accordance with the Guidelines for Animal Experimental issued by the Japanese Association for Laboratory Animal Science and approved by the provisions of Nippon Veterinary and Life Science University (2019K-14, 2020K-39).

Results and Discussions

The body temperature was significantly increased by the daily animal care and experimental procedures in all experimental groups. In addition, the most remarkable increase of body temperature was observed in cage-change treatment; however, the increased body temperature was significantly suppressed in mice pretreated with A/β_3R in the light phase, whereas A/β_3R did not suppress it after cage-change in the dark phase because of the elevated basal body temperature due to circadian rhythms. The effects of A/β_3R on the body temperature after light-off without cage-change also showed similar results and the antagonist did not affect the increased body temperature. In the experiment to examine the effect of odor factor, both the "with odor" and "without odor" groups showed significantly equivalent SIH. Moreover, the acclimatization to handling had no effects on SIH in response to any stimulations.

In conclusion, the present study reveals that mice rapidly increase their body temperature in response to daily animal care and experimental procedures, especially cage-change. Hyperthermia induced by cage-change was mediated by β_3R, but elevated basal body temperature due to circadian rhythms was not mediated by it. The factors of odor and acclimatization did not suppress SIH, and suggested that it was difficult to eliminate hyperthermia induced by the daily animal care, including cage-change. Further investigations are required to clarify the physiological significance of the increase in body temperature in response to daily animal care.

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Studies on muscle proteins contributing to the texture of dried horse mackerel meat

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Dried fishes have been used as a highly-preservative food in Japan for a long time, and are preferred as a daily food. In recent years, dried fishes with low salt concentration are increasing because of highly health-conscious consumers and even such dried fish have a unique texture and umami. It is thought that changes in actin and myosin which are the main proteins in myofibrils (MF) are major factors responsible for the texture peculiar to dried fish. However, recently there hasn't been studies on changes in proteins and texture of dried fishes processed at low concentration of salt. Therefore, purpose of this study is to elucidate behavior of actin and myosin during salting and drying, and those effects on texture of dried horse mackerel meat treated with low and high salt concentrations.

【Materials and Methods】

Ⅰ. Measurement of salt concentration of horse mackerel meat: Raw horse mackerel meats were cut into about 1×3×1㎝ portions, packed in dialysis-tubes, and then salted in 1.5 M or 3 M NaCl/20 mM Tris-acetate (pH7.0) (1.5 or 3 M salt solutions) at 4 °C. A 9-fold volume of pure water was added to the salted meat and homogenized. Ten g of the homogenate was put in a dialysis-tube and dialyzed against 90 mL of pure water at 4°C overnight. The salt concentration of the outer solution of dialysis was measured by the Mohr method. The salt concentration of commercial dried horse mackerel meat was measured by the same method.

Ⅱ. Measurement of moisture content of dried horse mackerel meat: The horse mackerel meats salted for 20 min according to the method I were placed on stainless net and dried in air circulation type incubator (Yamato Co., Ltd., IJ101 (W)) at 30 °C. The water content of the dried samples was measured with a heat-drying moisture meter (A & D Co., Ltd., MX-50 type) at 130 °C.

Ⅲ. Measurement of stress of dried horse mackerel meat : The samples salted and dried according to the method of II were cut into portions of a fixed size, and the stress change was measured using a cylinder or blade type plunger with a creep meter (YAMADEN, RE-33005s type).

Ⅳ. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of actomyosin (AM) of dried horse mackerel :

A 9-fold volume of Weber-Edsall solution was added to the samples salted and dried according to the method of II and then homogenized. The obtained homogenate was stirred at 4 °C for 21 h. A 2-fold volume of pure water was added to the homogenate to be 0.2 M KCl, and the mixture was centrifuged. The sample before centrifugation and the supernatant were subjected to SDS-PAGE on 10% gel.

V. Measurement of Mg^{2+}-and Ca^{2+}-ATPase activities of dried horse mackerel meat: A 49-fold volume of pure water was added to the sample salted and dried according to the method of II and homogenized. The Mg^{2+}-and Ca^{2+}-ATPase activities were measured according to the conventional method.

VI. SDS-PAGE of MF of dried horse mackerel meat in the presence of urea: MF was prepared from sample same as II according to the conventional method. Ten mg/mL MF was mixed with a 4-fold volume of 8 M urea / 2% SDS / 2% 2-mercaptoethanol / 20 mM Tris-HCl (pH 8.0) solution. The obtained mixture was subjected to SDS-PAGE on 2% gel containing 6 M urea.

【Results and Discussion】

I. The salt concentration of commercial dried horse mackerel meat was found to be 0.28 ± 0.1 M as a result of measurement. When raw horse mackerel

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meat was salted in 1.5 and 3 M salt solutions for 20 min, salt concentrations of the meats were about 0.3 and 0.5 M, respectively. Therefore, the former was set as the salting condition for producing recent dried mackerel meat with low salt concentration (LM), and the latter was set as the salting condition for producing the conventional dried mackerel meat with high salt concentration (HM).

Ⅱ. As a result of measuring water contents of raw horse mackerel meat and meats salted in 1.5 M and 3 M NaCl solutions during drying process, those of all meats decreased during drying process and there was no difference in decreasing rate among three kinds of meat. The relatively low salt concentration of the samples and small decreasing rate of water content were presumed to be responsible for the result.

Ⅲ. As a result of measuring the stress change of the horse mackerel meat sample with a cylindrical plunger, a fracture point was observed in the increasing process of the strain rate. In both LM and HM, the breaking stress increased with drying time. Regarding the effect of the salt concentration, the breaking stress of LM was significantly (P <0.05) larger than that of HM in drying for 1 h, but no significant difference was observed in other drying times.

The stress changes of the horse mackerel meat sample were also measured with a blade type plunger, which hit parallel and perpendicular direction to the body axis of fish. The stress increased in a curve until the strain rate reached 99% with any directions of blade and a fracture point was not observed. Therefore, we focused on the stress at a distortion rate of 80% (S80). When the blades were in perpendicular direction, drying process increased S80 in both salted samples. In drying times for 1 and 2 h, LM showed significantly larger (P <0.05 and P <0.01, respectively) S80 than HM, but there was no significant difference in other drying times. When the blades were in parallel direction, drying process raised S80 in both of LM and HM. In drying time for 0.5 h, LM showed a significantly larger (P <0.05) S80 than HM, but there was no significant difference between LM and HM in other drying times. On the other hand, in the comparison between perpendicular and parallel directions of blades, the perpendicular direction showed significantly larger (P <0.05 or P <0.01) S80 than the parallel direction in the case of LM dried for 0-1 h and HM dried for 0.5-1 h.

The above-mentioned results demonstrated that the horse mackerel meat becomes harder by drying after salting, but the difference in salt concentrations does not remarkably affect the hardness. In addition, the measurement by the blade type plunger showed stress differing in directions, which was considered to be influenced by the binding state of MF and the way the blade hits the myoseptum.

IV: Analysis of the AM dissociation status of the horse mackerel meat sample revealed that the bands of myosin heavy chain and actin had already appeared in the supernatant of the raw horse mackerel meat sample soon after purchased. The bands density of the actin and myosin heavy chain did not change during either of salting or drying process. These results suggested that AM had been dissociated into actin and myosin when raw horse mackerel was obtained, and actin and myosin were not markedly denatured by salting and drying.

V: The activities of Mg^{2+}-ATPase and Ca^{2+}-ATPase of LM tended to decrease gradually during drying process for 4 h. Neither of Mg^{2+}-ATPase activity nor Ca^{2+}-ATPase activity of HM decreased. From this result and the result of IV, it is considered that actin is not denatured to be insolubilized under 0.2 M KCl, but is denatured so that its interaction with myosin is affected during drying process after meat sample is salted in 1.5 M salt solution. On the other hand, it was suggested that denaturation of myosin induced by salting and drying is too small to decrease in Ca^{2+}-ATPase activity.

VI: As a result of SDS-PAGE in the presence of 6 M urea, multiple bands having a higher molecular weight than that of the myosin heavy chain were observed in both of LM and HM dried for 0 h, and they increased gradually during drying process. From this result, it is presumed that myosin molecules are polymerized by salting and the change is facilitated by drying.

All above-mentioned results clarified that there is little difference in physical properties and texture between LM and HM. The dissociation of AM is not involved in the change in texture during the drying process after salting, and mainly myosin denaturation and polymer formation was thought to contribute to the changes.

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Effects of the change in egg composition due to *in ovo* amino acid administration on the intestinal flora and immune gene expression in newly hatched chicks

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[Objective] It has been reported that at least amino acids among the components in eggs affect the formation of intestinal flora during hatching in birds. *In ovo* administration of Leu in birds also alters subsequent growth. The above is because the administered nutrients are sent from the egg yolk sac to the gastrointestinal tract, and then the bacterial flora is changed by using them by specific intestinal It shows multiple possibilities that the intestinal flora may also change. Therefore, in this study, we administer Leu, which has already been confirmed to cause muscle development due to insulin-like action, *in ovo*, examine the effect on the intestinal flora, and examine the interaction with Gln, which has a conjugate relationship.

[Materials and methods] In Experiments 1-1, 1-2 and 1-3, Experiments 2-1 and 2-2, Experiments 4, 5 and 6, each eighty Chunky strain broiler breeder eggs were divided into 4 groups of 20 so that the average egg weight was the same, and the eggs were incubated at a temperature of 37.8 °C and a relative humidity of 60% or more. Only in Experiment 3, eighty Julia-lite white leghorn breeder eggs, were used as same way as the other experiments.On Day 18 of incubation, 0.5 ml of distilled water and the same amount of Leu + Gln were administered *in ovo* to each of Leu and Gln. Only in Experiment 6, eggs was in ovo administered on Day 14 of incubation.In Experiment 2-2, the incubation conditions were the same as other experiments, and an AA mixture obtained by removing Leu and Gln from the whole egg composition amino acids was administered. Experiment 3 was performed in the same manner as Experiment 1-2, using only chicken breeds as white Leghorn. In Experiment 4, the same treatment was performed to elucidate the blood estradiol (E2) concentration in Experiment 1-1. Cardiac blood was collected at the time of hatching, the intestinal tissue was also collected.In Experiment 1-1, the body weight was measured immediately after hatching, and after euthanasia, the weight of the cecum including the contents was measured, and the contents were collected. Experiment 1-2 was performed in the same manner, and the cecal contents collected in Experiment 1-1 were separated by PCR and electrophoresis using specific primers that recognize Escherichia coli, Clostridium, lactic acid bacteria, bifidobacteria, Salmonella, and Campylobacter. The band was visualized. Treatments with different bands of detected intestinal bacteria were quantified by qPCR in Experiment 1-2. In Experiment 1-3, the weight of the superficial pectoralis major muscle was measured immediately after hatching, at 3 and 7 days of age, after euthanasia.

In Experiments 2-1 and 2-2, after euthanasia, the intestinal tissue at the base of the yolk stem was collected, and the mRNA expression levels of TLR2, TLR4, CD3, and IL2 were quantified by qPCR. The sampling of Experiment 3 was carried out in the same manner as in Experiment 1-2. In Experiment 4, the E2 concentration of blood was measured with a commercially available ELISA kit, and the expression level of estradiol receptor (ER) mRNA was examined in the intestinal tissue. In Experiment 5, in order to eliminate the influence of the elapsed time after hatching, the same treatment as in Experiment 1-1 was carried out, and after blood was collected from the embryo before the pipping of 20 eggs, the cecal

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contents and gastrointestinal tissue were collected by euthanasia. Plasma was measured for E2 content. DNA was extracted from the contents of the cecum, and intestinal bacteria were quantified by qPCR. In the gastrointestinal tissue, the ER expression level was quantified by qPCR in the small intestine, cecum, and large intestine.

[Results] In Experiments 1-1 and 1-2, 5 of the 6 gut flora bands (Escherichia coli, Clostridium, Lactic acid bacteria, Bifidobacterium, Salmonella) and Leu + Gln administration groups were electrophoresed. Clear bands of 3 species (Escherichia coli, Clostridium, Bifidobacterium), 2 species (Clostridium, Lactobacillus) in the Gln administration group, and 2 species (Clostridium, Salmonella) were confirmed in the control group. In Experiment 1-3, the weight was the heaviest in the control group, and it was the lowest in the combined administration.

Since Leu and Gln showed opposite effects in a series of Experiments 1, Experiment 2 examined the possibility that the bacterial flora was changed due to changes in gastrointestinal immunity that were first absorbed and metabolized in the host's gastrointestinal tract. As a result, in Experiment 2-1 the expression level of the immune gene tended to be different between males and females in the control group, the expression level tended to be suppressed only in females in the Leu administration group, and compared with males in the Gln administration group. The difference between males and females ($p < 0.05$) was confirmed to be significantly higher in females. No significant difference was observed in Experiment 2-2, but it tended to be higher in females in the group without Leu in TLR2 and TLR4, and lower in the group without Gln, and among the amino acids administered to whole eggs, Leu. And Gln may act alone on the immune gene.

In Experiment 3, which examined chicken species specificity in this phenomenon, the expression level of immune genes tended to be higher in females in white Leghorn as in broilers, but there was no change in the administration group. In Experiment 4, the effect of the administered amino acid on the amount of E2 in the blood and cecal contents and the gene expression level of the estradiol receptor (ER) in the gastrointestinal tract was investigated. As a result, no significant difference or tendency was observed in the E2 content in plasma and cecal contents in each administration group.From these results, it was considered that the receptor of sex steroid hormone may be involved in the action and immunity of amino acids.

No significant difference was found in Experiment 5, which examined whether the influx of steroid hormones into the intestinal tract was affected before hatching. From this, it was inferred that the difference in ER2 mRNA expression in the intestine was not due to the influence of the ligand before hatching.

「日本獣医生命科学大学研究報告」投稿規程（2019 年 7 月改正）

本誌は、日本獣医生命科学大学（以下「本学」という。）における自然・人文・社会科学分野の研究成果を「研究論文」「博士・修士論文の要旨」「梅野信吉賞受賞記念」「若手研究者支援」などに分類して掲載する定期刊行物である。

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2．論文は和文または英文とする。

(1) 原稿のデータ（USB, CD-R, メール添付等の方法で）を所属教室名・著者名を記入して提出する。提出した原稿は返却しない。

(2) 和文論文は A4 版横書きの用紙に記載し（1 行 25 字、1 頁 50 行）、英文論文は A4 版の用紙にダブルスペースでタイプする（1 頁 25 行）。両原稿とも周囲には幅 2 cm 以上の余白を残す。なお原則として、1 篇につき、図、表、抄録を含めて、刷上り 10 頁以内とする。

(3) 原稿第 1 頁に、表題・著者名・所属を記し、論文内容を端的に表現する和文または英文略表題（和文 25 字以内、欧文 8 語以内）を記載する。なお、原稿第 1 頁の下半分は余白とする。

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3．本学の教員と大学院学生が筆頭者となって 2 編以上投稿する場合、2 編目以降は実費を申し受ける。

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記　載　の　要　項

1．図・表はわかりやすく別紙に書き，本文中にそれを入れる場所を朱記する。図・表・写真の説明は，すべて欧文とする。ただし，人文科学・社会科学・外国語系は和文でも可とする。
和文中の「第○図」は「Fig. ○」とし，「第○表」は「Table ○」と書く。写真と図は「Fig. ○」に統一する。

2．図と表の内容が重複するような本文の表現は避けること。

3．和文論文中，外国語の人名は THOMAS のように書き，赤線 2 本のアンダーラインを引く。

4．数字はすべて算用数字を用いる。また，諸単位の略号は次の例にならう。
(km, m, cm, mm, μm, nm, l, ml, kg, g, mg, μg, etc.)

5．名詞並列の場合はナカグロ（牛・馬・羊・豚）とし，句読点はコンマ（,）を，終文点にはピリオド（.）を用い，ともに 1 字に数える。

6．本文中の文献番号は肩付きとする。：井上ら[4] によれば

7．引用文献は，筆頭著者の姓のアルファベット順，同一著者のものは年代順に列記し，番号をつけ，論文の最後にまとめて記載する。

記載例

1）竹山　弘・松原義江（1995）．心臓手術の合併症．胸部外科，**8**，854-860.

2）SMITH, H.W. and CRABB, W.E.（1961）. The faecal bacterial flora of animals and man. J. Pathol. Bacteriol., **82**, 53-66.

3）山村研一（1984）．ラットキメラ．哺乳類の発生工学（大沢仲昭・江藤一洋・舘　　鄰・御子柴克彦編）．ソフトサイエンス社，東京，pp. 149-153.

4）MCKELVIE, D.H.（1970）. Husbandry of the beagle-care and management. The Beagle, 1st ed.（ANDERSEN, A.C. ed.）. The Iowa State University Press, Iowa, pp. 16-21.

5）伊藤真次（1978）．性ホルモンと性行動．ホルモンと行動．共立出版，東京，pp. 193-219.

8．引用文献の略号は，Serial Sources for the Biosis Data Base に準ずる。特別のものを用いる場合は，その旨説明を付ける。

9．要約には原則として略記号を避けるが，もしそれを用いる場合は，本文の場合のように説明を付ける。

10．見出しの形式は，実験科学論文ではつぎの例の順序にしたがう。緒言の見出しは使わず，本文で書きはじめる。

和文論文	欧文	
要　　約	ABSTRACT	
実験材料	MATERIALS	番号をつけずに本文の中央に書く。これらの見出しを 2 ～ 3 まとめることは自由。たとえば実験材料および方法
実験方法	METHODS	
実験結果	RESULTS	
考　　察	DISCUSSION	
（謝　辞）	（ACKNOWLEDGMENTS）	
文　　献	REFERENCES	
ABSTRACT	要　　約	

11．内容の小見出しはつぎのようにする。（□印はあける字数 1 個）

□ 1.　□尿 PSP 排せつ

□ a.　健康群□……（本文）……

□排せつ量：……（本文）……

12．使用用語については「畜産学用語集」（日本畜産学会編．東京，養賢堂発行），「獣医解剖・組織・発生学用語」（日本獣医解剖学会編．東京，学窓社発行）および関連学会において規定された学術用語を参照すること。

図の作成要領

印刷効果および能率をあげるため，次の事項を厳守されたい。なお適当でない原図については，書き直しをお願いすることがある。

1．白紙または青色方眼紙にスミ書きすること（サインペンは避けること）。
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3．図の中には原則として文字は入れない。必要があれば，右の仕上り図(1)(2)(3)のように番号を右側につけ，図の下へ活字で組むこととして，－○－　－×－のように図示しない方が望ましい。
4．文字の大きさ，線の太さは 2/3 に縮小されることを考えて書く。

原図の例（実寸法）文字は白紙にタイプライターで印字したものを張り付けた例。もちろん直接印字する方が字の行・列がそろってきれいに仕上がる。

仕上り図の例　下の原図から実際の半頁幅の仕上り図を示したもの

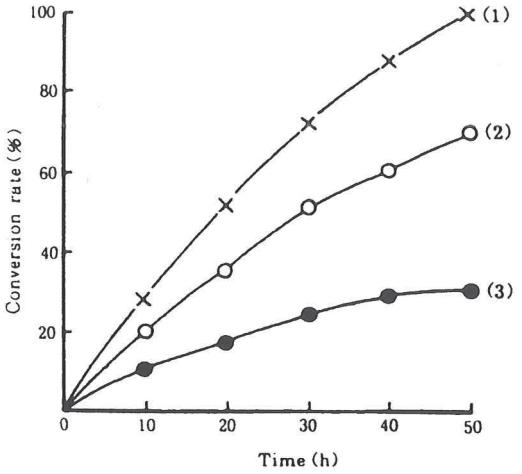
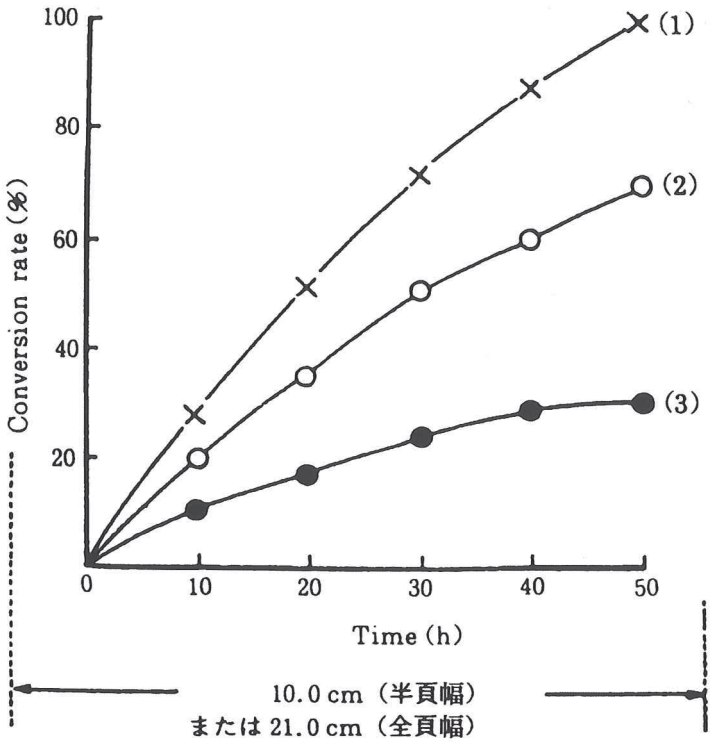


Fig. 1.　Effects of pH on the enzyme activities.

(1)：pH 8.0,　(2)：pH 7.0,　(3)：pH 6.0



1st March 2022

OUTLINE OF THE UNIVERSITY

Nippon Veterinary and Life Science University was founded by nine veterinarians in 1881. It was established in Otowa, in a corner of the Denzuin, a branch of the Gokokuji Temple, in Bunkyo Ward, Tokyo. Since that time, the university has maintained a high reputation as being a first-class institution for its Life Science education as well as other related disciplines. This is a reflection of the university's mission:"To develop highly qualified veterinarians, specialists, and researchers who not only have dedication for science but also have kind hearts." Embodied in this mission statement, there is the university's ethical approach of "Mutual Respect and Harmony" for and among its students. The university aims to have educated graduates who are individuals rich in humanity, full of creativity and strong in practical skills, yet filled with a sense of modesty, a spirit of cooperation, an aspiration for kindness and a sensitivity to humaneness.

Nippon Veterinary and Life Science University is comprised of two faculties, each of which as two schools. The Faculty of Veterinary Science is made up of the school of Veterinary Medicine and the school of Veterinary Nursing and Technology. The Faculty of Applied Life Science consists of the school of Animal Science and the school of Food Science and Technology. In addition to these two faculties and four schools, the university operates a Graduate School of Veterinary Medicine and Life Science which offers a post-graduate course in three major fields. They are respectively a Doctoral Course in Veterinary Medicine, Doctoral and Master's Course in Veterinary Nursing and Technology as well as Doctoral and Master's Course in Applied Life Science. Although the university is a relatively small educational institution with approximately 200 staff and 1,700 students, it has a long history with well-founded traditions which are a source of great pride. Notwithstanding, in this era of advanced veterinary medicine, technology and education, the university is keeping pace with the rapid changes in these areas Veterinary Medicine, Nursing and Technology through operating among others a Veterinary Medical Teaching Hospital on its Tokyo Campus and the Fuji Research Farm dedicated to animal husbandry and livestock veterinary medicine, research and education at the base of Mount Fuji. By such means the Nippon Veterinary and Life Science University is maintaining both its reputation and status as a first-class educational and research center spanning the fields of Veterinary Medicine, Veterinary Nursing and Technology as well as those of both Animal Science, and Food Science and Technology.

Zoonotic diseases, food shortage and the environmental change and damage, energy resources- these are the major global issues facing the world today. No long-lasting solutions to these enduring problems have yet been found, regardless of the efforts made to address them within the limited confines of traditional disciplines. It seems that since these global issues are interrelated, adopting an interdisciplinary approach towards them which combines agriculture and veterinary medicine with technology, engineering and science would better achieve breakthrough models and concepts to deal with them. Nippon Veterinary and Life Science University recognizes this challenge through all of its four schools and both of its faculties working together as one and acting in concert across this range of disciplines. The university centered on harmony between people and the environment through the efficient and effective but sustainable use (and reuse) of natural resources. Accordingly, the university is striving to tighten the mutual bands between disciplines and comprehensively apply the resulting approaches across a broad range of fields.

The university is committed to making contributions for the advancement of veterinary medicine on a worldwide scale as well as for securing a healthier world through maintaining biodiversity and providing more stable and safer food resources. Thus, Nippon Veterinary and Life Science University, though facing up to and tackling the pressing challenges confronting people today, aims to contribute to not only a more vibrant society but also a more prosperous world.

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