Journal of Integrated Field Science

Vol. 3

March. 2006

Field Science Center
Graduate School of Agricultural Science
Tohoku University

Journal of Integrated Field Science (JIFS)

Office : Integrated Field Science Center,
Graduate School of Agricultural Science,
Tohoku University,
232−3, Yomogida, Naruko-Onsen,
Osaki, Miyagi, 989−6711, Japan

Printed by Meirin-sha Company, Ltd., Sendai, Japan

Contents

Special Review

Original Paper

Nutritional Control of Inflammatory Responses in Broiler Chicken

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Key words : broiler chicken, CLA, cysteine, inflammatory response, xylitol

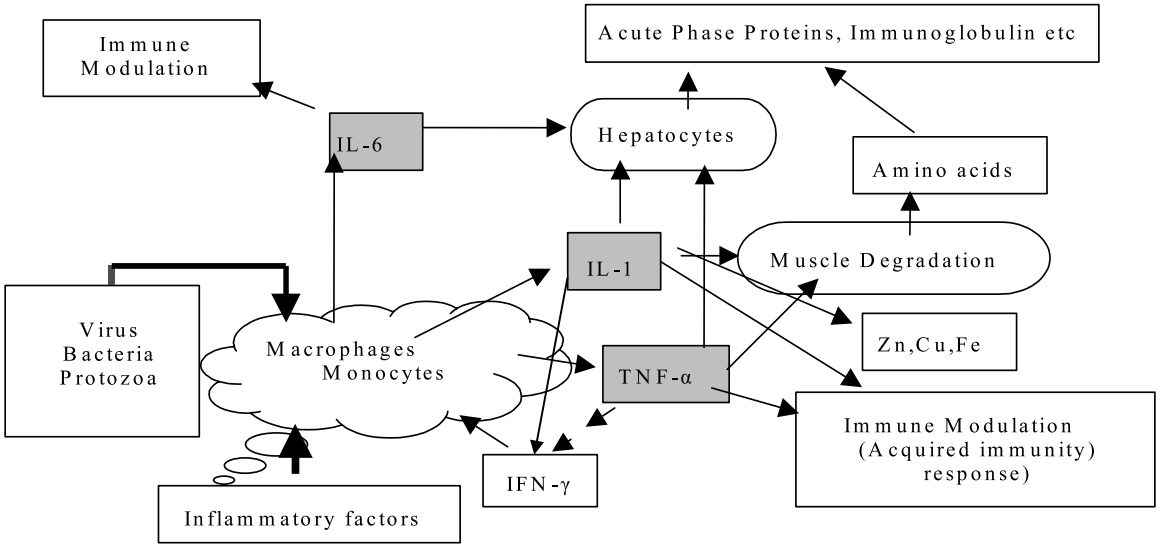
Introduction

Immunological stress(es) or stimulation generally results in decrease nutrient intake, increase in catabolic process of nutrients and change nutrient partitioning. Chickens under conventional raising conditions are exposed to many kinds of stressors, such as pathogenic or non-pathogenic microorganisms, transportation to the growing site, overcrowding, vaccination, chilling and/or overheating, etc. To maintain the self-defense functions, immunecompetent cells must proliferate, express receptors for the recognition of foreign molecules, produce cytokines to regulate the responses, produce antibodies and other effectors molecules. Production of effector molecules, such as reactive intermediates of oxygen and nitrogen may increase energy and nutrient demand. Thus nutrient requirements for achieving appropriate immunological status are not always identical with those for obtaining the maximum production under these circumstances. Since nutrient requirement for innate immune response is probably greater than for acquired immune response, it appear likely that control of innate immune response following production of pro-inflammatory cytokines such as iterleukin(IL)-1, 6 and tumor necrosis factor (TNF)-α have impact on chicken production during immune stimulation. However, TNF-α, a pro-inflammatory cytokine, is not cloned so far even though this cytokine is important not only in immune regulation, but also in metabolic changes.

Immune regulation and Nutrition

Klasing (1988) and Klasing and Johnstone (1991) have suggested that performance of poultry is adversely affected by immune stimulants and vaccination. Stimulation of selected cells of immune system triggers systemic metabolic changes, which include fever, anorexia, decreased net skeletal muscle protein deposition, and thus growth retradation. Improved sanitary condition and antibiotic therapy reduced the catabolic changes

Figure 1. Simple diagram of source and cause regarding pro-inflammatory cytokine (IL-1, 6 and TNF) release and its action in acute phase response



caused by immunological stimulation (Roura *et al*., 1992). On the other hand, nutritional modulation of specific immune responses or response to immune signals offers a simple method of regulating catabolic effects of immune stimulation. One of the goals for nutritional modulation of immune system is to alleviate loss of performance following immune stimulation without affecting the immune or inflammatory response *per se*. Feeding diets with essential nutrients excess or deficiency causes impairment of immune function and response in many aspects as reviewed by Katous and Klasing (2001). I introduce here how addition of certain nutrients to broiler diet affects immune and metabolic responses during immunological stimulation and some functions of chick TL1A as avian TNF-α.

Cysteine (Cys) or sulphur amino acid (SAA)

Cysteine (Cys) or sulphur amino acid (SAA) is of prime importance in increasing liver glutathione (GSH) in rats treated with TNF-α (Grimble *et al*.,1992; Hunter *et al*., 1994). Thus dietary sulfur amino acids also have certain influence on inflammatory and immune responses. Chicks fed on a Met sufficient diet had higher IL-1 activity, growth rate and feed intake compared to chicks fed on a Met-deficient diet when they received immunogen injections (Klasing and Barnes, 1994). Tsiagbe *et al*. (1987b) showed that antibody production against sheep red blood cell and delayed hypersensivity against phytohemagglutinin (PHA)-P in chicks fed a SAA-deficient diet was lower than those in chicks fed a SAA-sufficient diet. Although Hunter *et al*.(1994) showed production of several acute phase proteins in rats fed a Met-rich diet did not differ from that in rats fed on a Cys-rich diet when they were injected with TNF-α, it is likely that the function of Cys under stressful conditions is possibly different from that of Met. Tsiagbe et al. (1984a) showed that Cys was 70-84 % as efficient as Met in enhancing immunoglobulin-G (IgG) production and in delaying hypersensitivity to PHA-P stimulation, respectively, indicating that Cys is an essential nutrient for potentiating the immune response in broilers. Feeding L-Cys increases tissue GSH level (Graber and Baker, 1971) and an increase in tissue GSH concentration may be beneficial for growth of chicks reared in conditions with immunological stress by drug administration (Boebel and Baker, 1983). Furthermore, Cys and Cys derivatives have been shown to modulate lymphocyte and macrophage functions in the in vitro studies (Droge *et al*., 1991). As shown in Fig 2, a high Cys diet enhanced plasma α1-acid glycoprotein concentration, IL-1 like activity when chicks were single injected with *Escherichia coli* lipopolysaccharide (LPS), and lymphocyte proliferation (Takahashi *et al*., 1999). Our experiment also indicates that a high Cys diet enhanced mitogen-induced proliferation of lymphocytes compared to a high Met diet (Takahashi *et al*., 1997). Thus, a change in the ratio of Cys and Met in diet has an impact on the immune and inflammatory responses in chickens.

Conjugated linoleic acids (CLA)

Conjugated linoleic acids (CLA) are an isomeric mixture of 18:2 fatty acids that have conjugated

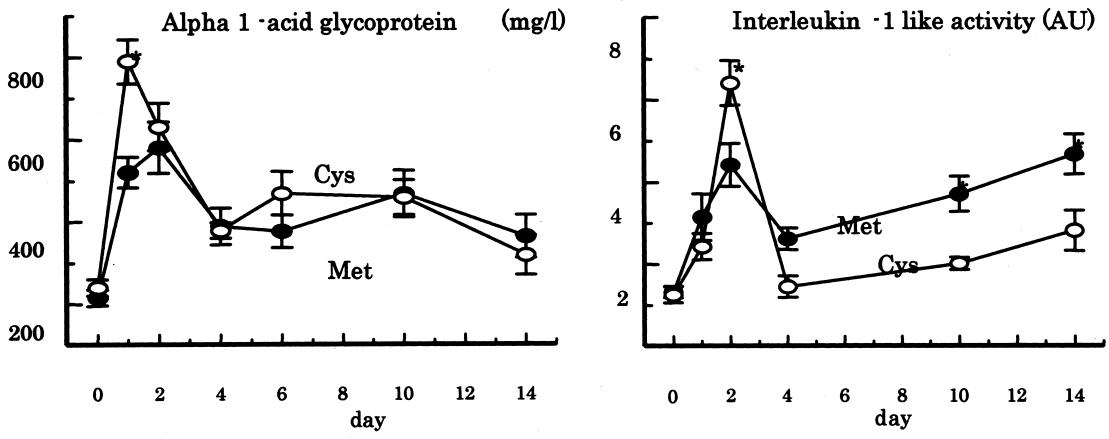


Figure 2. Effect of dietary methionine and cystine on plasma alpha-1 acid glycoprotein and interleukin-1 activity in chicks injected with LPS every other day (1 mg/kg body weight gain).

double bonds (Fritsche and Steinhart, 1998). The effects of CLA in animals are well reviewed by Fritsche and Steinhart (1998) and Pariza *et al*. (2000). There is a great interest in these fatty acid isomers because CLA has several unique proprieties that modulate the physiological and metabolic responses including immune response like lymphocyte proliferation in mice (Chew *et al*., 1997; Wong *et al*., 1997) and interleukin (IL)-2 production in mice (Hayek *et al*., 1999) and proinlfammatory cytokine production from macrophages (Turek *et al*., 1998). The immunomodulatory effects of CLA in animals have been recently reviewed by O'Shea *et al*. (2004). It has been suggested that CLA protected the catabolic responses against endotoxin in chicks and mammals (Cook *et al*., 1993; Miller *et al*., 1994). Takahashi *et al*. (2002) demonstrated that anti-inflammatory effect of dietary CLA in male broiler chickens by assessing the several inflammatory parameters, e.g. growth performance, blood heterophil to lymphocyte ratio, and plasma acute phase protein concentrations such as ceruloplasmin and α1 acid glycoprotein during lipopolysaccharide (LPS) and sephadex injections. Some results of this study are summarized in Table 1. CLA probably modulated immune responses in mammals although the effect has not been fully clarified (O'Shea *et al*. (2004). In addition, there are a few reports for effect of CLA on antibody or immunoglobulin (Ig) production. Dietary CLA enhanced Ig production in immunocompetent organs and plasma IgG concentration in rats (Sugano *et al*., 1998). Yamasaki *et al*. (2000) observed that CLA enhanced Ig production in spleen but did not affect serum Ig level in rats. Cook *et al*.(1993)showed that antibody production to sheep red blood cell (SRBC) was not affected by feeding CLA in chicks. We re-evaluated the effect of dietary CLA on antibody production to SRBC and IgG concentration in plasma using broiler chickens.

As shown in Fig 3, dietary CLA enhanced primarily antibody production to SRBC and IgG concentration in plasma (Takahashi *et al*., 2003). Our following study regarding effect of CLA on immune response suggests that mitogen-induced proliferation and IL-2-like activity in splenocytes was higher in splenocytes from chicks fed CLA-supplemented diet than those in chicks fed safflower-supplemented diet, which was comparable to those in chicks fed basal diet. Dietary CLA also had the potential to alter the T cell subpopulation in the spleen (submitted). Hence CLA

Table 1. Effect of dietary conjugated linoleic acid (CLA) on body weight gain and alpha 1 acid glycoprotein and ceruloplasmin in plasma during immunological stimulation induced by LPS (0.5 mg/kg body weight) and sephadex (250mg/ kg body weight) for 5 days[1]

	Saline		LPS and sephadex	
	Control	CLA	Control	CLA
Body weight gain (g,5days)	288 ± 18	276 ± 11	235 ± 17	262 ± 16*
Alpha 1 acid glycoprotein (mg/l)[2]	183 ± 17	203 ± 27	674 ± 51	511 ± 37*
Ceruloplasmin (mg/l) [2]	21.1 ± 2.9	25.6 ± 3.4	60.6 ± 6.9	44.1 ± 3.2*

[1] Male broiler chicks were used at 21 days of age. Mean ± standard error (n=10).
[2] Sample was obtained 24 hours after first LPS injection.
* Significantly different relative to the control ($p<0.05$)

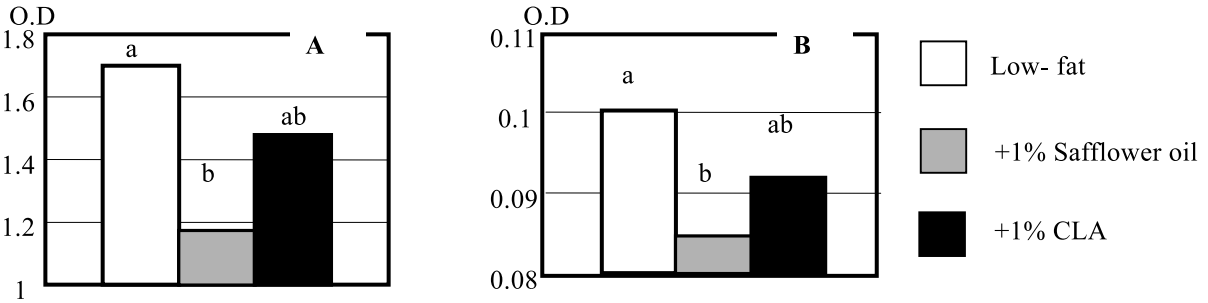


Figure 3. Effect of dietary conjugated linoleic acid on splenocytes proliferation to Con A (A) and interleukine-2 like activity of splenocytes induced by Con A (B). a,b<0.05.

may potentially be used as an alternative to feed antibiotics in chick's diet. Dietary CLA in chick also have potential as means of improving responses to vaccination and conferring disease resistance.

Xylitol

Van Heugten *et al*. (1996) showed that increasing energy density of diet did not alter the growth depression following LPS challenge, and that dietary addition of lard improved feed efficiency and efficiency of energy conversion in pigs. On the other hands, Benson *et al*. (1993) showed that increasing energy density by cornstarch, but not oil (corn oil), eliminated the growth depressing effect of immunogen in chicks when dietary energy level was above 13.4kJ/kg diet. It is known that xylitol supplemented parental nutrition has beneficial effects on nitrogen and glucose utilization during stress in mammals. Xylitol is a five-carbon polyol and an intermediate product in the glucuronic acid-xylulose cycle and the pentose phosphate pathway. It exerts a nitrogen-sparing effect without appreciable effects of insulin secretion, and is metabolized primarily in the liver, where it is converted via an insulin-independent pathway to glucose 6-phosphate. Dietary xylitol at the 20% level tended to lower insulin secretion, but did not change the plasma glucagon and corticosterone concentration in rats (Hamalainen & Makinen, 1985). Those observations may lead to an assumption that dietary xylitol has potential to alleviate the reduction in performance during immune stimulation.

Our serial studies (Takahashi *et al*., 1999, 2000 and 2005) shows that dietary xylitol prevents growth retardation (Table 2) without impairing acute inflammatory responses, e.g. IL-1 like activity, α-1 acid glycoprotein release (Table 2) under stressful conditions induced by LPS and Sephadex injections, but enhances pokeweed mitogen-induced nuclear cell proliferation of lymphocytes and antibody production against sheep red blood cell (Fig 4). In those experiments, metabolizable energy content of the experimental diets and energy intake did not differ among the dietary groups. This result suggests that dietary xylitol is a useful nutrient for controlling

Table 2. Effect of dietary xylitol (6%) on body weight gain and alpha 1 acid glycoprotein and interleukin-1 in plasma during immunological stimulation induced by LPS (0.5 mg/kg body weight) and sephadex (250mg/ kg body weight) for 5 days[1]

	Saline		LPS and sephadex	
	Glucose	Xylitol	Glucose	Xylitol
Body weight gain (g,5days)	254 ± 15	243 ± 13	181 ± 18	216 ± 12*
Alpha 1 acid glycoprotein (mg/l)[2]	247 ± 18	227 ± 31	1160 ± 79	1126 ± 67
Interleukin-1 activity (AU) [2]	1.24 ± 0.18	1.39 ± 0.05	1.89 ± 0.12	1.78 ± 0.20

[1] Male broiler chicks were used at 21 days of age. Mean ± standard error (n=10).
[2] Sample was obtained 24 hours after first LPS injection.
* Significantly different relative to the control ($p<0.05$)

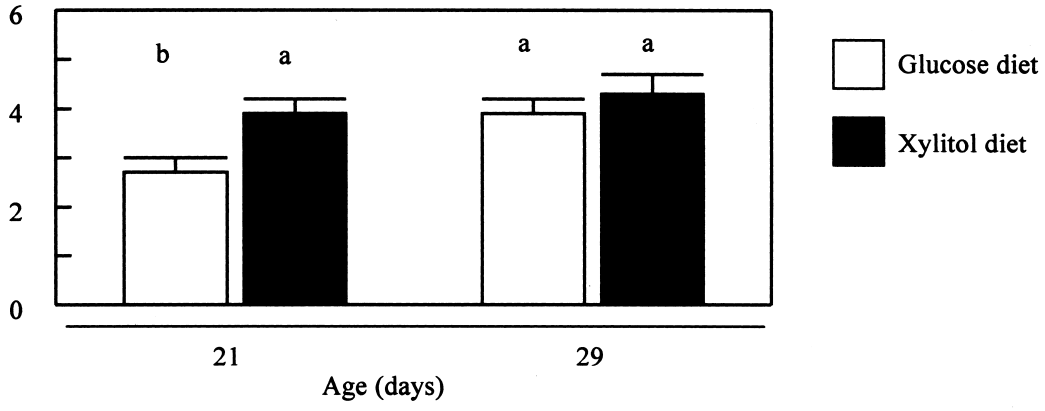


Figure 4. Antibody titers (log2) agnist Sheep Red Blood Cell in chicks fed the glucose or xylitol contained-diets. Chicks were challenged at 14 and 21 days of age. a, b <0.05.

growth performance during immune stress, but a mode of action of stress preventing effect of dietary xylitol may not be the same as those of increased energy concentration by glucose as previously reported by Benson *et al* (1993).

Tumor necrosis factor like ligand 1A (TL1A) as TNF-alpha substitute in chicks

TNF-α plays crucial roles in the immunological modulation of inflammation and cellular immune responses. In chickens, although TNF-α like activity has been detected only in the supernatant of chicken macrophages culture medium (Rautenschlein *et al*., 1999), molecular cloning of TNF ligand superfamily members in avian species has been unsuccessful so far. It is also notable that the possible sequence of a chicken homologue of mammalian TNF-α has not been identified to date in the chicken genome database. Recently, TL1A was cloned as a long form of vascular endothelial cell-growth inhibitor, another member of the TNF ligand super family, in mammals (Migone *et al*., 2002). Takimoto *et al*. (2005) first time showed the cloning and functional characterization of chicken TL1A. ChTL1A, a TL1A homologue in chickens, comprised of an ORF of 717 nucleotides that translated to produce a putative peptide of 239 amino acids. This is very similar to human pro-TNF-α that codes for 233 amino acids (Wang *et al*., 1985), whereas the human TL1A sequence encodes for a slightly longer polypeptide of 251 amino acids, translated from a 753 bp ORF (Migone *et al*., 2002). The phylogenic analysis suggests that molecule of chicken TL1A is closely related to that of human is nearer to TNF-α rather than any other molecules of chicken TNF ligand superfamily although the chicken TL1A was distantly related distant from to mammalians or teleosts TNF-α. The amino acid sequence predicted from ChTL1A revealed a TNF signature sequence similar to the TNF superfamily members reported in other animals and the cDNA sequence existing on chromosome 17 in the chicken.

Takimoto *et al*. (2005) showed that an increase in expression of ChTL1A mRNA following the induction of inflammation by LPS and addition of recombinant chicken TL1A to culture medium of L929 cells and chicken fibroblast cells reduced their survival. They also demonstrated decrease in feed intake, and increase in fever and nitric oxide production in chicks injected with TL1A as shown in Fig 5. Thus, chicken TL1A plays an important role in inflammation in chickens. Taken together these findings and observations, it is likely that chicken TL1A possibly substitutes for a part of function of TNF-α observed in mammals.

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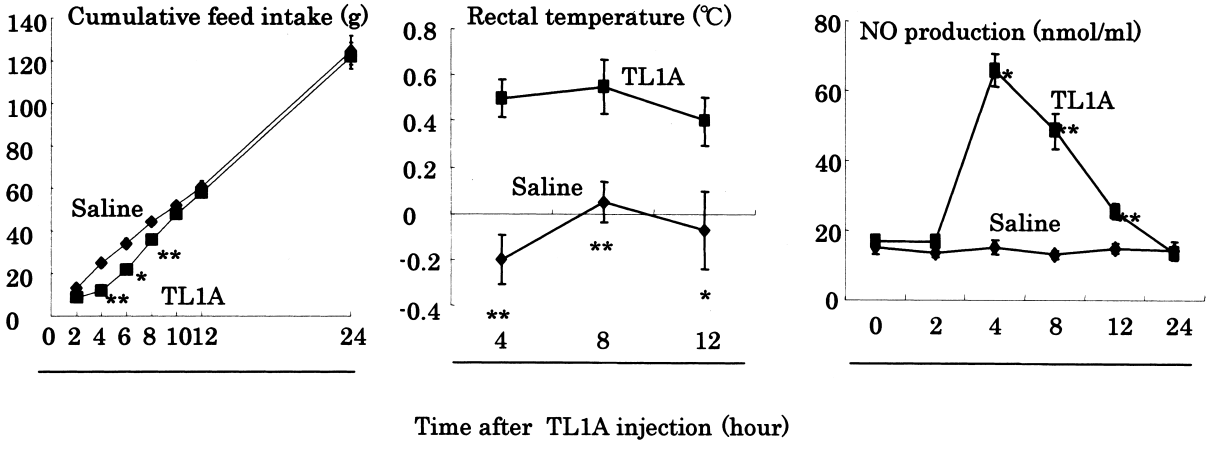

Figure 5. Temporal changes in feed intake, rectal temperature and NO production of chicks injected with TL1A. ** $p<0.05$

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Swine Intestinal Immunity via Toll-like Receptors and Its Advanced Application to Food Immunology

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Abstract

Recent interest has focused on the importance of intestinal immunity for the host defense, but to date, not much has been known about the underlying mechanisms. Toll-like receptor (TLR) family plays an important role in the defense through recognizing bacterial pathogen associated molecular patterns (PAMPs). Our research on the bioregulatory function of food products has investigated the immunoregulatory effects of lactic acid bacteria (LAB) via TLRs. Studies in swine, which is expected as a human model, have been examined intestinal immunoregulation by the LAB. Our research has now demonstrated modulation of intestinal immunity mediated by TLRs in Peyer's patches and the mesenteric lymph nodes. On the basis of our study, efforts have also been made to develop an immunoassay system for immunobiotic LAB DNA and cell wall components to evaluate immunoregulation by the LAB via TLRs. The findings in our research activities may provide important clues at the molecular level on TLR signal transduction pathways and recognition mechanisms. They also provide impetus to further delineate the activation mechanism of the innate immune response. In addition, identification of biofactors from LAB with immunoactivity, and better understanding of cytokine induction and intestinal immune regulation hold promise in basic research and development of “immunobiotic foods” to prevent specific diseases.

Introduction

The induction of the biological defense system begins with the recognition of pathogens. To date, 14 types of TLRs, which function as "pathogen sensors," have been identified (Takeda and Akira, 2005). The TLR family is important in all biological defense mechanisms and recognition of bacterial pathogen associated molecular patterns (PAMPs) which induce cytokine production and modulate the immune response in hosts. TLR2 and TLR9 recognize various cell wall components and cytosine-guanine sequences (CpG DNA) in bacterial genomes respectively (Takeuchi *et al*., 1999; Hemmi *et al*., 2000), triggers nuclear translocation of nuclear factor-kB (NF-kB) via the adapter molecule MyD88, induces production of inflammatory cytokines (e.g., TNF-α, IL-6, IL-12, IFN-α, and IFN-γ), and induces expression of cell surface costimulatory molecules (Fig.1). The development of vaccines containing this functional cell wall components and DNA shows promise in the prevention and treatment of infectious diseases, inflammatory diseases, cancer, and allergies (Shirakawa *et al*., 1997; Klinman, 2004a; Ulevitch, 2004; Ahmed *et al*., 2005; Prud’homme, 2005).

"Probiotics" refers to a category of microorganisms that provide beneficial health effects in the host by improving the intestinal flora. Research on probiotics has generally focused on controlling so-called "harmful bacteria" and increasing "beneficial bacteria" in the intestinal flora to improve "gut health." In addition to their intestinal regulatory effects, however, current research has also aimed at elucidating the effects of probiotics on intestinal mucosal immunity (Vaarala, 2003; Brown and Valiere, 2004; Cunningham-Rundles, 2004; Gill and Guarner. 2004). In 2003, Clancy proposed the concept of "immunobiotics" with reference to microorganisms that stimulate activation of mucosal immunity. This has prompted an interest in research and development of novel "immunobiotic foods" using the immunobiotics.

Our research on the bioregulatory function of food products, particularly in maintaining biological homeostasis via intestinal immunity, has investigated the immunostimulatory effects of LAB genomic DNA and DNA motifs and led to the identification of specific activation sequences (Kitazawa e*t al*., 2001, 2003; Iliev *et al*., 2005; Shimosato *et al*., 2005a, 2006). Furthermore, in line with other

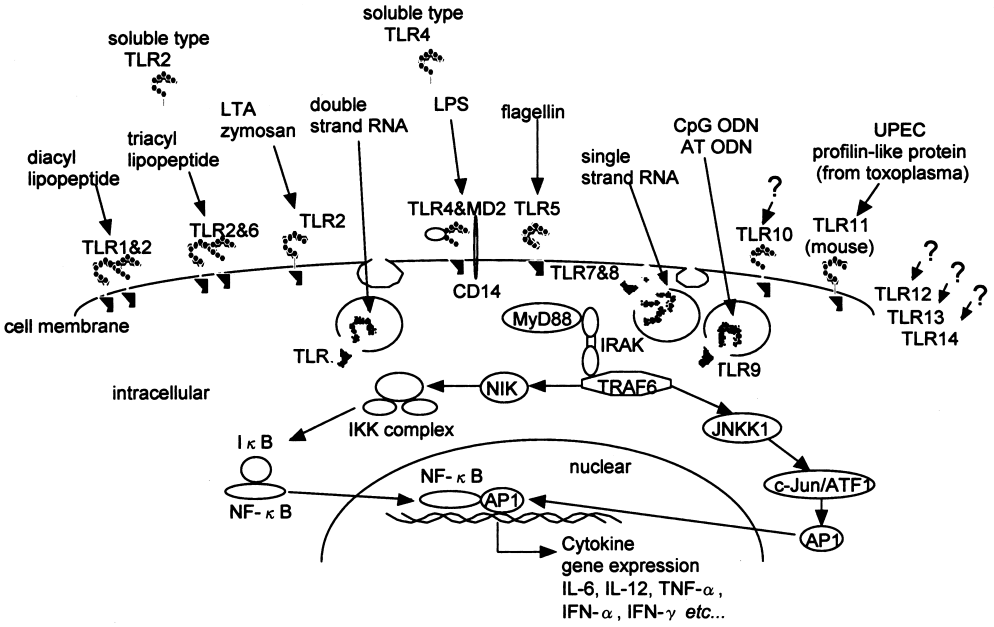

Figure 1. TLR family ligands and TLR9 mediated cell signaling. PGN(peptidoglycan), LTA(lipoteichoic acid), LPS (lipopolysaccharide), UPEC(uropathogenic. E. coli), MyD88(myeloid differentiation primary response gene 88), IRAK (IL-1 receptor-activated kinase), IKK(nuclear factor κ B (NF- κ B) kinase), JNKK1(c-JUN N-terminal kinase (JNK) kinase 1), ATF1(activating transcription factor 1), NIK(NF-kappa B-inducing kinase), AP1(activating protein 1).

recent publications, our previous study showed that structural, chemical, and conformational differences in cell surface constituents occurs even in genetically-related LAB strains containing functional cell wall components, affecting the differences of immunostimulatory effects among genetically-related LAB strains (Takeda *et al*., 1997; Schar-Zammaretti and Ubbink. 2003).

Studies in swine, which is often used as a model for organ transplantation in humans, have examined intestinal immune activation by the LAB in food products. Efforts have also been made to develop an immunoassay system for immunobiotic LAB DNA and cell wall components to evaluate immunoactivation by the LAB, with the objective of designing functional food products. In this review, we present some of our current research on swine intestinal immunity as a human model and the potential applications of immunobiotic LAB with immunoactivity mediated via TLRs particularly TLR2 and TLR9.

1. Expression of TLRs in swine intestine

Several studies have analyzed expression of the molecules of TLR family in various organs. Strong expression in the spleen has been used as a positive control to analyze TLR molecule expression in other tissues. We assayed TLR2 and TLR9 expression from 5 sites in the intestine in newborn and adult swine (duodenum, jejunum, ileum, ileal Peyer's patches, and mesenteric lymph nodes) to examine immune system development during growth and elucidate the mechanism of TLR2- and TLR9-mediated intestinal immunity (Fig. 2). In newborn swine, we found very strong expression of TLR2 in the thymus and TLR9 in the mesenteric lymph node, at least 5 to 7 times higher than in other tissues (Fig. 2A,C). In adult swine, in the ileal Peyer's patches and mesenteric lymph nodes, which play a major role in intestinal immunity, TLR2 and 9 expression was at least 3 times higher than in the spleen (Fig. 2B,D) (Shimosato *et al*., 2003, 2005b; Tohno *et al*., 2005a,b).In the analysis of TLR2, TLR2 mRNA was strongly expressed in the ileal Peyer's patches and mesenteric lymph nodes, and the expression of TLR2 in these two tissues is more than 8-fold higher than in other lymphoid tissues, including spleen and thymus (Fig. 2a) (Tohno *et al*., 2005a). Further analysis of TLR2 or TLR9 positive cells by immunohistochemistry using anti-swine TLR2 antibody or anti-swine TLR9 antibody showed the presence of TLR2 or TLR9 positive cells in the follicles of mesenteric lymph node and between the lymphoid follicles of Peyer's

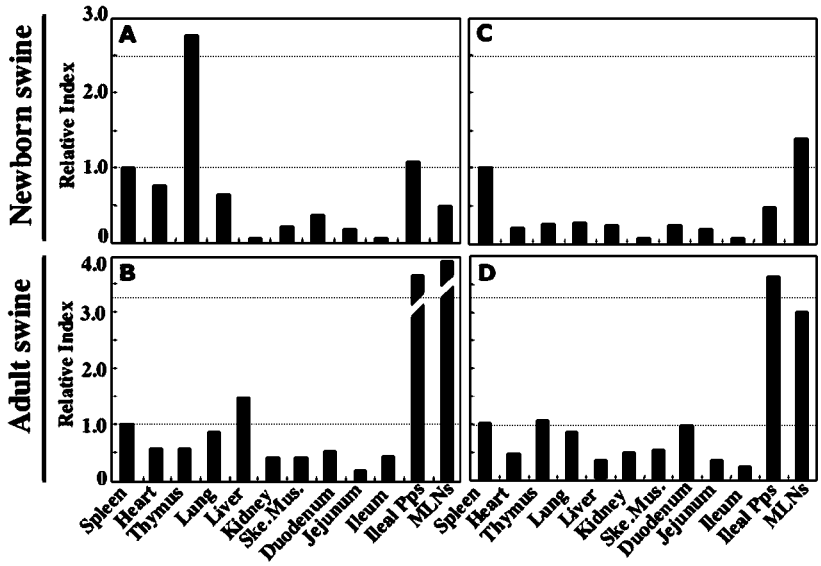

Figure 2. Real-time quantitative PCR analysis of sTLR2 mRNA in newborn (A) and adult (B) swine tissues and sTLR9 mRNA in newborn (C) and adult (D) swine tissues. The sTLR2 and sTLR9 mRNA levels were expressed as a relative index normalized against swine β-actin by the following equation: Relative index= sTLR2 or sTLR9 mRNA level of tissues/sβ-actin mRNA level of tissues. The results are presented relative to sTLR2 or sTLR9 mRNA levels in the spleen (1.0). Pps; Peyer's patches, MLNs; mesenteric lymph nodes.

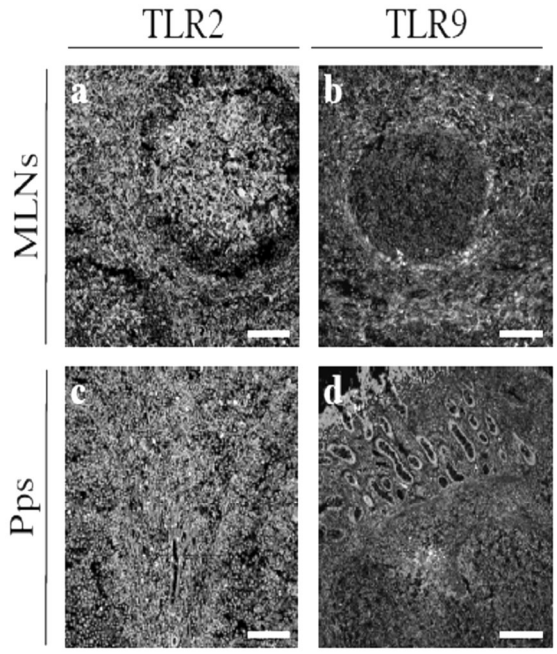

Figure 3. Immunofluorescent localization of TLR2 and TLR9 in longitudinal sections of swine MLNs and Pps. Frozen sections of MLNs and Pps were incubated with an anti-swine TLR2 or TLR9 polyclonal antibody. A panoramic view of MLNs, which was stained by anti-swine TLR2 polyclonal antibody (a) or anti-swine TLR9 polyclonal antibody (b). A panoramic view of Pps, which was stained by anti-swine TLR2 polyclonal antibody (c) or anti-swine TLR9 polyclonal antibody (d). Swine TLR2- or sTLR9-positive cells existed in and between the follicles of MLNs and Pps. Nuclei in panels a-d were stained with SYTOX orange. Original magnification = 200x. Scale bars = 100 μm. Pps; Peyer's patches, MLNs; mesenteric lymph nodes.

patchs (Fig. 3) (Shimosato *et al*., 2003, 2005b; Tohno *et al*., 2005a). During this research, we discovered a new finding of the strong TLR2 and TLR9 expression membranous (M) cells scattered in the follicular-associated epithelium (FAE) in the Peyer's patch dome epithelium (Shimosato *et al*., 2003, 2005b; Tohno *et al*., 2005a)

Recent interest has focused on the importance of intestinal immunity in medicine and immunology, but to date, not much has been known about the underlying mechanisms. Elucidation of this immune mechanisms would indeed be promising. Our research has now demonstrated modulation of intestinal immunity mediated by TLR2 and TLR9 in M cells in Peyer's patches and the mesenteric lymph nodes. Future studies will investigate in more detail the immunostimulating effects of cell wall components and LAB DNA on cells in each tissue. In addition, evaluation of the immune effects of LAB cell wall components and DNA using cell lines that express TLR2 or TLR9 will be important in elucidating the function of immunobiotic LAB and suggesting potential clinical applications.

2. Establishment of an immunoassay system for LAB DNA and cell wall components

We have shown that immunobiotic LAB cell wall components and DNA motifs can induce immunoactivation of intestinal lymphoid tissues. In addition, TLR2 and TLR9 are strongly expressed in this gut-associated lymphoid tissue (GALT). These findings demonstrate that TLR2 and TLR9 are able to recognize both pathogenic bacterial cell wall components and DNA, and dietary LAB cell wall components and DNA, thereby contributing to immunoactivation. Establishing immunoassay systems for these various components will enable researchers to evaluate both the "harmful" effects of pathogenic bacteria and the "beneficial" effects of dietary LAB. This will be an important tool in the development of functional food products. Studies must ultimately be conducted in human subjects, but basic research using animal cells and experimental animals is also essential. Therefore, to develop an immunoassay system for immunoactivity of functional cell wall components and DNA motifs from LAB, we constructed a transfectant of swine TLR2 and TLR9 with mammalian cells by the transfection of the swine TLR2 and TLR9 gene (Shimosato *et al*., 2004, 2005a; Tohno *et al*., 2005b).

The assay system for immunostimulatory cell wall components and DNA that we developed is a 3-step process for screening the TLR2- and TLR9-mediated immunoactivity of various DNA motifs and cell wall components by evaluating uptake, transcription activity of the intracellular signaling molecule NF-kB and cytokine induction. The cytokine assay combines both real-time quantitative PCR and ELISA to provide accurate evaluation of functional activity. This enables screening for motifs with potent immunoactivity from various DNA sequences and cell wall components in immunobiotic LAB. Elucidation of the TLR9- and TLR2-mediated immune response mechanism to LAB DNA and cell wall components are essential to future development of vaccines using normal flora and dietary LAB.

3. Future trends in immunobiotic LAB

LAB are technologically and commercially important and have various beneficial effects on the improvement of gut health through the control of intestinal ecosystem (Adolfsson *et al*., 2004). In many studies, whole cells, including live and heat-killed cells, cell wall and cytoplasmic fractions of LAB have been shown to have various biological functions. Especially, the surface cell wall properties of LAB are important in fermentation technology (Boonaert and Rouxhet, 2000) but they are also thought to play an important role in immunomodulation of the host.

In addiontion to the cell wall components, DNA in the cytoplasmic fractions, has been shown to be a major immunomodulatory substance. An initial report in 1984 by Tokunaga *et al*. on the antitumor effects of *Mycobacterium bovis* BCG DNA, and a later report in 1995 by Krieg *et al*. on stimulation of B lymphocytes by CpG DNA motifs, has prompted keen interest in immunoactivation by microbial DNA sequences. Further understanding of the effects of CpG DNA on the immune system came with identification in 2000 of TLR9 as the receptor molecule for CpG DNA (Hemmi *et al*., 2000). The immune effects of synthetic oligondeoxynucleotides (ODNs) containing various sequences of the 4 types of bases have been evaluated in experimental models using human PBMC and mice spleen cells. Sequences with potent immunoactivity have been identified in humans and mice. However, CpG ODNs, which are potent immunoactivators in primates (eg,

humans, rhesus monkeys, chimpanzees, orangutans), exert low immunostimulation in rodents such as mice (Klinman, 2004b; Shimosato *et al*., 2004). This mandates the use of animal models other than mice to evaluate the immune effects of ODNs for use in humans.

Functional food factors are thought to modulate intestinal immunity by contact and stimulation of immunocompetent cells in the gastrointestinal tract and induction of cytokine production. In this "new world" of food immunology, however, much remains unknown about the underlying mechanisms of intestinal mucosal immunity. Accordingly, many details remain unclear about the effects of food product components on intestinal immune responses. The findings in our research activities may provide important clues at the molecular level on TLR2 and TLR9 signal transduction pathways and recognition mechanisms. They also provide impetus to further delineate the activation mechanism of the innate immune response. In addition, identification of LAB cell wall components and DNA with immunoactivity, and better understanding of cytokine induction and intestinal immune regulation hold promise in basic research and development of “immunobiotic foods” to prevent allergies, infectious and inflammatory diseases. Our research results can enable the design of functional food products that contribute greatly to disease prevention. This can benefit mankind by offering immunobiotic foods as a safer alternative to conventional drug therapy (Kitazawa *et al*., 2005).

Acknowledgements

These studies were supported by a Grant-in-Aid for Scientific Research (C)(2) (No. 11660265, 13660265), (B)(2) (No. 13556018, 15380183) from the Japan Society for the Promotion of Science (JSPS) and the Uehara Memorial Foundation to Dr. H. Kitazawa.

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Recent Advances in Disease Control by Natural Products in Animals and Birds in Bangladesh

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Abstract

Use of herbs for curing diseases is well documented in the history of all civilizations. Because of the increasing reports of the possible hazards in using synthetic products in animals, natural products are rapidly establishing their credibility. At least 60 different varieties of plants, herbs and shrubs grown in Bangladesh have recognized medicinal properties, and are being used by Village Doctors like Hekims and Quacks for treating diseases in man and animals. A number of natural products are commercially available in Bangladesh most of which are used as feed additives, though few have antibacterial and anticoccidial use. Recent studies revealed that ethanol extract of Ata (*Annona reticulata*) at 2% concentration as spray showed highest efficacy (100%) followed by aqueous extract of Bishkatali (*Polygonum hydropiper*) against *Boophilus microplus*. Ethanol extracts of Neem (*Azadirachta indica*), bitter gourd (*Momordica charantia*) and Padmagulancha (*Tinospora tomentosa*) were highly effective against common stomach worm *Haemonchus contortus* in both *in vitro* and *in vivo* studies. Bitter gourd (*Momordica charantia*) has been found very effective against chicken coccidiosis. Birds receiving bitter gourd powder with feed had almost a similar weight gain compared with uninfected control chicks, and both these groups of chicks gained significantly higher ($P<0.05$) weight compared with chicks receiving sulphaclozine sodium. Anticipating the bright prospect, our research is now targeted mostly on the use of different plants against different parasitic infections in animals and birds. This presentation will cover the details of the currently used natural products in Bangladesh and our efforts in revealing the greatness of these natural products.

Introduction

The plant kingdom is a rich source of botanical anthelmintics, antibiotics and insecticides (Satyavati *et al*., 1976; Lewis and Elvin-Lewis, 1977). Use of herbs for curing diseases is well documented in the history of all civilizations. Despite the tremendous success in research in medicine and discovery of newer drugs, herbs as medicine still has a great role especially amongst the rural people in the third world countries in particular. Because of the increasing reports of the possible hazards in using synthetic products in animals grown for human consumption, natural products are rapidly establishing their credibility as feasible alternative in the recent years. However, there are arguments that natural products may not be safe or equally effective or may not comply with pharmaceutical grade products. Some of these are very valid questions and need to be answered before widespread acceptance of these products. Bangladesh has a long history of using different plants for treating diseases in animals and man. At least 60 different varieties of plants grown in Bangladesh have recognized medicinal properties, and are being used by Village Doctors like Hekims and Quacks for treating diseases in man and animals. But very little attention has been given on the study of these plants as of their efficacy in curing and/or controlling disease in animals and man, and of course the safety in using these products in Bangladesh. This communication will cover the recent studies done in the Department of Parasitology of Bangladesh Agricultural University, Mymensingh, Bangladesh. However first of all the products those are being commercially available in Bangladesh will be mentioned with a brief note about their use in animal medicine.

Herbal products in current use:

A number of local pharmaceutical industries are either producing or importing herbal drugs for use in animals and birds in Bangladesh. Some of these are reported to have a good efficacy. Following is the table listing the herbal drugs available in Bangladesh with their uses and dosage (Table 1).This could be a good beginning, only if steps are taken to study the efficacy and safety of these products in extensive field trial.

Bitter gourd against chicken coccidiosis:

Increasing report of resistance against the commonly used sulphonamides (Siddiki *et al*, 2000) prompted this work in which we used bitter gourd (*M. charantia*) in chickens experimentally infected with *Eimeria tenella*. Dried bitter gourd was pulverized, and a group of chicks infected with 10^4 sporulated oocysts each at the age of 26 days received this powder in feed from 3-6 day post infection @ 10 gm/kg. Results were assessed on the basis of weight gain and oocysts output of birds (Table 2). Chicks received bitter gourd had almost similar weight gain ($P>0.05$) with that of uninfected control chicks, and both these groups of chicks gained significantly higher ($P<0.05$) weight compared with chicks received Esb_3® (sulphaclozine sodium). Chicks receiving bitter gourd had a relatively higher oocysts count ($p<0.01$) compared with chicks treated with Esb_3®, but lower than the untreated chicks, although statistically it was not significant ($p>0.05$). Despite relatively large oocysts counts, significantly higher weight gain in chicks receiving treatment with bitter gourd is very interesting, but difficult to explain. Products bitter in taste are reported to enhance the immune response in chickens (Deepika *et al*., 2002). Therefore it is possible that bitter gourd, because of its bitter taste, might have contributed in a better immune response in the birds. This preliminary study suggests that bitter gourd can be a good alternative to the common coccidiostats. It is also highly economic, since only a fraction of the cost of sulphonamides is needed. However, further study for titration of the doses and on any possible adverse effect on the chickens needs to be carried out.

Herbs against the common cattle tick:

Some synthetic acaricides have residual effects

Table 1. List of commercially available herbal products for use in animals and birds in Bangladesh

Trade name	Indications
Hepavet	(Herbal Liver tonic) as Feed Supplement (Poultry: 1 ml/liter water; Large animal: 0.5ml/kg wt)
Enzyvet liquid	Digestive enzyme as feed supplement (Poultry: 1 ml/liter water; Large animal: 0.5ml/kg wt)
Herbomix-DS	Feed grade Liver tonic (125 g/ton feed)
Galactovet	Milk Enhancer (1 g/10 kg wt)
Ayucal-D	Herbs + V- D to prevent cannibalism &improve egg shell quality (5g/100 chicks; 10g/100 grower; 15g/100 layer)
Toxiroak	Mold inhibitor, Toxin binder, Mycotoxin biosynthesis inhibitor, Mycotoxin bio-neutralizer (100-200g/100 kg feed)
Superliv	Increases efficiency of liver, digestive tract.Act as a renal tonic (1ml/1-2 liter)
Livfit Vet	Increases detoxification capacity of liver (5g/100 chicks10g/100 grower, 15g/100 layer)
Herban Liquid	Increases feed intake, FCR, egg production, hatchability and fertility (Poultry: 3ml/10 L water, Large animal: 1ml/10kg wt)
Coxynil- Feed Additive	Anticoccidial (250-300g/Ton feed)
Respowell - Feed additive	Organic broad-spectrum antimicrobial against Mycoplasma, Staphylococcus, Streptococcus, Salmonella , Campylobacter, Pasteurella, Actinobacillus, Clostridium,
Fertiwell- Feed Additive	Active against Streptococcus and Staphylococcus (Chicken: 1kg/ton, Bull: 100mg/kg wt)
Growell -Feed Additive	Immune stimulant, Toxin remover, Growth promoter, Anti-stress, Liver and kidney stimulant and Productivity enhancer.

Table 2. Mean weight gain and oocysts output of chickens infected with 10^4 oocysts of *E. tenella*.[1]

Group	Bitter gourd Powder	Metronidazole (Filmet®)	Sulphonamides (Esb_3®)	None	Control (uninfected)
Mean wt. gain (gm)	$84.41^a + 9.98$	$63.60^b + 13.44$	$67.96^b + 13.17$	$67.42^b + 12.92$	$85.12^a + 7.36$
Total oocysts out put x 10^6	$39.06^a + 7.80$	$40.79^a + 5.55$	$16.33^b + 5.33$	$66.44^a + 12.49$	0

[1]Rahman, *et al. 2005*

Table 3. Acaricidal efficacy of aqueous extracts (2%) of different plant materials against adult *B. microplus* after 72 hours exposure (N=15)[1]

Name of the plants	Scientific name	Application	%Mortality
Neem	*Azadirachta indica*	Spray on	86.67
		IFP	80
Bishkatali	*Polygonum hydropiper*	Spray on	93.33
		IFP	86.67
Ata	*Annona reticulate*	Spray on	86.67
		IFP	80
Sharifa	*A. squamosa*	Spray on	80
		IFP	60
Durba	*Cynodon dactylon*	Spray on	80
		IFP	53.33

[1]Nahar *et al*, 2005

Table 4. Acaricidal efficacy of ethanol extracts (2%) of different plant materials against adult *B. microplus* after 72 hours exposure (N=15)[1]

Name of the plants	Scientific name	Application	(%) Efficacy
Neem	*Azadirachta indica*	Spray on	66.67
		IFP	60
Bishkatali	*Polygonum hydropiper*	Spray on	80
		IFP	66.67
Ata	*Annona reticulata*	Spray on	100
		IFP	80
Sharifa	*A. squamosa*	Spray on	86.67
		IFP	53.33
Durba	*Cynodon dactylon*	Spray on	60
		IFP	46.67

[1]Nahar *et al*, 2005

and are accumulated in environment, which induce resistant strains of pests including the tropical cattle (O' Sullvian and Green, 1971; Howell, 1977). The acaricidal effects of various plants have been studied in many countries of the world (Khudrathulla and Jagannath, 1998; Mulla and Su, 1999; Abdel-Shafy and Zayeed, 2002). This was the first attempt to study the acaricidal effects of a few indigenous plants of Bangladesh (Nahar, *et al* 2005). Leaves of Neem (*Azadirachta indica*), Bishkatali (*Polygonum hydropiper*), Ata (*Annona reticulata*), Sharifa (*A. squamosa*) and Durba ghas (*Cynodon dactylon*) as paste, aqueous and ethanol extracts were tested *in vitro* against *B. microplus*, applied either as thin layer of paste or as spray or as impregnated filter paper. Fresh leaves were made into paste with pestle and

Table 5. Acaricidal efficacy of freshly prepared pastes of different plant materials against adult *B. microplus* after 72 hours (N=15)[1]

Name of the plants	Method of application	% Mortality
Neem	Thin layer of paste	60
Bishkatli	Do	80
Ata	Do	86.67
Sharifa	Do	60
Durba	Do	80
Control	Moistened with water	0

[1]Nahar *et al*, 2005

Table 6. Percent non-motile (dead) adult (A) and infective larvae (L_3) of g/i nematodes exposed to different increasing concentrations of aqueous extracts *in vitro*[1]

Name of Plants		% mortality @ 100 mg/ml	
Common name	Scientific name	A	L_3
Jute leaves	*Corchorus olitorious*	92*	84
Pineapple leaves	*Ananas comosus*	100*	53
Amrul	*Oxalis corniculata*	100*	18
Biskathali leaves	*Polygonum hydropiper*	100*	15
Padmagulancha	*Tinospora tomentosa*	96*	82
Karola	*Momordica charantia*	100*	70
Neem leaves	*Azadirachta indica*	92*	36
Hatishur leaves	*Heliotropium indicum*	100*	67
Katakhura	*Amaranthus spinosus*	100*	10
Lazzabati	*Mimosa pudica*	100*	26
Garlic whole	*Allium sativum*	100*	56
(Morantel citrate)		100*	100*
Albendazole		100*	100*
PBS		04	04

[1]Rahman, 2004

mortar. Ten gram powder made from dried plants was suspended in 100 ml distilled water or ethanol, filtered and concentrated to 10 ml by evaporation in water bath. Extracts were used in 0.5%, 1% and 2% concentrations and the percent mortality of the ticks was recorded at 12, 24 and 72 hours. Ethanol extract of Ata at 2% concentration showed highest efficacy (100%) followed by aqueous extract of Bishkatali (93.33%) at same concentrations and ethanol extract of Ata at 1% concentration in spray on method (Tables 3-5).

Herbs against the common stomach worm:

Benzimidazole and Probenzimidazole groups of anthelmintics are widely used in Bangladesh for controlling parasitic infections in animals. Indiscriminate and unscrupulous use of these anthelmintics leads to the development of resistant strains of helminths (Karim, 2005). Screening of medicinal plants for their anthelmintic activity got a momentum in the recent past (Akhtar *et al*. 2000), though very little attention has been given on this in (Mostofa, 1983; Begum, 1997). This prompted a study on the use of aqueous and ethanol extracts of twelve indigenous plants for *in vitro* and *in vivo* anthelmintic effect against gastrointestinal nematodes of goat origin (*Haemonchus contortus, Trichostrongylus* spp., *Trichuris* spp., *Strongyloides papillosus, Oesophagostomum columbianum, Cooperia* spp. and *Bunostomum trigonocephalum*) and their infective larval stage (L_3) obtained from *in vitro* culture (Rahman, 2004) (Tables 6 and 7).

Table 7. Percent non-motile (dead) adult (A) and infective larvae (L_3) when exposed to an increased concentration of ethanol extract of indigenous plants *in vitro*[1]

Name of Plants		% mortality @ 50 mg/ml	
Common name	Scientific name	A	L_3
Jute leaves	*Corchorus olitorious*	100*	82.0
Pineapple leaves	*Ananas comosus*	100*	95.75*
Amrul	*Oxalis corniculata*	88	47.75
Biskathali leaves	*Polygonum hydropiper*	92*	49.0
Padmagulancha	*Tinospora tomentosa*	100*	100*
Karola	*Momordica charantia*	100*	100*
Neem leaves	*Azadirachta indica*	100*	100*
Hatishur leaves	*Heliotropium indicum*	96*	80.0
Katakhura	*Amaranthus spinosus*	100*	80.75
Lazzabati	*Mimosa pudica*	90*	60.75
Garlic whole	*Allium sativum*	100*	81.0
(Morantel citrate)		100*	100*
Albendazole		100*	32
PBS		07	3.25

[1]Rahman, 2004

Selected plants were processed for use as mentioned earlier. Aqueous and methanol plant extracts at various concentration (10, 25, 50, 100 mg/ml) levels were screened by using both adult worms and L_3 stage larvae. Twenty five adult worms (both male and female) or 100 L_3 in 200 µl PBS was added with 800 µl of extracts at different concentrations, after 3 hrs at room temperature, the percent non- motile (dead) parasites were counted.

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Heterogeneous Impacts of Grazing Animals and Vegetational Change in Japanese Native Pastures

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Keywords : defecation, grazing management, invading plants, *Miscanthus*-dominant pasture, selective grazing, trampling.

Abstract

Defoliation, defecation and trampling are the major modes whereby grazing animals give impacts on vegetation. Due to the uneven distribution, such grazing behavior can have profound effects on vegetation. For extensive grazing systems in native pastures, understandings of the plant-animal interaction are vital for adequate control of vegetation and animal conditions and sustainable use of natural resources. This paper reviews recent studies of the grazing impacts on vegetation in Japanese native pastures. Most of the studies were carried out in the Kawatabi Field Science Center (Kawatabi FSC), Tohoku University.

1.Native pastures in the Kawatabi FSC are composed of 61–155 plant species, of which cattle graze upon 26–76 species. Among these species, *Miscanthus sinensis* (Japanese plume-grass) was the most frequently grazed by cattle. The spatial distribution of available forage is a major factor affecting diet selection and consumption of cattle. Such selective grazing results in significant reduction of *M. sinensis* in native pastures.

2.Seed dispersal of plants by defecation of grazing animals can also result in significant vegetational change. Recent studies have shown that *Carex* spp. is the major plant whose seeds are dispersed by defecation of animals rotationally grazed in a native and a sown pasture. The mechanisms of the seed dispersal and its possible effects on vegetational succession are discussed.

3.Heavy trampling is known to degrade vegetative ground cover. Our research has shown that trampling by cattle promotes the invasion of a shrub, *Weigela hortensis* into *Miscanthus*-dominant pastures. Because the seeds of *W. hortensis* are light sensitive germinators, trampling by removing ground cover promotes its seed germination.

These findings provide new perspectives on plant-animal interactions in Japanese native pastures and help estimate the impact of animals on plant succession. They also contribute to efforts to ensure sustainable grazing use of pastures.

Introduction

It is well known that behavior of grazing herbivores including defoliation, defecation and trampling affect pasture vegetation (Vallentine 2000). The actual impact of such behavior is spatially heterogeneous, and can give profound effects on vegetation where it concentrates.

In native pastures in Japan, large herbivores such as beef cattle and horse have been allowed to graze for more than 150 years. In such grazing systems, understandings of the impacts of behavior of herbivores on vegetation are vital for the sustainability of both animal production and the conservation of natural resources and biodiversity.

Studies on plant-animal interactions have been carried out in native pastures grazed with beef cattle at the Kawatabi Field Science Center (Kawatabi FSC), Graduate School of Agricultural Science, Tohoku University, to develop a grazing system for mountainous regions in Japan. Early research focused on vegetational characteristics and forage production (e.g., species composition, canopy structure, and forage production) and behavior and productivity of animals. Recent research has focused on the impacts of grazing animals on vegetation; i.e., 1) diet selection of grazing animals under diverse vegetation, 2) seed dispersal by defecation of animals, 3) effects of trampling of animals on vegetational changes.

The objective of this paper is to review recent studies of the impacts of grazing herbivores on

Table 1. The number of plant species growing and grazed by cattle in native pastures at the Kawatabi Field Science Center.

Year	Growing		Grazed by cattle		Reference
	No. of sp.	Dominant sp.	No. of sp.	Dominant sp.	
1955	155	*M. sinensis*[1]	76	*M. sinensis*	Iizumi et al. (1956a)
1963	80	*M. sinensis*	44	*M. sinensis*	Sato (1996)
1972	-	-	N 75[3]	-	Hasegawa et al. (1973)
			B 55	-	
			H 47	-	
1992	61	*P. aquilinum*[2]	48	*M. sinensis*	Matsumoto and Sugawara (1995)
2002	66	*M. sinensis*	26	*M. sinensis*	Takahashi et al. (2003)

[1] *Miscanthus sinensis* (Japanese-plume grass)
[2] *Pteridium aquilinum* (Blackenfern)
[3] N: Japanese Shorthorn, B: Japanese Black, H: Holstein.

vegetation in native pastures in Japan. Most of the studies described were carried out at the KFSC.

1. Selective grazing: damage of defoliation to Miscanthus *sinensis*

Selective grazing is one of the major factors affecting vegetation in grazing systems, exerting its effects at several hierarchical levels; i.e., from a small patch or a feeding station to landscape (Senft et al. 1987; Vallentine 2000). In native pastures, animals encounter a variety of plant species of differing canopy structures, quality and phenology. These vegetational characteristics affect diet selection and consumption of animals.

The Kawatabi FSC has a large native pasture dominated by *Miscanthus sinensis* (Japanese plume-grass). Previous studies have shown that the pasture is composed of 61-155 plant species (Iizumi et al. 1956a; Matsumoto and Sugawara 1995; Sato 1996; Takahashi et al. 2003), of which cattle graze upon 26-76 species (Iizumi et al. 1956a; Hasegawa et al., 1973; Matsumoto and Sugawara 1995; Takahashi et al. 2003) (Table 1). Among these species, *M. sinensis* is one of the most frequently grazed (or strongly selected) by cattle, suggesting that *M. sinensis* is valuable foliage cattle in the grazing system.

The frequent grazing, or strong selectivity, to *M. sinensis* can lead to reduction of the species. Takahashi et al. (2000a, 2000b) showed that *M. sinensis* declined for 3 consecutive years, even under a low grazing intensity (39.3–66.7 heads・day/ha/year), with reduced the stand size and the number of tillers per stand rather than population density. To evaluate the damage inflicted by grazing animals on *Miscanthus*-dominated pastures, recent research has focused on the mechanisms of selective grazing. Investigations of plant structure and ingestive behavior of cattle suggested that *M. sinensis* offers an abundance of available leaves at different heights of each stand (Takahashi et al. 2005) (Figure 1), enabling animals to take more bites from different heights within a feeding station compared to other native grasses (Takahashi et al., unpublished data).

Further studies, including investigation of the damage of tillers of *M. sinensis* by foraging of large herbivores, will provide useful information on the effects of selective grazing on *M. sinensis* populations in pastures.

2. Seed dispersal by defecation of grazing animals: contribution to diffusion of Carex albata

Seed dispersal by the defecation of grazing animals is another significant factor in vegetational change (e.g., Janzen 1984; Archer and Pyke 1991). It is well known, for example, that the seeds of certain plant species are ingested by grazing animals and disseminated through the digestive tract. Recent studies have shown that grazing cattle dispersed seeds of monocotyledons and forbs such as *Carex albata, Cerastium holostoroides*, *Rumex obtusifolius* and *Viola* sp. (Obara et al. 2006) (Table 2). *Carex albata* is on of the species most commonly dispersed by defecation of cattle (Watanabe et al. 2002; Obara et al. 2006). This phenomenon can have wide-ranging effects on vegetation in cases in which animals are rotationally grazed from a pasture where such plants are dominant to other pastures.

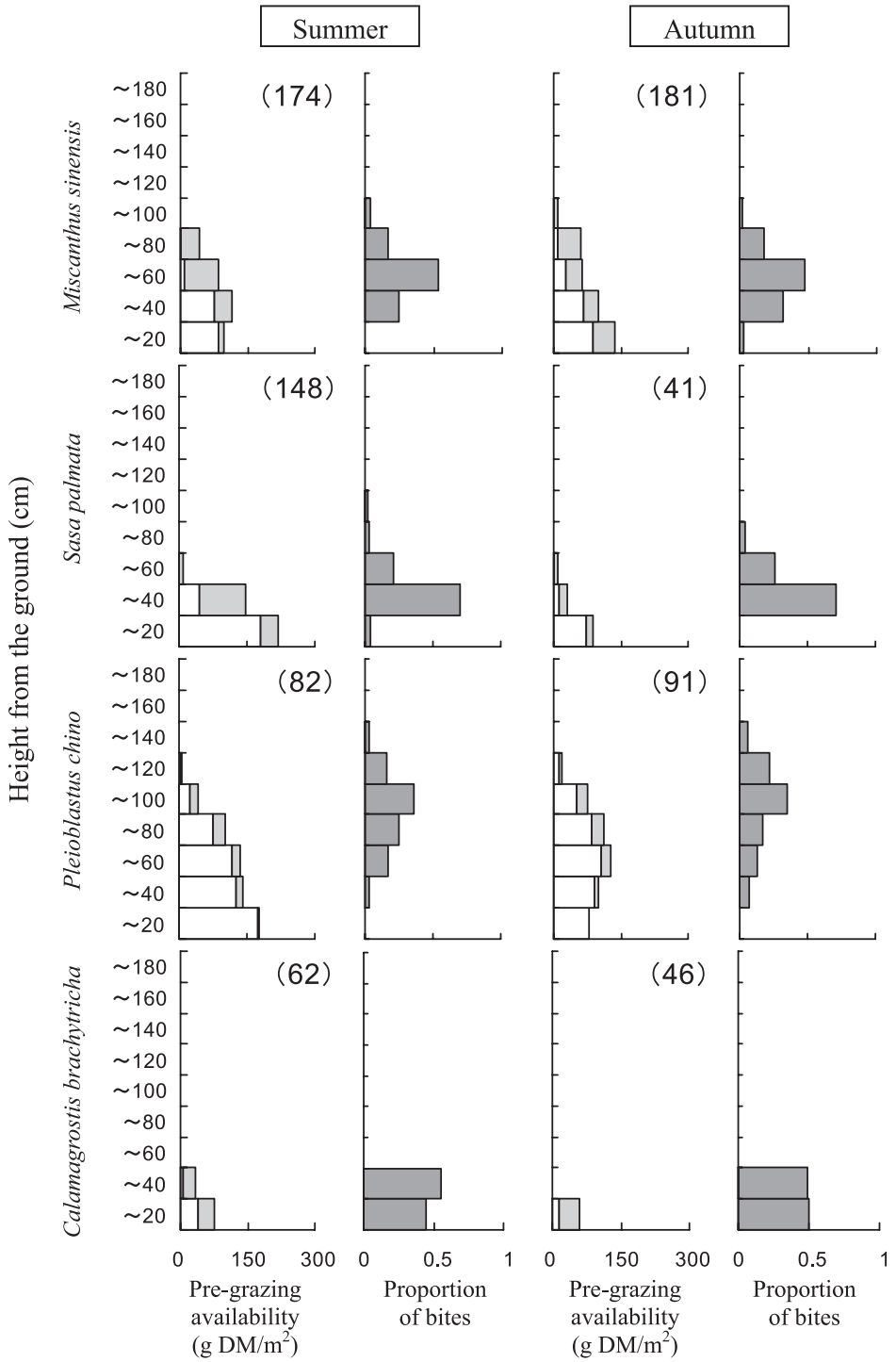

Fig. 1. Vertical distribution of leaves (gray color) and stem (white color) and proportion of bites taken by grazing steers in 4 major native grass species. The number in each parentheses represents the total amount of available leaves (g DM/m^2) (Takahashi et al. 2005).

Seed dispersion via defecation requires the ingestion of seeds by animals as a first step. This means selective grazing is an important factor in understanding the mechanism underlying this phenomenon. Sward-based measurements showed that *Carex albata* was grazed by cattle throughout the grazing seasons, even in heading stage (Obara et al. 2006). It would also appear that since the height of *Carex albata* seed heads are nearly equal to that of the leaves (Obara et al. 2006), cattle may have difficulty completely avoiding the seed heads while grazing, even if they would prefer to do so.

The location of dung and its environment also affects vegetation. Dung in grazing pastures tends to aggregate in certain areas – around a corral, a watering and feeding place, on a resting place of animals such as a ridge and under a tree (e.g., Hirata et al. 1987; Tajima et al. 2002). At such locations, plant seeds enter the soil seed bank from dung and may result in dramatic vegetational change (Harasawa et al. 1987). A recent study, however, suggested that both sunny areas (e.g., resting areas on a ridge) and shaded areas (e.g., under trees) are unsuitable for germination of seed from (Obara et al., unpublished data). Dung patches may be suitable for germination and establishment of plant seeds in dung because the environmental conditions of sunshine and moisture are moderate, and because these areas tend to be protected from defoliation and trampling by animals.

To fully understand the effects of seed dispersal by defecation on vegetational change, further study is required of on plant (seed) – animal interactions, including investigations of the effects of mastication and digestion in the digestive tract on seed survival and germinability.

3. Effects of trampling: promotion of Weigela horetensis *invasion into a* Miscanthus*-dominant pasture*

Trampling by grazing animals damages the aboveground portions of plants, enhances soil compaction, and removes litter covering the ground. All the factors result in vegetational change. While grazing cattle in Japanese native pastures tend to convert tall (*Miscanthus*-type) grasslands to sod (*Zoysia*-type) grasslands (e.g., Iizumi et al. 1956b; Hayashi et al. 1968), cattle grazing in a *M. sinensis* pasture appears to increase a shrub species (*Weigela hortensis*) in certain areas in the Kawatabi FSC. The invasion of *W. hortensis* is noteworthy on concave slopes rather than convex slopes (Nishiwaki et al. 1993).

Recent research suggests some possible reasons the shrub species invaded and proliferated in the tall pasture. First, the decline in aboveground biomass (Shinsho and Sugawara 2002) and seed production (Nishiwaki et al. 1996) of *M. sinensis* due to selective grazing promotes the invasion and survival of the shrub. Second, the removal of ground litter by

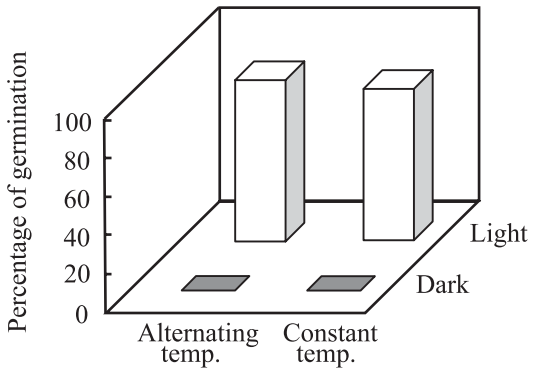

Fig.2. Percentage of germination in Weigela hortensis in artificially controlled conditions (Shinsho et al. 2000). Alternating condition: 25/15ºC (12 hrs interval), Constant condition: 25ºC.

Table 2. Total number of seedlings germinated from feces of cattle rotationally grazed in a sown and a native pasture at the Kawatabi Field Science Center (Obara et al. 2006).

Plant species	No. of seedlings germinating (/kg feces)[2]	
	Mean	SD[3]
Carex albata	129.8	355.3
Cerastium holosteroides	7.3	22.4
Other Cyperaceae[1]	3.3	8.5
Rumex obtusifolius	3.0	9.7
Viola sp.	0.4	3.0
Other dicotyledons	17.8	29.5
Other monocotyledons	11.6	21.7
Not identified	0.7	4.4
Total	173.9	370.6

[1] Excluding *Carex albata.*
[2] n=91.
[3] Standard deviation.

trampling increases the germination of the seeds of the shrub, because *W. hortensis* is light sensitive germinator (Shinsho et al. 2000) (Figure 2). In fact, the removal of ground litter (i.e., creation of bare areas) was promoted by cattle grazing (Shinsho et al. 2000), and seedlings of *W. hortensis* were germinated in the case that both aboveground portion of vegetation and ground litter were removed (Shinsho and Sugawara 2002).

These examples indicate that trampling has profound effects on vegetational changes in native pastures.

Conclusions

The findings reviewed in this paper indicate that grazing of animals reduces *M. sinensis* and increases *Carex albata* and *Weigela hortensis* in *Miscanthus*-dominant pasture. The mechanisms of these changes are selective grazing, defecation and trampling. These findings provide new perspectives on plant-animal interactions in Japanese native pastures and will help estimate the impact of animals on plant succession, thereby promoting sustainable grazing use of pastures. However, the complex interrelationships of various factors remain to be elucidated, and predicting the impacts and vegetational change precisely will be difficult until these factors are better understood. Construction of a predictive model based on the results of earlier studies will help evaluate the effects of the impacts by herbivore grazing on vegetational change, thereby establishing practices that promote the sustainable use of native pastures for grazing.

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* In Japanese only. Title translated by the author.

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The Role of Diet Selection in Sustainable Agriculture

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Abstract

Recent research has shown that domestic ruminants have clear diet selection goals. They eat mixed diets and show consistent diurnal patterns of diet preference. Current theoretical explanations for these observed patterns of behaviour focus on evolutionary traits. Grazing ruminants will have evolved a foraging strategy that optimises their fitness (which is the ultimate currency driving natural selection). Whilst this strategy will have been modified by the process of domestication, modern domestic ruminants appear to retain many aspects of their foraging strategy from their wild forebears. These include optimising the efficiency of nutrient capture and the associated need to maintain rumen function, whilst at the same time reducing the risk of predation and the risk of poisoning from plant toxins. These diet selection characteristics need to be taken into account in the development of grazing management strategies, both those aimed at optimising their nutrient capture whilst at the same time minimising the environmental impact of the animal, as well as strategies that aim to promote biodiversity in semi-natural grazed pastures. Research in this area indicates that an understanding of the diet selection characteristics of grazing ruminants has an important role to play in the development of grazing management strategies that are both environmentally and economically sustainable.

Introduction

The intensification of grazing systems in many parts of the world over the last few decades has led to the increased use of ryegrass monocultures. These single monoculture swards restricted the dietary choice of the grazing animals to a single plant species. More recently, the incorporation of legumes into swards, principally to fix atmospheric Nitrogen, has led to an interest in the study of diet selection, as these swards contain at least two plant species allowing the animal to select a diet consisting of a mixture of the two. More recently still, the desire to maintain and promote biodiversity has seen an interest in studying diet selection from semi-natural pastures (Rook *et al*., 2004), which offer the grazing animal a diverse range of plant species from which to select their own diets. This paper summarises research into diet preference and selection in grazing domestic sheep and cattle, considering the theoretical basis of mixed diets as well as the potential production and environmental benefits resulting from allowing the animals the opportunity to select their own diets.

Preference versus selection

It is important to differentiate between an animal's *preference* (i.e. what it 'wants' to eat) and what it actually eats (*selects*) due to some external constraint (Parsons *et al*., 1994). The difference between the two is best illustrated by example. Grass and clover swards grazed by domestic ruminants are usually offered as an intimate mixture of the two plant species, often along with other weed plant species mixed in the sward. In this situation, the grazing animal has to search through the mixture in order to find and then prehend i.e. take a bite of the preferred plant species. The need to search through the mixture imposes a constraint on the animal and consequently is an example of selection (Hodgson, 1979). In order to study preference, the constraint of needing to search through the mixture needs to be removed. This is typically achieved in diet preference studies by offering and grazing the two plant species as separate, conterminal monoculture swards (e.g. Parsons *et al*., 1994).

General results

In a recent review of diet preference for grass and legumes in domestic sheep and cattle, Rutter (in press) concluded that both animal species do not graze at random, but select mixed diets that generally consisted of 75% ±5% legume for lactating animals and 65% ±5% legume for non-lactating animals. This

can be generalised such that both lactating and non-lactating sheep and cattle generally select 70% ± 10% legume. Studies of the intake rates of grass and clover show that both sheep (Penning *et al*., 1991) and lactating dairy cows (Rutter *et al*., 2004a) eat clover more quickly than grass, but the intake rates of the two herbages were the same in non-lactating dairy heifers (Rutter *et al*., 2002).

Where studied, diet preference studies for grass and clover show a consistent diurnal pattern of preference, with the animals showing a strong preference for clover in the morning, but with the proportion of grass in the animals' diets increasing as the day progresses and reaching a maximum at the end of daylight hours (Figure 1).

Cost of selection

Champion *et al*. (2004) demonstrated that there are selection costs associated with grazing intimately mixed grass/clover swards. Although the sheep grazing mixed ryegrass/white clover swards had the longest grazing times (compared to sheep grazing ryegrass only, white clover only or separate, adjacent ryegrass/white clover monocultures), they had the lowest intakes. This can be attributed to the time taken as the sheep searched through the mixed sward looking for their preferred herbage at that moment in time. This searching time was, ultimately, at the expense of eating time resulting in a reduction of daily intake compared with the animals on the other treatments. The highest daily intakes in the study were achieved when the sheep could select their own diets from the spatially separate but adjacent grass and clover monocultures.

Optimum spatial scale of separation

The majority of studies into diet preference using separate, adjacent monocultures have generally had a spatial scale of separation at the paddock scale i.e. each paddock typically had one contiguous area of pure clover and another of pure grass, representing the opposite extreme of spatial separation from an intimately mixed sward. The effect of the spatial scale of separation of the two herbages on diet preference and selection was studied by Rutter *et al*. (2005). They presented beef heifers with adjacent strips of

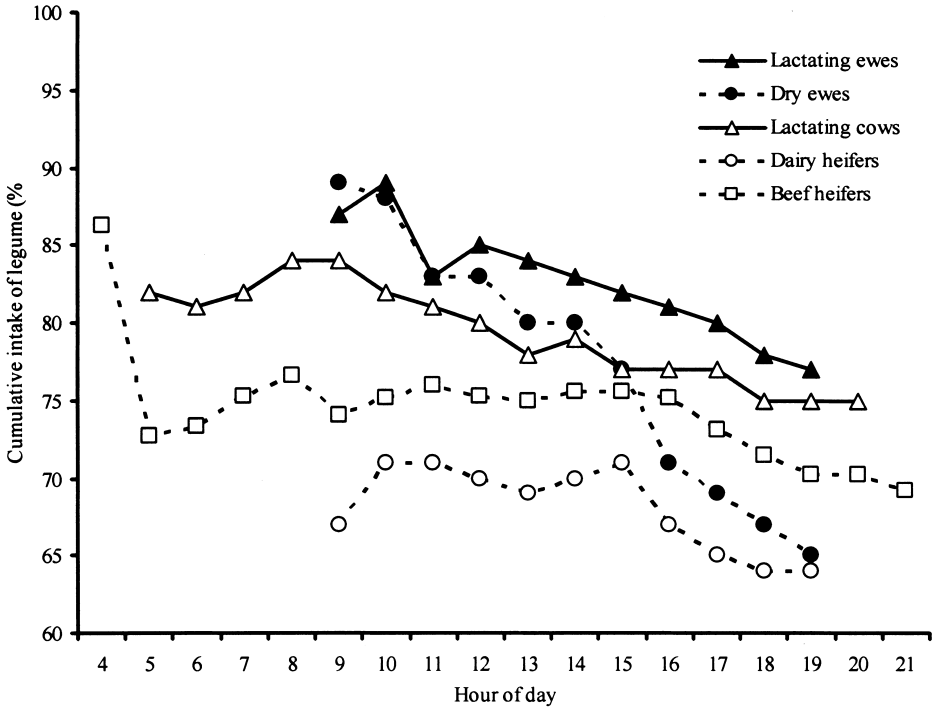

Fig.1. Diurnal patterns of preference for clover (vs perennial ryegrass) in lactating and non-lactating (dry) sheep (Parsons *et al*., 1994), lactating dairy cows (Rutter *et al*., 2004a), for non-lactating dairy heifers (Rutter *et al*., 2004b) and for non-lactating beef heifers (Rutter, unpublished data). In all cases, the lines represent the mean diet selected from animals that were offered 50% clover and 50% grass (by ground area).

grass and clover at different widths: 108 cm, 36 cm and 12 cm, as well as an intimately mixed sward. The cattle selected approximately 60% from the two wider strips but only approximately 37% clover from the 12 cm width strips and the mixed sward, indicating that the animals could select their preferred diets in strips of 36 cm and wider, but not from the 12 cm strips. This suggests that the critical scale of spatial separation to allow the cattle to select their preferred diet lies between 12 and 36 cm.

Why do ruminants eat mixed diets?

Various theories have been proposed for why ruminants eat mixed diets (Rutter, in press). Whilst several theories have been discounted, those relating to the animals evolutionary traits to optimise its fitness appear to warrant further research, and these are summarised in this section.

One of the most important functions an animal undertakes is the acquisition of nutrients. Eating a single food that has an excess of a particular nutrient can be less than optimal for an animal's fitness. This is because the excretion of the excess nutrient is likely have an energetic cost associated with it, putting the animal at a competitive disadvantage compared with an animal that optimises its nutrient capture such that by balancing its nutrient intake it avoids the excess energetic costs. This is probably one reason why ruminants, when given a choice, do not eat purely clover diets, as these have a higher proportion of nitrogen (compared with carbon) than grass (Whitehead, 1995), and there is an energetic cost associate with the excretion of excess nitrogen. There is evidence to support this hypothesis from *in vitro* studies that showed that the optimum level of microbial protein synthesis in an artificial rumen is achieved with 70% clover and 30% grass (Merry *et al*., 2002) i.e. the same proportion that grazing animals prefer when given free choice from separate, adjacent grass and clover monocultures, suggesting that this ratio of grass and clover provides the optimum balance of nutrients.

Related to the need to optimise the efficiency of nutrient capture is the need to maintain rumen function as another possible explanation of mixed diets. Although sheep and cattle can live on clover only diets, this is likely to lead to a change in their rumen micro-flora such that their ability to digest cellulose is less than that of animals that maintain cellulose-rich grass in their diet. Again, this would place these animals at a competitive disadvantage compared with those that maintain grass in their diets, and there is strong evolutionary pressure for animals to maintain the ability to cope with change.

Plants contain a variety of secondary compounds, many of which are toxic to animals. This could account for the diurnal pattern of preference seen in grazing ruminants, as the accumulation of one or more such compounds from clover could lead to the animal incorporating a greater proportion of grass in its diet in an attempt to dilute the toxins form the clover.

Another possible explanation for the diurnal pattern of preference is that the animal is filling its rumen with grass in the evening as it is a bulky feed with a slower passage rate than clover. Consequently, the animal is less likely to need to graze at night if it adopts this strategy. This has been proposed as a possible anti-predator strategy. Although modern domesticated animals are usually protected from predators, and their domestication as lead to a reduction in anti-predator responses (Mignon-Grasteau *et al*., 2005), they still exhibit some anti-predator behaviours similar to wild animals (Biossy *et al*., 2005).

Of the four possible explanations given above for why ruminants eat mixed diets, that latter two (toxin and predator avoidance) appear to be the most compelling as they account for both mixed diets and the diurnal pattern of preference. However, it is possible that all four explanations play a role, and that the animal has to make trade-offs between these (sometimes competing) goals as it attempts to optimise its survival and fitness. Further research is needed to explore all of these hypotheses as we attempt to understand the ultimate basis of mixed diets in ruminants.

Production benefits

Given the higher daily herbage intakes associated with grazing separate, adjacent clover and grass monocultures demonstrated in Champion *et al*.'s (2004) study (reported earlier), it would seem logical that this approach could be used to improve the production of grazing livestock. This has been demonstrated to be the case in dairy cattle. Both Nuthall *et al*. (2000) and Cosgrove *et al*. (2001) found that dairy cows grazing separate grass and clover monocultures under

continuous stocking produced at least 11% more milk than cows grazing a mixed grass/clover sward. Rutter *et al*. (2001) demonstrated that continuous free choice was not necessary, and that the intake and production benefits of grazing separate monocultures could be achieved by allowing the animals to graze clover only following morning milking and grass only following afternoon milking i.e. mimicking their natural diurnal pattern of preference (a treatment they called 'temporal allocation'). Rutter *et al*. (2003) then demonstrated that dairy cows on a temporal allocation treatment under strip grazing produced 14.6% more milk than those receiving a twice a day allocation of a mixed grass clover sward under rotational grazing. Rutter *et al*. (2003) argued that temporal allocation could provide a practical way to exploit the production benefits of grazing grass and clover as separate swards on farms.

Environmental benefits

The higher production levels from dairy cows grazing separate grass and clover monocultures reported in the previous section were originally attributed to higher daily intakes. However, some recent results from Australia (Venning, pers. comm.) show that sheep grazing separate grass and clover monocultures showed similar daily intakes but higher production levels than those grazing a mixed sward or a ryegrass only monoculture. This clearly indicates a higher feed conversion efficiency from the separate monocultures, with a more efficient capture of nutrients i.e. potentially less pollution than from animals grazing either mixed swards or ryegrass monocultures. High feed conversion efficiencies are also associated with lower methane emissions (DeRamus *et al*., 2003), resulting in another potential environmental benefit of allowing the animals the possibility to select their own diets i.e. a reduction in a potent 'greenhouse gas' (and a contributing factor in climate change) from grazing livestock.

Summary

Domestic ruminant livestock appear to have clear diet selection goals that are related to evolutionary traits inherited from their ancestors aimed at optimising their fitness. These include the need to optimise the efficiency with which they capture nutrients, maintaining rumen function and to avoid the danger of consuming toxins and the risk of predation. Grazing domestic sheep and cattle show a partial preference of approximately 70% for clover and show a diurnal pattern of preference, with a stronger preference for clover in the early part of the day, with the proportion of grass in their diet increasing towards the evening. Such a high proportion of clover in the diet is best achieved if the two herbages are offered as separate, adjacent monocultures, with the critical spatial scale of separation between the two lying between 12 and 36cm for heifers. By allowing sheep and cattle the opportunity to easily select their own diets from spatially separate monocultures, they achieve higher daily herbage intakes and higher levels of production than animals grazing grass only, clover only or a mixed sward. The higher levels of production also appear to be associated with a higher feed conversion efficiency, with reduced levels of pollutants per unit product, including methane (a potent 'greenhouse gas') emissions, giving positive environmental benefits. The combined benefits of enhanced production with reduced pollution show that the animals diet selection characteristics should be taken into account in the design of sustainable livestock grazing systems. An understanding of the factors influencing diet selection is also a pre-requisite for the development of grazing management strategies for the maintenance and promotion of biodiversity (both floral and dependent faunal biodiversity) in semi-natural pastures grazed by domestic ruminants.

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Environmental Impacts of Grazing Grassland

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This study is intends to assess the use of grasslands for grazing and nitrogen pollution in order to determine the environmental impact of the utilization of grasslands for grazing.

1. Characteristics of grazing grassland and water nitrogen pollution

There are specific characteristics of grasslands used for grazing such as heterogeneously-distributed nutrient input and tread pressure, which are the direct results of cattle grazing on the grassland. Since the pollution of water with nitrogen occurs when the amount of excess nitrogen in an area exceeds a certain level, the presence and intensity of the pollutant depends on the amount of nitrogen being applied in the area. This is true for grasslands being used for grazing or cutting. In the case of grazing grasslands, since the amount of nitrogen application depends on the amount of chemical fertilizers being used and cattle density or intensity, cattle density control is obviously an essential factor in nitrogen pollution prevention.

Grassland utilization methods of grazing and cutting were compared in terms of grass yield and the soil's chemical composition (Figs 1 and 2). The results of the grassland utilization experiment that compared grazing grassland and cutting grassland under the same application rate of chemical fertilizers were as follows: 1) Grass yield was higher in grazing grasslands than in cutting grasslands, 2) Soil nutrient accumulation was also higher in grazing grasslands than in cutting grasslands. These results are due to the extra nutrients the soil receives with the additional application of the grazing cattle's manure. The results establish that grass productivity increases under conditions of grazing as a result of the additional nutrient enriched manure being applied to the grasslands. Similar data are being published in "Nature" (Ryden et al. 1984). Under the same conditions of nitrogen application (420 $kgNha^{-1}$), perennial ryegrass grassland was used as cutting grassland or grazing grassland. Obviously higher concentrations of nitrate nitrogen in the soil solutions were observed in the grazing grassland (Fig. 3). Owens et al. (2004) and Cuttle et al. (1998) also reported the relations between grazing and water pollution.

However, this does not necessarily mean that there is a higher risk of nitrogen pollution in grazing grasslands compared to cutting grassland. There is no difference between them in terms of risk if the nutrient enriched manure applied by grazing cattle is taken into consideration when determining the amount of chemical fertilizer that is to be applied. Recent unpublished IGER data shows interesting results; the nitrate concentration of the leachate was higher in plots that had been grazed except for those using tactical treatment. This result indicates not only the occurrence of water nitrogen pollution in grazed plots but also the potential for avoiding nitrogen pollution through the proper management of the soil nitrogen level.

The amount of nitrogen runoff or leaching in grazing grasslands and cutting grasslands was compared in Konsen AES (Table 1) (Kouda 1999). Calculations were based on farm records and other relevant documentation. The comparative study displayed that the potential for water pollution through leaching and surface runoff is higher in grazing grasslands than cutting grasslands. The nitrogen concentration in shallow ground water in different methods of land use was as follows: forests< cutting grasslands< grazing grasslands< crop fields (Table 2) (Hayakawa et al. 1997). From these results, it is undeniable that the risk of water nitrogen pollution by grazing utilization of grassland increase.

Primal cause of this is of course on manure of the grazing cattle, but it is not deniable that the intake-rate (water permeability) of the surface layer can be increased due to the influence of heterogeneous tread pressure and water pollution caused by unevenly distributed nutrients. However, it was also shown that the risk of water nitrogen pollution can be avoided by adjusting the amount of nitrogen application through

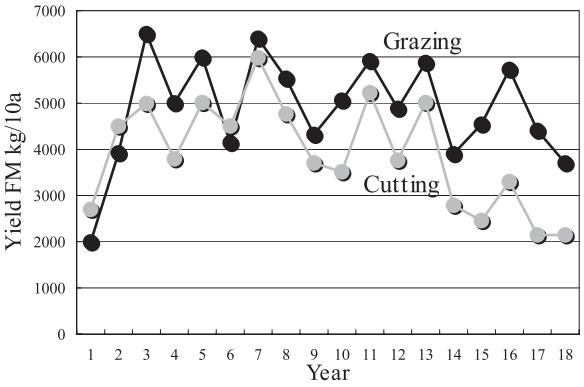

Fig. 1. Yield comparison between cutting and grazing grassland.Orchardgrass (*Dactylis*) + Perennial ryegrass (*Lolium*), 100kgNha^{-1}yr^{-1}, 5-6 cuts or graze yr^{-1}

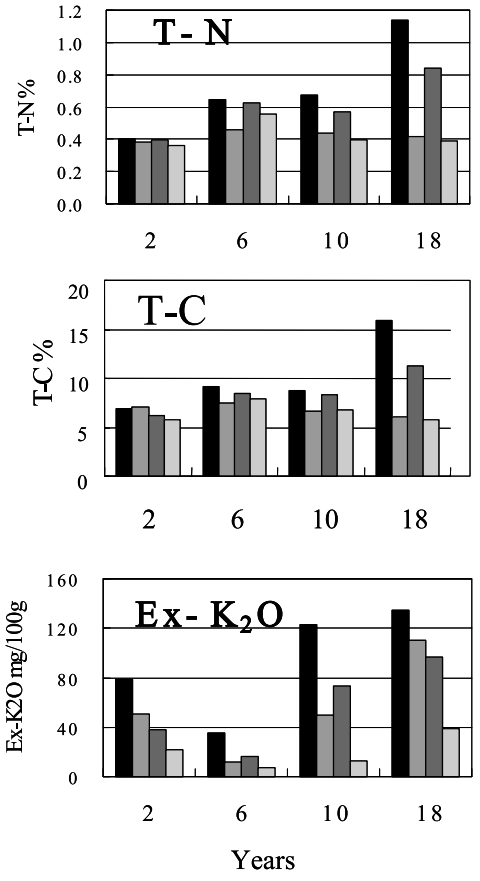

Fig. 2. Differences in soil nutrition accumulation by grazing or cutting

chemical fertilizer control.

2. Environmentally advantageous aspects of grazed grasslands

However, it can be stated that the use of grazing contributes to environmental conservation in that it decreases the amount of manure that needs to

Table 1 Nitrogen loading on the water in Konsen AES (Estimation)

Water category	Land utilization	Loading kgN/yr	Area ha	Loading/ha kgN/ha/yr
Surface runnoff	Cutting grassland	218	68	3
	Grazing pasture	252	60	8
	Both	101	17	6
	Total	571	125	5
Percolating	Cutting grassland	461	68	7
	Grazing pasture	403	30	14
	Both	174	17	10
	Facilities	1160	9	129
	Total	2199	125	18
Sum	Cutting grassland	679	68	10
	Grazing pasture	655	30	22
	Both	275	17	16
	Facilities	1160	9	129
		2769	125	22

Kouda et al.,

Table 2 Nitrate concentration of shallow ground water

Land utilization	NO3-N mg/L
Forest	0.000
Cutting grassland 1	1.453
Cutting grassland 2	1.571
Grazing pasture	4.142
Arable crop 1	3.890
Arable crop 2	21.433

Hayakawa 1997, average of 17 sampling

be treated. The amount of manure produced in the barn decreases in accordance with increases in the amount of time spent grazing (Bando 1996) (Fig. 4). This is definitely advantageous for environmental conservation because it can reduce the time and labor dedicated to manure treatment. However, the amount of manure that needs to be treated is not reduced to zero and manure is naturally spread over the grassland.

In other words, allowing livestock to graze on grasslands reduces the amount of manure being generated in barns, therefore reducing the time and energy that word otherwise be spent treating manure, which, in turn, enables farmers to dedicate more time and attention to environmental concerns and, consequently, works to further environmental conservation. In this way, from an environmentalist standpoint, the use of grasslands as grazing lands is advantageous.

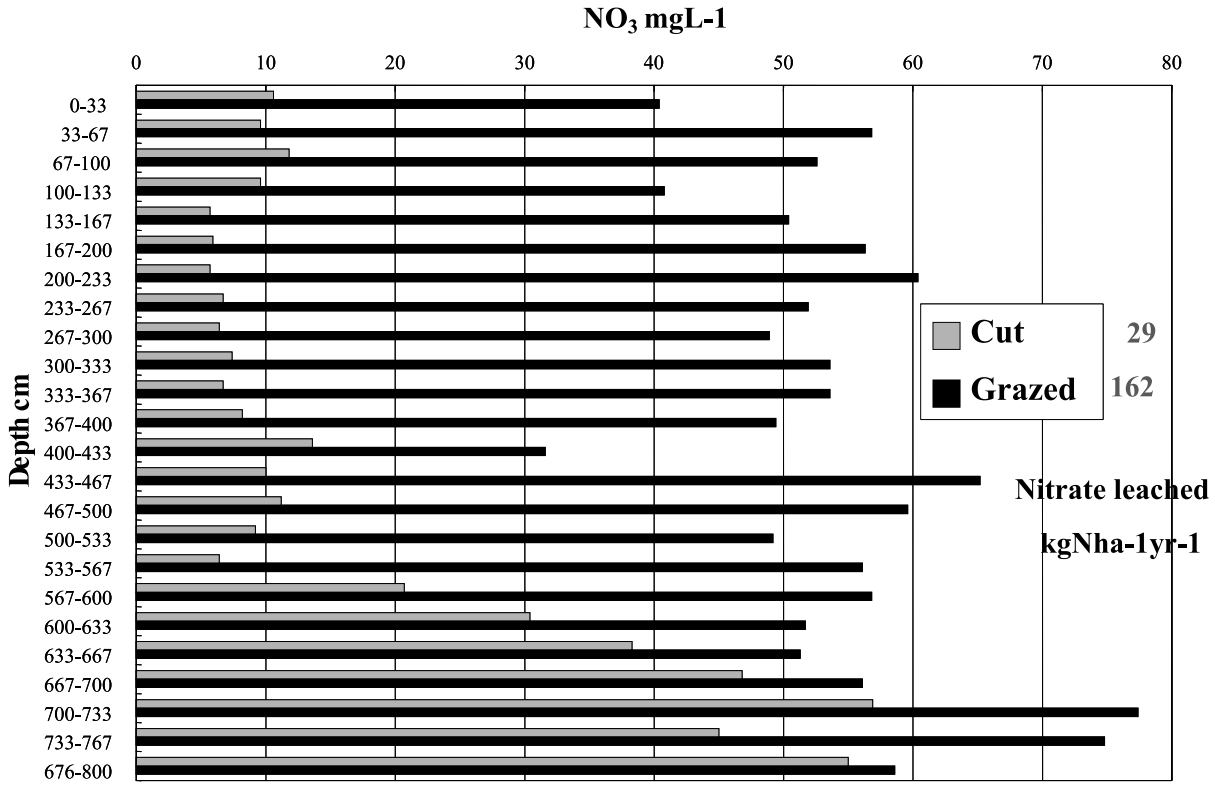

Fig. 3. Nitrate concentration of soil solutions from successive depth below cut and grazed ryegrass sward, after 5 years treatment (420kgNha^{-1} + 9.3headha^{-1})
J.C Ryden *et al*., Nature 331 (1984)

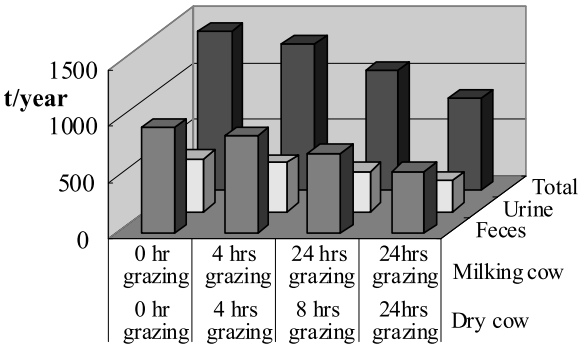

Fig. 4. Effects of grazing hour on manure production in barn (estimation) Bando 1996

3. Difficulties in analyzing the environmental impacts of grazing grassland in sloped areas

Since Japan is limited in terms of arable land, grazing grasslands have primarily been limited to hilly or sloped areas. Therefore, it is necessary to differentiate the impact of maintaining grasslands through gazing from factors resulting form an area' s sloped conditions. Heterogeneously-distributed soil nutrients exist in sloped areas for a variety reasons. According to the data for sloped areas and valleys, nutrient accumulation often occurred at the bottom of the valley (Sakai and Hojito 2001). However, there were few cases that were contrary to this. Comparing soil nutrients at the time of seeding time and one month later showed that the movement of nutrients in the soil is caused by precipitation that occurs just after the seeding. This means that grasslands maintained through grazing in sloped areas have risk of nutrients escaping due to surface runoff during periods of precipitation shortly after seeding.

Creating a buffer zone is an effective way to address this problem. Establishing 5 m-wide at lower part of a sloping grassland resulted in decreases in nutrient loss (Sakai and Hojito 2001). On the other hand, the nutrient distribution pattern in the sloping grazed grassland highly corresponded to the location of the grazing cattle's feces (Yamada 2000). Since the nutrient accumulation is more pronounced at areas in which the cattle gather, risks of nutrient leaching increase there. Although soil diagnosis and fertilizer management is said to counteract these risks, actually conducting them is somewhat difficult.

In this way, factor analysis of heterogeneously-distributed soil nutrients in grazed grasslands is

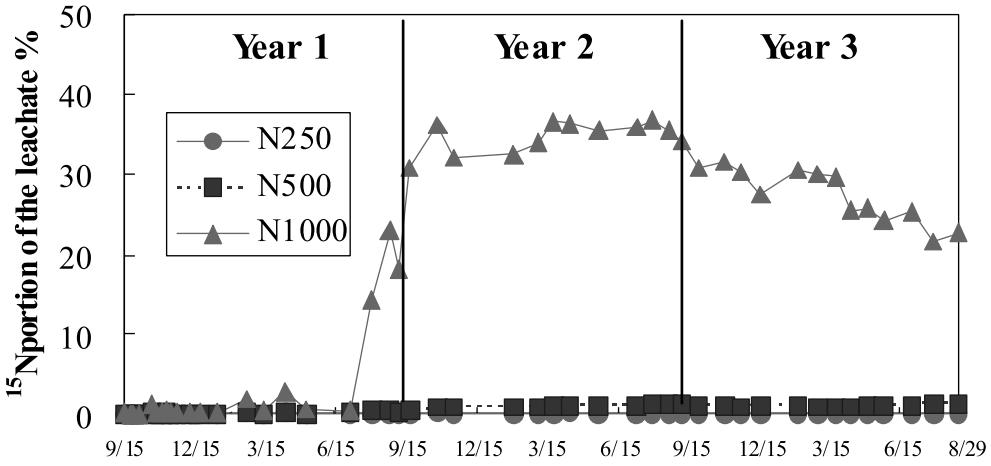

Fig. 5. 15N proportion in the leachate after N application-Lysimeter experiment- (Matsunami and Hojito 2005)

complicated since factors associated with cattle behavior overlap with those connected to sloping.

4. Difficulties in verifying water pollution

Even though the risks of water pollution from grazing grasslands exist, providing sufficient data to verify them is not easy. For instance, a case of the investigation of shallow ground water quality when grazing is introduced to fallow paddy field is discussed. Most sites failed to show increases in nitrogen concentration during grazing, but displayed decreases as a result of the water management techniques being applied to the surrounding paddy fields. However, despite being at very low levels, the tendency of ammonium nitrogen to increase during grazing was recognizable. This phenomenon is representative of the actual conditions of grazed grasslands. One major reason for the difficulties in tracing nitrogen pollution in grazed grasslands is that a considerable amount of time lapses between the excretion of feces and the dissolving of leachate. For example, with conditions of 240 cm deep lysimeter, it took almost one year to get nitrogen from the fertilizer applied even in 1000 $kgha^{-1}$ level to the leaching water (Fig. 5) (Matsunami et al. 2005). In addition, results have yet to be obtained in 250 and 500 $kgNha^{-1}$ levels in three years of experiments. This results show that it is necessary to consider the time lag associated with how grazing effects leachate in order to accurately analyze its influences. Therefore, demonstrative data regarding practical grazing grassland and the resulting levels of water pollution are limited in certain cases. The reason for this limitation is that it is difficult to take water samples and identify and separate influences that directly result from grazing and those of other possible variables. It is only possible to identify direct result and influences of grazing in areas where shallow ground water can be taken or where very effective impermeable soil layers exist making it possible to collect whole leachate through the fields. In the absence of such conditions, it is difficult to collect and analyze data in terms of the direct effects of grazing on leachate on a practical scale.

A capillary lysimeter can be installed in soil layer as a means to achieve this (Fig. 6). This is a 40cm diameter and 100 cm long polyvinyl chloride tube, used to collect water permeating 40-100cm into the soil layer. This device sets the capillary to have a very weak negative pressure for water suction. This technique is useful and inexpensive however, setting it up is labor intensive and needs to be replicated a number of times because of the variations in the collected water quantity. The 'grazing lysimeter'

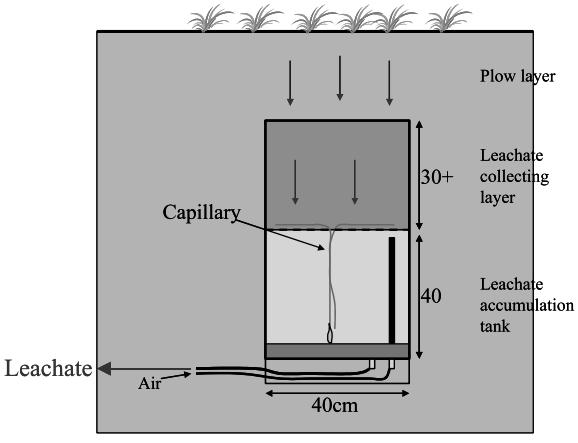

Fig. 6. Capillary lysimeter

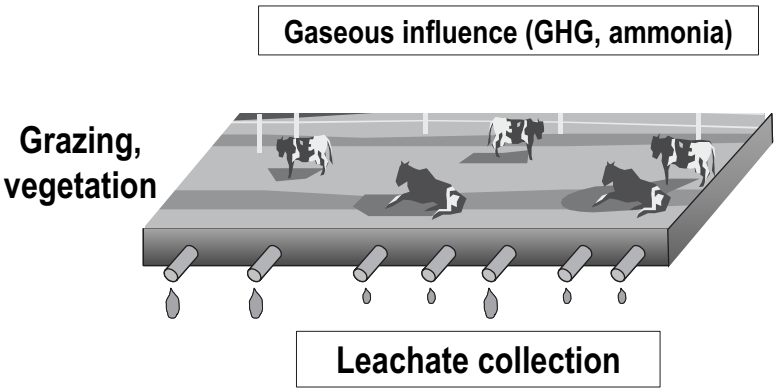

Fig. 7. Grazing lysimeter

should be regarded as a useful piece of equipment (Fig. 7). It can be used to develop a practical scale model of a grazed grassland with a pool made of concrete that traps leaching water which can then be collected from bottom of the pool. This method is of course costly, but it is nevertheless useful for collecting leaching water. There is a good grazing lysimeter in IGER NorthWyke, Rowden Moor, UK, where the top soil is very argillaceous and impermeable to water (Scholefield et al.). Consequently most of the drainage water can be collected by artificially draining it with a mole drain. The drainage water volume was measured using weir chambers and 14 plots, each 1ha in area. (Unfortunately, no data the effects of maintaining grasslands through grazing has been taken as of yet.)

5. Other environmental impacts of maintaining grassland through grazing

In terms of global greenhouse gasses (GHG_s), grazing grasslands have negative relationships to nitrous oxide (N_2O) and methane (CH_4) (Flessa et al. 1996, 2002). Grasslands are primarily considered to be methane absorption sites. However, due to the accumulation of cattle manure, grazed grasslands are generally regarded as a source of methane release. On the other hand, ammonia (NH_3) emissions are also regarded as a concern causing characteristic of manure. However, recent data concerning air ammonia concentration attained through the passive sampler method showed no difference of emissions between cut and grazed grassland. The effects of chemical fertilizer application were alternatively observed noticeable. In addition to this, the potential for pathogenic microbe pollution was reported in the study.

Consequently, grazing is a reasonable and rational method of cattle production. In order to perform grazing in the absence of risks of environmental pollution, it is important to develop a proper understanding of the characteristics of grazing grasslands and the measures required to counter its negative effects. Features of grazing grasslands include heterogeneously-distributed soil nutrient, problem associated with sloped areas, tread pressures and unstable vegetation, which was unaddressed here. Each characteristic has its own corresponding environmental pollution problem. Hence, in solving these issues, from a scientific perspective, separating the impact of factors directly related to cattle from variables and effect associated sloping is important. In addition to the effectiveness of orthodox grassland management, controlling cattle density and grazing intensity is important in terms of nitrogen loading control.

Acknowledgement

Author wish to thank Dr Toshiya Saigusa, for his cooperation of writing manuscript, particularly his chapter in the booklet as written follows was very helpful: Is grazing frienly to einvironment? *In* Milk production by grazing, ed., T.Matsunaka, Rakuno Sogo Kenkyusyo, Sapporo 2003, p119-134*

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A Review on Feeding System for Deer Production

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Keywords : Deer farming, velvet antler production, feed source, medicinal herb.

Abstract

Deer farming has been continuously expanding because it requires less investment, land, animals, and labor than other livestock enterprises. The system of velvet antler production is relatively new, however, it may continue to grow until its full potential. High growth rate and feed availability can enhance deer farming. Understanding the feeding system for deer production is essential in achieving maximum productivity. Deer have mainly been grazed in perennial pasture for venison and velvet antler production in western countries, including New Zealand, Australia, Canada, and America, where they have intensively been fed a variety of food sources for velvet production in oriental countries, including Korea and China. It is well known that deer belong to intermediate eater and have a good feed availability. Furthermore deer show a seasonal physiological digestive system and their feed availability differs seasonally. Deer farming industry in Korea has mainly depended on imported feed sources, such as oak leaf hay and alfalfa bale, owing to small plow land and increased labor fee. However, oak leaf hay which was greatly acceptable by deer farms had a low feed availability and comparatively high cost. Therefore, they demanded increasingly positive development of feed source which can reduce cost and increase availability. Forest by-product which is included trees, wild grasses and shrubs collected from the reforestation areas, agricultural by-products including soybean cured meal and brewer's grain, and forages including corn, sorghum, and rye silages are expected to adapt well for deer. Furthermore, it was proven that there is a possibility to produce high quality velvet antler by feeding several different feed sources including medicinal herbs.

Introduction

Deer (Cervidae) have been valuable to humans as a sources of meat (Takatsuki, 1996), clothing, tools and weapons (Jarman, 1972), the important game and sporting animal (Kay and Staines, 1981) over the world. Especially, velvet antlers have been part of an important traditional oriental medicine for a long time. With increasing demand for velvet antler, deer farming is becoming a rapidly growing industry as an alternative form of animal industry in many parts of the world, including North America, Europe, Oceania, and Korea (Sim, 1987). Because deer are now becoming more and more valuable farm animals, there are abundant demands in many temperate areas. Much research has been done recently on their management and nutrition due to increasing demands for deer farming. Some researchers have reviewed the nutritional requirement of deer (Kay and Staines, 1981; Adam, 1996) and have formulated feed guidelines for the deer farmers (Jeon et al., 1995). However, reliable data on feeding system, nutrient requirements and feeding standards for deer are scanty by comparison to other ruminants such as cattle and sheep.

Feeding system for deer is variable depending on production system and feeding condition of each country. New Zealand which is the most productive country for velvet antler and venison has traditionally adopted a grazing system based on the perennial pasture while Korea which consumes the most for velvet antler has mostly adopted a pen feeding system (Intensive feeding system). Deer farming in Korea is developing well in contrast to other agricultural businesses. This is because of the special features of deer farming namely low cost, low labor, environmentally friendly stock breeding and high profitability (Yerex and Speiers, 1993). However, it is estimated that feeding cost make up the highest portion of overall production cost in an intensive deer farming industry (Moon, 2000) and also there is little information of value regarding suitable

Table 1. A feeding system for deer production.

Country	Species	Feeding system	Feed source
Korea	Spotted deer Red deer Elk	Pen system (Intensive)	Wild grasses, Shrubs, Tree, Agricultural by-products, Medicinal herbs, Grains, Concentrate
China	Spotted deer Maral deer	Pen system (Intensive)	Corn silage, Grains
New Zealand	Red deer Elk	Pasture system (Grazing)	White clover, Perennial ryegrass, Oat, Chicory
Canada	Elk Red deer	Pasture system (Grazing)	Oat
Australia	Red deer Elk	Pasture system (Grazing)	Grasses

Table 2. Seasonal dry matter intake (DMI) in spotted deer.

Item	Spring	Summer	Autumn	Winter
DMI (g/d)	2,685^{a}	2,255^{ab}	1,997^{ab}	1,929^{b}
DMI (g/kgW$^{0.75}$/d)	85.5^{a}	70.6^{ab}	70.9^{ab}	65.1^{b}

^{ab} values with different superscripts in the same row differ ($p<0.05$).

Table 3. Seasonal dry matter intake (DMI) and digestibility by feed sources in spotted deer.

Item	Summer		Winter	
	FBS[1]	CS[2]	FBS	CS
DMI (g/day)	1269.7 ± 265.2^{aA}	1110.9 ± 212.8^{aA}	1130.6 ± 67.4^{aA}	1040.6 ± 104.6^{aA}
Digestibility				
Dry matter (%)	57.3 ± 8.9^{bA}	63.9 ± 7.1^{aA}	50.8 ± 8.0^{bB}	59.4 ± 6.7^{aA}
C. protein (%)	32.4 ± 3.8^{bB}	48.7 ± 9.9^{aA}	42.2 ± 10.1^{bA}	50.2 ± 9.1^{aA}
C. fiber (%)	55.5 ± 16.6^{aA}	40.2 ± 13.0^{bA}	30.7 ± 21.4^{aB}	26.1 ± 16.2^{bB}

[1]FBS: Forest by-product silage, [2]CS: Corn silage
^{ab} Means with different superscripts in the same row are significantly different ($P<0.01$).
^{AB} Means with different superscripts in the seasonal row are significantly different ($P<0.01$).

feeding system or standards for each production system. Therefore, it is necessary to establish an effective feeding system in order to obtain successful management of deer farming.

In this paper we would like to review our research results on feeding systems for velvet antler production and present the prospects for available feeding systems for farmed deer under intensive farming condition.

A feeding system for deer production

A feeding system for deer production is largely divided to two main systems. One is the grazing system which is mainly adopted in the countries of Europe, Oceania, and North America and another is a pen feeding system which is generally adopted in the countries of Orient including Korea, Japan, and China (Table 1). The former has low cost but low productivity, and the latter has high productivity or high quality of production but high cost. Deer farming industry in Korea is characterized by using variable feed sources for high productivity and quality of velvet antler, such as forages, shrubs, wild grasses, grains, oak leaf hay and medicinal herbs.

Digestive physiology and seasonality

The main species of farmed deer for velvet antler production are Elk (*Cervus canadensis*), red deer

(*Cervus elaphus*), and spotted deer (*Cervus nippon*), classified morphophysiologically as ruminants (intermediate feeding types) selecting a wide range of browses, forbs, shrubs, and grasses (Hoffman, 1988; Blair and Brunett, 1980). Also it is well known that deer have seasonal intake and digestibility (Suttie et al., 1983; Barry et al., 1991; Moon et al., 2000). Deer show marked seasonal feed intake (Moon et al., 2000), the volume of rumen, feed passage rate (Kato et al., 1989), and forming ammonia in rumen (Freudenberger et al., 1994) with lower values in winter and higher values in summer. This seasonal change in feed intake would be thought to be associated with their seasonal basal metabolic rate (Blaxter and Boyne, 1982). Therefore, it is necessary to conduct research on feeding management considering the unique digestive physiology of deer. We also reconfirmed the same research results with high value in summer and low value in winter for internal availability of feed source. (Tables 2 and 3). (Moon et al., 2000; Moon et al., 2004).

Feeding behavior of spotted deer in pen feeding system.

Jeon and Moon (2002) researched eating and ruminating time of spotted deer on Pen Feeding system. They figure out that deer do feed-eating and ruminating frequently by bits (Figures 1 and 2), unlike cows that eat feed intensively two or three times during daytime and ruminate primarily at night. In consequence, the way that deer can always eat feed is good for the feeding system.

Development and utilization of diversified feed source

Oak leaf hay, mainly imported from China, has been used as roughage source for deer because of high palatability and containing tannin that can have positive (Aerts et al., 1999) or negative effects (Natis and Malechek, 1981) on animal performance in Korea deer farming. However, our previous researches indicated that imported oak leaf hay had low nutritive value and internal availability compared with corn silage, rye silage and forest by-product silage (Kim et al., 1996; Jeon et al., 2003) and was comparatively expensive. Our study also shows that forest by-products including whole browses, shrubs, and wild grasses that are produced in reforestation areas and agricultural by-products are valuable feed sources for deer (Jeon et al., 1998, 2002). Meanwhile, we have conducted the related researches to reduce the feed cost which was estimated as possessing the highest portion of the total production cost. Moon et al. (1999) studied that soybean cured meal and brewer's grain was available for deer with form of silage and Jeon et al. (2001, 2003) reported that deep stacked broiler litter could be applied to about 30% of the total feed source for deer with no reduction of feed

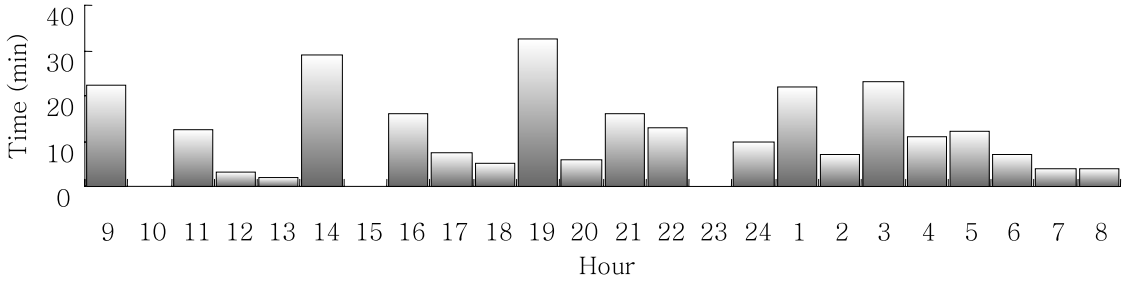

Fig 1. Circadian distribution of eating time in Korean spotted deer.

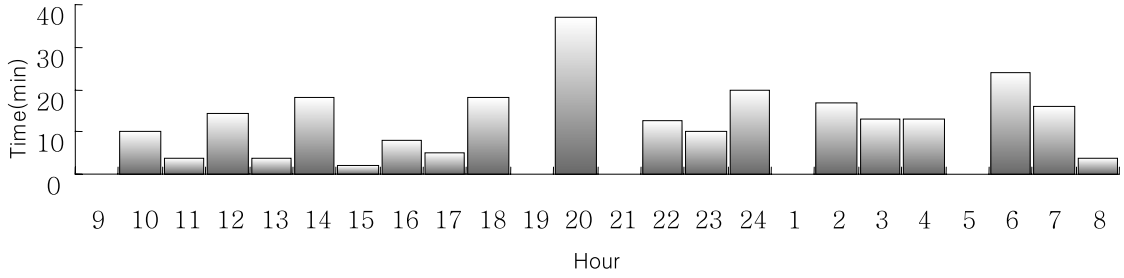

Fig 2. Circadian distribution of ruminating time in Korean spotted deer.

Table 4. Comparison of nutritive value on various roughage in spotted deer.

Item	Feed				
	Oak leaf hay	Corn silage	Rye silage	FBS[1]	Arrow-root silage
Dry matter (%)	88.0	25.4	21.9	34.4	22.7
CP (%)	9.7	7.3	12.9	8.2	14.8
DM intake (g)	1386.4	954.3	1111.4	1102.7	985.7
DM digestibility (%)	47.6	72.2	72.4	65.5	70.9
DDM intake (g)	659.9	689.4	804.1	722.2	698.9
CP digestibility (%)	35.1	63.1	74.5	62.6	71.6
CP intake (g)	64.3	81.7	136.3	119.3	139.6

[1]FBS: Forest by-Product Silage

utilization. Also Jeon et al. (2000) reported that forest by-products produced in reforestation areas are to be a good feed source having high palatability, nutritive value, huge produce, and economical efficiency (Table 4).

High quality velvet antler production.

Velvet antler consumption has largely increased all over the world and consumers have a high interest in the quality, quantity and content of velvet antler. Producers and consumers will then focus more on velvet antler quality and stability in the future and it is extremely essential to determine not only the improvement of velvet antler production but also the differences in velvet antler quality by breed, age, growth stage and feeding condition. Recently, several researchers have carried out related researches. Moon et al. (2004) have been heavily involved in determining the variations of velvet antler content by feeding condition and carried out several experiments for variation of velvet antler productions and content by different feed sources and nutrition level. Results indicate that blood and some composition of velvet antlers were analyzed showing large differences by feed sources. (Figure 3, Ha et al., 2003) It is then obvious that the quality of velvet antler partially depends on the source of feed and feeding conditions.

Thus, it is partly possible to control velvet quality with feed sources.

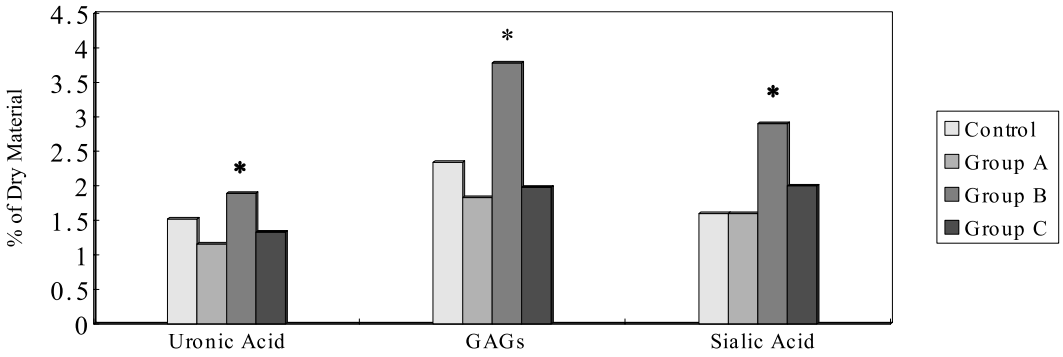

Fig. 3. Comparison of carbohydrate amount in the top sections of Velvet antlers after treatment of special fodder. Group A : mulberry, Group. B : Lycii Fructus, Group. C : Complex of herbs, % of dry material x10. * : $p<0.05$.

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JIFS, 3 : 45 − 52 (2006) Special review

Recent Advances in Animal Breeding Theory

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Abstract

Recent developments in animal breeding theory have been driven largely by computer science and molecular biology. Several of the theories designed for practical application to animal breeding are heavy computing requirements. The development of computer hardware and of many algorithms for constructing and solving mixed model equations (MME) has enabled breeding values to be estimated from data on a huge number of animals by best linear unbiased prediction (BLUP) procedures. It has also enabled genetic parameters to be estimated by restricted maximum likelihood (REML). However, because the size of the MME that can be analyzed by REML depends on the genetic model and the numbers of traits and animals, all kinds of data cannot be used to estimate genetic parameters by even the latest supercomputers. For this reason, traditional theories are still being improved and new theories studied for practical applications to animal breeding. Traditional quantitative genetics theory has been based almost exclusively on the assumption that genetic variation in quantitative traits of interest is controlled by polygenes. On the other hand, molecular genetics techniques have made it possible to estimate individual genotypes. Because DNA marker information can be obtained by using these molecular biological techniques, theoretical studies of linkage mapping, detection of quantitative trait loci (QTLs), and the potential for marker-assisted or genotype selection have been developed in the last decade. Information on QTLs with large effects will be used for the genetic improvement of animals in the near future. These new technologies will not replace existing animal breeding practices, but will be blended with them through the use of breeding program design and genetic evaluation methods that cover both known and unknown QTLs.

1. Introduction

Population genetics and quantitative genetics are the sciences behind animal breeding theory. Fisher (1918) demonstrated that measured correlations between relatives could be explained by the contributions of a large number of Mendelian factors (now called polymorphic loci), each with a small effect. The advancement and use of animal breeding theory have been based almost exclusively on this assumption. The classical model of inheritance based on this assumption seems to be quite robust in the predicted response to selection and simulation studies. The development of computer hardware has enabled this traditional theory to be applied to actual animal breeding programs. On the other hand, molecular genetics techniques have made it possible to detect quantitative trait loci (QTLs). This information may increase the efficiency of genetic selection programs for livestock. For these reasons, computer science and molecular biology have largely driven recent advances in animal breeding theory.

The purpose of this review is to introduce the traditional and recent animal breeding theories that are widely applied to animal breeding. In addition, I will discuss the effect of including information on currently identified QTLs. Finally, I will briefly discuss the nature of animal breeding in the near future.

2. Effects of development of computer science on animal breeding theory

2.1 Application of best linear unbiased prediction (BLUP) to genetic evaluation

Figure 1 is an example of a basic system of genetic improvement by selection in livestock. In this system, genetic improvement in the population is strongly affected by selection rather than by constructing base population, mating system, or first-stage selection at weaning. The basic problem in animal improvement through breeding is to choose animals that have the greatest genetic value as parents of the next generation. To evaluate the genetic value of candidates for selection, we need to divide the

phenotypic value into component parts attributable to different causes. The simplified model for the relationship between an animal' phenotype and its genotype is:

Phenotype = **G**enotype + **E**nvironment
= (Additive genetic effect + Other genetic effects) + **E**.

Before 1970s the selection index was the major tool used in genetic improvement programs in most countries for estimating individual genotypes although it is dependent on the animal species. The selection index is a tool for estimating the breeding value of an animal by combining all information available on the animal and its relatives (Hazel, 1943). The theory of restricted selection indices, whereby the aim is a genetic change of zero in one or some traits (Kempthorne and Nordskog, 1959) or predetermined relative changes in some (Harville, 1975) or all traits (Yamada et al., 1974) under selection, was developed after the introduction of selection index theory by Hazel. A detailed discussion can be found in a review by Brascamp (1984). The restricted selection index theory is still studied today and new theories are still being developed (e.g. Lin, 2005).

The application of best linear unbiased prediction (BLUP) to dairy and beef cattle breeding continued to develop rapidly during the 1970s. The theory of BLUP was originally developed by Henderson (1949, 1973) for the genetic evaluation of sires in the dairy industry. Since then, it has evolved from application to sire and maternal grandsire models for genetic evaluation in the early years to multiple-trait animal models (Henderson and Quaas, 1976) and random regression models (Henderson, 1982), which are used to analyze of longitudinal data or repeated records on individuals over time, in recent years. BLUP has become the most widely accepted method of genetic evaluation of domestic animals.

Some of the reasons for the greater genetic gain achievable by using BLUP are summarized by Long et al. (1991), as follows:

- BLUP uses information from all known relatives of an individual and facilitates comparisons of the genetic merit of animals by using differing amounts of information.
- It facilitates comparisons of genetic merit among animals for which data have been recorded in different management regimes or over different periods of time, and it facilitates comparisons between animals from different herds.
- It allows comparisons to be made among animals that have undergone different amounts of prior selection.
- It partitions genetic and non-genetic effects on performance into their respective components, thus enabling breeders to assess genetic change over time.

We need to solve mixed model equations (MME) to obtain BLUP of breeding values. The size of the MME depends on the genetic model (Figure 2). One of the main advantages of the genetic models used lately is the fact that they increase the accuracy of evaluations. For example, although the breeding values can be predicted by solving MME with several dozen or a few hundred unknowns in a sire model, one multiple-trait, random regression model in a country with an advanced dairy cattle evaluation system led to MME with near 100 million unknowns (Lidauer et al., 1999).

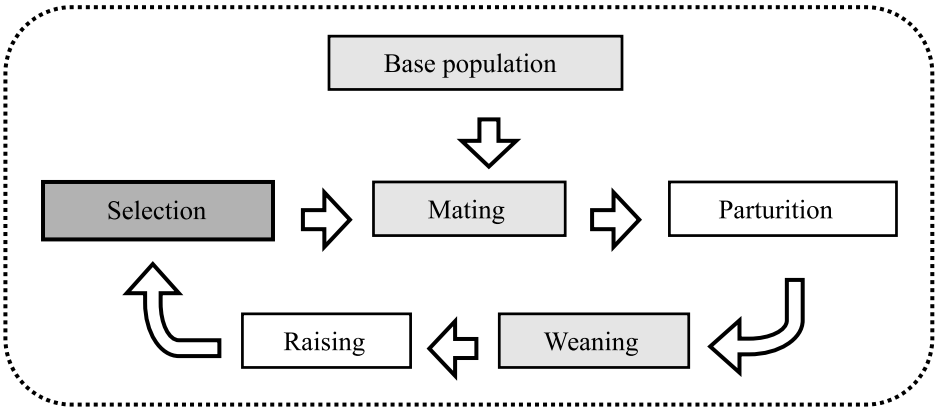

Fig.1. A basic system for genetic improvement by selection.

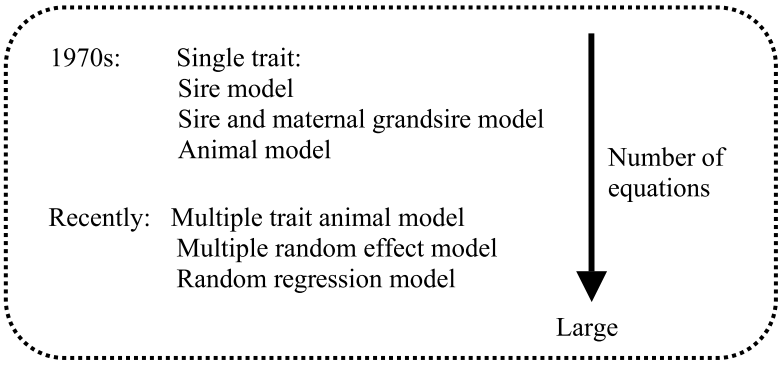

Fig. 2. Relationship between size of MME and mathematical model.

Genetic evaluation of livestock owes what its current status to increasing computer power. Currently we can solve MME with a few million unknowns by using only a personal workstation. The development of computer science has facilitated stochastic computer simulation studies for comparing genetic models or animal breeding systems by selection using BLUP. The genetic evaluation systems are supported not only by computer hardware but also by software. After the 1970s, many studies on BLUP methodologies, such as methods for computing the inverse of a relationship matrix (Henderson, 1976; Quaas, 1976), rules for calculating the coefficients associated with genetic group effects of MME (Westell et al., 1988; Quaas, 1988), or iterative algorithms and their application in solving MME (e.g. Schaeffer and Kennedy, 1986; Tsuruta et al., 2001; Strandén et al., 2002), have been developed. As a result, many programs for calculating the BLUP of breeding values are now offered worldwide. For these reasons, the development of computer hardware and software is enabling us to apply important breeding theory to practical animal breeding systems.

2.2 Problems in applying BLUP to genetic evaluation

Some basic assumptions of the linear model are demanded for predicting breeding values by using BLUP methodology. For example, the distributions of observations, breeding values, and random residual effects are assumed to be multivariate normal, implying that traits are determined by many additive genes, each with infinitesimal effects, at many infinitely unlinked loci; and the base population is assumed to be unrelated, unselected, and sampled randomly from a conceptually infinite population. However, actual data are not based on these assumptions. Several problems, therefore, arise when breeding values are predicted by the BLUP method on the basis of actual data. Two of them—genetic parameter estimation and selection with constraints—are introduced in this subsection.

In the BLUP method, genetic parameters in the base population are assumed to be known. However, the genetic parameters are generally unknown. Accordingly, we have to estimate genetic parameters in the population beforehand. Before the 1980s, Henderson's Method III (Henderson, 1953) was widely used for estimating the variance component. A disadvantage of this method for animal breeding application is that it was difficult to estimate genetic covariances. Restricted maximum likelihood (REML: Patterson and Thompson, 1971) was then adopted by animal breeders for estimating genetic parameters. More recently, Bayesian inference via Gibbs sampling (Geman and Geman, 1984; Gianola and Fernando, 1986) and Method R (Reverter et al., 1994) have been proposed for estimating variance components.

The effects of selection (e.g. van der Werf and de Boer, 1990; Schaeffer et al., 1998), statistical models (e.g. Clément et al, 2001; Satoh et al., 2002), incomplete pedigree information (e.g. Schenkel and Schaeffer, 2000; Roughsedge et al., 2001), and different methods of estimating genetic parameters (e.g. van Tassell et al., 1995; Cantet et al., 2000) have been studied for a few decades. There have also been many studies on the empirical comparison of REML algorithms. A detailed discussion can be found in a review by Hofer (1998).

The REML technique is the most accurate method available, because it takes account of all genetic relationships between the animals and the effects of selection. Larger data sets and more complex models can be analyzed by REML, owing to the increased power of computers and advances in computing algo-

Table 1. An example of comparison with methods for calculating restricted BLUP[1]

Selection method: Author and (Year)	Constraints	Number of nonzero elements[2]	CPU time[3] (second)
Satoh (1998)	All animals	144,161	0.72
Quaas and Henderson (1976)	Some animals	204,561,480	6,916.91
Satoh (2004)	Some animals	17,620,448	169.97

[1] Total number of animals used for calculation is 31,650 and the number of restricted animals is 6,000.
[2] Number of nonzero elements in the upper triangular matrix on the left-hand side of restricted BLUP equations.
[3] Central processing unit time.

rithms. However, the computational requirements for variance component estimation are still demanding, and continued efforts at improvement are still necessary.

In Japan, selection for desired change in all traits has been frequently used in swine or poultry breeding programs. At first, this selection procedure was based on a restricted selection index, achieving predetermined relative changes in all traits (Yamada et al., 1974). After that, restricted selection indices were applied to restricted BLUP (Quaas and Henderson, 1976). The original method for calculating restricted BLUP of breeding values has been improved by Itoh and Iwaisaki (1990) and Satoh (1998). More recently, Satoh (2004) derived a new procedure for estimating restricted BLUP of breeding values when constraints were imposed on the additive genetic values of only some animals in a population. This method requires several hundred times more computing power than usual multiple-trait BLUP (Table 1).

Because the population size of closed herds in swine or poultry breeding in Japan is generally small, calculation of restricted BLUP is not difficult. However, if all the animals in the whole country were to be evaluated, the computing requirement would be quite heavy. Because restricted selection includes selection with zero change in one or a few traits, if some economic trait is optimized by selection, then restricted selection with zero change will be conducted. Egg weight in laying hens and backfat thickness and meat quality (e.g. intramuscular fat content and meat color and pH value) in swine and beef cattle have optimum levels in terms of economics or consumer requirements, and these traits may reach their optimum levels in the near future. Traits that required balance, such as milk and milk fat yields in dairy cattle, body weight and leg weakness in swine, and composition of fatty acids in meat will be also used for selection with constraints. Calculation of restricted BLUP as well as REML will require increased computing power.

3. Effects of development of molecular biology on animal breeding theory

3.1 Detection of QTLs segregation

Over the last few decades, we have developed molecular biology techniques and the associated analytical genetic tools. These advances have increased interest in using genotypic information to improve response to selection. In particular, theoretical studies on the detection of QTLs by using polymorphic DNA markers and on the use of QTL information for genetic improvement in livestock have developed rapidly in the last decade. These are a series of problems, including genetic marker linkage analysis and mapping, identification of marker loci linked to QTLs, and identification of QTLs. Interest of the next step can be divided into two directions. One is the analysis of gene function and genetic mechanisms by molecular geneticists; the other is the use of linkage associations or QTLs directly in the genetic improvement of economic merit by animal breeders and quantitative geneticists.

Dense marker linkage maps have been constructed for the most important domestic species. There are few theoretical problems in terms of yield. DNA polymorphisms are used as linked or direct markers to detect QTLs segregating in particular populations with specific allele frequencies. More recent practical studies have investigated polygenic traits with the aim of identifying QTLs for production traits such as growth, meat, milk, wool, fertility, and disease resistance, and the results of a large number of studies have been summarized in species-specific QTL maps (e.g. Bidanel and Rothschild, 2002; Khatkar et al, 2004). Finally, all QTLs with economic merits will

be identified and the location of the QTLs in the genome and the size of the QTL allele effects will be estimated.

Accurate estimation and improvement of the accuracy of estimation of QTL allele effects are most important in the use of QTL information for genetic improvement in livestock. The accuracy of this procedure depends on the number of animals and the heritability of the trait affected by the QTLs. In this respect, estimation of the genotype of the QTL allele is similar to breeding value estimation using BLUP methodology. After all, estimation of the genotype of the QTL allele is affected by the phenotype itself.

3.2 Selection efficiency of direct use of QTL information

Genetic markers can be used to identify specific regions of chromosomes where genes affecting quantitative traits are located. Marker-assisted selection (MAS) uses information about these regions in livestock selection programs to identify individuals with favorable combinations of QTL. Most researchers agree that MAS is likely to complement, rather than replace, conventional selection systems, leading to increased rates of genetic change. However, there is a risk of reduced genetic response if the marker association information is inaccurate, since MAS is a form of indirect selection. On the other hand, if assumed, error-free QTL information is used directly to predict response to selection, the genetic improvement is expected to be greater than that by using MAS and QTL information with errors. However, if the response to selection by using identified error-free QTL information is inferior to that by using conventional selection, information on the identified QTL will not be useful for genetic improvement. The potential benefit from using information on identified QTLs in selection is discussed in this and the next subsections.

The benefits of combining both the genotype and performance information have mostly been assessed in terms of the short- and medium-term genetic responses relative to traditional mass or BLUP selection. However, the benefits decrease in long-term selection (e.g. Larzul et al., 1997; Pong-Wong and Woolliams, 1998; Villanueva et al., 1999). The general conclusions are that the use of QTL information from genes with large effects or from markers linked to these genes significantly increases the short-term genetic response but has lower cumulated gain than the use of traditional selection methods such as phenotypic or BLUP selection. Figure 3 shows the typical responses to selection using QTL information. Loss of long-term response with genotypic selection or with BLUP selection based on genetic markers linked to a QTL is caused by a reduction in the effective intensity of selection that is applied to polygenes (Gibson, 1994). The loss in polygenic response is not offset by the increased response for the major gene. Build-up of gametic-phase disequilibrium between the major gene and the polygenes is also a reason why the result of genotypic selection is less than that of phenotypic selection in the medium to long term. The most important conclusion is that there is no large difference in response to selection whether or not QTL information is used for genetic evaluation.

3.3 Selection efficiency indirectly using QTL information

Much of the breeding of commercial plants such as rice, wheat, and soybean is based on pedigree selection, by which elite inbred lines are crossed and then self-fertilized for several generations to produce a large number of recombinant inbred lines that are tested to select a new set of elite inbreds.

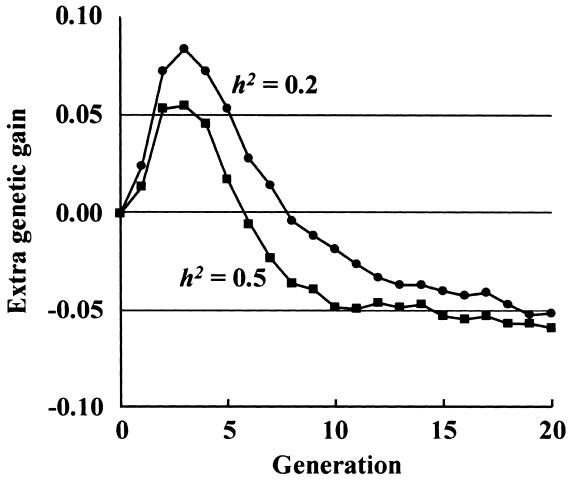

Fig. 3. Total genetic gain over 20 generations of selection using BLUP with the genotype information when selecting a performance trait with heritability (h^2) of either 0.2 or 0.5. Results are expressed as deviation from the predicted cumulated gain achieved with the conventional BLUP selection.

This breeding system may be effective in using QTL information. Furthermore, each plant species has many genetic resources that may include some important QTLs. Therefore, the advantages of using QTL information in animal breeding are smaller than in plant breeding, particularly if the allele effect of the identified QTL is not large.

Candidates for performance testing in cattle or in swine being grown for breeding stock are generally selected by information on relatives and by visual inspection. Additionally the use of information on a QTL identified in the first-stage of selection in a multi-stage selection system is more effective than the direct use of information on the identified QTL (Figure 4), even if the total genotypic variance of the identified QTL is around 20% or less of the total genetic variance of the trait. The use of QTL information may also be effective in evaluating genetic performance when it is applied to traits such as those that:

- have low heritability and/or are unmeasurable before sexual maturity
- are expressed late in life, i.e. lifetime productivity
- are sex-limited, i.e. reproductive or maternal performance
- are expensive and difficult to measure, i.e. disease resistance.

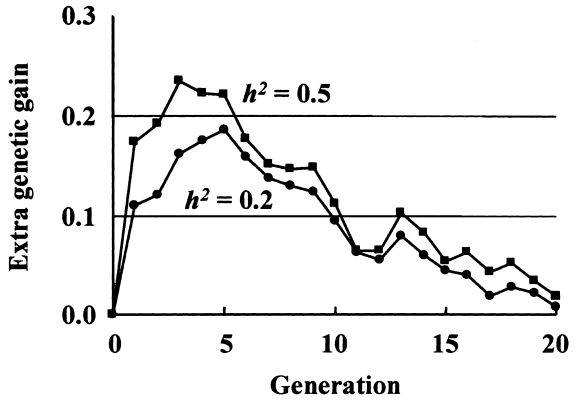

Fig. 4. Total genetic gain over 20 generations of two-stage selection using BLUP with the genotype information. First-stage selection was carried out within families on the basis of QTL information, and second-stage selection was based on BLUP selection. Results are expressed as deviations from the predicted cumulated gain achieved with conventional BLUP selection.

Little attention has been paid to research into unproductive traits, such as disease inheritance. However, owing to the high selection intensity of recent animal breeding systems, studies on disease inheritance or inbreeding depression will become important. In fact, single recessive genes that cause inherited diseases and have major negative impacts on productive traits have now been identified by using molecular genetic approaches (Raadsma and Tammen, 2005). Consequently, the design of breeding programs and genetic evaluation methods to exploit properly the benefits of major genes is urgently required.

4. Conclusions and implications

Almost one century has passed since the first step in animal breeding theory was taken. Recent advances in animal breeding theory have been driven largely by computer science and molecular biology. Conventional animal breeding systems will gradually change with these advances. The genetic model for evaluating candidates for selection will become more complex, and evaluation of combining both information on identified major genes (such as those for inherited diseases) and conventional performance in economic traits will be used in breeding systems. New molecular biological technologies will not replace existing animal breeding practices. Indeed, these new techniques will blend in with conventional methods that are used in breeding program design and genetic evaluation methods which covering both known and unknown QTLs. However, since genetic improvement of economic traits is very rapid, even if we were to continue to use traditional genetic evaluation systems, some traits would approach their selection limits in the near future. We need to consider the development of animal breeding theory aimed at genetic improvement by balancing genetic merit and genetic diversity.

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JIFS, 3 : 53 − 58 (2006) Special review

Challenges of Pig Breeding in Japan

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Abstracts

Genetic breeding systems for pigs over the last 30 years in Japan are roughly classifiable into three. Local government experiment stations and the national improvement center have used a closed-line breeding system. A second is an open breeding system used by private breeders and breeding companies. A third is breeding through introduction of hybrid pigs from foreign countries. The closed line breeding system has produced many excellent breeds from lines that have been selected not only for meat production traits, but also for reproductive traits and meat quality traits. Tokyo-X and Shimofuri-red were completed respectively in 1996 and in 2002. These two lines are breeds for which intramuscular fat was adopted as a direct selection trait. These successes achieved through such breeding are attributable to improved measuring methods of meat production traits and meat quality traits, along with improved statistical breeding methods. Now and in the future, breeding for disease resistance will become an important subject that attracts pig breeders' research efforts. One method of breeding for disease resistance is to select for morbid state as a direct selection trait. Aside from this, an indirect selection exists for immunity traits that are related genetically to disease resistance traits. We examine a breeding strategy that unites these two methods, and are performing selection now. Future selection methods might use marker-assisted selection with markers obtained through QTL analysis, for which candidate gene analyses might be effective.

Introduction

Japan has 55% pork self-sufficiency. The number of pig farms decreased to 9,430, representing a decline of about 20% over the 15 years preceding 2003. Pork with a high added value of safety and deliciousness is demanded to compete with increased pork imports. The average number of breeding sows per farm has expanded to 1031, concomitant with a decreasing number of farms, which has improved production efficiency in Japan. Local official experimental stations in Japan have undertaken improvement of pig breeding stock over the last 30 years. The closed line breeding system has produced many excellent breeds from lines that have been selected not only for meat production traits, but also for reproductive traits and meat quality traits. The Tokyo Metropolitan Livestock Experiment Station has developed a synthetic line called TOKYO-X (Hyodo, 1996). Base breeds of TOKYO-X were Berkshire, Duroc breed, and Chinese Beijing black pigs. This line was selected over five generations for meat production traits and intramuscular fat content (IMF). The author performed selection experiments for meat production traits and IMF for the Duroc breed at the Miyagi Prefecture Livestock Experiment Station (Suzuki et al., 2005a, 2005b). These breeding successes are attributable to improved measuring methods of meat production traits and meat quality traits, along with improved statistical breeding methods. Since December 2004, addition of antifungal agents to domestic animal feed has been planned for prohibition in Japan. Sub-therapeutic antibiotics that are added to pigs' feed engender bacillus' increased resistance to antibiotics. Such practices present the dangerous possibility of bacillus infection of humans. Moreover, antibiotics and antifungal agents present the danger of remaining in the meat after processing. The market also demands meat produced without use of potentially harmful agents. Corresponding to such a situation, breeding for disease resistance is demanded. The Miyagi Prefecture Livestock Experiment Station, along with Tohoku University, has begun a selection experiment of the fifth generation for atrophic rhinitis (AR) and mycoplasma pneumonia (MPS). Furthermore, potential relationships among candidate genes for health and immune response will be investigated. We introduce the outline of the anticipated breeding improvement in our country, especially, improvement

Table 1. Completed and progressing line breed in Japan (1975–2005)

Breeds	Completed Line	Maintained Line	Progressing Line		Total
	a	b	c	a+c	b+c
Landrace	39	18	3	42	21
Large white	21	12	6	27	18
Hampshire	5	0	0	5	0
Duroc	8	5	3	11	8
Berkshire	3	3	0	3	3
Synthetic line	1	1	1	2	2
Total	77	39	13	90	52

of meat quality traits and disease resistance, and the possibility of genetic improvement using DNA markers.

Current situation of pure line breeding

As for pig breeding in our country, the closed population breeding that the late Dr. Takeo Abe advocated has been done for the past 30 years (Abe et al., 1981). The method to produce fattening pigs with crossing of the improved pure breed line has been adopted. The breeds of the line that had been improved in the past 30 years are shown in Table 1 along with the number of lines. The features of line breeding can be summarized as follows. Genetic lines with highly improved growth rates were produced during ten years. Backfat thickness was constant thickness. This reflects the carcass grading in Japan. Recently, selection methods have changed from index selection to use of breeding values estimated by BLUP method (Suzuki, 2004). Selection traits have changed from meat production traits to reproductive traits, meat quality traits and disease resistance traits. Selection traits with feature and number of lines were as follows. Reproductive traits (Litter size): four Landrace line and four Large White line in these 5 years, meat quality traits (intramuscular fat): One synthetic line (TokyoX) and one Duroc line (Shimofuri Red), leg soundness trait (direct selection trait and independent culling trait): seven lines, Disease resistance: one line (Miyagi prefecture).

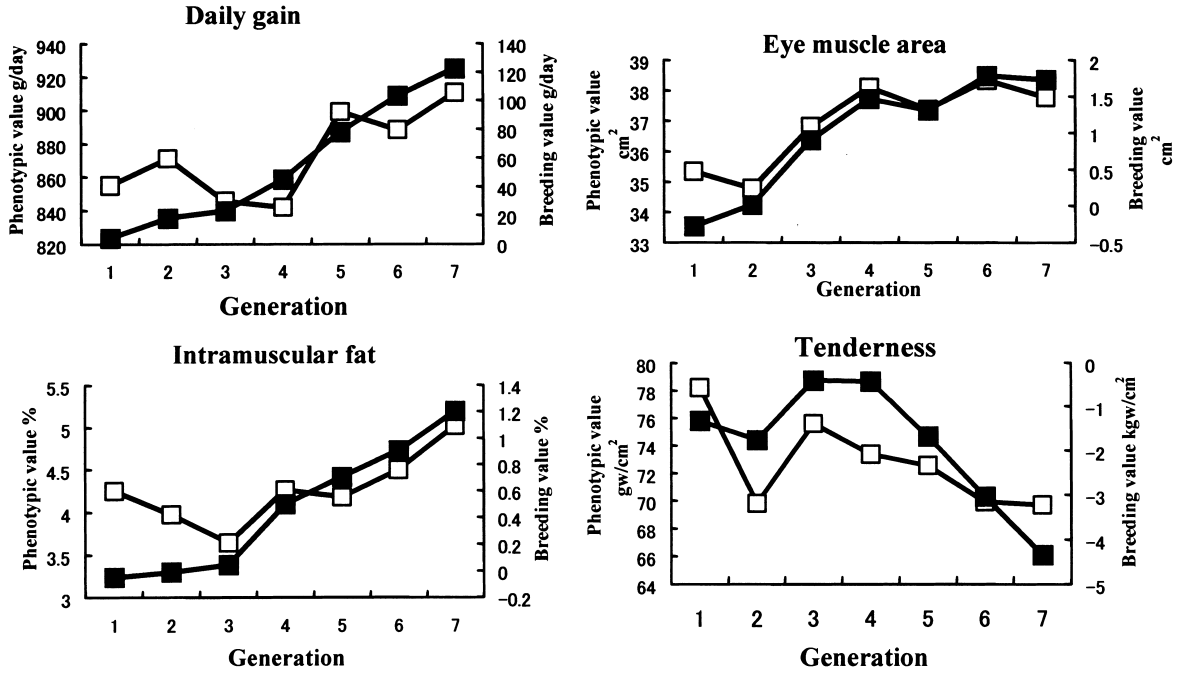

Fig. 1. Changes of phenotypic and breeding value over seven generations of selection.
□ – phenotypic value, ■ – breeding value.

Selection experiment for high meat quality

To develop an excellent line of Duroc breed in performance traits and meat quality traits, selection was conducted over seven generations for growth rate (DG), real-time ultrasound loin eye muscle area (EM), backfat thickness (BF), and IMF. Meat quality traits were IMF and tenderness (TEND), which were measured after slaughter in *M. longissimus* taken two sections above the last rib. Selection was based on a selection index that comprised four traits (DG, EM, BF, and IMF), at the first and second generations of selection. After the third generation, expected breeding values (EBVs) for four traits were obtained from analyses of performance and meat quality data. Selection of boars and gilts was based on an aggregate genotypic value that was evaluated by the sum of cross products of each EBV and the corresponding relative economic weight. Although the desired gains were not achieved completely for DG, EM, and BF, the total genetic gain of IMF at the seventh generation (1.20%) exceeded the initially intended desired gain (0.7%), and the phenotypic mean of the IMF level reached about 5.0%. Estimates of genetic parameters are useful in aiding selection for pig meat quality. The present experiments also showed that meat quality traits were improved effectively through a selection process that used sibling information regarding IMF and TEND.

Table 2 shows heritability estimates. Respective heritability estimates of DG, EM, and IMF were moderate and around 0.5. That of BF was estimated to be high (0.72). Concerning the meat production trait under *ad libitum* feeding, the respective average estimates of heritability for DG and BF were 0.31 and 0.49 (Clutter and Brascamp, 1998). Recent heritability estimates for BF reported by Kuhlers et al. were 0.56 for Landrace (2001) and 0.58 for Duroc (2003). In addition, 0.47–0.48 heritability has been reported for EM (Sellier, 1998; Kuhlers et al., 2001). The present estimate was of the same order as that reported previously, but estimates for DG (0.48) and BF (0.72) were higher than previously reported estimates. Therefore, heritability of these traits is moderate or high and improvement of these traits appears possible. The heritability of IMF was moderate (0.46). Hovenier et al. (1993), NPPC (1995) and Sellier (1998) presented mean heritability estimates of around 0.50 for intramuscular fat. Recent estimates were 0.38 for Large white, 0.67 for Landrace, and 0.42 for Pietrain by Knapp et al. (1997), 0.44 for Large white by Larzul et al. (1997), 0.35 for Large white and Landrace by Hermesch et al. (2000a), and 0.25 for Iberian pigs by Fernandez et al. (2003). In addition, Knapp et al. (1997) reported high common environmental effects for IMF (0.14 for Large white and 0.16 for Pietrain). In the present experiment, the common environmental effect for IMF was 0.06. Moderate heritabilities of 0.52 and 0.34 were estimated, respectively, for the daily feed intake and feed conversion ratios (Clutter and Brascamp, 1998). Heritability estimate for Tenderness of 0.45 were higher than estimates presented by Lo et al. (1992), de Vries et al. (1994) and NPPC (1995) of 0.17, 0.20 and 0.20, respectively. These heritabilities for tenderness were assessed using shear force measurements by a Warner-Bratzler or Universal

Table 2. Numbers of animals (N), means, standard deviations (SD), heritabilities (h^2) ± standard errors (SE), common environmental effects (c^2) ± standard errors (SE).

Traits		N	Mean	SD	h^2 ± SE	c^2 ± SE
Daily gain,	g/day	1,642	873.6	109.3	0.48 ± 0.02	0.04 ± 0.01
Eye muscle area,	cm^2	1,639	37.00	4.05	0.45 ± 0.03	0.02 ± 0.01
Backfat thickness,	cm	1,642	2.37	0.43	0.72 ± 0.03	0.01 ± 0.01
Intramuscular fat,	%	543	4.25	1.46	0.46 ± 0.03	0.06 ± 0.02
Tenderness,	kgf/cm^2	544	72.51	12.71	0.45 ± 0.04	0.06 ± 0.02
Feed conversion ratio		379	2.65	0.17	0.34 ± 0.04	0.19 ± 0.03
Daily feed intake,	kg/day	379	2.62	0.23	0.52 ± 0.03	0.06 ± 0.01
Drip loss,	%	543	2.21	1.31	0.14 ± 0.01	0.17 ± 0.02
Cooking loss,	%	545	24.7	3.33	0.09 ± 0.02	0.16 ± 0.02
Pork color standard		541	3.42	0.46	0.18 ± 0.02	0.08 ± 0.01
L* value		543	48.44	3.16	0.16 ± 0.02	0.15 ± 0.02
pH		515	5.97	0.43	0.07 ± 0.02	0.22 ± 0.02

Testing machine. Further, Hovenier et al. (1993) reported that heritabilities for tenderness assessed by shear force measurement and by taste panels vary from 0.21 to 0.37. In addition, Sellier (1998) reported a 0.26 average heritability for tenderness by instrumental determination, and 0.29 for that of sensory panel scores. High heritability in the present study suggests that the Tensipresser is an appropriate device to evaluate meat tenderness (Nakai et al., 1992). Low heritabilities for water-holding capacities of DL and CL (0.14 and 0.09, respectively) were also estimated. Hovenier et al. (1993) reviewed a wide range of heritability estimates of 0.00–0.63, probably because of the different methods used to measure the trait in their review. The average heritability for water-holding capacity is about 0.20. In his review, Sellier (1998) also reported average heritability of 0.16 (0.01–0.31) for drip loss and 0.16 (0.00–0.51) for cooking loss. Heritability estimates for meat color of PCS and L (0.18 and 0.16, respectively) were lower than the estimate (0.29) presented in a recent study by Hermesh et al. (2000a) as well as mean heritability estimates presented in reviews by Hovenier et al. (1993) and Sellier (1998); those respective averages were 0.30 and 0.28. However, Lo et al. (1992) and Knapp et al. (1997) reported a lower estimate of 0.11 for Landrace and Duroc pigs and 0.12 for Landrace, which are approximately the estimates found for Duroc in this study. Heritability estimates for ultimate pH (0.07) were lowest in this study and lower than estimates presented in reviews by Hovenier et al. (1993) and Sellier (1998) and other estimates presented by Knapp et al. (1997), Larzul et al. (0.13, 1997), Sonesson et al. (1998), and Hermesh et al. (2000a).

This pure breed Duroc line (named "Shimofuri-red") produces high-quality pork. Therefore, it sells at a stable price (570–600 yen/dressed carcass kilogram). Production of 10,000 fattened animals per year is planned with this pure breed. It is possible to specifically address production as a farmer because of the steady price offered for this pork. Moreover, the consumer can taste high-quality pork that is improved in a scientific manner. Its value to the consumer is proven.

New approach for genetic improvement using DNA marker within breed

1) Association of IMF with candidate genes in Duroc pigs

We investigated the association of intramuscular fat with candidate genes in Duroc pigs. The relation between IMF and Heart Fatty Acid Binding Protein (H-FABP) gene, which was the candidate gene, was examined for efficient IMF selection. First, the change in each RFLP frequency of the H-FABP gene according to the selection of generation was examined using the Duroc breed, in which IMF was improved by repeated selection. Next, we examined whether a difference of IMF content existed between H-FABP gene polymorphisms. Moreover, the effect of the genotype on the IMF was presumed; the way in which RFLP of the H-FABP genes influenced IMF content was examined. The H-FABP genotype frequencies genotyped for the HaeIII, HinfI and MspI PCR RFLP changed significantly according to the generation of the selection. A significant difference was detected in the breeding value of IMF between the H-FABP PCR RFLP genotypes. The AA genotype has a significantly larger positive effect on the IMF breeding value than that of the Aa and aa genotypes in MspI RFLP. In addition, the DD genotype has a significantly greater positive effect on IMF breeding value than the Dd and dd genotypes in HaeIII RFLP. In HinfI RFLP, the hh genotype has a significantly larger positive effect on IMF breeding value than the HH genotype. Multiple regression analyses that used the IMF breeding values have referred to the three H-FABP genotypes as independent variables. The dependent variable was the IMF breeding value. Results revealed that the contribution rate of the genotypes was about 40% (Table 3). These results demonstrated that H-FABP RFLPs affect IMF in this Duroc population.

Table 3. Effect of H-FABP genotype on the breeding value of intramuscular fat

Dependent variable	Independent variable	R^{2a}
IMF breeding value	MspI BV[b], HaeIII BV, HinfI BV	0.394
IMF breeding value	MspI BV, HaeIII BV,	0.389

[a]R^2: Coefficient of determination of multiple regression.
[b]BV: Breeding value.

Table 4. Results of QTL analysis for selection and correlated traits

Traits	Maximum LOD score	Position of maximum LOD (cM)
Daily gain	0.11	80
Backfat thickness	0.55	81
Loin muscle area	2.77**	70
Intramuscular fat	0.13	15
Tenderness of meat	0.82	71

2) Using DNA marker information (QTL analysis)

Quantitative traits loci (QTL) analysis was executed within Duroc breed on the seventh chromosome for meat production and meat quality traits. Regarding this seventh chromosome, the significant QTL region for intramuscular fat has already been reported (Sato et al., 2003). The polymorphism of 12 microsatellite markers that was arranged at about 20-cM intervals was investigated in about 1004 pigs. A significant region of QTL for intramuscular fat was not detected by QTL analysis using the data corrected with BLUE. Therefore, it was suggested that the QTL does not exist in the seventh chromosome related to intramuscular fat. However, significantly maximum LOD was detected in the 70-cM region for the loin eye muscle area (EM) (Table 4), which suggests that the intra-breed QTL analysis was effective using the population of line breeding of pigs. However, the QTL heritability was 0.07, and the heritability by polygene, aside from the effect of QTL was 0.46. Further QTL analysis is necessary to search the region for other chromosomes that are related to intramuscular fat.

Genetic improvement of chronic disease resistance in pigs

The potential for genetic improvement of resistance against chronic infectious diseases was investigated in Duroc pigs. Five immunities were measured for each. Furthermore, morbid changes caused by AR and MPS were measured in two full-brothers of the candidate. Daily body weight gain was measured. Genetic and phenotypic parameters of delayed type hypersensitivity, phagocyte activity, antibody productivity, morbid changes engendered by AR and MPS, daily body weight gain, back fat thickness, and eye muscle area were estimated using restricted maximum likelihood (REML). Two selection indices were made based on those parameters to delete morbid changes attributable to AR and MPS from the population. One index was based on morbid changes in the two full-brothers of the candidate; another index was based on the three immunities of the candidate itself. Intensities of selection were assumed as unity in the indices. Seven or eight generations of selection based on the indices were inferred to be sufficient to yield a population showing almost no morbid changes by AR and MPS (Nishida et al., 2001). Based on these results, a selection experiment with Landrace pigs for disease resistance traits and growth began in 2004 at the Miyagi Prefecture Livestock Experiment Station. Selection traits are the degree of morbid states by AR and MPS. In addition, some immune response phenotypes will be used as selection criteria and QTL analyses will be used to investigate immune traits within breeds.

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Selection for Growth and Feed Efficiency – The Australian Experience

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Abstract

Profitability in beef production is influenced by a number of traits, including growth and feed efficiency. This paper reviews key Australian selection experiments on growth and feed efficiency, and the beef industry adoption of selection for these traits. Response to selection for growth has been demonstrated by a divergent selection experiment. Five generations of divergent selection for growth resulted in 19% divergence in yearling weight and 18% in weaning weight, and no effect on carcass composition at maturity. Selection for growth in industry herds started in the 1980s, with a steady rate of genetic gain being achieved. In Australian Angus seedstock population, for example, the annual genetic gain in estimated breeding values (EBVs) for 400-day weight was 0.15 standard deviation units from 1998 to 2003. As with growth, selection for feed efficiency has been demonstrated by a divergent selection experiment for residual feed intake (RFI). Two generations of selection produced an annual divergence of 0.25 kg/day of 10MJ ME feed with no correlated responses in growth and meat quality of young cattle. Cow weight and reproduction were not affected, however, High RFI cows tended to have higher subcutaneous fat depth relative to Low RFI cows. Testing for RFI in industry herds started in 1996, and from 2002, RFI EBVs are provided for seedstock Angus and Hereford cattle. Several studies have indicated substantial economic benefit from selection for low RFI, however the initial high cost of testing to identify superior animals is an impediment to industry adoption.

Introduction

Beef cattle breeders world-wide place considerable emphasis on selection for growth. It is relatively easy to measure, and is closely related to profitability of beef enterprises, especially under feedlot conditions (Dickerson et al., 1974). Also related to profitability is feed cost. Providing feed for cattle is the single largest input cost in most livestock production enterprises (Schmidt et al., 2001). Hence improvement in the efficiency of feed utilisation is essential in reducing production cost. The objective of this paper is to review key selection experiments in beef cattle in Australia on growth and feed efficiency and the adoption of selection for these traits in the beef industry.

Selection for growth

In the early 1970s beef producers in Australia began to place considerable emphasis on selection for growth and size. While it was known that selection for growth rate would result in faster growing and heavier animals, there was little information on the expected changes in other economically important traits such as reproductive performance, milk production, carcass quality and other components of herd profitability. To complement the cattle experiments already in progress (listed in the review by Mrode, 1988), selection experiments were set up in Australia and New Zealand (listed in the review by Parnell and Morris, 1994) in the 1970s to address some of these issues under pasture conditions.

Divergent selection for growth rate at Trangie

An experiment to evaluate the effects of selection for yearling growth rate (average daily gain from birth to yearling age) on the major components of herd profitability was conducted at the Agricultural Research centre at Trangie, Australia from 1973 to 1992. Two divergent selection lines (High line and Low line) and an unselected Control line were created in a closed Angus herd. Seventeen years of selection resulted in 5.5, 5.0 and 5.1 generations of selection for High, Control and Low lines, respectively. The rate of inbreeding (0.74% for High line, 0.53% for

Control line and 0.88% for Low line) was low due to the high effective population sizes of 67, 106 and 71 per generation for High, Control and Low selection lines, respectively. Average selection differentials per year were 0.016 kg/day and -0.018 kg/day for the High and Low selection lines, respectively, resulting in a corresponding direct selection response per year of 0.006 kg/day and -0.007 kg/day. Using animals recorded prior to 1964 as the base, the mean estimated breeding value (EBV) for yearling growth rate at the start of the experiment (1974) was calculated to be 0.015 kg/day. After 17 years (1991) of selection, average yearling growth rate EBV for High line calves was 0.115 kg/day, compared to 0.030 kg/day for the Control line and -0.060 kg/day for the Low line calves. Realised heritabilities for yearling growth rate were 0.37 ± 0.09 for the High line and 0.38 ± 0.09 for the Low line (Parnell et al., 1997). These results are also in agreement with expectations from published genetic parameters as reviewed by Koots et al. (1994a,b).

The annual correlated responses in calf growth traits up to yearling age are presented in Figure 1. Control Line means for birth weight, weaning weight, and the weight, height, girth, length, pelvic area and scrotal circumference at yearling age were 31 kg, 197 kg, 274 kg, 102 cm, 153 cm, 129 cm, 185 cm^2 and 33 cm, respectively (Arthur et al., 1997). Mature weight (kg) and height (cm) of cows were 540 ± 6 kg and 119.3 ± 0.7 cm for the High Line, 497 ± 6 kg and 115.7 ± 0.7 cm for the Control Line, and 418 ± 6 kg and 108.3 ± 0.6 cm for the Low Line. No significant differences between the lines were found in rate of maturation of weight, height and girth (Archer et al., 1998b). These positive responses to liveweight at all ages are in agreement with other selection experiments reviewed by Mrode (1988). In a serial slaughter study, the mean mature empty body weight (body weight minus urine and gut content) was 666 ± 17 kg, 588 ± 18 kg and 512 ± 16 kg for High, Control and Low line steers, respectively. However, no significant selection line differences were obtained in carcass composition at maturity (Perry and Arthur, 2000). These results indicate that selection for yearling growth rate leads

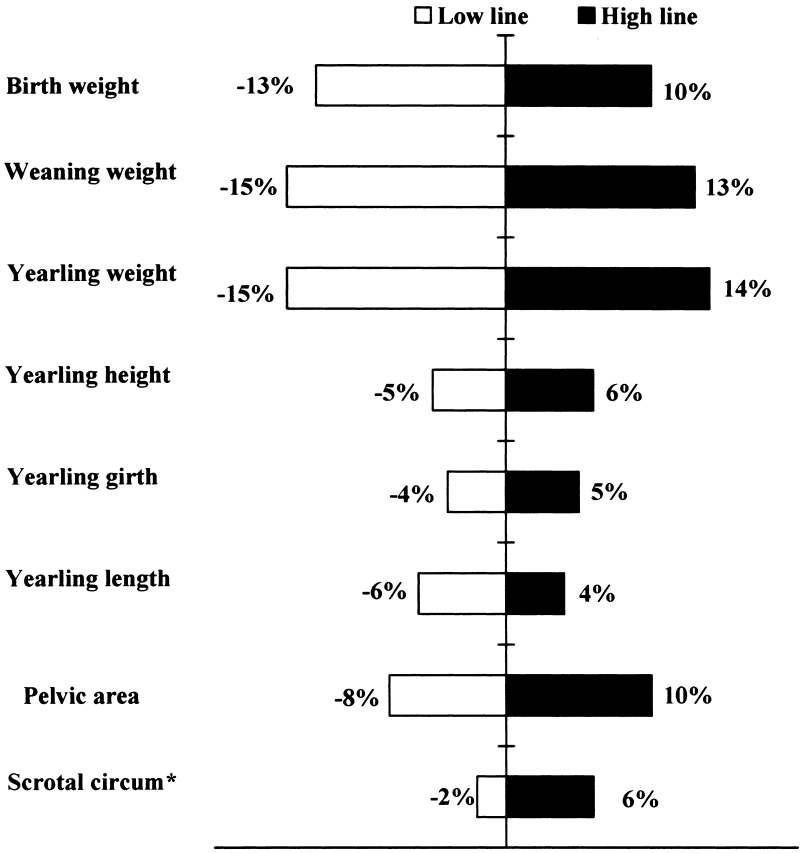

Fig. 1. Correlated responses to selection for yearling gain, as deviations from Control Line means. *Denotes scrotal circumference. [Arthur et al., 1997].

to a change in body size at all ages but no change in body composition at maturity. Recent analyses of data from about 4,000 cattle indicate favourable genetic associations between growth rate and several meat quality traits of cattle in temperate Australia (Reverter et al. 2003).

Heifers from the High Line reached puberty at a younger age (324 days) than Control Line heifers (336 days), who in turn reached puberty at a younger age than Low Line heifers (355 days). Cows from the High line had similar reproductive rates (per cow mated), at calving (86%) and at weaning (83%) compared to the Control Line (means of 84% and 78%, respectively), while the Low Line (means of 77% and 69%, respectively) cows had significantly lower reproductive rates (Archer et al., 1998a). Similar responses in reproductive rates were obtained in a High and Control growth rate selection experiment in New Zealand (Morris et al., 1992). These results show that selection for high growth rate did not compromise reproductive performance.

Application in industry - Growth

BREEDPLAN is a genetic evaluation system for beef cattle, for Australia, New Zealand and other international countries. It generates EBVs for growth, carcass, fertility and calving ease trait complexes of animals from performance recorded seedstock herds of all the major cattle breeds (Graser et al. 2005). BREEDPLAN EBVs for growth traits have been available since 1985. Initially all available Australian data were used to generate genetic parameters (Meyer et al., 1990; Meyer 1994) that underpinned BREEDPLAN evaluations. Figure 2 shows the rate of genetic gain in EBVs for Australian Angus cattle over four periods from 1980 to 2003 (Barwick and Henzell, 2005). It shows a clear trend of increasing rate of genetic gain in 200-day and 400-day weights. The rate of genetic gain in birth weight increased in the first two periods (1980-85) but declined during the last two periods (1992-2003). The changes observed in the industry rate of genetic gain reflect the improved ability of seedstock breeders, over time, to select for other traits at the same time as selecting for growth. The uptake of multi-trait selection for economic merit was enhanced by the development of the selection index program, BreedObject, and the subsequent release of the "BreedObject on the web" version (www.breedobject.com).

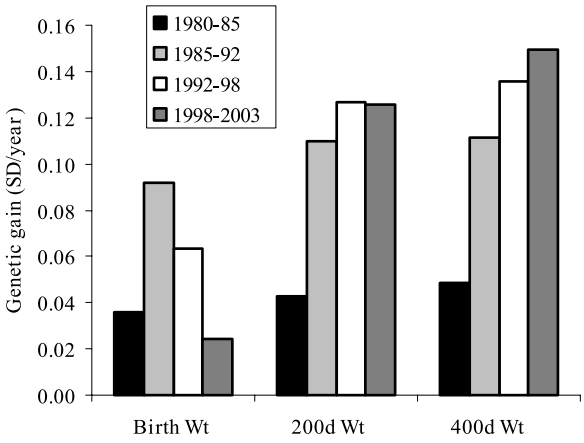

Fig. 2. Rate of genetic gain in growth in estimated breeding values for growth in Australia Angus over four periods: 1980-85 (pre-BREEDPLAN), 1985-92 (early BREEDPLAN – growth traits only); 1992-98 (BREEDPLAN – growth, carcass and fertility); 1998-2003 (BREEDPLAN and Selection indexes). [Adapted from Barwick and Henzell, 2005]

Selection for feed efficiency

The importance of feed efficiency to profitability in beef production is obvious. However, measuring individual animal feed intake is difficult and expensive, and this constraint has been responsible for the paucity of research into genetic variation in feed intake and efficiency. With recent advances in computing and electronics, reliable automatic feed intake recorders have been developed which make it easier to measure feed intake. This has resulted in renewed interest in research in this area.

Feed intake is correlated with liveweight and level of production. Hence to relate feed intake to production system efficiency, several feed efficiency traits have been developed over the years. These traits include feed conversion ratio (FCR), partial efficiency of growth, maintenance efficiency, efficiency of lactation and residual feed intake. The definitions, computational formulae and the relationships among the feed efficiency traits and with growth traits have been reported by Arthur et al. (2001b). Residual feed intake (RFI) is defined as the difference between an animal's actual feed intake and it's expected feed intake, based on it's size and level of production (eg. growth rate in beef cattle). Residual feed intake is phenotypically independent of size and level of production, and it is fast becoming the trait of choice in studies on efficiency of feed utilisation.

There have been three major integrated projects in Australia since 1993 on feed efficiency. They are the Trangie, Beef CRC I, and Beef CRC II projects, and have been described by Arthur et al. (2004). The projects include a divergent selection experiment and the generation of data for estimation of genetic parameters.

Divergent selection for feed efficiency at Trangie

An experiment to evaluate the effects of selection for postweaning RFI in Angus cattle was started in 1993 at the Agricultural Research Centre at Trangie, Australia. It was the first RFI single-trait selection experiment in beef cattle in the world. Starting with the 1993-born animals, two divergent selection lines were created: the Low RFI (more efficient in feed utilisation) and High RFI (less efficient in feed utilisation) selection lines. Five years of selection (ending with the 1999-born animals) resulted in 1.73 and 1.96 generations of selection for the Low and High RFI lines, respectively. The inbreeding rate was low (0.6% for dams and 1.6% for calves) due to the high effective population sizes of 42 and 43 per generation for the Low and High RFI lines, respectively. Average selection differentials per year were -0.318 kg/day and 0.387 kg/day for the Low and High RFI lines, respectively, resulting in a significant average annual response (divergence between the lines) of 0.249 kg/day of feed with 10 MJ ME (Arthur et al., 2001a). During the period of study the cost of feed was $200 per 1000 kg, hence the divergence between the selection lines in the 1999-born progeny (1.247 kg/day) represents savings of $27 in feed costs per animal over a 100 day feeding period. Mean EBV for RFI for the 1993 foundation animals were used as the zero base (Fig. 3), and by 1999 the means were 0.562 kg/day and -0.508 kg/day for the High and Low RFI lines, respectively.

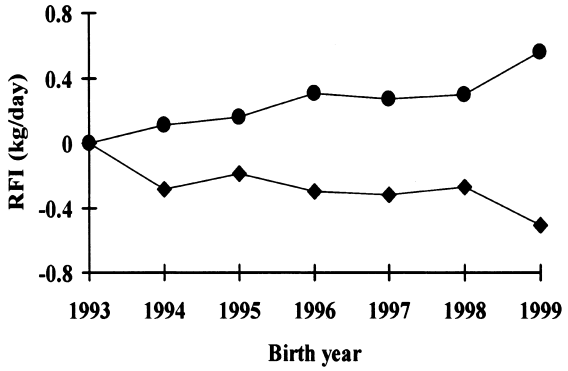

Fig 3. Trends in estimated breeding values for residual feed intake (RFI) for Low (♦) and High (●) RFI selection lines [Adapted from Arthur et al., 2001a]

Correlated responses in yearling weight (mean of 383 kg) and average daily gain (mean of 1.4 kg/day) were not significant. However, the annual correlated response of 0.24 kg/day for feed intake (mean of 10 kg/day) and 0.24 for FCR (mean of 7.2.) were significant (Arthur et al., 2001a). These results indicate that selection for low RFI results in improvement in postweaning efficiency of feed utilisation with minimal effect on growth.

The 1997-, 1998- and 1999-born females were used to study the effect of divergent selection for RFI on maternal productivity across three mating seasons, starting from 2000. Rib fat depth on the cows was measured by ultrasound at the start of mating season and six months later at the weaning of their calf each year. Differences in fatness were not significant except for those measured at the start of the 2000 (10.8 ± 0.4 mm *v*. 9.3 ± 0.4 mm), 2001 (11.3 ± 0.4 mm *v*. 9.8 ± 0.4 mm) and 2002 (7.0 ± 0.5 mm v. 5.7 ± 0.5 mm) mating seasons, where High RFI cows had significantly higher rib fat depths. There were no selection line differences in cow weight (measured four times a year), pregnancy (mean of 90.4%), calving (mean of 88.7 %) and weaning (mean of 80.8 %) rates, milk yield (mean of 7.7 kg/day) and weight of calf weaned per cow exposed to bull (mean of 195 kg). These results indicate that after 1.5 generations of divergent selection for residual feed intake there are no significant selection line differences for maternal productivity traits (Arthur et al., 2005). Feed intake was not measured during this study, however results from an earlier study on mature cows (Archer et al., 2002) suggests that the Low RFI cows would have consumed less feed relative to the High RFI cows.

Application in industry – Feed Efficiency

By 1996, there was interest by the Australian beef industry to test animals for feed efficiency, and a number of centralised test stations have developed across southern Australia. A Standards Manual, with detailed procedures for feed efficiency testing, was developed, and test stations were required to be

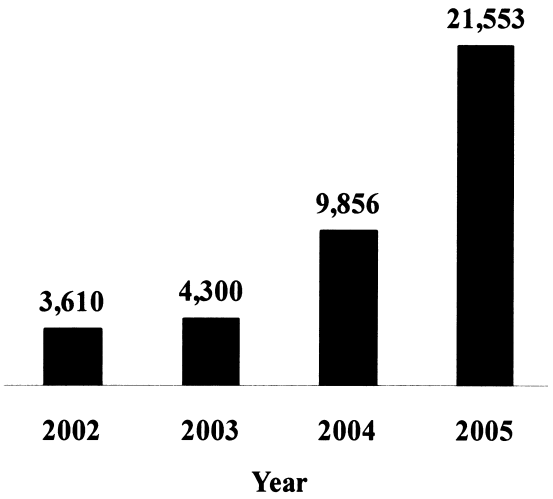

Fig.4. Number of Angus cattle with estimated breeding values for residual feed intake.

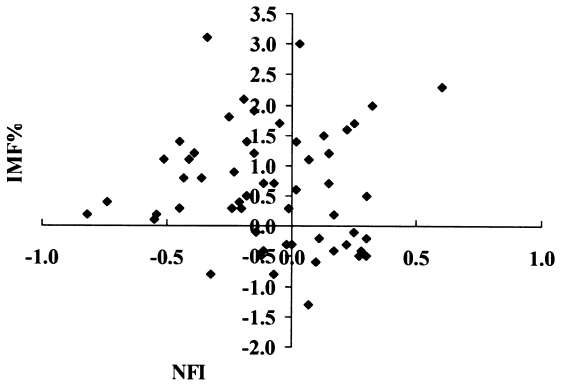

Fig 5. Estimated breeding values for intramuscular fat (IMF) and net (residual) feed intake of industry Angus bulls in Australia.

accredited before data can be used in BREEDPLAN. By 2002, there were enough data collected on industry animals for BREEDPLAN to start providing EBVs for RFI (also known as net feed intake or NFI in the industry). From 2004, plasma concentration of insulin-like growth factor-I (IGF-I) was included in the generation of the NFI EBVs (Moore et al., 2005). The number of cattle (Angus and Hereford breeds) with EBVs for RFI is shown in Figure 4.

Genetic parameters for feed efficiency

A comprehensive review of information available pre-1996 on the genetics of feed efficiency in cattle was provided by Archer et al. (1999), and subsequent new information was included in the review by Arthur et al. (2004a). Recent studies by Robinson and Oddy (2004) and Schenkel et al. (2004) have provided additional genetic information. These results have been confirmed in Japanese Black (Wagyu) bulls in a recent publication by Hoque et al. (2005). All the reviews and major studies highlight the existence of genetic variation in feed efficiency and the fact that most feed efficiency traits are moderately heritable, hence the potential for genetic improvement. Whereas there is some information on the genetic relationships between carcass and meat quality traits with FCR (Koots et al. 1994b), information on their relationship with RFI is limited. Genetic correlations have been reported between RFI and carcass lean percentage of 0.17 by Jensen et al. (1992) and -0.47 by Herd and Bishop (2000); and between RFI and subcutaneous rib fat of 0.48 by Robinson and Oddy (2004) and 0.30 in Wagyu cattle by Hoque et al. (2006). The differences among the studies, in the value of the genetic correlations may be due to the maturity level at which the RFI and the fatness were measured. Results on cattle, after 1 generation of divergent selection, for RFI indicate that there is no correlated response in meat tenderness (shear force) and marbling (McDonagh et al. 2001). However, Hoque et al. (2006) have reported a favourable and moderate genetic correlation between RFI and marbling in Wagyu cattle. Data from the 2005 Angus BREEDPLAN analysis indicate a significant number of bulls which have low EBVs for NFI (high efficiency) and high EBVs for marbling (Fig 5; Exton 2005, per. comm.).

Challenges to adoption of feed efficiency technology

The high cost associated with the identifying cattle that are superior for feed efficiency is the major impediment to adoption. Simple, low-cost alternatives need to be developed. Although physiological markers like plasma IGF-I do help, gene markers for feed efficiency, if discovered will go a long way to improve adoption. Although there are studies world-wide to find such genes, few results have been published. Pitchford et al. (2002) reported five QTL for feed intake and efficiency in cattle. Recent studies have shown associations between polymorphisms in the leptin gene and feed intake and energy balance in dairy and beef cattle (Liefers et al. 2002, Nkrumah et al. 2004a, 2005).

Benefits from selection for feed efficiency

A number of comprehensive economic analyses on the benefit of selection for feed efficiency, based on RFI, have been conducted, and all indicate that in spite of the high cost of testing for feed efficiency, the economic benefits to the individual producer and to the beef industry is substantial (Exton et al., 2000; Archer et al., 2004; Wood et al., 2004). In addition to the economic benefits, recent studies have highlighted the potential to use selection for RFI as a greenhouse mitigation strategy. Studies by Nkrumah et al. (2004b) and Hegarty et al. (2005) indicate that methane production from Low RFI steers was approximately 6-8% lower than those from High RFI cattle.

Gaps in knowledge

There are clear gaps in our knowledge which need to be filled. Most of the information currently available is on the growing animals. There is the need for knowledge of the phenotypic and genetic relationships of feed efficiency in different parts of the production system with all other economically important traits. There is also the need for information on possible interactions of feed efficiency with different levels of feed quantity and quality. Related to this is the lack of information on genotype by environment interactions on feed intake and efficiency. Finally there is a need for information on non-additive genetic effects, such as heterosis, on feed efficiency.

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The Long Road to a Representative *In Vitro* Model of Bovine Lactation

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Introduction

The biology of the mammary gland of the dairy cow has long been the focus of scientific investigation. The mammary gland represents a unique system for the study of organogenesis, and it has the distinctive ability to enter a cyclical process of development and regression along with successive cycles of pregnancy and lactation. As yet, however, the mechanisms controlling this are poorly understood, particularly for cattle (Chotteau-Lelievre *et al*. 2003). Moreover, during lactation the mammary gland is involved in the mass transfer of nutrients from the blood into milk, though the transport of a number of these is poorly understood. For example, the calcium in cows' milk is important in human nutrition, but relatively little is known about its transfer into milk (Neville, 2005). Furthermore, *in vivo* experimentation has not been able to shed a great deal of light on these mechanisms (e.g. Barlet *et al*. 1992).

There are two fundamental disadvantages of studying lactation *in vivo*: Firstly, because of the ability of mammals to maintain a constant internal environment, treatments that are applied can have systemic effects that confound the net effect at the mammary gland. Controlling the environment of the milk secreting epithelial cells *in vivo* in a predictable way is therefore very difficult. In contrast, an accurate model of mammary function would allow the mammary component to be isolated and its environment to be controlled independently of systemic effects. Secondly, there is an unease amongst the general public (in the UK at least), over the use of animals for experimentation, particularly when that research is not for medical purposes (e.g. MORI, 2002). Taking these arguments together, lactational physiologists would therefore find a representative model of bovine lactation of major use in gaining a greater understanding of mammary biology.

A Brief Critique of In vitro *Systems of Ruminant Mammary Gland Biology*

A number of attempts have been made to replicate the function of the bovine mammary gland *in vitro*. The methodologies involved have been reviewed (Blum *et al*. 1989; Ip and Darcy, 1996; Matitashvili *et al*., 1997; Shaw *et al*. 2004). The earliest attempts to model the mammary gland in culture used rodent explants containing whole alveoli (Elias, 1957). This methodology has been used several times in the intervening years for cattle tissue (e.g. Feuermann *et al*. 2004; Yang *et al*. 2005). The major advantage of explant culture is that the cellular composition of the mammary tissue, including the extracellular matrix, is (at least initially) similar to that of *in vivo* tissue. Accordingly, the effects of hormones and growth factors etc. may be investigated under a comparatively native environment. However, the interpretation of the results of explant cultures can be difficult. Firstly, there is the potential of carry-over effects from the animal from which the mammary tissue was obtained, including latent growth factors and hormones activated during the incubation. Secondly, there is a difficulty of identifying the primary cellular target of factors added to the media. An additional difficulty is that of determining changes that occur in each cell type within the tissue. Finally, explants remain viable in culture only for a limited period of time.

An alternative approach is to use mammary epithelial cells (MEC) that have been isolated and separated from the extracellular matrix of mammary tissue. These are then plated onto cell culture-ware, usually existing as a monolayer of cells. Freshly isolated cultures of primary MEC have been used extensively in this way. More recently, immortalised as well as clonal lines of bovine MEC have also been used. Methodologies for the maintenance of primary cultures of bovine MEC were first developed over two decades ago (Mackenzie *et al*. 1982; 1985), and since then there has been a constant development of techniques to replicate the biology of the mammary

gland *in vitro*. However, even now, a good many of these attempts may be regarded as deficient in one or more of the following ways:

Experiments are often performed on cells that are relatively undifferentiated (that is, they do not have the intracellular biochemical processes that occur in the natural state). This is because many studies have used MEC plated on cell culture plastic-ware (e.g. Cheli *et al*. 2003). When MEC are cultured in this way, they form a monolayer attached to the plastic that excludes the possibility of cellular polarisation. Crucially, the cells in this state generally do not synthesise any milk components, nor do they have the cellular responses of MEC found *in vivo* (Blum *et al*. 1989).

Another problem of attempts to replicate the biology of the mammary gland *in vitro* is that the cells used have often been transformed (that is, they are unnaturally immortal; e.g. Silva *et al*. 2002). Immortalisation can occur spontaneously, or it may occur as a result of deliberate transfection of viral genes. Either way, immortal cell lines have a reduced dependence on hormones and factors for growth in culture, due in part to their abnormal secretion of these or other growth factors (Todaro and Delarco, 1978). Furthermore, immortalised cell lines often have other abnormalities not seen in the untransformed cell (Matitashvili *et al*. 1997). For example, the immortal MAC-T bovine mammary cell line, most often used in bovine mammary research *in vitro*, is not a single homogeneous cell type (Zavizion *et al*. 1994). Additionally, this cell line has very low levels of milk specific protein production, relative to the levels seen in untransformed cultures (German and Barash, 2002). There is also some evidence that the MAC-T cell line is not dependent on the *in vivo* factors (hormones or extracellular matrix) known to regulate differentiation *in vivo* (Huynh *et al*. 1991; Berry *et al*. 2003), suggesting that their receptor mechanisms differ from the *in vivo* state. Studies of other immortal bovine MEC lines, 'BME-UV' and 'HH2a', have also indicated that these too have an abnormal physiology when attempts are made to bring them to the differentiated state (Matitashvili *et al*. 1997)

To date, attempts to model the biology of the bovine mammary gland have nearly always used MEC that have been cultured in isolation from the other cells types known to be important to its development and function. For example, the fibroblast growth factors (secreted by fibroblasts) are thought to greatly influence the proliferation and morphogenesis of MEC *in vivo* (Powers *et al*. 2000). Additionally, fibroblasts are thought to secrete at least part of the extracellular matrix and basement membrane for the parenchyma, and are a site of action of various hormones, in addition to synthesising their own growth factors (Hovey *et al*. 1999). During the early stages of development of the mammary gland, adipose tissue is thought to be crucial. However, despite this, these cell types are very rarely included in *in vitro* culture systems.

A further problem relates to the fact that the hormones and growth factors that have been shown to regulate MEC function *in vivo* are often absent from the culture media (or are undefined in the media because of the use of foetal bovine serum (FBS)). FBS is often used because it contains (undefined) cell attachment factors, growth factors and nutrients. The removal of FBS from culture media has the advantage of allowing the media to be free of (unknown) confounding growth factors, hormones and other components. Additionally, FBS can be of variable composition between batches, and it can be a way of introducing infection into cell cultures.

Some *in vitro* investigations have used pre-formed extracellular matrices (e.g. that from the Engelbreth-Holm-Swarm mouse sarcoma). These result in substantial morphological differentiation of the MEC as well as milk component synthesis (e.g. Rose *et al*. 2002). These matrices are rich in the extracellular proteins, which enable MEC to form lobule like structures, reminiscent to those seen *in vivo*, into which milk components are secreted (Rose *et al*. 2002; McConochie 2004a). However, the problem is that the contents of the lumina are difficult to obtain without destroying the lobule. A further problem is that as milk components accumulate in the lumina, there may be feedback inhibition of further milk synthesis (Peaker and Wilde 1996). Finally, Matrigel is also rich in a range of murine growth factors that may confound experimental results obtained.

Cell Culture Insert Methodology

Virtually all reports of *in vitro* models of bovine mammary function published to date can be criticised in one or more of the ways outlined above. In Aberystwyth, we have pursued an alternative

methodology that avoids some of these criticisms. We have isolated an untransformed (mortal) clonal cell line from lactating mammary tissue and have frozen it in numerous aliquots (Rose *et al*. 2002). These aliquots of cells can be defrosted as required and used for a number of passages. This methodology allows for several years of experimentation on the same batch of cells. Nevertheless, the cells are not immortalised by transformation with viral genes.

The cells are plated onto two-dimensional, porous, cell culture well-inserts. These allow free and repeated access to products secreted by the cells, whether to their apical or basolateral side (Delabarre *et al*. 1997; McConochie *et al*. 2004b, 2005). Additionally, because the cells are plated onto a porous membrane, the cells may be treated with different media on each side, reflecting more accurately the *in vivo* situation. Moreover, it is possible to co-culture other cell types, such as fibroblasts, in the lower chamber while the MEC are on the porous membrane.

We plated clonal bovine MEC onto collagen I coated porous membrane inserts and treated them with media in both the upper and lower chambers containing Dulbecco's Modified Eagle's Medium supplemented with either 10 ml/l of FBS or 10ml/l FBS with 5 mg/l each of the lactogenic hormones prolactin, dexamethasone and insulin (PDI). We found that significantly greater levels of the milk proteins α-casein and α-lactalbumin were produced with the PDI treatment (Figure 1). Furthermore, a substantially greater proportion of the α-lactalbumin secreted was found in the upper chamber (Figure 1b), suggesting that a degree of polarisation had occurred. However, in contrast, the α-casein was found to a significantly greater extent in the lower chamber (Figure 1a). Despite this, in terms of the concentration of α-casein in the media, approximately the same concentration of α-casein was found in the lower chamber as in the upper chamber. The greater total amount in the lower chamber therefore reflects the greater volume of media present there. Accordingly, we also concluded that α-casein might have had a greater mobility between the chambers than the α-lactalbumin. Certainly, the cells on the porous membrane were not wholly confluent, but did recede from the edges slightly in this experiment, allowing diffusion from the upper chamber to the lower chamber. The reason for the relative immobility therefore of the α-lactalbumin is unclear, but it is known that α-lactalbumin is at least partially associated with the apical membrane of MEC (Sasaki *et al*. 1978). Finally, it was noticeable in this experiment that even without the lactogenic hormones (but with the inclusion of the FBS), a degree of milk specific protein secretion was observed, possibly suggesting that FBS has some lactogenic properties.

Next we determined the effect of the coating of the porous membrane (either no coating, collagen I coating, or laminin coating) on the secretion of α-casein by the MEC clone when cultured with either FBS alone, the lactogenic hormones (PDI) or FBS plus PDI. The surprising finding was that there was

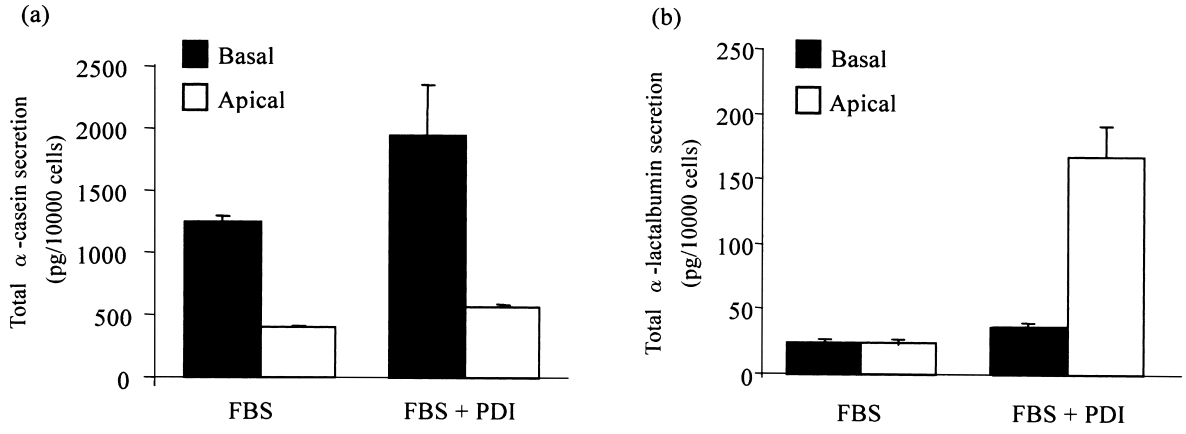

Fig. 1. Secretion of (a) α-casein and (b) α-lactalbumin into the apical (□) and basal (■) chambers by clonal bovine MEC plated onto collagen-I coated porous membrane inserts in media containing either 10 ml/l of foetal calf serum (FBS) or 10 ml/l of FBS supplemented with 5 mg/l of prolactin, dexamethasone and insulin.

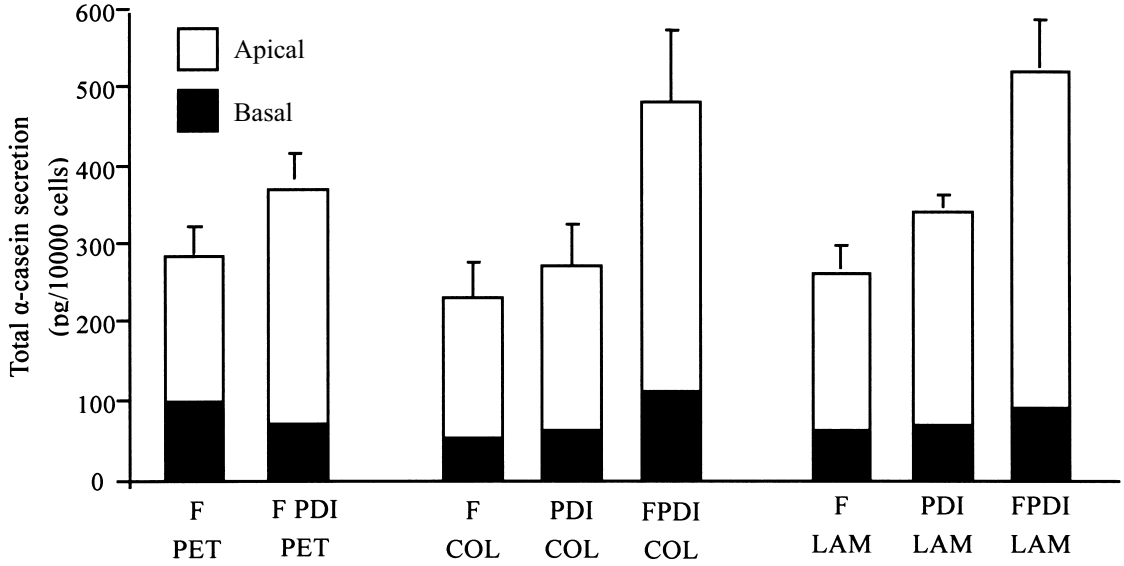

Fig. 2. Secretion of α-casein into the apical (□) and basal (■) chambers by clonal bovine MEC plated onto either uncoated (PET), collagen-I coated- (COL), or laminin coated- (LAM) porous membrane inserts in media containing either 10 ml/l of foetal calf serum (F), 5 mg/l of prolactin, dexamethasone and insulin (PDI), or both (FPDI).

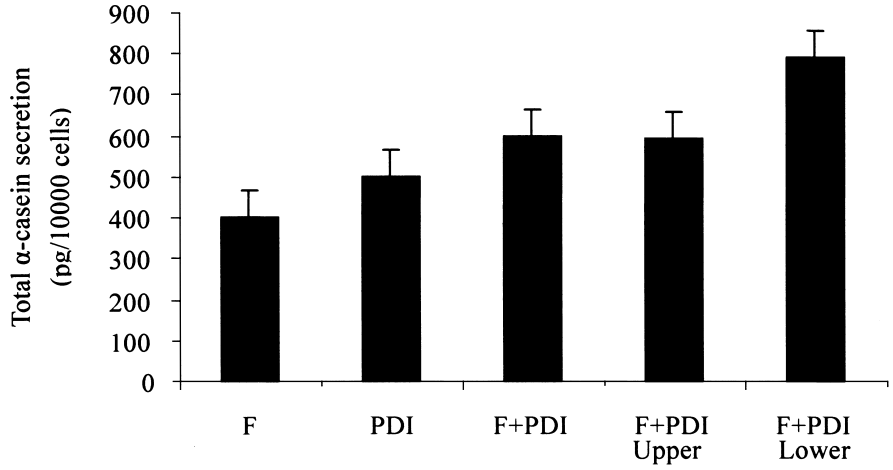

Fig. 3. Total secretion of α-casein by clonal bovine MEC plated onto collagen-I coated- porous membrane inserts. The media contained either 10 ml/l of foetal calf serum in both the upper and lower chamber (F), 5 mg/l of prolactin, dexamethasone and insulin in both the upper and lower chamber (PDI), F and PDI in both the upper and lower chamber (FPDI), F and PDI in the upper chamber only (FPDI upper), or F and PDI in the lower chamber only (FPDI lower).

no effect of the coating of the porous membrane on the secretion of α-casein (Figure 2). All treatments resulted in the secretion of α-casein to a similar extent. Again, milk protein was secreted in the absence of lactogenic hormones, but in the presence of FBS. However, the lactogenic hormones alone resulted in generally (but non-significantly) higher levels of secretion of α-casein. The combination of FBS and the lactogenic hormones resulted in significantly higher rates of secretion of α-casein, relative to either treatment alone. However, we found that this occurred to a lesser extent for the uncoated inserts. When we stained the uncoated membranes for the presence of laminin and collagen I, we found that both proteins had been deposited on the membrane. As these proteins were absent from the

uncoated membranes we concluded that these extracellular matrix proteins had been secreted by the MEC. *In vivo* it is thought that fibroblasts are largely responsible for the secreting extracellular matrix.

In a further experiment we again measured the total α-casein secretion by the MEC plated onto collagen-I coated- porous membrane inserts. In this experiment, the media contained either 10 ml/l of foetal calf serum, 5 mg/l of prolactin, dexamethasone and insulin (PDI) or F plus PDI in both the upper and lower chambers of the cell culture well inserts. A further two treatments had the F plus PDI in the upper chamber only, or F plus PDI in the lower chamber only, with unsupplemented DMEM in the other chamber, respectively. In this experiment, the secretion of α-casein was significantly greater when F+PDI was in the lower chamber only, relative to being in the upper chamber only, or in both chambers (Figure 3). This may indicate an increased level of polarisation when hormones and growth factors are present on one side only.

Conclusions and Perspective

While the methodology we discuss in the second half of this review is clearly open to further improvement, in particular with regard to the continued need by the cells for FBS, it nevertheless does represent an improvement over some of the methodology discussed in the earlier part. In particular, we have shown that the cells are responsive to lactogenic hormones and that the cells do reach a state of functional differentiation when plated on the porous membrane. Furthermore, the insert methodology allows the repeated sampling of the substances synthesised by the cells without the destruction of the morphological integrity of the cells. The methodology allows different treatments to be applied to the cells in the upper and lower chambers, and it also allows another cell type to be cultured in the lower chamber, separate from but in reasonably close proximity to the MEC. Unfortunately, our cells retain the requirement for FBS. This is required for the attachment of the cells to the porous membranes and thereafter for elevated levels of milk protein synthesis. Further research will concentrate on eliminating FBS from the culture media.

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Modulation of Sperm Function during Sperm Transport in the Female

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Keywords: Capacitation, Cow, Oviduct, Sow, Mare, Sperm reservoir, Sperm transport.

Abstract

In the cow, sow and mare, a functional tubal sperm reservoir is established before ovulation to ensure availability of suitable numbers of viable spermatozoa for fertilization. Although identification of subpopulations reaching this reservoir has been attempted, it is still unclear whether this recruitment is programmed or fortituous. Those spermatozoa not reaching the oviduct are generally destroyed by phagocytosis of invading leukocytes. While the type of ejaculate differs in these species, seminal plasma proteins and/or the spermatozoa appear to act as leukocyte chemoattractant both *in vitro* and *in vivo*. Those spermatozoa in the sperm reservoir not only escape phagocytosis or rejection by the female immune system but sustain viability and potential fertilizing capacity by not capacitating or acrosome-reacting while residing in the oviduct. Sperm numbers in the reservoir diminish gradually in relation to ovulation, spermatozoa being continuously redistributed towards the upper isthmus. *In vitro*, only uncapacitated spermatozoa bind to epithelial explants, suggesting that the reservoir milieu modulates sperm capacitation. *In vivo*, most viable spermatozoa during preovulatory spontaneous standing oestrus are uncapacitated, with capacitation significantly increasing after ovulation. In either species, there seems to be different components of the oviductal fluid effecting capacitation, and bicarbonate appears to be common denominator for the membrane destabilizing changes that encompasses the first stages of the process. Such effects can be blocked or even reversed by co-incubation with isthmic fluid or specific glycosaminoglycans such as hyaluronan. Although the pattern of response to *in vitro* induction of sperm capacitation is similar for all spermatozoa, the capacity of response and its speed is very individual. Such diverse individual response to capacitation would not only confirm capacitation does not occur massively in the reservoir but clearly insures full sperm viability before ovulation and the presence of spermatozoa at different stages of capacitation in the upper oviduct, thus maximizing the chances of normal fertilization.

Introduction

Sperm transport in the female genitalia follows a general pattern in domestic animals with spermatozoa being sequentially exposed to various genital environments, before encountering the oocytes and participating in fertilization. While the transport is usually rapid through the cervix and uterus, depending on the place of sperm deposition among species, most of the lengthy phases of sperm transport occur in the oviduct. The mammalian oviduct provides suitable environment for sperm transport, storage and capacitation, oocyte pick-up, transport and maturation, fertilization and ultimately, early embryonic cleavage (Hunter & Rodriguez-Martinez 2004). During their tubal permanence, spermatozoa bathe in the isthmic secretion in contact with oviduct-specific proteins, enzymes, glyco- and lipoproteins and become eventually associated, to various degrees, to the epithelial lining (Smith, 1998). Secretion flow, ciliary beating and myosalpinx contractions all play a major role in the formation and mixing of a tubal fluid where gametes meet. Oviductal fluid (ODF) differs from blood plasma in terms of ionic composition, pH, osmolarity and macromolecular content (Leese 1988), originating from selective transudation (mostly at the ampullar segment) and, a minor part, as secretion from the lining epithelium (particularly the isthmus segment) thus establishing regional differences in composition that might relate to the process of gamete preparation for fertilization. The present paper reviews aspects of the interactions

between spermatozoa and the surrounding tubal milieu in relation to the modulation of capacitation and fertilization.

The ejaculate

The bull ejaculates a single fraction of rather highly concentrated spermatozoa in a single jet of about 5 mL (range 2-10 mL) after secreting a pre-sperm secretion from the bulbourethral glands that cleanse the urethra. Such ejaculate contains a mean of 1 billion spermatozoa/mL bathing in a mixture of the cauda epidydimal fluid and secretions from the ampullae deferens, the prostate rich in electrolytes and carbohydrates and a basic protein-rich secretion from the seminal vesicles. Most of these proteins adhere to the spermatozoa (so-called spermoadhesins) of which a large part are able to bind to glycosaminoglycans (GAGs) such as heparin sulphate and heparin. Some proteins, including the heparin-binding proteins and osteopontin have been related to the fertility of the males (Killian et al. 1993). Other males, such as the stallion or the boar, ejaculate distinct fractions, with spermatozoa being located in a second-third fraction (the so-called sperm-rich) coincidental with the different jets ejected by ejaculatory thursts. Also here, the seminal plasma contains a large amount of proteins that interact with spermatozoa. In the stallion, the mean total number of spermatozoa ascends to 9 billion, suspended in a seminal plasma fluid that contains as much as 14 different types of major proteins (Frazer & Bucci 1996). Although stallion seminal plasma has its own typical protein profile in SDS-PAGE-gels, the relative protein amounts varies between stallions (Kareskoski et al. 2005). Calvete et al. (1994) isolated eight types of horse seminal plasma proteins (HSP-1 – HSP-8) by reverse phase HPLC. All the identified proteins had a low molecular mass of 14-30 kDa, and all of the proteins except HSP-4 were also peripherally bound to the sperm surface. The major proteins in stallion seminal plasma, HSP-1 and HSP-2, are heparin-binding proteins. HSP-3 is a member of the cysteine-rich secretory protein (CRISP) family, and it could be involved in membrane-fusion processes or act as an anti-infectious agent in seminal plasma (Calvete et al. 1997, Magdaleno et al. 1997). HSP-7 is a zona-pellucida-binding protein that is the sole member of the spermadhesin protein family in the stallion (Reinert et al. 1996).

The boar ejaculates also a high number of spermatozoa (mean 60 billion) in a large volume of seminal plasma (200 to 250 mL) mainly (95-98%) built by the secretions of the accessory sexual glands whose secretion is verted in a sequential manner, providing a series of easily recognizable fractions that can be separated during manual collection of a boar (Einarsson 1971, Lavon & Boursnell 1975). These fractions are usually called *presperm* (dominated by the secretion of the urethral and bulbourethral glands, as well as the prostate), *sperm-rich* (SRF, in which the vast majority of spermatozoa are present and where the emitted epididymal fluid in which they originally bathe is diluted with a fluid derived from the seminal vesicles and the prostate), and *post-sperm-rich* (PSRF), in which few spermatozoa are present and the fluid is primarily derived from the increasing secretion of the seminal vesicles, the prostate and, by the end of the ejaculation, the bulbourethral glands. The latter produces the tapioca-like floccula that coagulates the seminal plasma, as seen shortly after the ejaculate is collected in a receptacle, and that serves *in vivo* to retain the ejaculate *in utero*, thus minimizing retrograde flow through the cervix. The spermatozoa are therefore ejaculated with a maximum concentration in the first portion of the SRF, decreasing thereafter in number along this fraction to virtually disappear by the PSRF. The secretion of the seminal vesicles, albeit present at the moment at which spermatozoa are first emitted, increases in volume toward the PSRF.

More than 90% of the seminal plasma proteins in boars belong to the spermadhesin protein family comprising five members: AQN-1, AQN-3, AWN, PSP-I, and PSP-II, with different biological activities, depending on their sequence, glycosylation or aggregation state, and ability to bind heparin (Calvete et al. 1995a-c). The remaining components are proteins of low molecular mass (5-10 kDa) of which the acrosin inhibitor is the best identified. Although AQN-1, AQN-3, and AWN are sperm-coating molecules that can bind heparin, they seem to stabilize the plasma membrane over the acrosome but are mainly released during capacitation (Dostàlova et al. 1994, Calvete et al 1997); the other two proteins, PSP-I and PSP-II (Kwok et al. 1993), account for >50% of the total proteins, forming a non-heparin-binding heterodimer of glycosylated spermadhesins (Calvete et al. 1996) that displays immunostimulatory

activity *in vitro* by binding to porcine lymphocytes (Yang et al. 1998) and modulating their activity *in vitro* (Leshin et al. 1998). Since the various seminal plasma proteins originate from the testis, the epididymides, or the sexual accessory glands, they are present in the entire ejaculate but their relative concentration therefore varies with the different fractions of the boar ejaculate the lowest concentrations of spermadhesins being recorded by the end of the pre-sperm fraction, and particularly by the first portion of the SRF, compared to samples collected in the PSRF, where the seminal vesicles deliver both heparin-binding proteins (HBP) and PSPI/II (Rodriguez-Martinez et al. 2005).

Sperm transport in the female genital tract

The site of sperm deposition differs among species. In the bovine, semen is deposited in the cranial segment of the vagina, close to the cervical opening during mating. However, when artificial insemination (AI) is used, spermatozoa are deposited intra-utero, waiving the cervical barrier. In swine, spermatozoa are -during mating or during conventional AI- deposited in the narrow cervical canal, thus entering the uterine cavity rather quickly. In the equine species, the stallion (or the operator during AI) deposits the spermatozoa directly in the uterine cavity, owing to the large opening provided by the cervix. Despite these differences in sperm deposition, the process of sperm transport through the female genitalia is comparable in these three exemplified species and therefore can be divided in three phases: a) a rapid trans-uterine transport immediately after semen deposition, b) the colonization of a sperm reservoir in the lower oviduct, and c) a slow release from the reservoir towards the site of fertilization (ampullary-isthmic junction, AIJ), in relation to ovulation (Barrat & Cooke 1991).

Are there subpopulations among the ejaculated spermatozoa?

The spermatozoa present in a given ejaculate are part of an aliquot stored, for different periods, in the cauda epididymides. They represent, therefore, a heterogenous population of cells released by tubuli seminiferi that underwent sperm maturation along the ductus epididymidis at various intervals. Although highly seeked, identification of sup-populations has been restricted to morphological and functional attributes separating abnormal from normal (e.g. potentially fertile) spermatozoa. Determination of which spermatozoa actually participate in the fertilization process among those potentially fertile has yet not being possible. On the other hand, the ability of spermatozoa to interact with different seminal plasma proteins has indicated that some spermadhesins are able, when present in very low concentrations, to maintain the viability and fertilizing ability of boar spermatozoa (Vazquez et al. 2001, Centurión et al. 2003, Caballero et al. 2004a, 2005) with significant variation among boars (Caballero et al. 2004b).

Once ejaculated, a certain proportion pass the cervix-uterus (bovine) or the uterus (horse, pig), while the majority of the spermatozoa are rapidly eliminated from the genital tract, either by way of retrograde flow or by intrauterine (Einarsson 1985, Rodriguez-Martinez et al. 2005), with the exception of a small subpopulation of spermatozoa that is rapidly (in minutes [Hunter 1981]) transported by the myometrial contractions towards the uterotubal junction (UTJ) during the so-called *rapid phase* of the process of sperm transport in the female internal genital tract and that colonizes the sperm reservoir in the oviduct (rev by Rodriguez-Martinez et al. 2001, 2005).

Our own studies have tested the hypothesis that there are sperm subpopulations in the boar ejaculate, one of which first colonizes the sperm reservoir during natural mating (Rodriguez-Martinez et al 2005), where spermatozoa from the first portion of the SRF made up the bulk of spermatozoa in the oviductal sperm reservoir under the experimental conditions used, when fractionated sperm deposition was mimicked. Spermatozoa from this particular first portion of the SRF sustain better handling in the laboratory, such as storage at room temperature, cooling, or freezing-thawing, than the spermatozoa of the rest of the ejaculate (Sellés et al. 2001, Peña et al. 2003, 2005a-b). It seems, therefore, that a window of opportunity exists for a particular, albeit fortuitous, sperm subpopulation (e.g., bathing in a particular SP fraction) that not only presents the best viability but also escapes leukocyte phagocytosis. Interestingly, the seminal plasma present in the above-mentioned first portion of the SRF differs significantly in its relative contents of total protein and, particularly, in protein compositions compared to the rest of

the ejaculate. This first SRF-portion of the seminal plasma is characterized by major components of 5, 7 (acrosin inhibitor) and 10 kDa as well as a relative low amount of the glycosylated heterodimer PSP-I/PSP-II. The first named are not present in the later fractions of the ejaculate where instead we found high concentrations of spermadhesins (including the glycosylated heterodimer PSP-I/PSP-II) (Rodriguez-Martinez et al. 2005).

Fate of the spermatozoa in the female genital tract

As already mentioned, the spermatozoa (and the seminal plasma that surrounds them) that do not colonize but are retained in the uterine cavity are eliminated, both by vaginal reflux and by phagocytosis by leukocytes that migrate from the endometrium to the uterine lumen and, partially, by macrophages in the endometrial epithelium, phenomena best studied in pigs (Viring & Einarsson 1981, Einarsson 1985), but recorded in most species, including the bovine (Cobb & Watson 1995) and the horse (Kotilainen et al. 1994, Tunón et al. 2000). The entry of semen into the uterine cavity provokes, by means that are not yet known in detail, a massive invasion of leukocytes (mostly PMN) into the uterine lumen. These PMN migrate from the lamina propria, subjacent to the lining epithelium, where they accumulate after extravasation, presumably as a result of the high levels of estrogens that dominate the proestrus in the sow (Lovell & Getty 1968). Interesting to note is the fact that the PMN do not reach the uterine lumen immediately after semen deposition, at least not during the first 10 min. A massive presence of PMN is first detected 30 min after semen deposition (Lovell & Getty 1968), increasing in a sustained manner for the following 2 to 3 h (Viring & Einarsson 1981). Through this primary leukocytic reaction (phagocytosis), the majority of the ejaculated spermatozoa and the proteins surrounding them, both considered foreign by the female, are eliminated from a uterine lumen that should be cleansed and prepared to host and nurture the early embryos; in the pig, these can already reach the uterus 48 h after ovulation. Several factors have been implicated as mediators of the PMN recruitment toward the uterine lumen, including the uterine distension *per se* (Matthjis et al 2003), the spermatozoa (Kotilainen et al. 1994, Rozeboom et al. 1999), or the seminal plasma (Claus 1990, Bischof et al. 1994, Hadjisavas et al. 1994).

The spermadhesin PSP-I/PSP-II and its isolated subunits can induce migration of PMN *in vitro* and *in vivo* in rodents (Assreuy et al. 2002, 2003) as well as in pigs *in vivo* (Rodriguez-Martinez 2005), at doses five-fold lower than those present in the boar ejaculate. Associated the data presented thus far, spermatozoa that would temporarily (and fortuitously) be present in the fist portion of the SRF of pigs would benefit, under *in vivo* conditions from the absence of signal substances (such as PSP-I/II) at levels needed for stimulation of leukocyte migration to the uterine lumen (and the resulting sperm phagocytosis). Entry of the rest of the ejaculate into the utero, having higher levels of the heterodimer, would stimulate PMN migration and eliminate spermatozoa from the lumen. Such a period of latency for PMN migration to the uterine lumen (<30 min) suggests that there may be a window of opportunity for a certain subpopulation of ejaculated spermatozoa to traverse the uterine lumen during the first phase of sperm transport, without risking phagocytosis. Such sperm phagocytosis would start when a relevant number of spermatozoa had already colonized the sperm reservoir of the oviduct.

The oviduct and sperm transport

The mammalian oviduct is anatomically divided into three main segments; e.g. the isthmus, ampulla and infundibulum, counted from the ad-uterine to the ovarian end. Connecting areas are also described, i.e. the uterotubal (UTJ) and the ampullary-isthmic (AIJ) junctions, as well as a terminal section connected to the ovarian fimbriae and bursa in the abdominal opening (ostium; Beck & Boots 1974). The histoarchitecture is very simple, with a non-glandular mucosa (endosalpinx), covered by a lining epithelium composed of non-ciliated (secretory) and ciliated cells, an underlying double-layered smooth muscle (myosalpinx) and a covering serosa (mesosalpinx) continuous with the peritoneal covering. While the thickness of the internal, circular smooth muscle becomes thinner, the longitudinal mucosal plicae gain complexity (with secondary and tertiary plicae) and the number of ciliated cells increases dramatically towards the ostium (Rodriguez-Martinez et al. 2001). This histoarchitecture defines the presence of tubal compartments each one with a specific

function, providing the best environment to sustain and regulate gamete preparation, fertilization and the first steps of zygote development while transported through the tubal lumen (Boatman 1997). Those spermatozoa that ascended the uterus in the first phase of sperm transport colonise rapidly (minutes to 1-2 hours, second phase of sperm transport) the UTJs and the adjacent tubal segment in very reduced, albeit significant numbers (from thousands to 1-2 10^9 spermatozoa) compared to the original sperm population contained in the AI-dose or ejaculate, thus depending on the species. This segment marks the building up of a pre-ovulatory sperm reservoir as a consequence of several concerted factors, both mechanical and biochemical, and whose functionality is theoretically prolonged in pigs up to 30 h from onset of estrus (rev by Rodriguez-Martinez et al. 2001, 2005). These sperm numbers in the reservoir remain basically unchanged during the pre-ovulatory period for up to 18 hours in the cow (Hunter & Wilmut 1984, Hawk 1987) or 24 hours in the pig (Hunter 1995a, Mburu et al. 1996), immersed in the tubal fluid or contacting the lining epithelium. Most spermatozoa present in the pre-ovulatory sperm reservoir remain viable and potentially fertile (rev by Rodriguez-Martinez et al. 2005) until they ascend to the upper tubal segments either shortly before ovulation (Hunter & Wilmut 1984, Hunter 1995a) or as a continuous stream during the peri-ovulatory period (Larsson & Larsson 1985; Mburu et al. 1997).

Composition of the tubal fluid

The intraluminal ODF is, as stated above, created by secretion from the epithelium and by transudation from the blood through the lamina propria (Leese et al. 2001) varying in volume and composition with the stage of the oestrous cycle (Carlson et al. 1970, Buhi 2002, Rodriguez-Martinez et al. 2001). The ODF of bovine, equine and porcine species contains, among other compounds, GAGs either non-sulphated (hyaluronan) or sulphated (S-GAGs, eg chondroitin sulphate, dermatan sulphate, keratan sulphate, heparan sulphate and heparin) (Lee & Ax 1984, Varner et al. 1991, Tienthai et al. 2000, Bergqvist et al. 2005, Bergqvist & Rodriguez-Martinez 2005). The mean concentrations of total S-GAGs in tubal fluid differ between species; being larger in cows (Bergqvist & Rodriguez-Martinez 2005) than pigs (Tienthai et al., 2001). However, in either species levels and variations in concentrations are different between isthmus and ampulla and vary also in relation to the moment of the cycle. While in the cow the concentrations in the ampulla are significantly higher than in isthmus, the situation is reversed in pigs, probably owing to the larger secretory capacity of the latter. S-GAG-levels increase significantly in isthmus during preovulatory oestrus, to decrease towards metaoestrus. While not differing between sides in pigs, the concentration is higher in the "ovulatory" side in cows, compared to the contralateral oviduct. Regarding hyaluronan, this non-sulphated GAG is present in the ODF in both species, without segmental differences, but with a tendency to increase during standing oestrus, highest around ovulation. Absolute concentrations were significantly higher in pigs compared to cows. Both HA synthases, HA-binding proteins and specific membrane receptors are present in the epithelial lining, particularly in the sperm reservoir (Tienthai et al. 2001, 2003a-b, Bergqvist et al. 2005), where mucus accumulate pre-ovulation (Rodriguez-Martinez et al. 1998a-b, Johansson et al. 2000). These GAGs seem to be beneficial for sperm survival and capacitation (Rodriguez-Martinez et al. 2001, 2005).

Modulation of sperm capacitation in the oviduct

Sperm capacitation is a gradual, essential event pre-requisite for fertilization that takes place *in vivo* during the sequential exposure of spermatozoa to the different compartments of the female genital tract that occurs during sperm transport (Yanagimachi 1994). It can also be mimicked during incubation of spermatozoa *in vitro*, although our knowledge of the different steps of the process are yet to be fully unveiled. Capacitated spermatozoa are endowed with a number of abilities, including release from the sperm reservoir, penetration of the cumulus layers, and binding to the ZP, that permit the occurrence of the acrosome reaction (reviewed by Rodriguez-Martinez et al. 2001). In order to reach this status in ejaculated spermatozoa, bound proteins from the cauda epididymidis and the seminal plasma SP are removed from the sperm surface, particularly over the acrosomal region. When this sperm surface domain is exposed, it becomes accessible to lipid-binding components of the female intra-luminal fluids either in the uterus, but mainly from the oviduct.

These in turn are able to remove cholesterol from the sperm plasma membrane, thus enhancing membrane fluidity, which in turn causes lipid scrambling, and initiates further capacitation changes such as the uptake of extracellular Ca^{++}, tyrosine phosphorylation and the reorganization of the sperm membrane (Töpfer-Petersen et al. 2002, Tardif et al. 2003). During capacitation, intracellular pH and Ca^{++} rise, adenylate cyclase is activated, resulting in a rise in cAMP levels, and specific proteins (including extracellular signal-regulated cyclases) are then tyrosine phosphorylated (Tardif et al. 2003). In parallel, lipid redistribution in the plasma membrane and membrane destabilization result in a more fusogenic membrane with the exposure, and perhaps also the hiding, of specific receptors (Jaiswal et al. 1999). Therefore, sperm capacitation can be triggered *in vitro* by specific signals such as certain GAGs or changes in pH or Ca^{++} (Rodriguez-Martinez et al. 1998), clearly imitating events occurring *in vivo*. In the pig, horse and bovine, bicarbonate (a stimulator of adenyl cyclase) appears to be the effector molecule that is able to trigger the lipid scrambling seen in the lipid bilayer of the plasma membrane, and thus it is considered to be one of the earliest signs of capacitation (Harrison 1996, 1997, Harrison et al. 1996, Gadella & Harrison 2000, Harrison & Gadella 2005). All these modifications of the fluidity of the sperm membrane precede specific changes in Ca^{++} movement and of motility patterns (such as the associated hyperactivated motility), that predispose to the acrosome reaction and the penetration of the ZP during fertilisation.

Capacitation lasts for different times depending on the ability of the environment to cleanse the surface of the spermatozoa, and the exposure of these to environments which enable the sequence of events listed above, to occur. Since spermatozoa are exposed to uterine and tubal fluids, these ought to be able to regulate the speed of the process *in vivo*. Capacitation can be elicited more rapidly, for instance, if boar spermatozoa are deposited directly in the caudal isthmic portion of the oviduct, rather than in the UTJ or the upper ampulla (Hunter & Rodriguez-Martinez 2004). Not only in pigs, but also in all other species of mammals studied, the sperm reservoir in the oviduct appears to retard, rather than promote sperm capacitation (Smith & Nothnick 1997), apparently intending the extension of the viability and fertilizing capacity of those spermatozoa retained in this environment (Murray & Smith 1997, Rodriguez-Martinez et al. 2005). When spermatozoa leave this segment, either continuously or following a signal during ovulation, the suppressive effect of the sperm reservoir disappears or spermatozoa are confronted to a more favourable environment, triggering capacitation. In either case, and in all species studied thus far, capacitation is to be considered a peri-ovulatory process (Rodriguez-Martinez et al. 2001).

Our own experimental evidence, obtained *in vivo* by way of the flushing of specific tubal segments at well-defined stages of standing estrus (pre-, peri- or immediately post-spontaneous ovulation), indicate that the majority of boar spermatozoa retained in the SR during the period from 10-8 h before ovulation to 8-10 h after ovulation maintain a stable plasmalemma and are therefore not to be considered as undergoing capacitation (monitored as the significant increase in lipid scrambling at the membrane level by flow cytometry of Merocyanine 540/Yo-Pro-1-loaded spermatozoa (Rodriguez-Martinez et al. 2001), except for a significant increase (on the order of 10%) in the percentage of sperm capacitation after ovulation (Tienthai et al. 2004).

In following studies in the bovine, spermatozoa were exposed to bovine oviductal fluid surgically collected *in vivo*, to different glycosaminoglycans (GAGs) as well as to bicarbonate-enriched media. Following different exposure length, the spermatozoa were stained either with Chlortetracycline (CTC) or loaded with Merocyanine 450-Yo-Pro-1, and evaluated with epi-fluorecent light microscopy or flow cytometry, respectively for events related to sperm capacitation (Bergqvist et al. 2005, unpublished). When extended, but not chilled bull spermatozoa were exposed between 30 min and 2 hours to oviductal fluid (ODF), from either the isthmic or the ampullar regions and collected either during standing oestrus or at the day of ovulation, there were significant increases ($p<0.05$) in capacitation as measured by Merocyanine and CTC, indicating factors present in the ODF can trigger sperm capacitation. When exposing bull spermatozoa to the different GAGs known to be present in the ODF of the cyclic cow, hyaluronic acid was the only GAG that seemed to cause a slight capacitation, as detected by CTC, with a significant increase in B-pattern sper-

matozoa (p=0.012) compared to the negative controls. The only GAG that gave a significant increase in the Merocyanine high fluorescence sperm population was dermatan sulphate (p=0.035). Such effects were somewhat puzzling regarding the S-GAGs, since heparin is routinely used to induce sperm capacitation of bull spermatozoa *in vitro*. However, these results were obtained using an exposure to specific substances one by one, loosing eventual synergistic effects or dose response-interactive effects that ought to be the ones reflected by the ODF. Hyaluronan has been able to induce similar early changes in boar spermatozoa (B-pattern of CTC, for instance) but without leading to the acrosome reaction (Rodriguez-Martinez et al. 1997). That hyaluronan was able, *in vitro*, to induce capacitation (monitored via Merocyanine or CTC) but without eliciting the acrosome reaction has comparative interest. The presence of sperm reservoir fluid or hyaluronan during the incubation of boar spermatozoa flushed from the sperm reservoirs during pre-ovulation has prevented the induction of sperm capacitation after exposure to bicarbonate *in vitro* (Tienthai et al., 2004). These data indicate, albeit indirectly, that the SR fluid before ovulation maintains sperm viability without causing sperm capacitation, perhaps because of its HA content. When the same treatment was used with spermatozoa collected from the SR after ovulation, however, the presence of HA increased the rate of sperm capacitation, suggesting the temporal nature of the effect.

When exposed to a bicarbonate-enriched medium for 30 min, the number of bull spermatozoa depicting a higher degree of lipid disorder in the plasma membrane (Merocyanine with high fluorescence, Mero high) increased significantly (p<0.0001), compared to before bicarbonate addition and independent of the treatment before the exposure (including pre-exposure to GAGs, both sulphated or non-sulphated. There was no significant difference in the number of spermatozoa depicting B-pattern when bicarbonate was added, compared to negative controls, but an increase in the proportion of spermatozoa with AR-pattern (acrosome reacted spermatozoa) was registered (p<0.0001). As well, exposure to solubilised homologous ZP proteins significantly increased the proportion of acrosome-reacted spermatozoa (p=0.016). These results indicate that bicarbonate is also the effector molecule for bovine spermatozoa, as it has been the case for boar or stallion spermatozoa (Harrison & Gadella 2005). Moreover, it points out that bull spermatozoa exposed to a hyaluronan-rich medium can undergo sperm capacitation if further exposed to a low concentration of bicarbonate (30 mM).

The results with bull spermatozoa were, moreover, fitting with previous studies where Tienthai et al. (2004) were able to initiate the process of capacitation in boar spermatozoa, retrieved from the sperm reservoir, when exposed *in vitro* in a medium containing the effector bicarbonate (HCO_3^-) at concentrations similar to those recorded *in vivo* in the AIJ/ampulla segment of the peri-ovulatory pig oviduct (e.g., 33–35 mM/L (Rodriguez-Martinez et al. 1998). Taken together, the results suggest that the immersion of boar or bull spermatozoa in homologous ODF is not, *per se*, able to induce sperm capacitation unless the exposure is done with peri-ovulatory ODF. The triggering of the process of membrane destabilization that capacitation implies, does not seem to occur until spermatozoa are exposed to a specific effector, such as bicarbonate that leads the spermatozoa to acrosome exocytosis. Taking into consideration that the bicarbonate levels used were on the same order of magnitude as those in the AIJ, it seems possible, although speculative, that the progression of individual spermatozoa out of the sperm reservoir (as an expression of the innate heterogeneity of the ejaculate) is sufficient to induce capacitation when adequate levels of the effector are encountered outside of the sperm reservoir area has been proven for boar spermatozoa (Rodriguez et al. 2005).

A progressive and continuous release of spermatozoa from the sperm reservoirs in the oviducts, already occurring before ovulation (albeit increasing, but not massively, after ovulation) may be related to the gradual induction of capacitation following exposure to the fluid of the upper tubal segments. In this case, the numbers of capacitated spermatozoa at one particular time would be low, and because the capacitated state in the spermatozoon is transient and eventually leads to cell death due to its irreversibility *in vivo*, they would exocytose their acrosome contents and die if they are not near an oocyte. Following this hypothesis (Rodriguez-Martinez et al. 2005), there should be a continuous replacement of capacitated, short-lived spermatozoa leading to low sperm numbers per area

at any one time, albeit ensuring the availability of capacitated spermatozoa for such an extended time that could cover the very long interval between sperm deposition and ovulation.

Acknowledgements

The author wish to express his best thanks to those co-authors whose cooperative research has built a significant part of this review. Funding of the author´s research has been provided by the Swedish Council for Forestry and Agricultural Research (SJFR), the Swedish Farmer´s Foundation for Agricultural Research (SLF) and The Swedish Foundation for International Cooperation in Research and Higher Education (STINT; SLU-Japan Programme on Reproductive Biotechnology), Sweden.

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JIFS, 3 : 85 − 94 (2006) original paper

Basic Framework of Research on the Establishment of Iwate Recycling Basin Economic Area

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Keywords : renewable energy, regional resources, discarded timber, methane gas, barren ground, charcoal, rice, ethanol, social system, regional innovation

Received 1 September 2005; accepted 15 October 2005

Abstract

For human society to find methods of solving global environmental problems and maintain a sustainable society, it is necessary to recycle materials to minimize environmental burden. Agriculture, forestry and fishing industries and rural areas that can produce renewable energy, especially biomass energy, will be able to make significant contributions to solving global environmental issues. The question is what should be done to bring out the latent potential of regional resources.

There are three main points that must be considered in relation to formulating fundamental strategy to achieve this aim. The first point is the development of basic technology to facilitate recycle of regional resources (Regional innovation). The second point is the establishment of new social system to make material recycling commercially viable (Business model). The third point is the creation of an entity that can promote regional innovation and establish the new social system.

The purpose of this research is to analyze the basic condition of establishing basin recycling economic area. For the purpose of this research, we started survey over three regions in Iwate prefecture. Problems these regions are facing from the standpoint of use of regional resources are as follows: The first issue is that there is a large amount of discarded timber from thinning in the mountains. The second issue is the problem facing farmers of disposal of animal waste. The third issue is that 30-40 % of paddy fields are forced to use as non- rice crop field. The fourth issue is demise of marine forest (barren ground).

We promoted the following types of regional innovation in an attempt to solve these problems. The first initiative is to extract methane gas from animal waste and use this energy to carbonize discarded timber (Methane stock system). The second initiative is to use the charcoal for a wide range of objectives, including for the artificial reefs for marine algae. The third is to make ethanol from rice. And an entity which will establish and operate new social system must be considered continuously.

1. Key issues and basic strategy of this research

(1) Global environmental issues and agriculture, forestry and fishing industries/rural areas

Global environmental issues are among the most serious problems currently faced. Particularly grave is global warming, which is a result of increasing levels of carbon dioxide brought about by excessive use of fossil fuels. Hiroshi Komiyama predicts that if the current trend continues unchecked, by the end of the 21st century, atmospheric levels of carbon dioxide will double, average temperatures will rise by 2.5°C and sea levels will rise by more than 60 cm. The Kyoto Protocol, the international framework aiming to reduce carbon dioxide emissions, came into force on February 16, 2005, following ratification by Russia. However, Komiyama states that even if carbon dioxide levels are stabilized within the next few decades, it will take several hundred years for the warmed earth to revert to its original state (Hiroshi Komiyama, 1999).

Economist Hiroji Baba attributes the crisis resulting from environmental issues to the contradiction between humans' inclination to maintain life and their creation of capitalism, a system that is bringing about social destruction (Hiroji Baba, 1997). In other

words, Baba is stating that environmental problems are caused in particular by the capitalistic economic system and are not merely a result of business activities by humans. The extent of the problem shows that the capitalistic system is at breaking point.

For human society to find methods of solving global environmental problems and maintain a sustainable society, it is necessary to recycle materials to minimize environmental burden. In consideration of the fact that global warming is caused mainly by the use of fossil fuels, it is essential to replace such fuels with carbon-neutral, natural forms of energy (renewable energy sources) as much as possible.

If this transition can be achieved, agriculture, forestry and fishing industries and rural areas that can produce renewable forms of energy such as biomass energy, will be able to make significant contributions to solving global environmental issues, simply by tapping their own latent potential. That means that in addition to providing food and other multiple functions to the external economy, agriculture, forestry and fishing industries and rural areas will also able to produce energy, thus fulfilling, important and new function for the sustainable development of human society.

However, it would not be going too far to say that agriculture, forestry and fishing industries and rural areas in Japan are currently facing a survival crisis. In addition, the cost of energy produced by agriculture, forestry and fishing industries and rural areas is predicted to be significantly higher than that from conventional fossil fuels. This fact makes commercialization difficult in the market economy. The question is what should be done to unlock the latent potential of agriculture, forestry and fishing industries and rural areas to realize a human society capable of sustainable development. That is the very issue that we hope to address in this research.

(2) Fundamental strategy to unlock the latent potential of local resources

Ever since Japan's period of rapid economic growth, the rate of growth of labor productivity in agriculture has been almost equal to that of the industrial sector. However, as revealed in the course of enactment of the Basic Law on Food, Agriculture and Rural Areas in 1999, Japan's calorie-base food self-sufficiency ratio has decreased significantly to its current level of 40%. The number of agricultural workers is rapidly decreasing because of the ageing society of Japan and lack of successors to take over farms. It is predicted that the number of agricultural workers will halve in the next ten years or so. In addition, in mountainous regions, which produce 40% of total agricultural output, the population is falling, agricultural production is decreasing and the multiple functions of agriculture are being reduced. These results are the consequences of the downsizing of unprofitable production facilities amid an environment in Japan where there has been pressure to increase agricultural productivity as a result of market logic. The situation is the same in both the forestry and fishing industries. As a result, rural areas of Japan where primary industry forms the mainstay of the economy have become commercially stagnant and there remain a significant amount of unused or discarded local resources in such areas, i.e. local resources with a negative value.

In response to this situation, it is necessary to fully exploit the potential of such local resource to the maximum to realize a recycling-based system that causes minimal environmental burden. There are three main points that must be considered in relation to formulating fundamental strategy to achieve this aim.

The first point is the development of basic technology to facilitate new material recycling, in other words, technology that does not place a burden on the environment. Rather than development of new technology, what is needed most is the development of elemental technology to meet the specific needs of each region, or a new combination of resources, i.e. regional innovation.

The second point is the establishment of a social framework (new social system) to make material recycling commercially viable. If the market economy and capitalism continue to rule the economic system, the cost of overcoming this crisis that human society is facing must be borne socially by the market mechanism. In other words, the cost to achieve new material recycling must be borne by the market mechanism. In the new social system, it is necessary to build a framework and enhance infrastructure so that the provision of renewable energy from agriculture, forestry and fishing industries and rural areas will be commercially viable.

The third point is the creation of an entity that can promote regional innovation and establish and

operate the new social system. This entity would have the role of a regional coordinator. It is likely that the most appropriate entity to fulfill this role would be neither an official body such as the national, prefectural or municipal governments nor a wholly private company. The best choice would be a semi-private entity intermediate between public and private status.

If the potential of local resources can be fully exploited and a system that makes use of the blessings of nature could be put into place, agriculture, forestry and fishing industries and rural areas, which have always been at disadvantage in the conventional market economy, would be on an equal footing with industry for the first time. It is even conceivable that they could surpass industry. At the same time, this would contribute to the solving of global environmental issues and revitalize regional societies.

What we must do is to formulate such fundamental strategies and implement them to achieve realization. Let's look at an overview of our research project, which we are conducting based on the above principles.

2. Research project and Iwate Galactic Environmental Network

(1) Overview and basic policy of Iwate Galactic Environmental Network

Iwate Galactic Environmental Network was established in June 2002 with the aim of establishing a sustainable society where the environment and economy can coexist in harmony. The mission statement was written quoting ideas from *Natural Capitalism* by Paul Hawken et al. (Paul Hawken et al. 1999), with whom we agree on many issues. Members were sought from various walks of life. Iwate was incorporated into the name since Iwate Prefecture was selected as the base for study and research activities. This prefecture has been actively tackling environmental issues for a long period. The status of non-profit organization (NPO) was acquired in September 2004 so that the network could be the operating body for activities. Research has been carried out through the activities of this NPO. First, let us take a look at an overview of the NPO and its basic stance.

There are many research organizations that conduct study and research on environmental issues. What differentiates Iwate Galactic Environmental Network from conventional organizations is the fact that far from merely conducting research and making proposals, it attempts to prove results and construct actual business models. This is because, although the approach is somewhat experimental, it was hoped to show the local population that it is possible to achieve the social framework that will facilitate the establishment of businesses that can enhance the environment, and through this initiative, contribute to solving the problems at hand. Accordingly, the fundamental policy of the network was decided as follows at the time of establishment:

The first policy is the emphasis of collaboration between industry, government, and academia. In order to formulate a functional business model that actually works in the market economy, it is absolutely essential to enlist the cooperation of businesses, and also people that are connected with research and the government that provide support to such enterprises. There are approximately 70 members of the network altogether. Industry consists of over 10 companies and individuals. Membership in the industrial sector totals just over 20 people from a wide variety of companies ranging from large corporations to small and family-run enterprises. Membership in the governmental sector consists of just about 30 people, mainly employees of Iwate Prefecture and municipalities within the prefecture. Iwate Prefecture has stood out from other prefectures in Japan by actively tackling environmental issues. The awareness level of prefectural employees is also high. Municipal employees number just over 10. The majority of these employees work for municipalities that are also actively tackling environmental issues. The details of such initiatives follow later in the paper. Membership from academia totals 10 people, from Tohoku and other universities and research institutes of the Ministry of Agriculture, Forestry and Fisheries.

The second policy is to define the field of study, research, and pilot programs as the area extending from the watershed to the coast, i.e. the economic area corresponding to the basin of one river. The vast majority of research and environmental initiatives has focused on the use of local resources by each municipality and has treated agriculture, forestry and fishing industries separately. However, in this project, we have decided to remove such limitations and treat

the area in a unified way with the region and industry linked together. This is because in order to realize the fundamental strategy of fully extracting the potential of local resources, it is necessary to expand the area and industries/types of businesses involved. The new concept of "the region as an industry" encompassing different industries was proposed at a meeting at Tohoku Regional Agricultural Administrative Office by thinking first about revitalizing the region. Our way of thinking is basically the same.

The third policy is to place emphasis on the ideas and wishes of the region. To put local resources to effective use, it is necessary to examine the problems the region faces from the standpoint of solving them, develop the technology that is required for that particular region (Regional innovation), and develop social and economic support systems suited to the region to make the implementation of this technology viable as a business. To establish the social system it is necessary to appeal to related government entities, research organizations, local residents and companies. Specifically, we must evaluate a broad range of issues including what kinds of existing subsidy schemes can be used, what kind of support can be received from national and other testing laboratories, and whether individual municipality support and support schemes can be established. To achieve these aims, rather than simply applying general existing technologies and systems, we are striving to empathize with the problems local residents are facing.

The fourth policy is to conduct pilot programs and field tests. The reason field tests are given such high priority is, to construct a new business model, the most effective way in terms of scrutinizing problems and issues. The field tests referred to in this paper is the tests in which many stakeholders participate, and problems and challenges are scrutinized, aiming to achieve commercialization. Results achieved by the network and the feasibility of commercialization will be judged by the results of such pilot programs and field tests

(2) Operation of the network

Based on the basic policy outlined, the Network is operated as follows. The ultimate power of decision lies with the Operating Committee, which is made up of about 10 Directors. Under this committee there is the Planning and Policy Research Group and the Technical Support Committee. The former is a study forum that holds debates about the direction of research of the network, technical and research results presentations by members, and talks by external lecturers.

There are 6 special interest groups (SIGs) within the research group: (1) reforestation and effective use of woody biomass, (2) river basin cleanup, (3) next-generation eco farms, (4) next generation waste disposal, (5) environmental materials and (6) coastal environment and fishing systems. Although the research group is divided into specialist groups, the

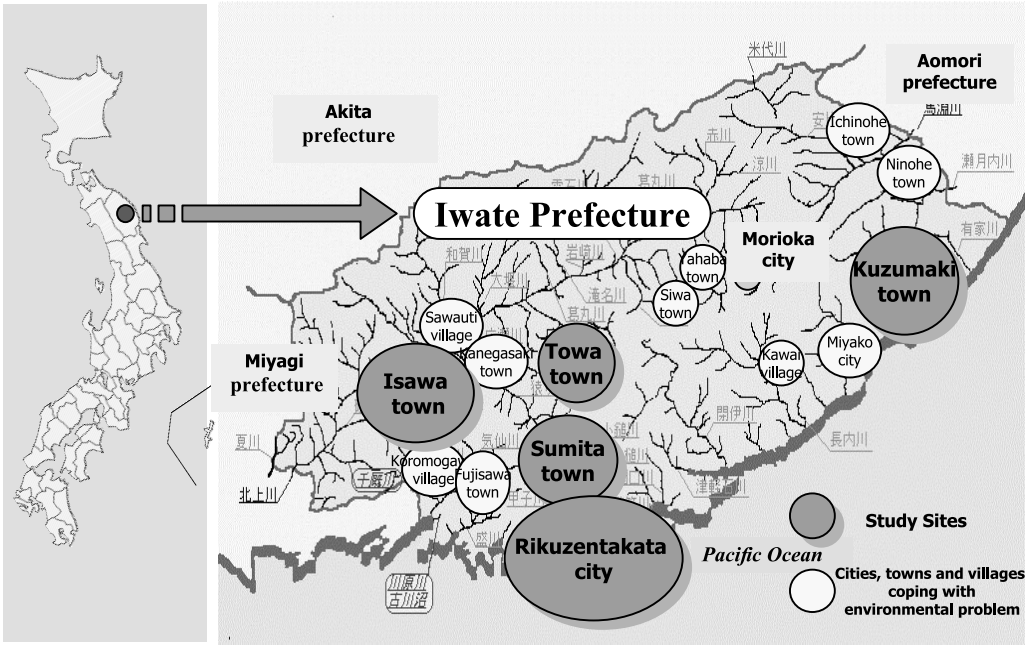


Fig. 1. Fields of study and research in Iwate Prefecture

SIGs are closely linked to each other, and meetings comprising several SIGs are held frequently. Most members belong to several SIGs.

The latter, the Technical Support Committee, is mainly responsible for supporting the evaluation of elemental technologies necessary for the development of technological innovation (regional innovation) and also for examining and solving problems and other issues. In principle, the network's policy is to only use existing technology and never newly developed technology. In addition, the network actively aims to use traditional technology and low-technology alternatives as much as possible and avoid entropy-increasing large-scale facilities and high-risk high-tech methods.

3. Progression of research project and current state

(1) Philosophy regarding recycling and the social system in the research project

Figure 1 shows our field of study and research in Iwate prefecture.

Figure 2 shows conceptually a social and economic system that can coexist in harmony with the environment. Realization of such a system is the very aim of this project. The municipalities in Iwate Prefecture scheduled for the field study are shown within the large dotted circle. The environmental material at the center of the figure is charcoal. All the basic technologies involved are linked by the environmental material charcoal.

The area enclosed by the large dotted circle represents the basin economic area. There are several social and economic frameworks, i.e. systems, mechanisms and movements, embedded within the circle so that use of local resources represents business. At the border of the basin economic area and the general market economic area represented by the line, if the value of a certain product in the two areas is different, adjustment will be necessary. Iwate Galactic Environment Network at the bottom of the figure represents the pivotal role of controlling the social and economic framework within the circle. For example, if a regional currency is to be used within the circle for transactions, the network will have to issue currency and oversee and operate currency exchange between the regional and regular market currencies.

Of the municipalities shown in the figure, we are conducting full-scale studies and research in Kuzumaki Town, Isawa Town, and Rikuzentakata City. We are examining what problems these regions are facing from the standpoint of use of local resources and solution of environmental problems. We have decided to look at the following three issues common to each of these municipalities.

The first issue is that there is a large amount of discarded timber from thinning in the mountains and

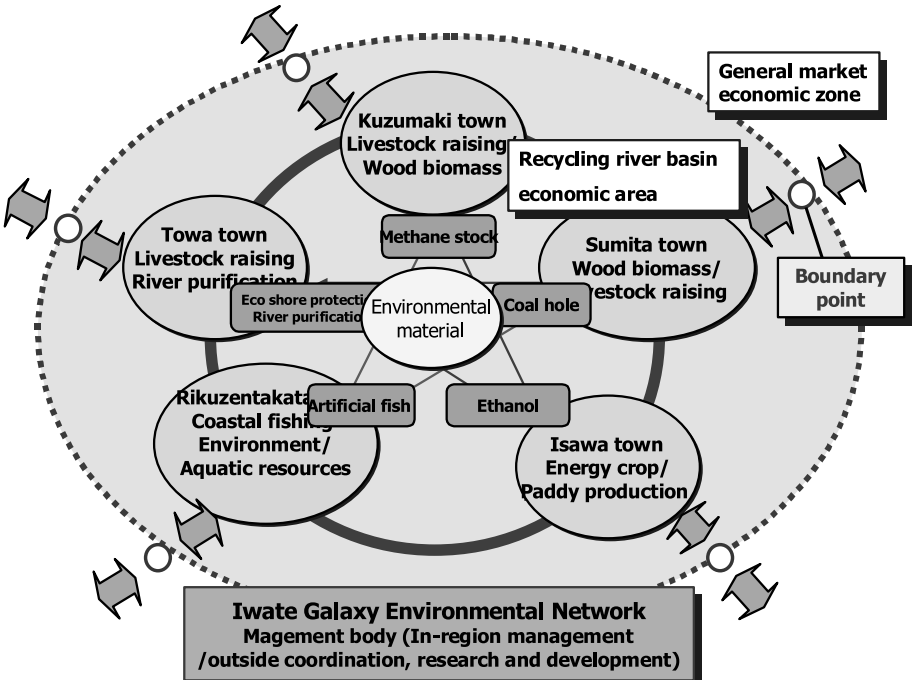


Fig. 2. Social system supporting coexistence of environmentalism and economics

by the forest industry. This problem is hampering the revitalization of forests and the forestry industry. In the event of floods and other events, such timber can drift into rivers, causing a great deal of damage to the basin.

The second issue is the problem facing farmers and municipalities of the disposal of animal waste (urine and feces). This problem has escalated in line with the expansion of the livestock industry. The waste often flows into rivers, contaminating water.

The third issue is that 30-40% of paddy fields are forced to use as non-paddy crop fields through the whole area of Japan. Under the guidance of central government soybean, wheat, vegetable, etc. are planted in the paddy fields. It is caused by overproduction of rice in Japan. It is difficult to fully exploit the potential of paddy fields.

The fourth issue is the demise of marine forests in the sea and coasts ("barren ground" or "coralline flat"), which are necessary for cultivating sea urchin, abalone and other organisms and for fish spawning. There are many possible reasons for this phenomenon, but it is most likely due to the effects of global warming.

Accordingly, in this research project, we decided to promote the following types of regional innovation in an attempt to solve these problems.

The first initiative is to extract methane gas

from animal waste and use this energy to carbonize discarded timber taken from the mountains. This process is referred to as methane stock since methane gas is stored in the form of charcoal. Methane extraction is conducted using a wooden anaerobic fermentation facilities made from discarded timber while carbonization is carried out using a carbonation facilities. Experiments have shown that the wooden fermentation facilities are effective in producing methane gas, due to the action of methane bacteria.

The carbonization facilities can be used not only to effectively carbonize discarded timber but also to turn other kinds of forest material including branches and leaves into charcoal. In addition, dry distilled gas is produced from the timber during carbonization, which is another source of heat in addition to methane gas. It is also possible to utilize the large amount of heat generated from carbonization to heat farmhouses, livestock barns and greenhouses. The total quantity of heat per day that can be produced from the carbonization of the methane from animal waste and discarded timber from a dairy farm of 70 cattle is equivalent to the amount of heat produced by 500 liters of kerosene. It is theoretically possible to heat 2 greenhouses of size approximately 1000m^2 with this quantity of heat.

In Europe, the generation of electricity from agricultural methane gas has already been commercialized, although this is because there are legal schemes to buy electricity generated from such sources. In fact, in Germany, there has been the emergence of many farmers who sell electricity to make a living. So-called "electricity farmers" totaled about 2000 households in 2003.

The second initiative is to use the charcoal produced for a wide range of objectives in addition to its conventional purpose, including for artificial reefs and for all kinds of environmental materials. The use most expected to benefit this region in terms of facilitating material recycling is use in coastal areas as artificial reefs for fish and marine algae. The artificial reefs for marine algae made from charcoal boards are used for nurturing the initial implant for the propagation of marine forest beds. Charcoal is used for algal reefs because the remaining animal waste slurry from which methane has been extracted (digestive juices) can be impregnated in it, the nutrients of which can be used to nurture marine forests. Furthermore, accumulation of charcoal will not contaminate the sea. The relationship between nurturing marine forests and nutrients has been researched in detail by Professor Kazuya Taniguchi et al. of Tohoku University Graduate School of Agriculture. Our experiments are based on the results of this research.

In addition, charcoal can be used as other environmental material, for example as a building material (using carbonized boards for walls and other purposes to solve sick house syndrome), to improve the environment of livestock barns, to clean up rivers and as a soil conditioner. It is also possible to use charcoal as a nanotechnology material.

(2) Characteristics and current state of progress in each research field

The above ideas basically apply to regional innovation in all three regions. However, there are some differences in the three fields of study and research in terms of resource endowment and the occurrence of problems (In Figure 2, Towa town are

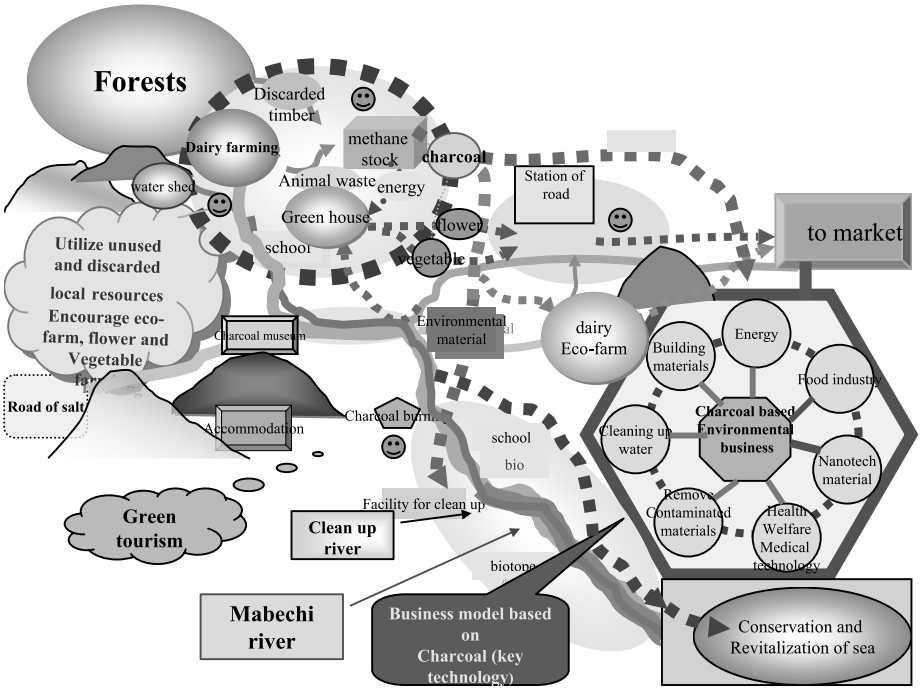
listed as a field of this research project. However at this stage we do not start a study in Towa town). Let's take a brief look at each of the three fields in turn.

1)Kuzumaki Town

The material recycling system in Kuzumaki is shown in figure 3. There are about 30 specialist dairy farms of scale 60-70 dairy cows. All these farms face difficulties over the disposal of animal waste. Forests cover approximately 80% of the area of the town and there is a significant amount of unused discarded timber. Because of this factor, the key issue in this town is exploring the possibility of adopting the methane stock system outlined above. In addition, in light of the fact that the Mabechi River that flows through the town reaches the sea via the Hachinohe City in adjacent Aomori Prefecture, we are looking at the environmental system of the basin economic region as far as the coast.

In fiscal 2003, an investigation was carried out on the methane stock system in Kuzumaki as part of an initiative of the *New Energy and Industrial Technology Development Organization* (NEDO). The members of our NPO made up the majority of the researchers participating. The investigation is entitled Kuzumaki Town, Iwate Prefecture-field test on animal waste and woody biomass composition methane stock system in mountain areas (March 2004) and detailed results are available. Based on these results, discussions are currently proceeding among sections of the town hall, dairy farmers in the town, forestry cooperatives, and the chamber of commerce regarding the feasibility of methane stock. In addition, the feasibility of carbonization facilities, necessary for the methane stock system, is being debated among related companies and other parties.

A system to promote implementation has been partially put in place in the town hall. Related sections in the town hall include those in charge of energy policy, agriculture-forestry and planning. We have also been discussing the establishment of a cooperative framework in the municipality since 2003. Confirmation was received in August that these sections would collaborate to facilitate the establishment of such a framework. Subsequently, through the town hall section in charge of energy, there have also been discussions with just under ten dairy farmers in the Hoshino area, the main dairy farming district of the town, concerning the adoption of methane stock system, financial allowances, and the merits and demerits of implementation. While most of them support the adoption of this scheme, more concrete proposals regarding financial allowances and usability are needed to spur current



-Illustrated by Nobuo Tomura-

Fig. 3. Recycling river basin economic zone : Kuzumaki town

discussion.

2) Kesen River Basin (Sumita Town and Rikuzentakata City)

A representation of material recycling in the Kesen River basin economic area, which includes Rikuzentakata City and Sumita Town, is shown in figure 4. In this region, the use of charcoal made from discarded timber from forests of the upper reaches of the Kesen River and methane from animal waste (beef and dairy cattle, pigs and chicken) is being evaluated. Part of this would be used as algal reef in Hirota Bay. It is currently being considered whether the seaweed forests produced can be further processed for use in the seaweed business. It is predicted that through this initiative, the sea will be enriched, leading to a revitalization of the livestock industry and mountain areas.

A Rikuzentakata Cluster has been formed in this area comprising people in Rikuzentakata that support the project and some of the NPO members. This cluster is the basis for promotion of research. Efforts are currently being focused on the formation of seaweed (*eisenia bicyclis*) forests by burying algal reef produced from charcoal in the sea. These forests are necessary for cultivating sea urchin and abalone. In March 2003 thanks to assistance from Ofunato Regional Development Bureau (Director Ken Abe), a symposium was held on this new recycling system and the merits of implementation in the Kesen River basin. The seminar was attended by about 100 local residents and other parties.

Since then, close collaborative relationships have been established step by step between the network and regional fishery, agricultural and forestry cooperatives; local residents; city assembly members and other relevant parties, both collectively and with each group individually. Rikuzentakata City has achieved significant results from implementation of environmental measures. For example, it has been tackling the issue of effectively utilizing local resources such as garbage for a long time. This city has also been actively promoting local studies and research by local residents on subjects such as local resources and history. Many of the members of the Isawa Cluster have also been involved in similar initiatives.

The most pressing matter in this region was burying of algal reefs in Hirota Bay in winter. Thanks to the cooperation of Secretary Shimizu and colleagues of Hirota Bay Fishery Cooperative, the laying of algal reefs and an experiment were commenced on January 28, 2005. The algal reefs were constructed in November 2004 based on a proposal by members

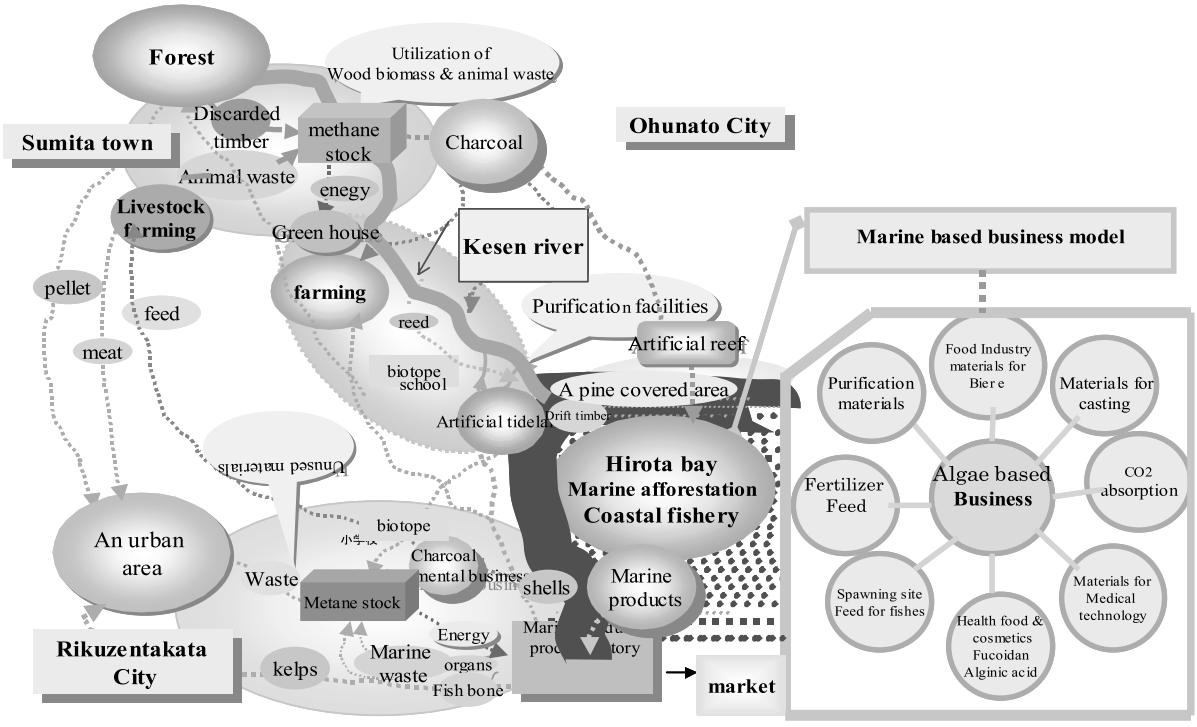


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Fig. 4. Recycling river basin economic zone : Kesen area (Sumita town & Rikuzentaka City)

to use charcoal chips, with cooperation from Iwate Industrial Research Institute. Other materials used in constructing the reefs included carbonized chicken waste and porous concrete, which was purchased separately.

Going forward, we plan to adopt the methane stock system in the region with the cooperation of local livestock farmers. Digestive juices will be added to the charcoal produce algal reefs. We are also planning to conduct experiments on other methods of nurturing the initial implant for the propagation of seaweed beds in addition to the current method (longline method intermediate breeding). The problems in this case are whether farmers would actually use the methane stock system and how the expenses would be covered. We plan to examine these issues with cooperation from Rikuzentakata Agricultural Cooperative.

3) Isawa Town

Figure 5 shows a model of Isawa Town. Adoption of the methane stock system is also a major issue in this municipality. However, whereas discarded timber from thinning would be used as a raw material for this purpose in the other regions, in this municipality trees felled in the process of construction of Isawa Dam (scheduled to be completed in 2010) or driftwood would be used.

Another feature of Isawa Town is its vast expanse of fertile fields (approximately 5,000 hectares). About 1,800 hectares of this total is suited is to crop rotation. However, excluding the fields where soybeans are grown, maximum productivity is not being achieved in all the fields. Accordingly, in this region the cultivation of high-yield rice and production of ethanol is being investigated.

However there are many problems to overcome in achieving this objective. Such challenges include whether it would be possible to achieve a steady high yield of rice (approximately 120-150% of the current level- maximum of 900 kg of rice used for feeding livestock per annum are according to data from Iwate Agricultural Research Center), the production cost of rice and whether the production cost of ethanol can be significantly reduced. Accordingly, we are conducting an investigation into the feasibility of ethanol production as part of a fiscal 2004 Ministry of Economy, Trade and Industry grant program. The government is promoting an increase of the concentration of ethanol in gasoline to 3% in 2005

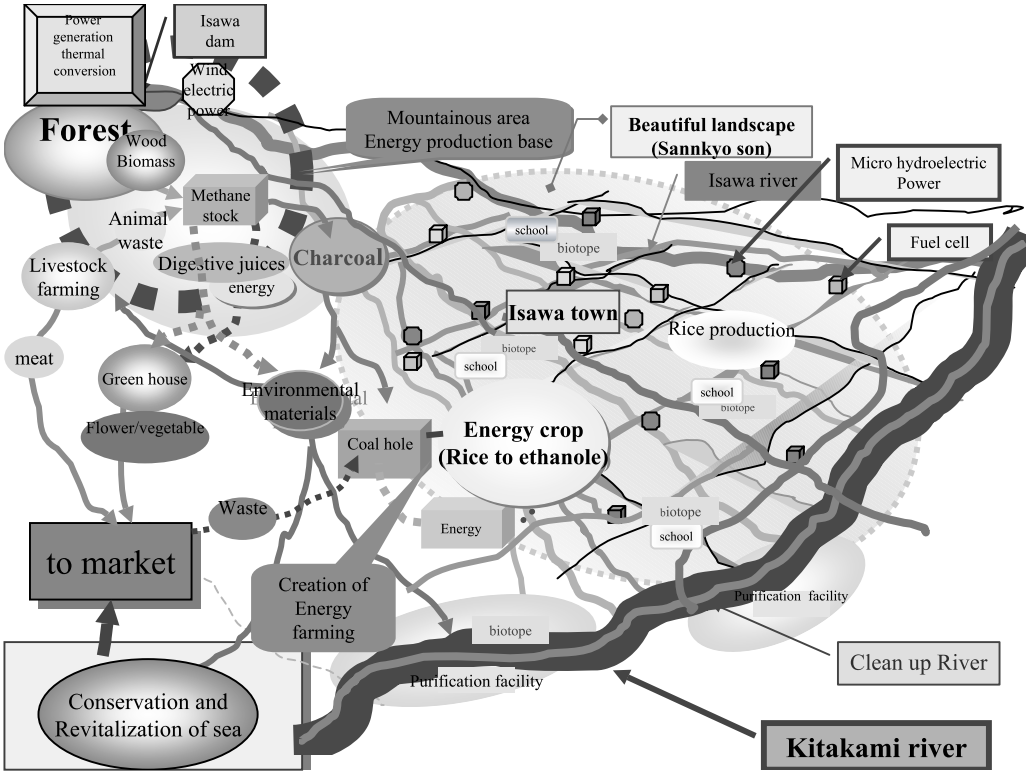

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Fig. 5. Recycling river basin economic zone: Isawa town

(and subsequently to 10%). This policy will of course provide support, meaning that commercialization may be possible in the near future.

It is also predicted that a significant amount of powder will be produced during the production of charcoal. Accordingly, we are also investigating producing emulsion fuels (called *coal hole*) by mixing this powder with either waste oil or oily plants. This would allow all the charcoal to be utilized. In addition, it seems likely that micro hydro power generation is possible from drainage ditches running horizontally and vertically along and around expansive rice paddy fields. Basic research was conducted in both 2003 and 2004 about the quantity of water and electricity used.

As part of a NEDO grant project, Isawa Town laid the foundations to make maximum use of local resources by constructing a new energy vision and investigating energy use in the town. We are conducting our research project based on this groundwork.

4. Future issues-Aiming to establish the social system and operating body

Technical preparation to facilitate regional innovation is currently being made, with efforts concentrated on the above three regions. Members of the network have traveled to field sites on many occasions in an attempt to refine combining such elemental technologies. Also, we have held a symposium in which local residents participated and created many chances for debate with workers in the agricultural, forestry and fishing industries; the general public and main related organizations. Further, we have formed regional clusters to cooperate with the network.

The next problem to be overcome is formulation of the social framework to allow implementation of regional innovation. Specifically, issues that need to be solved include how to use local resources, who will bear the cost, and how and whether the government will provide financial support. In other words, we need to formulate a concrete approach to construct a specific business model and establish an operating body.

For example, in order to implement the methane stock system, it is necessary to consider the following issues: from where discarded timber will be hauled, which livestock farmers will provide animal waste, where charcoal produced will be processed into algal reefs, how much the costs will be and who will cover expenses. At the same time, it is necessary to provide concrete results on the environmental benefits of this system, i.e. to show to what extent it will reduce the burden on the environment.

Unless a functional social system is established, no progress will be made with experimental studies and pilot programs. The regional revitalization aimed for in this project will be nothing more than mere theory. The question that needs to be asked is who in the region can gather ideas and wishes from local people and solve the problems that are being faced. It is thought agricultural cooperatives could be the most effective bodies in rural areas to fulfill this role.

Agricultural cooperatives are the largest community organization in rural areas, own land and are economic entities, operating with the aim of generating profit. Agricultural cooperatives have also been involved to a large extent with the management of social overhead capital (including infrastructure, local resources and various systems) (Uzawa,2000). In regard of this fact, agricultural cooperatives are likely to be the most effective operating body to establish and operate the regional social system. However, there are many problems to be overcome for agricultural cooperatives to undertake this new role, regarding their objectives, structure and operating system. We have to clarify the role that agricultural cooperatives should play, including reference to these issues, and also assess whether they are really capable of undertaking such a task. This is one of the biggest challenges that must be overcome.

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Case Studies on the Seasonal Changes of Diatom Community in Paddy Fields

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Key words : Freshwater algae, Terrestrial ecosystem, Andosol, Fulvisol, Rice production, Silicon nutrition

Received 10 January 2006 ; accepted 9 February 2006

Abstract

As a case study, the seasonal changes of diatom community in flooded water of paddy fields were discussed. The investigations were conducted on Andosols (Field 1) at Field Science Center, Tohoku University, and on Fluvisols (Field 2) at Furukawa Agricultural Experimental Station, located at Miyagi Prefecture, Japan, during the rice-growing season of 2004. The results obtained are as follows:

1) Diatom cell density at Field 1 ranged between $2.1x10^5$ and $1.1x10^6$ cells L^{-1}. There was no large difference during the experimental period in Field 1. Diatom cell density in Field 2 ranged between $4.3x10^5$ and $5.3x10^6$ cells L^{-1}. Diatoms in Field 2 were low in the end of May, and increased gradually thereafter.
2) Nineteen genera were observed in Field 1 and twenty-four genera in Field 2. In general, *Nitzschia* was predominated genus in the both fields. In Field 1, *Melosira* became predominated from June to August. In Field 2, *Navicula* was also predominated in July.

Introduction

Rice is a known silicon (Si) accumulator, and the plant benefits from Si nutrition. There is a definitive need to consider Si as an agronomically essential element for increasing and sustaining rice production (Savant et al., 1997).

Recently, Si concentration in rivers and coastal region had decreased. Kumagai et al. (1998) reported that Si in the river that irrigated to paddy fields has been decreased significantly in last 40 years. Humborg et al. (1997) investigated long-term data sets of water and nutrient discharge from the River Danube to the Black Sea. This data revealed a reduction in the dissolved Si load of the river by about two-thirds due to dam construction in the early 1970s.

It is reasonable that diatom (*Bacillariophyceae*) is responsible for the process of reduction in the dissolved Si load. They are very important primary producer in aquatic ecosystems, and often became predominant among phytoplanktons (Iwasa, 1976). Planktonic diatoms take dissolved Si from water column to make their frustules. Much of the Si taken by diatoms would be difficult to return to the water column because it is deposited on the bottom and is not readily dissolved (Miyajima et al., 1995).

Above phenomena may also happen in the paddy fields, which use a large amount of irrigation water and act as water reservoir. This can be supposed with the investigation on the changes of Si concentration in flooded water particularly in terraced and enlarged paddy fields where rice plants were cultivated (Saigusa and Kobayashi, 2002, Saigusa et al., 2004). In the terraced paddy field conducted plot-to-plot irrigation, the Si concentration of the flooded water decreased from the upper field to the lower field (Saigusa and Kobayashi, 2002). Likewise, the enlarged paddy field, Si concentration decreased gradually with a distance from the water inlet to the water outlet (Saigusa et al., 2004). These phenomena seem to result from Si consumption by diatoms or rice plants and might be one of the reasons to decrease Si concentrations in the river and the coastal regions.

Biomass and seasonal growth pattern of paddy field diatoms provide a basis for estimating their Si consumption. Nevertheless, only few studies

have focused on the diatom community in paddy field. Thus, this study was conducted to describe the seasonal changes of diatom communities in two paddy fields.

Materials and Methods

Experimental design

The research was conducted in two paddy fields located in Miyagi prefecture, northeastern Japan.

Field 1 was located at the Field Science Center of Tohoku University (38°44.4 N, 140°45.3 E, altitude 180m) in Naruko town. It was a terraced paddy field composed of six fields. This investigation was conducted at second field from top field. The soil in the paddy field was Andosols and the area was 2,640m^2. Paddy field irrigation was conducted from 26 April to 30 August 2004. Water was maintained at 2–7 cm depth and irrigated from a neighbor stream through the fallow paddy field. Rice seedlings were transplanted with an average density of 24.2 seedlings m^{-2} on 18 May. Coated fertilizers (N:P_2O_5:K_2O=14:20:14) were applied at the rate of 70kgN ha^{-1} at transplanting time. Herbicides were applied on 27 May (pretilachlor) and 30 June (pyriminobac-methyl). Fungicide (probenazole) was applied on 23 July. Rice was harvested on 12 October.

Filed 2 was located at Furukawa Agricultural Experiment Station (38°35.5 N, 140°54.5 E, altitude 26m) in Furukawa city. The soil in the paddy field was Fluvisols and area was 96,000m^2. Paddy field irrigation was applied from 20 May to 25 August 2004. Water was maintained at 3-5.5 cm depth and irrigated from agricultural water through the pond. Direct seeding culture of rice in the paddy field was conducted. Rice seeds were sowed 15 May. Coated fertilizers (N:P_2O_5:K_2O:Mg=12:16:14:3) were applied at the rate of 50kgN ha^{-1}. Herbicide was applied on 4 June (pyriminobac-methyl) and 29 June (bentazone). Fungicide was applied on 2 July (probenazole) and 23 July (pyroquilon). Rice was harvested 4 October.

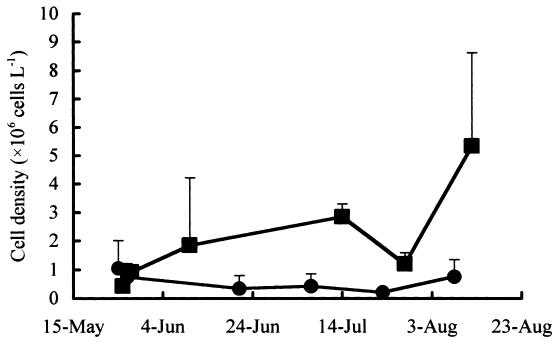

Fig. 1. Seasonal changes of diatom cell density in Field 1 and 2

Methods of sampling and counting diatoms in flooded water

Flooded water was collected six times in the field water inlet during May–August. Samplings at Field 1 were conducted on 25 May, 27 May, 21 June, 7 July, 23 July and 8 August. Samplings at Field 2 were conducted on 26 May, 28 May, 10 June, 14 July, 28 July and 12 August. Water samples of 250 ml were taken with three replications and fixed using formalin (37%). These were concentrated 8.3-25 times. They were put into prepared slide of 100mm^3 volume for counting algae cells (MATSUNAMI Co. MPC-200). Living diatom cells, as distinguished from empty frustules by the presence of chloroplasts, were counted under an inverted light microscope (LM) (Nikon Co. ECLIPSE TE300) at magnification of 400. Diatoms were identified at the genus level by using following references: Round et al. (1990), Round and Bukhutiyarva (1996) and Cox (1996). The concentrated samples were heated with 1 mol L^{-1} hydrochloric acid and hydrogen peroxide to clean the diatom frustules from organic materials. Micrographs of the diatom frustules were taken with a scanning electron microscope (SEM) (HITACHI Co. S-4206).

Results

The seasonal changes of diatom cell density in flooded water of Field 1 and 2 are shown in Fig.1. Diatom cell density ranged between 2.1x10^5 and 1.1x10^6 cells L^{-1} at Field 1 and between 4.3x10^5 and 5.3x10^6 cells L^{-1} at Field 2. Diatom assemblage in Field 2 had a low cell density in flooded water just after rice transplanting, and increased gradually thereafter. In contrast, the diatom cell density in Field 1 did not largely fluctuate during experimental period. The diatom cell density in Field 2 was from two to five times higher than that at Field 1, except in May.

The list of diatom genera and the changes of diatom cell density in genus level are shown in Table 1. Nineteen and twenty-four diatom genera were found in Field 1 and Field 2, respectively in this study. *Stauroneis*, *Neidium*, and *Eunotia* were observed just only in Field 1, whereas *Asterionella*, *Aulacoseira*, *Gyrosigma*, *Rhoicosphenia*, and *Cymbella* were

observed just only in Field 2.

In Field 1, *Nitzschia* was generally predominant; it was $7.2x10^4$ – $6.6x10^5$ cells L^{-1} in cell density and occupied 18 – 89 % of the total diatom cells. It was the most numerous just after rice transplanting, and decreased rapidly thereafter (Table 1). From June to August, *Melosira* was also dominant with the cell densities of $6.8x10^4$ – $3.8x10^5$ cells L^{-1} (Table 1). Eight genera were also abundant with the cell densities of more than $1.0x10^4$ at least in one sampling. *Navicula*, *Pinnularia*/*Caloneis*, and *Surirella* were abundant throughout the sampling period. *Cyclotella* was abundant in May and August. *Rhopalodia* was abundant only in June. *Fragilaria* (sensu lato), *Synedra* and *Diploneis* were abundant only in August.

In Field 2, *Nitzschia* was also predominant with $9.4x10^4$ – $2.7x10^6$ cells L^{-1} in cell density and occupied 7 – 61 % of the total diatom cells. Unlike in Field 1, it was not abundant in May and increased thereafter (Table 1). *Navicula* was also dominant in July and August with the cell density of $5.2x10^5$ – $2.6x10^6$ cells L^{-1} (Table 1). Seven genera were also abundant with the cell densities of more than $1.0x10^5$ at least in one sampling. *Pinnularia*/*Caloneis* was abundant throughout the sampling period, except May. *Asterionella* and *Alulacoseira* were abundant only in May and occupied 68-77% of the total diatom cells. *Surirella* was abundant only in June. *Fragilaria* (sensu lato), *Melosira* and *Synedra* were abundant only in August.

Figure 2 shows the SEM and LM micrographs

Table 1. List of diatom genera and the changes of diatom cell density ($x10^4$ cells L^{-1}) in genus level in Field 1 and Field 2.

		Field 1						Field 2					
	Genera	25-May	27-May	21-Jun	7-Jul	23-Jul	8-Aug	26-May	28-May	10-Jun	14-Jul	28-Jul	12-Aug
1	*Nitzschia*	74.3	66.1	7.8	23.9	7.2	13.3	9.4	11.1	113.0	19.4	28.3	266.3
2	*Navicula*	4.9	1.2	0.5	4.4	1.7	5.8	0.2	1.2	10.7	255.2	52.3	124.1
3	*Fragilaria*	0.4	0.4		0.6	+	2.4		1.0	1.5			64.8
4	*Melosira*	0.1		20.6	6.8	8.6	37.7	1.3	3.0			0.4	13.5
5	*Asterionella*							18.5	48.3	+	0.2	0.2	0.4
6	*Pinnularia/Caloneis*	11.6	0.8	0.5	1.7	1.1	2.9	0.4	0.3	34.2	6.0	19.7	20.5
7	*Surirella*	10.6	0.5	0.5	1.1	0.5	1.0	1.3	1.5	13.4	0.4	1.0	0.7
8	*Aulacoseira*							10.7	22.5	3.0		2.6	1.7
9	*Synedra*	0.8	0.8	0.5	0.9	0.6	6.8	0.3	0.4	3.5	0.3	3.2	11.9
10	*Cyclotella*	0.3	3.7	+	+	+	1.5	0.4	0.8	1.1	0.1	5.3	1.1
11	*Diploneis*	0.3		+	0.1	0.1	1.2			0.2		0.2	5.4
12	*Rhopalodia*			2.7	0.4	0.1		+		2.0		1.8	1.8
13	*Encyonema*	+	0.1	0.2	0.5	0.2	+	0.1	1.0	0.6		1.0	8.4
14	*Placoneis*	0.2			0.2	0.1	0.7	+	0.1	+	0.2	0.1	4.5
15	*Gyrosigma*									0.2	1.3	1.1	3.7
16	*Amphora*	0.2	+	+	0.2	0.2	0.2	+	0.1	0.2		0.2	1.5
17	*Gomphonema*	0.1	0.3	0.4	0.2	0.1	0.1	0.1	+	0.1	0.1	0.5	0.3
18	*Tryblionella*	0.2		0.1		0.1	0.2					0.5	
19	*Planothidium*									0.3	0.1	0.3	0.5
20	*Rhoicosphenia*												0.5
21	*Cymbella*											0.3	
22	*Stauroneis*	0.2				+							
23	*Neidium*	0.1	+										
24	*Sellaphora*									0.1			
25	*Cocconeis*											0.1	
26	*Eunotia*	+	+			+	+						

* + This symbol means that the diatom cell density is below $1.0x10^2$ cells L^{-1}.

of predominant and characterized diatom genera in Field 1 and Field 2. Fig.2-1, 2-2, and 2-3 show *Nitzschia*, it was the most predominant genus in both fields. *Nitzschia nana* (Fig.2-3) was abundant in Field 2. Fig.2-4 and 2-5 show *Navicula*. It was also the predominant genus in Field 2, and was abundant during experimental period in Field 1. *Navicula lanceolata* (Fig.2-4) is taken from Field 1, and *Navicula gregaria* (Fig.2-5) is taken from Field 2. *Melosira* (Fig.2-6) was also dominant in Field 1, *Melosira varians* was observed in both fields. *Pinnularia* (Fig.2-7), *Caloneis* (Fig.2-8) and *Surirella* (Fig.2-9) were abundant in both fields. *Cyclotella* (Fig.2-10), *Rhopalodia* (Fig.2-11) and *Diploneis* (Fig.2-12) were abundant in Field 1. *Rhopalodia gibba* (Fig.2-11), *Stauroneis* (Fig.2-13) and *Neidium* (Fig.2-14) were observed just only in Field 1. *Asterionella formosa* (Fig.2-15) and *Aulacoseira* (Fig.2-16) were observed just only in Field 2, and were dominant in May.

Discussion

In previous papers, diatom cell density in flooded water has been reported as on the order of 1.0×10^5

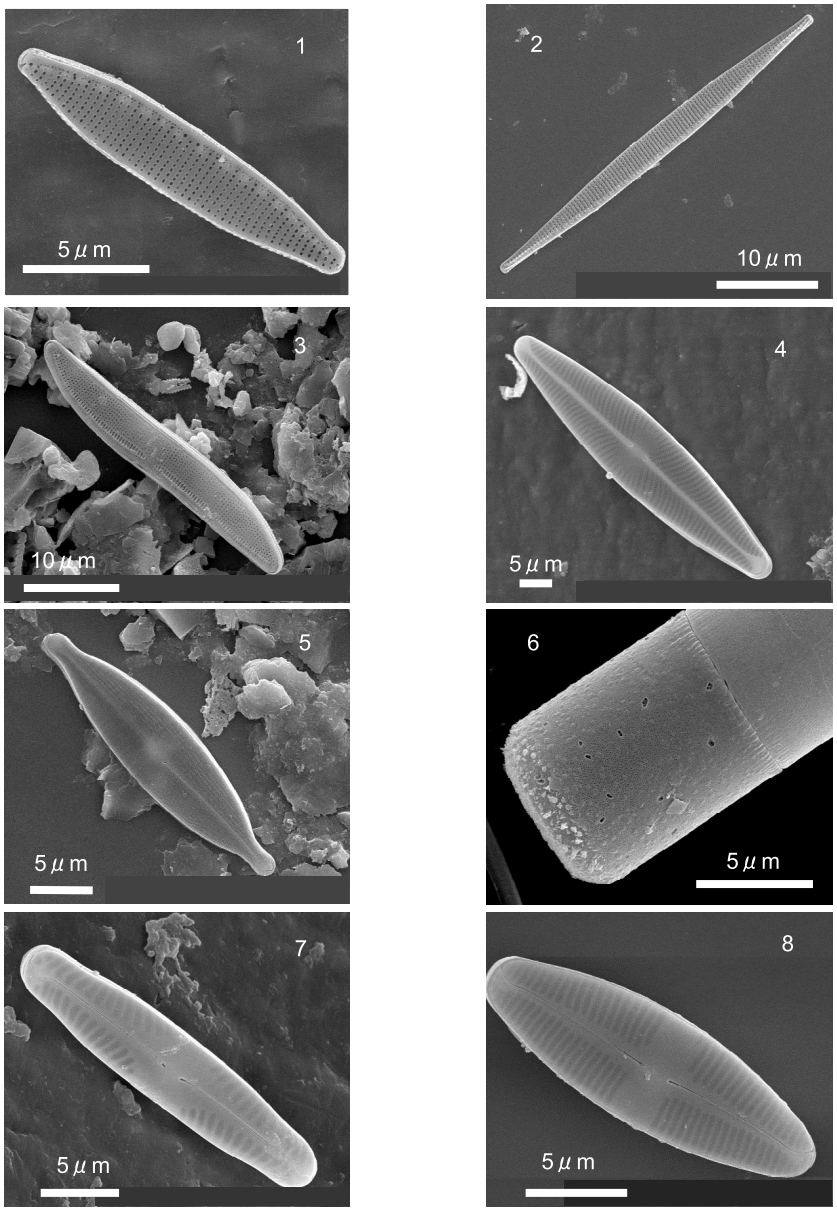

Fig. 2. The SEM and LM micrographs of predominant and characterized diatom genera in Field 1 and Field 2. 1: *Nitzschia hantzschiana* (SEM x5000). 2: *Nitzschia subacicularis* (SEM, x2000). 3: *Nitzschia nana* (SEM, x2000). 4: *Navicula lanceolata* (SEM, x1300). 5: *Navicula gregaria* (SEM, x2500). 6: *Melosira varians* (SEM, x4500). 7: *Pinnularia sp.* (SEM, x3000). 8: *Caloneis bacillum* (SEM, x4000).

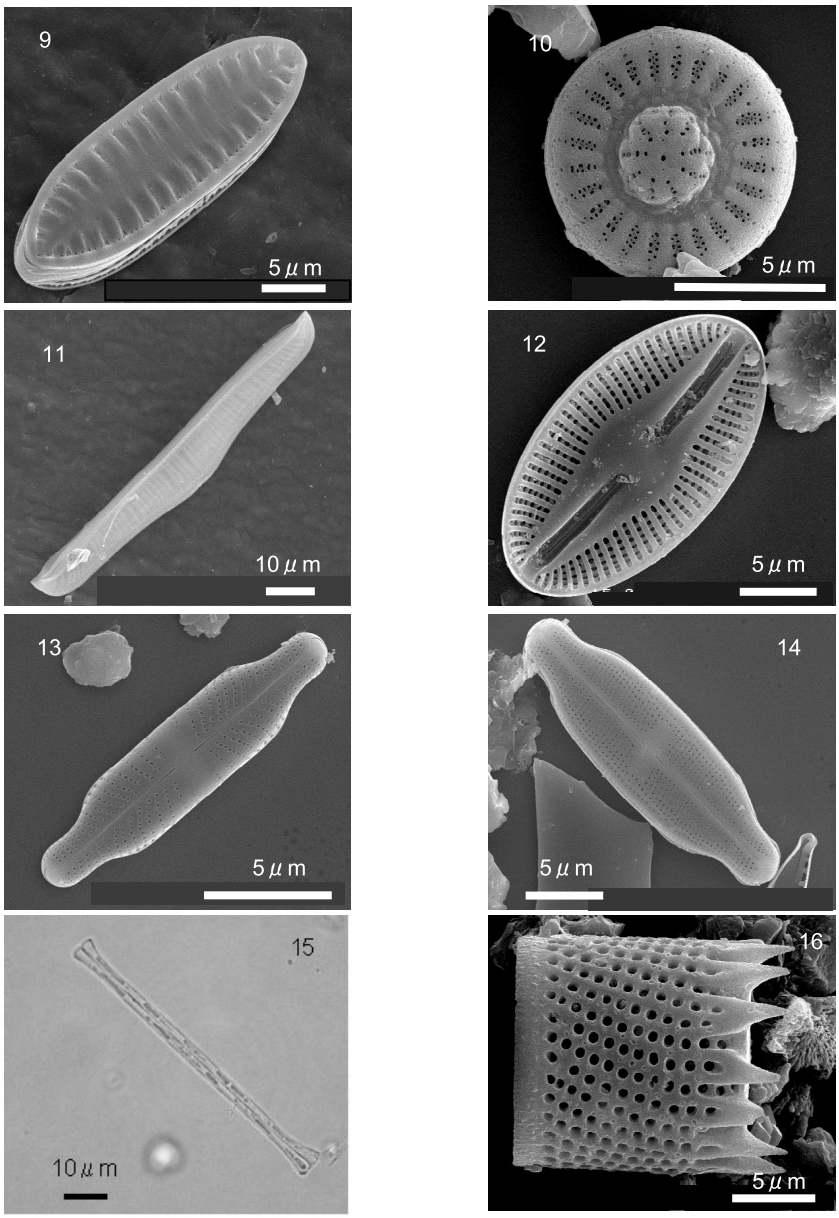

Fig. 2. continued. 9:*Surirella angusta* (SEM, x2500). 10: *Cycloella pseudostelligera* (SEM, x6000). 11: *Rhopalodia gibba* (SEM, x1000). 12: *Diploneis* sp. (SEM, x3000). 13:*Stauroneis thermicola* (SEM, x5000). 14: *Neidium longiceps* (SEM, x3000). 15: *Asterionella formosa* (LM, x800). 16: *Aulacoseira* sp. (SEM, x5000).

and $1.0x10^6$ cells L^{-1} (Kurasawa, 1957, Taira et al., 1987). Results of this study showed within the range as stated above, diatom cell densities ranged from $2.1x10^5$ to $1.1x10^6$ cells L^{-1} in Field 1 and from $4.3x10^5$ to $5.3x10^6$ cells L^{-1} in Field 2. In the present study, seasonal pattern of diatom cell density in the flooded water were largely different between Field 1 and 2. In previous papers, the diatom cell density was the highest just after rice transplanting and decreased thereafter (Kurasawa, 1957, Taira et al., 1987, Fujita and Nakahara, 1999). Whereas, in Field 2, diatoms had a low cell density in flooded water just after rice transplanting, and increased gradually thereafter, which showed unusual pattern for flooded water in paddy fields. The difference of diatom cell density between Field 1 and 2 might be caused the irrigation method. Because Field 1 was conducted the plot-to-plot flow irrigation. On the other hand, Field 2 was stored the flooded water, and irrigated it when it decreased. However, we could not give sufficient interpretation for this difference of seasonal patterns with reference to the environmental factors

or agricultural managements.

Diatom genera reported from paddy fields were both planktonic and benthic, and among them, epipelic ones were usually predominant (Kobayasi, 1950, Negoro, 1954, Kanetsuna, 1957, 1958, 1960, 1961, Mori, 1963, Negoro and Higashino, 1986, Ohtsuka and Fujita, 2001). The diatom genera identified in this study was 90% consistent with the previous studies conducted. However, *Asterionella* and *Rhoicosphenia* in Field 2 were not observed in previous studies.

Most studies reported that *Nitzschia* were predominant genus in the paddy field (Kurasawa, 1957, Taira and Hougetsu, 1987, Fujita and Nakahara, 1999, Ohtsuka and Fujita, 2001). From this study we also supported that *Nitzschia* is usually the most dominant genus in paddy fields.

The difference of genera composition between two fields was partly explained by the different origin of irrigation water. In Field 1, the water was irrigated from small stream gushed from neighbor mountains, which is thought to contain small amount of diatoms. On the other hand, Field 2 was irrigated from the large river with a dam; probably the irrigation water contains many planktonic and stream benthic diatoms. Among the genera observed just only in Field 2, *Asterionella* and *Aulacoseira* were representative planktonic genera (with some exceptional species) which are common in eutrophied lakes containing reservoirs (Krammer and Lange-Bertalot, 1991). *Rhoicosphenia abbreviata*, general species among *Rhoicosphenia*, was periphyton and abundant in the river. Therefore, the seed populations of these genera might be brought through the irrigation water.

This study has provided the basis to clarify the influence of diatoms on Si concentration of flooded water in paddy fields. To clarify the influence, however, it needs more study, especially quantitative studies on Si assimilation by diatoms and accumulation of their frustules in the paddy soil. We will elucidate them in the next studies.

Acknowledgement

We sincerely thank Mr. Tsuruji Sato (Tohoku University) for supporting to take the SEM-micrographs. We are also grateful to Dr. Yuko Fujita (Lake Biwa Museum), Dr. Kinuko Ito (Tohoku University), Dr. Hisao Sasaki (Miyagi Prefectural Institute of Public Health and Environment) and Mr. Kazuhiko Ohba (Miyagi Prefectural Institute of Public Health and Environment) for valuable advice.

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JIFS, 3 : 103 − 114 (2006) original paper

Estimation of Isozyme Marker Genes and Genetic Variability in Shijimi Clam, *Corbicula japonica* in Japan

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Keywords : *Corbicula japonica*, Isozymes, genetic variability

Received 20January 2006 ; accepted 17 February 2006

Abstract

In order to estimate the isozyme marker genes in Shijimi clam, *Corbicula japonica*, starch gel electrophoresis was carried out about 27 enzymes for three tissues. As a result, twelve isozyme loci, *Aat-1, Aat-2, Ak-2, Fdp, Gpi, Idh-1, Mdh-1, Mdh-2, Mdh-3, 6Pgd, Sod-1* and *Sod-2* coding for eight enzymes were estimated as genetic markers. Tissue specific patterns were not observed, but foot tissue was better used for isozyme analysis based on clarity of bands.

Using the above marker genes, genetic variability was calculated as follows: the proportion of polymorphic loci ranged from 0.17 to 0.42 with a mean of 0.31, expected average heterozygosity was ranged from 0.041 to 0.103 with a mean of 0.071, and mean number of alleles per locus ranged from 1.8 to 2.0 with a mean of 1.9. The results revealed that genetic variability in *Corbicula japonica* was higher than fishes, crustacean and squids, but lower than the average of marine mollusks. These isozymes could be useful genetic markers for analyzing population structure of this species.

Introduction

The shijimi clam, *Corbicula japonica*, which has inhabited in brackish lakes and estuary around Japan, is one of the most commercially important species in inland fisheries (Nakamura 2000). Illegal ingressions of the other exotic *Corbicula* species from East Asian countries have been occurred. Ecological and genetic disturbance have been worried.

Isozymes are one of the most useful genetic markers for determination of species or subspecies in many aquatic animals. In *Corbicula* sp. of Japan, Sakai et al. (1994) and Hatsumi et al. (1995) reported the genetic difference among the three species using 12 isozyme loci. But, the genetic control of isozymes used in their reports was not necessarily the same. In fact, Hatsumi et al. (1995) estimated two loci controlling AAT and IDH, but one in Sakai et al. (1994), while Sakai et al. (1994) estimated two loci controlling SOD but one in Hatsumi et al. (1995). This means it is necessary to estimate the genetic control of isozymes before using as genetic markers.

The aims of the present study are to estimate the genetic control of isozymes and to evaluate the genetic variability in *C. japonica* using estimated isozyme marker genes.

Materials and Methods

Specimens of *Corbicula japonica* examined were collected from 3 localities, Lake Jusanko, Lake Ogawarako and Lake Shinjiko, from March to June 2003, as shown in Table 1. Samples were kept cool

Table 1. Sampling data of *Corbicula japonica* examined.

Location	Abbreviation	Date (d-m-y)	Sample No.	Shell height (mm)	Shell length (mm)	Shell width (mm)	Total weight (g)
Jusanko (Aomori)	JYU	3-Mar-03	128	19.5 ± 1.6	22.1 ± 2.0	13.7 ± 1.0	3.7 ± 0.9
Ogawarako (Aomori)	OGA	20-May-03	60	21.1 ± 3.1	22.8 ± 2.8	13.7 ± 2.0	4.5 ± 2.1
Shinjiko (Shimane)	SHI	11, 15-Jun-03	100	18.7 ± 1.4	20.2 ± 1.6	12.8 ± 0.9	3.1 ± 0.7

Shell height, Shell length, Shell width, Total weight : Mean ± S.D.

and transferred to laboratory where they were stored at -30°C until dissection.

Isozymes were detected by horizontal starch gel electrophoresis following the procedure of Fujio and Ikeda (1999). Adductor muscle (AM), digestive caecum (DC), and foot including siphon (FS), were used to analyze the tissue specific patterns. Approximately 200mg of tissue was minced in a 1.5 ml microtube with 30µl deionized water and frozen at -30°C until the electrophoretic run. After thawing the minced tissues at 4°C and centrifugation at 15,000rpm for 10min at 4°C, the tip of the filter paper (4×10mm) was dipped into the supernatant. These tips were used as electrophoretic samples.

Electrophoresis was carried out using 5mm thick 11% (W/V) starch gels under the constant voltage at 240V and 10mm thick gel at 300V. After electrophoretic run for 6 hours, the thick gel was cut into 1.5mm slices for staining. The electrode buffer systems were Tris-citrate buffer (135mM Tris, 43mM citrate, pH 7.0) for all the enzymes and citrate-aminopropylmorpholine buffer (40mM citrate, 0.96% aminopropyl -morpholine, pH 6.0) for AAT, GPI and IDH.

A total of 27 enzymes were examined, as shown in Table 2. The enzyme activity and convergence of bands were estimated by visual observation. The allele estimated from the most frequently appearing band in the sample of Lake Jusanko, was designated as allele *100* at each locus. Other alleles were named according to their relative mobility with respect to the band of allele *100*.

Chi-square tests were carried out to examine whether observed genotype number agreed with expected one under Hardy-Weinberg equilibrium (HWE). Genetic variability was estimated by the proportion of polymorphic loci (P^*), the proportion of variant loci (V^*) less than polymorphism, the average

Table 2. Enzymes surveyed (27enzymes).

Enzyme	Abbreviation	Enzyme Commission Number
Aspartate Aminotransferase	AAT	EC 2.6.1.1
Acid Phosphatase	ACP	EC 3.1.3.2
Alcohol Dehydrogenase	ADH	EC 1.1.1.1
Adenylate Kinase	AK	EC 2.7.4.3
Alkaline Phosphatase	ALP	EC 3.1.3.1
Creatine Kinase	CK	EC 2.7.3.2
Diaphorase	DIA	EC 1.6.*.*
Esterase	EST	EC 3.1.1.-
Fructose-1,6-Diphosphatase	FDP	EC 3.1.3.11
Fumarase	FH	EC 4.2.1.2
Galactose Dehydrogenase	GAD	EC 1.1.1.48
Glutamate Dehydrogenase	GDH	EC 1.4.1.-
Glucosephosphate Isomerase	GPI	EC 5.3.1.9
Glucose-6-phosphate Dehydrogenase	G6PDH	EC 1.1.1.49
Glycerol-3-phosphate Dehydrogenase	αGPD	EC 1.1.1.8
Hexokinase	HK	EC 2.7.1.1
Isocitric Dehydrogenase	IDH	EC 1.1.1.42
Laucine Aminopeptidase	LAP	EC 3.4.11.1
Lactate Dehydrogenase	LDH	EC 1.1.1.27
Malate Dehydrogenase	MDH	EC 1.1.1.37
Malic Enzyme	ME	EC 1.1.1.40
Mannose-6-phosphate Isomerase	MPI	EC 5.3.1.8
Octanol Dehydrogenase	ODH	EC 1.1.1.73
Phosphoglucomutase	PGM	EC 2.7.5.1
6-Phosphogluconate Dehydrogenase	6PGD	EC 1.1.1.44
Sorbitol Dehydrogenase	SDH	EC 1.1.1.14
Superoxide Dismutase	SOD	EC 1.15.1.1

number of alleles per locus (A/L), and observed and expected average heterozygosity (Ho and He). The observed heterozygosity was the direct count of heterozygous individuals in the sample, and the expected heterozygosity was calculated from the formula, $1-\sum\sum x_{ij}^2 / n$, where x_{ij} was the frequency of j-th allele of i-th locus and n was equal to number of examined loci. Genetic differentiation among localities was examined using the Genepop ver. 3.1 (Raymond and Rousset 1995a; 1995b; Goudet et al. 1996).

Results

Genetic control of isozymes

A total of 27 enzymes were examined in the adductor muscle (AM), digestive caecum (DC), and foot including siphon (FS). Electropherograms of the three tissue samples were shown in Fig. 1-1, 1-2, 1-3. Enzyme activity was observed in 23 enzymes out of 27. Tissue specific bands were not obtained though activity and clarity were not the same. Adequate activity was obtained in foot for the 23 enzymes. Enzyme activity and convergence of bands among the three tissue samples were shown in Table 3. As a result, converged bands were observed in 11 enzymes out of the 23. Finally, clear and stable expression bands were obtained in eight enzymes, that is, AAT, AK, FDP, GPI, IDH, MDH, 6PGD and SOD. In order to estimate the isozymes as a genetic marker, suitable electrophoretic condition was examined in the eight enzymes. Comparing the buffer system, clearer bands were observed by C-APM buffer system in AAT, GPI and IDH. In AK, FDP, MDH, 6PGD and SOD, T-C buffer system was suitable for electrophoresis.

In FDP, GPI and 6PGD, single zone was observed on the gel, indicating genetic control of single locus. In AAT, IDH and SOD, two zones always appeared on the gel, revealing two loci systems. Three active zones were observed in AK and MDH. Three loci coding for MDH were estimated, but only one locus was detected in AK because only one zone was always observed stably.

From the above results, twelve loci coding eight enzymes, namely *Aat-1, Aat-2, Ak-2, Fdp, Gpi, Idh-1, Mdh-1, Mdh-2, Mdh-3, 6Pgd, Sod-1* and *Sod-2*, were estimated (Table 4). These twelve loci were used as genetic markers for estimating genetic variability in the present study.

Typical electrophoretic patterns of allozymes of *C.*

Table 3. Tissue specific enzyme activity in the *Corbicula japonica* (n=5).

		T-C[*1] pH7.0		
Enzyme	Isozyme	AM[*2]	DC[*3]	FS[*4]
AAT	AAT-1	++ ○	+ △	++ ○
	AAT-2	nd	nd	++ ○
ACP	(ACP-1)	+ ▲	+ ▲	+ ▲
	(ACP-2)	+ △	± ▲	+ △
ADH	—	-	-	-
AK	(AK-1)	+ △	+ △	+ △
	AK-2	++ ○	+ ○	++ ○
	(AK-3)	+ △	+ △	+ △
ALP	(ALP)	± △	± △	± △
CK	(CK)	++ △	+ △	++ △
DIA	(DIA-1)	+ △	± ▲	± ▲
	(DIA-2)	+ △	± ▲	± ▲
EST	(EST-1)	+ ▲	± ▲	± ▲
	(EST-2)	+++ ▲	+++ ▲	++ ▲
FDP	FDP	++ ○	+ △	++ ○
FH	(FH-1)	+ △	± ×	± ▲
	(FH-2)	+ △	± ×	± ×
GAD	—	-	-	-
GDH	—	-	-	-
GPI	GPI	++ ○	++ △	++ △
G6PDH	(G6PDH)	++ △	++ △	++ △
αGPD	(αGPD-1)	+ △	+ △	+ △
	(αGPD-2)	+ △	+ △	+ △
HK	(HK-1)	++ △	+ △	++ △
	(HK-2)	+ ×	± ×	+ ×
IDH	IDH-1	++ ○	++ ○	++ ○
	(IDH-2)	+ ×	+ ×	+ ×
LAP	(LAP-1)	++ ○	++ ○	++ ○
	(LAP-2)	++ ○	++ ○	++ ○
LDH	—	-	-	-
MDH	MDH-1	++ ○	++ ○	++ ○
	MDH-2	++ ○	++ ○	++ ○
	MDH-3	+ ○	+ ○	+ ○
ME	(ME-1)	+ △	+ △	+ △
	(ME-2)	++ ×	++ ×	++ ×
MPI	(MPI)	+ △	+ △	+ △
ODH	(ODH)	± ○	± ○	± ○
PGM	(PGM-1)	+ △	+ △	+ △
	(PGM-2)	+ ○	+ ○	+ ○
6PGD	6PGD	++ ○	++ ○	++ ○
SDH	(SDH)	+ ×	+ ×	+ ×
SOD	SOD-1	++ ○	++ ○	++ ○
	SOD-2	++ ○	++ ○	++ ○

∗ Activity	∗ Convergence
+++ : very high	○ : veryclear
++ : high	△ : unclear
+ : low	▲ : hard to converge
± : unstable	× : diffused
- : nothing	nd : no data

[*1]T-C : tris-citrate buffer
[*2]AM : adductor muscle
[*3]DC : digestive caecum
[*4]FS : foot (including siphon)

Table 4. Expected loci and available as genetic marker in the *Corbicula japonica* of Jusanko in Aomori prefecture.

Enzyme	Tissue	Buffer[*1]	pH	Expected loci	Available as genetic marker[*2]
AAT	Foot	C-APM	6	*Aat-1*	○
	Foot	C-APM	6	*Aat-2*	○
AK	Foot	T-C	7	*(Ak-1)*	×
	Foot	T-C	7	*Ak-2*	○
	Foot	T-C	7	*(Ak-3)*	×
FDP	Foot	T-C	7	*Fdp*	○
GPI	Foot	C-APM	6	*Gpi*	○
IDH	Foot	C-APM	6	*Idh-1*	○
	Foot	C-APM	6	*(Idh-2)*	(○)
MDH	Foot	T-C	7	*Mdh-1*	○
	Foot	T-C	7	*Mdh-2*	○
	Foot	T-C	7	*Mdh-3*	○
6PGD	Foot	T-C	7	*6Pgd*	○
SOD	Foot	T-C	7	*Sod-1*	○
	Foot	T-C	7	*Sod-2*	○
				15 loci controlling 8 enzymes	12 loci controlling 8 enzymes

[*1]C-APM : citrate-aminopropylmorpholine buffer

[*1]T-C : tris-citrate buffer

[*2]being able to be used as genetic marker

japonica are shown in Fig.2-1 and Fig.2-2. In AAT, one or three bands were observed both on the anodal and cathodal zones individually, estimating three alleles at *Aat-1* and two at *Aat-2*. In AK, one or two bands were observed individually, indicating a typical genetic variation of monomeric enzyme structure. Based on the banding positions, five alleles were estimated. The FDP showed only one band in all individuals, revealing monomorphic at the one locus, *Fdp*. In GPI and 6PGD, typical genetic variations of dimeric enzyme structure at one zone were observed. According to the banding positions, three alleles were estimated at *Gpi*, and four alleles at *6Pgd*. In IDH, two banding zones were observed separately on the anodal (IDH-1) and the cathodal areas (IDH-2), but IDH-2 bands were not always detected. In the IDH-1, one or three bands were observed, indicating a typical genetic variation of dimeric enzyme structure, and then three alleles were estimated at the *Idh-1*. At least three bands and maximally five bands were observed in MDH. The first and second bands from origin were always appeared but at the most anodal zone, one or two or three bands were observed individually. The third band from origin was always appeared. Therefore, three loci controlling MDH were estimated. The *Mdh-2* and *Mdh-3* loci were monomorphic while *Mdh-1* was polymorphic with three alleles. The band controlled allele *100* at *Mdh-1* appeared at the same position with that of *Mdh-2*. In SOD, two active zones were observed on the anodal area, indicating two different loci (*Sod-1* and *Sod-2*). Both two loci were monomorphic.

Genetic variability of isozymes

Allele frequencies at the 12 loci in the 3 localities of *C. japonica* are shown in Table 5. Significant deviation from HWE was not observed at all loci in all localities. Homogeneity tests for allele distribution at each locus between every pair of localities were done. Significant difference was observed in every pair of localities, indicating that an independent local population was constructed in each location.

Genetic variability of *C. japonica* was estimated based on the 12 loci, as shown in Table 6. Genetic variation was observed in 6 to 7 loci out of 12. The number of polymorphic loci, defined as the locus that had the maximum allele frequency of $\leqq 0.95$, was 4, 5 and 2, in Lake Jusanko, Lake Ogawarako and Lake Shinjiko, respectively. The proportion of polymorphic loci (P^*) and the proportion of variant loci (V^*) ranged from 0.17 to 0.42, with a mean of 0.31, and from 0.17 to 0.42 with a mean of 0.25, respectively. The

Table 5. Gene frequency at 12 loci in the 3 local lots for *Corbicula japonica*.

locus	*allele*	JYU	OGA	SHI
Aat-1	N*	90	55	98
	150	-	0.082	0.005
	100	0.950	0.918	0.990
	80	0.050	-	0.005
Aat-2	N	82	57	100
	-70	0.061	0.088	0.020
	-100	0.939	0.912	0.980
Ak-2	N	90	60	100
	120	-	-	0.005
	110	-	0.017	0.005
	100	1.000	0.950	0.970
	90	-	0.017	0.020
	80	-	0.017	-
Fdp	N	90	60	97
	100	1.000	1.000	1.000
Gpi	N	90	59	100
	100	0.900	0.856	0.925
	90	0.083	0.119	-
	80	0.017	0.025	0.075
Idh-1	N	90	44	100
	140	0.006	-	-
	100	0.894	0.875	0.995
	75	0.100	0.125	0.005
Mdh-1	N	90	60	100
	140	0.111	0.208	0.020
	110	-	-	0.005
	100	0.889	0.792	0.975
Mdh-2	N	90	60	100
	100	1.000	1.000	1.000
Mdh-3	N	90	60	100
	100	1.000	1.000	1.000
6Pgd	N	90	60	100
	140	0.011	-	0.090
	100	0.978	0.992	0.905
	90	0.006	-	-
	65	0.006	0.008	0.005
Sod-1	N	90	60	100
	100	1.000	1.000	1.000
Sod-2	N	89	60	100
	100	1.000	1.000	1.000

*N : sample size

proportion of total variant loci (P*+V*) from 0.50 to 0.58 with a mean of 0.56. Mean number of alleles per locus (A/L) was counted as 1.8 and 2.0 with a mean of 1.9. The observed average heterozygosity (Ho) and expected one (He) were distributed from 0.042 to 0.108 with a mean of 0.073, and 0.041 to 0.103 with a mean of 0.071, respectively. Ho/He values were 1.006 to 1.050 with a mean of 1.025. Genetic variability was roughly similar among localities.

Discussion

Detection of Isozymes

In *C. japonica*, Sakai et al. (1994) estimated isozyme loci used adductor muscle and mid-gut gland

(equal to digestive caecum) and Hatsumi et al. (1995) used mid-gut gland, respectively. In the present study, tissue specific pattern was not observed, and then any tissues can be used for isozyme analysis. Therefore, the present data could be compared with their reports on the view point of used tissues.

In the present study, 12 loci coding for 8 enzymes were estimated in *C. japonica*. In the same species, Sakai et al. (1994) reported 12 loci, *Aat, Acp, Cap, Gpi, Idh, Mdh-1, Mdh-2, Pgd, Pgm-1, Pgm-2, Sod-1* and *Sod-2* coding for 9 enzymes, and Hatsumi et al. (1995) reported 12 loci, *Aat-1, Aat-2, Cat, Idh-1, Idh-2, Lap, Mdh-1, Mdh-2, 6Pgd, Pgm-1, Pgm-2* and *Sod* coding for 8 enzymes, respectively. Different kinds of loci were estimated in these studies. This is probably caused by the difference of electrophoretic condition such as buffer systems, kinds of chemicals for staining, and condition of samples storage. Actually different buffer systems were used as shown in Table 7.

Table 6. Gene variability of isozymes in 3 localities for *Corbicula japonica*.

Locus	JYU	OGA	SHI
Aat-1	V(2)*	P(2)	V(3)
Aat-2	P(2)	P(2)	V(2)
Ak-2	M(1)	V(4)	V(4)
Fdp	M(1)	M(1)	M(1)
Gpi	P(3)	P(3)	P(2)
Idh-1	P(3)	P(2)	V(2)
Mdh-1	P(2)	P(2)	V(3)
Mdh-2	M(1)	M(1)	M(1)
Mdh-3	M(1)	M(1)	M(1)
6Pgd	V(4)	V(2)	P(3)
Sod-1	M(1)	M(1)	M(1)
Sod-2	M(1)	M(1)	M(1)
P^*	0.33	0.42	0.17
V^*	0.17	0.17	0.42
P^*+V^*	0.50	0.58	0.58
A/L	1.8	1.8	2.0
H_o	0.069	0.108	0.042
H_e	0.069	0.103	0.041
H_o/H_e	1.006	1.050	1.019

*( ) : No.of alleles

P : Polymorphic (maximum allele frequency ＜ 0.95)

V : Variant (maximum allele frequency ≧ 0.95)

M : Monomorphic

P^* : Proportion of polymorphic loci

V^* : Proportion of variant loci

P^*+V^* : Proportion of P+V

A/L : Average Number of alleles per locus

Ho : Average heterozygosity (observed)

He : Average heterozygosity (expected)

Genetic variability

Genetic variability of *C. japonica* was reported using 12 loci by Sakai et al. (1994) and Hatsumi et al. (1995). The proportion of polymorphic loci, the average number of alleles per locus and expected average heterozygosity were reported as 0.33 to 0.42, 1.8 to 2.0 and 0.111 to 0.132 by Sakai et al. (1994) and 0.42 to 0.58, 1.7 to 2.1, 0.127 to 0.206 by Hatsumi et al. (1995), respectively. Compared with the present result, genetic variability was almost the same, suggesting that obvious change in genetic variability has not been occurred during recent decade.

Genetic variability, especially expected average heterozygosity was reported in many fishes and shellfishes, including crustacean. In fish species, average heterozygosity is reported as 0.059±0.007 (Fujio and Kato 1979), 0.043±0.028 in decapod crustacea (Chow and Fujio 1987), 0.033±0.010 in squids (Fujio and Kawada 1989) and 0.147±0.011 in marine mollusks (Fujio et al. 1983). Compared with *C. japonica* including the data from Sakai et al. (1994), Hatsumi et al. (1995) and our result (He = 0.073±0.033), average heterozygosity of *C. japonica* is higher than fishes, crustacean and squids, but lower than an average of the other marine mollusks.

In the present study, observation of genetic differentiation among localities would mean the existence of local populations. Therefore, 12 allozyme loci estimated in the present study will be useful genetic markers for analyzing population structure of the species.

Acknowledgments

We wish to express our sincere thanks to Mr. Minoru Oonaka, I-fish, Inc. and Mr. Kesao Yagihashi, Hirosaki-Maruuo for supporting the specimens of *C. japonica*.

Table 7. Comparison of isozyme loci detected among the three independent papers

Enzyme	Sakai et.al (1994)		Hatsumi et.al (1995)		Present study	
	Locus	buffer* (pH)	Locus	buffer (pH)	Locus	buffer (pH)
AAT	*Aat*	T-C (8)	*Aat-1*	T-C (7)	*Aat-1*	C-APM (6)
			Aat-2	T-C (7)	*Aat-2*	C-APM (6)
ACP	*Acp*	AC (6)	nd		*(Acp)*	T-C (7)
AK	nd		nd		*Ak-2*	T-C (7)
CAT	nd		*Cat*	others (8)	nd	
CAP	*Cap*	RW (8.5)	nd		nd	
FDP	nd		nd		*Fdp*	T-C (7)
GPI	*Gpi*	T-C (8)	nd		*Gpi*	C-APM (6)
IDH	*Idh*	T-C (8)	*Idh-1*	C-APM (6.2)	*Idh-1*	C-APM (6)
			Idh-2	C-APM (6.2)	*(Idh-2)*	C-APM (6)
LAP	nd		*Lap*	T-C (7)	*(Lap-1)*	T-C (7)
					(Lap-2)	T-C (7)
MDH	*Mdh-1*	T-C (8)	*Mdh-1*	C-APM (6.2)	*Mdh-1*	T-C (7)
	Mdh-2	T-C (8)	*Mdh-2*	C-APM (6.2)	*Mdh-2*	T-C (7)
					Mdh-3	T-C (7)
PGM	*Pgm-1*	RW (8.5)	*Pgm-1*	C-APM (6.2)	*(Pgm-1)*	T-C (7)
	Pgm-2	RW (8.5)	*Pgm-2*	C-APM (6.2)	*(Pgm-2)*	T-C (7)
6PGD	*6Pgd*	T-C (8)	*6Pgd*	T-C (7)	*6Pgd*	T-C (7)
SOD	*Sod-1*	RW (8.5)	*Sod*	T-C (7)	*Sod-1*	T-C (7)
	Sod-2	RW (8.5)			*Sod-2*	T-C (7)
No. loci	12		12		12	

*T-C : tris-citrate buffer
*AC : amine citrate buffer
*RW : tris-citric acid, lithium hydroxide-boric acid buffer
*C-APM : citrate-aminopropylmorpholine buffer
*others : tris-EDTA-borate buffer
nd : no data

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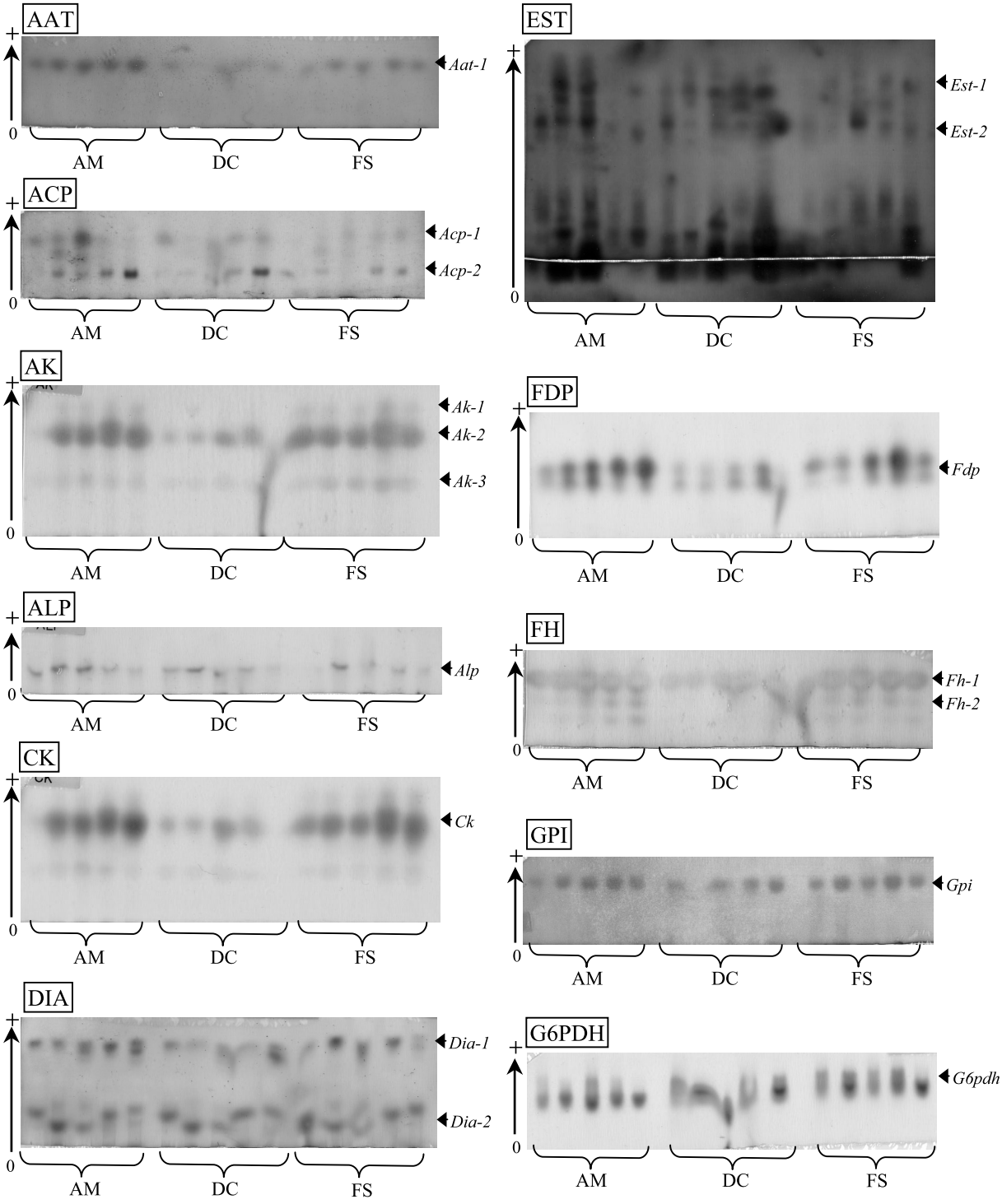

Fig. 1-1. Tissue specific isozyme patterns in *Corbicula japonica*.
AM:adductor Muscle, DC:digestive caecum, FS:foot including siphon

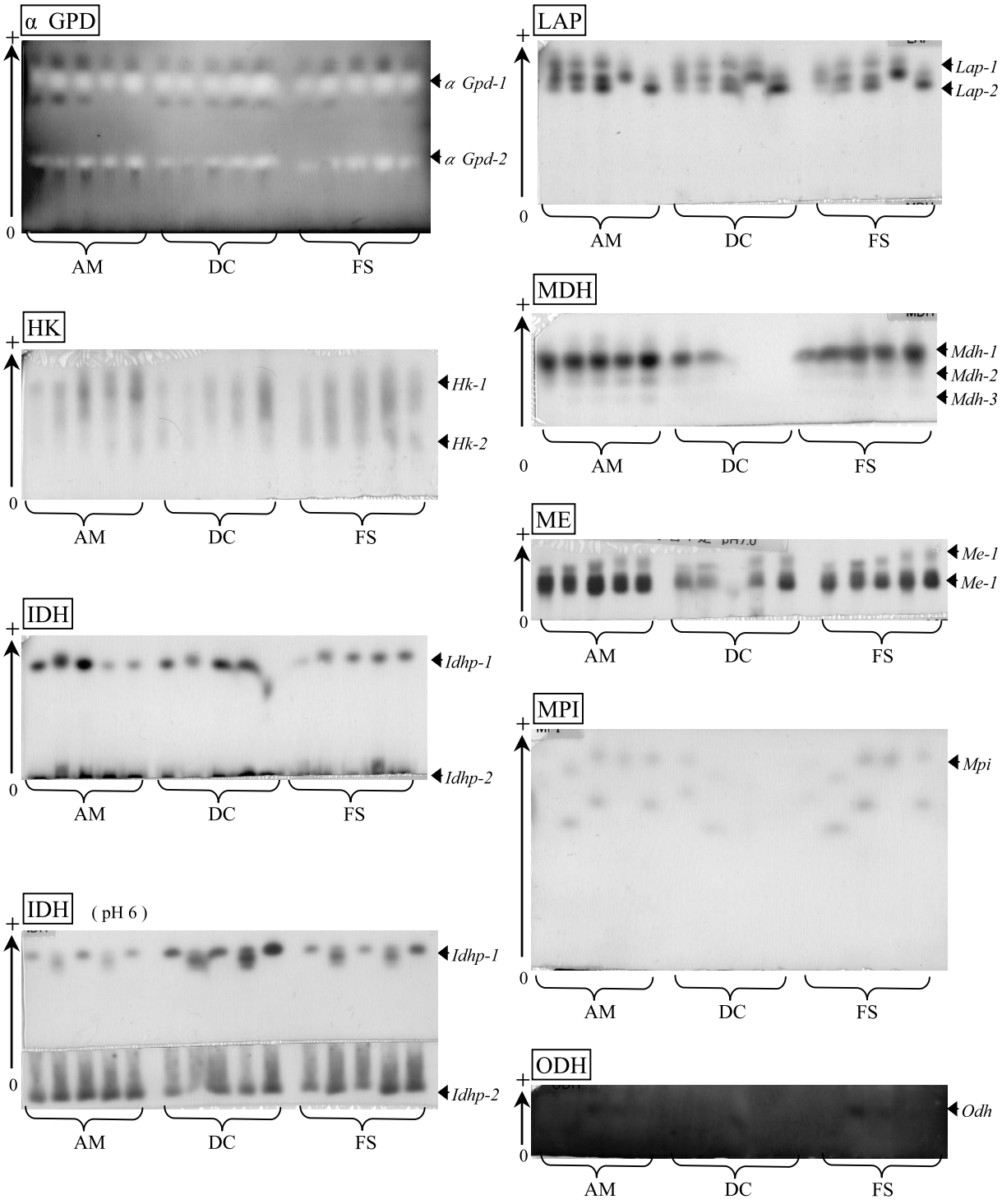

Fig. 1-2. Tissue specific isozyme Patterns in *Corbicula japonica*.
AM:adductor Muscle, DC:digestive caecum, FS:foot including siphon

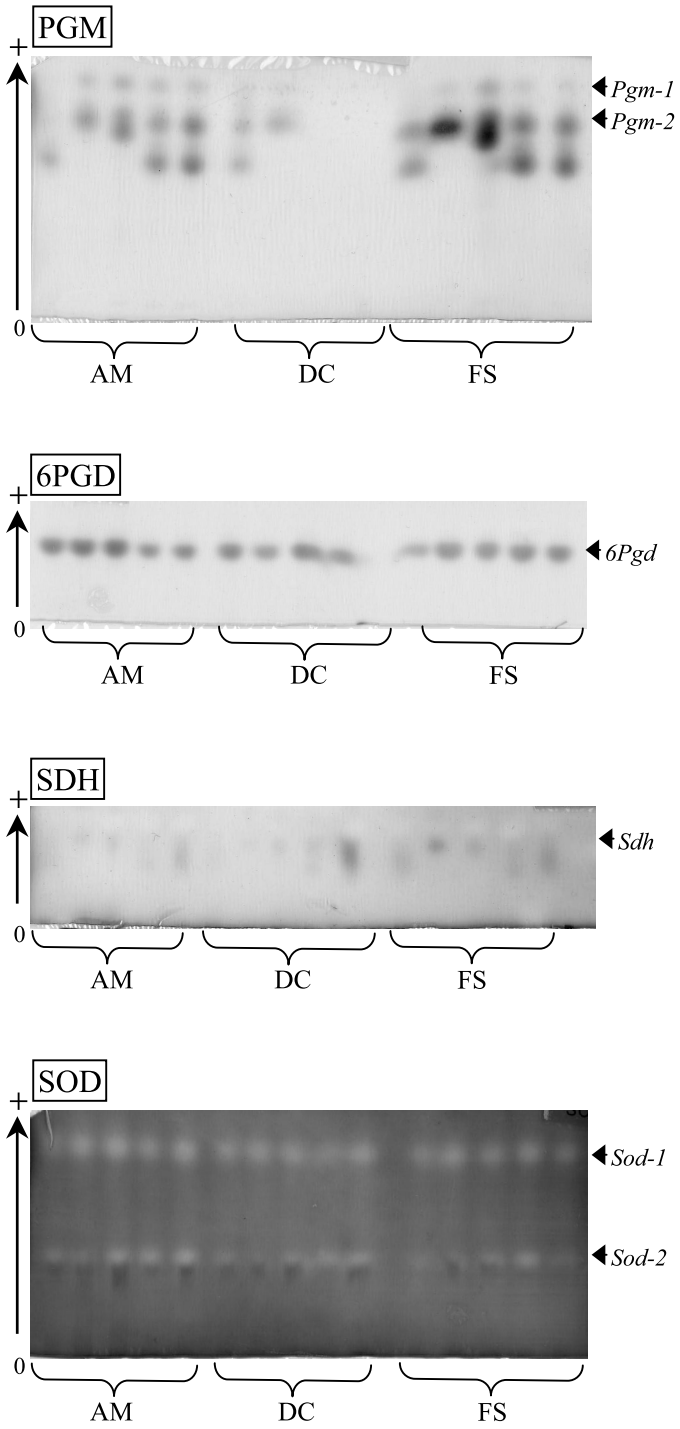

Fig. 1-3. Tissue specific isozyme Patterns in *Corbicula japonica*.
AM:adductor Muscle, DC:digestive caecum, FS:foot including siphon

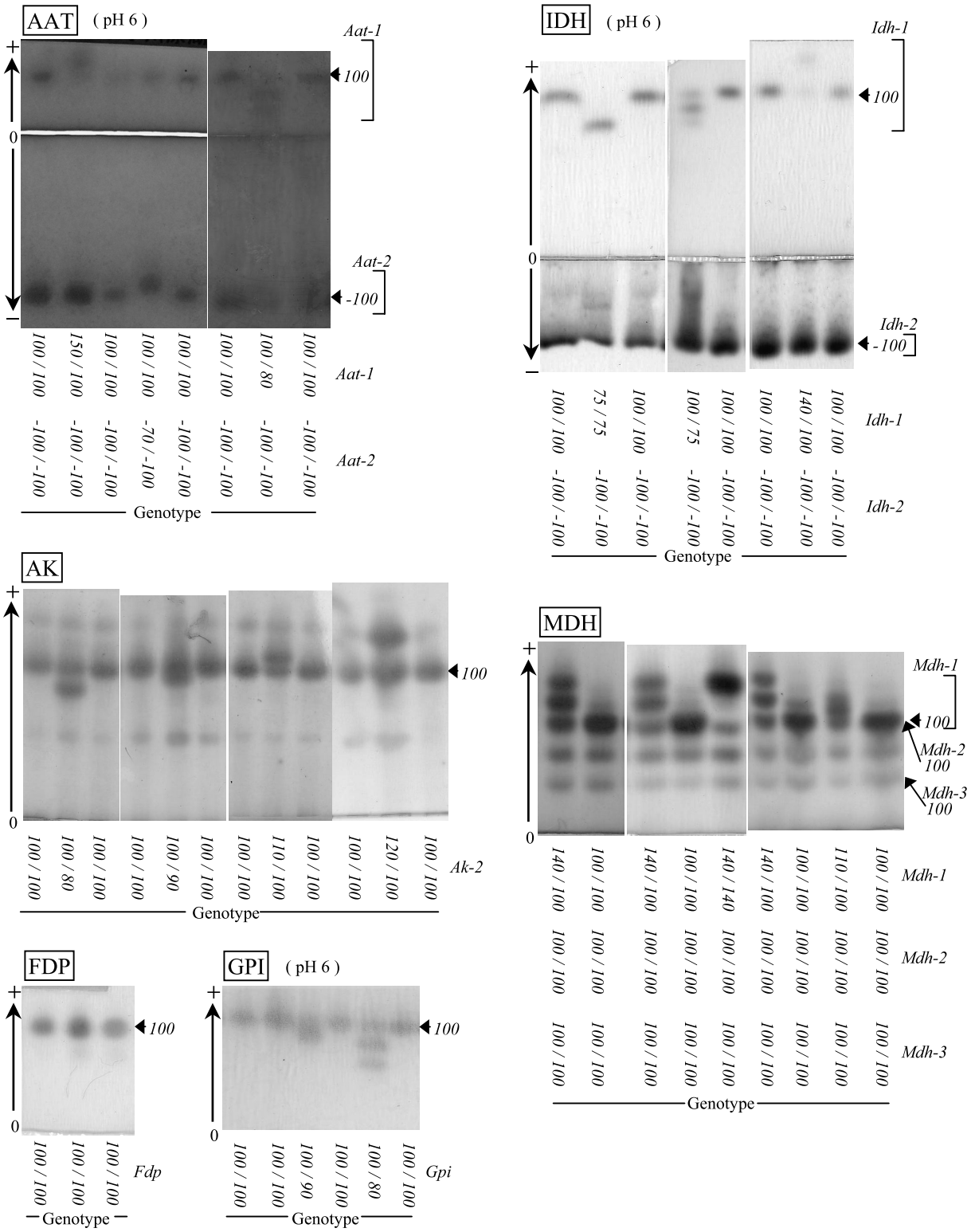

Fig.2-1. Electropherograms of 12 isozymes in *Corbicula japonica*.

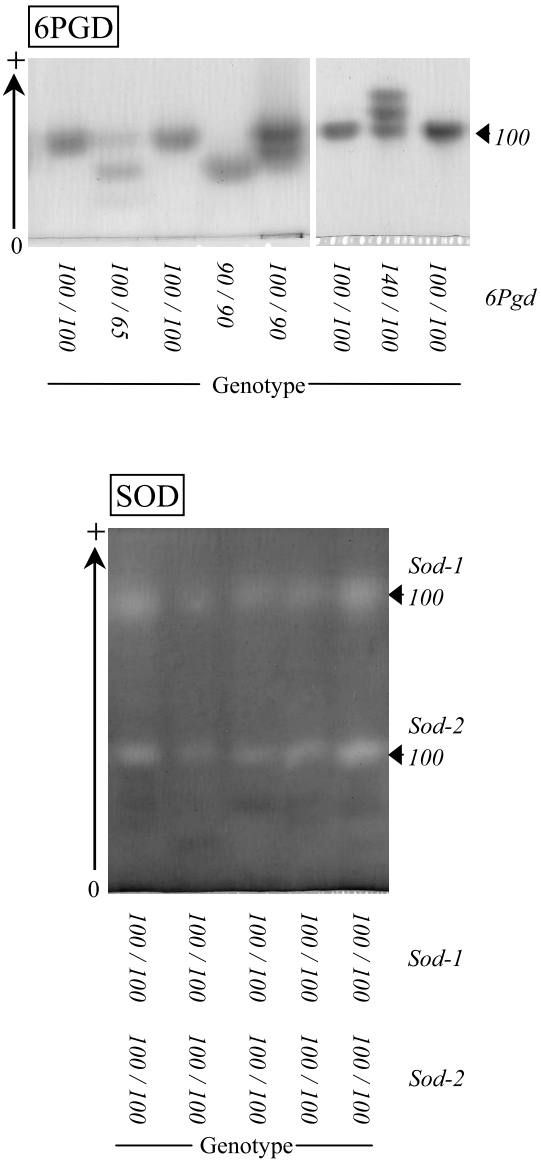

Fig.2-2. Electropherograms of 12 isozymes in *Corbicula japonica*.

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