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Doctoral Thesis

Glycomics Approach of Livestock Management Toward the Discovery of Novel Biomarkers Indicating an Environmental Stress

(糖鎖解析による家畜管理法の試み–環境ストレスを反映するバイオマーカーの探索)

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March, 2016

Abstract

Because various stresses strongly influence the food productivity of livestock, biomarkers to indicate unmeasurable environmental stress in domestic farm animals are of increasing importance. Thermal comfort is one of the basic principles of dairy cow welfare that enhances productivity, so that the more attention needs to be placed on the principles of The Welfare Quality® Assessment protocol, especially in summer and autumn seasons, in order to enhance the performance and production. However, the expression levels of two major sialic acids, notably Neu5Ac and Neu5Gc, in the glycoproteins/glycosphingolipids synthesized by common mammalian cells except human cells might be a critical posttranslational modification indicating drastic changes of the metabolic immune balance as well as seasonal alteration of the total serum *N*-glycan profiles; as an abundant and major class of glycoconjugates in animal serum glycoproteins. Thus, I considered that advent of novel and sensitive serum biomarkers using glycoblotting combined with MALDI-TOF/MS and DMB-HPLC allowed for comprehensive *N*-glycomics of animal model to indicate not only their productivities but significant changes in total healthy condition caused by unmeasurable stresses would contribute greatly to the improvement of their management, healthy performance and high-quality productivity.

Keywords**:** *glycoblotting, serum glycoproteins, N-glycan structure, sialic acids, environmental stress, immune responses, Holstein dairy cow welfare, serum biomarker.*

Acknowledgements

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Sapporo, 2016

Mr. Ibrahim Farag Mohammed Rehan

Table of Contents

List of Figures

List of Tables

Abbreviations

ABC	ammonium bicarbonate 99%
AVG	Average
AcOH	acetic acid
AFP	α-fetoprotein
BOA	*O*-benzylhydroxylamine hydrochloride
CEA	carcinoembryonic antigen
DCSIGN	dendritic cell-Specific intercellular adhesion molecule-3-grabbing non-integrin
DHex	Deoxyhexose
DMB	1,2-diamino-4,5-methylenedioxy-benzene
DTT	Dithiothreitol
Fc	fragment crystallizable
Fcγ/FcRn	Fc-gamma/neonatal Fc receptor
Fu	Fucose
Gal	Galactose
GlcNAc	*N*-acetyl-D-glucosamine
HeNAc	*N*-acetylhexosamine
Hex	Hexose
HPLC	high performance liquid chromatography
IAA	Iodoacetamide
ICAM-3	intercellular adhesion molecule 3
IgG	immunoglobulin G
IS	internal standard
JMA	Japan metrological agency
KDN	2-keto-3-deoxynonulosonic acid
m/s	meters per second
MALDI-TOF/MS	matrix assisted laser desorption ionization-time of flight/mass spectrometry
Man	Mannose
MeOH	Methanol
MJ/m^2	megajoule per square metre

MS	mass spectrometry
MTT	3-Methyl-1-*p*-tolyltriazene
Neu5Ac	5-*N*-Acetylneuraminic acid
Neu5Gc	5-*N*-Glycolylneuraminic acid
PHM	sodium 2-hydroxy-3-tetradecanamidopropane-1-sulfonate [1-propanesulfonic acid, 2-hydroxyl-3-myristamido, sodium salt (1:1)]
PNGase F	peptide *N*-glycosidase F trypsin
PRRs	pattern recognition receptors
RH	relative humidity
Siglec	sialic acid-binding immunoglobulin-type lectins
T_H2	T helper cell2
THI	temperature−humidity index
CMAH	cytidine monophosphate *N*-acetylneuraminic acid hydroxylase
CMP	cytidine monophosphate

Chapter 1

General Introduction and Review of Literatures

1.1. Stress and Farm Animal Welfare

From an analytical viewpoint, the farming system can be seen as the primary condition determining the living circumstances and thereby the welfare including the health of livestock. The welfare of an animal is its state as regards its attempts to cope with its environment. The animal welfare contains four principles (good housing, good feeding, good health, and appropriate behaviours) and twelve criteria (absence of prolonged hunger, absence of prolonged thirst, thermal comfort, comfort around resting, ease of movement, no painful management procedures, no disease, no injuries, positive emotional state, good human-animal relationship, and expressing other behaviors), as shown in (Figure 1.1). These principles and criteria should be considered to deal with animal farm. Coping means maintaining control of mental and bodily stability of animal in response to a challenge. Welfare therefore includes the extent of failure to cope, which may lead to disease and injury, but also the ease or difficulty in coping. Both the long-term and short-term costs of coping are important for an animal‘s welfare. For example, an animal may cope with an acute stress in the short-term by taking action that results in a long term reduction in its welfare. For example, dairy cattle may be able to cope with the stresses associated with high milk in lactation, but this coping may result in long-term health problems that are apparent in later lactations.[1] Stress is defined as an organisms total response to environmental demand or pressures.[2,3] Environmental stressor is not limited to climatic factors but extends to nutrition, housing and any stimuli that demand a response from the animal to adapt to new circumstances.[4] However, the main stressors in an animal's life is changing in routine/environment, over-stimulation which is the opposite of boredom.[3] For instance, to get a proper management of dairy cows welfare thermal comfort,[5] must be considered in those animals to make adjustments of the metabolism in order to maintain homeothermy.[6]

At this time, most of the animal welfare researchers were in zoology or animal production departments in universities and research institutes. Although not often aware of the wide range of welfare topics, many veterinarians were aiming to benefit the animals and improve animal welfare by trying to cure or prevent animal disease. Some of these used their clinical knowledge to ensure that the health of animals was properly considered in evaluation of welfare whilst others carried out experimental work. Veterinarians who contributed to more general aspects of animal welfare science included Andrew Fraser, Ingvar Ekesbo, Henrik Simonsen, Robert Dantzer, Roger Ewbank, Barry Hughes and John Webster. Andrew Fraser was one of the founders of the Society for Veterinary Ethology (later the International Society for Applied Ethology), which is the major scientific society for animal welfare science. He was also editor of the journal then called ‘‘Applied Animal Ethology’’ and now called ‘‘Applied Animal Behaviour Science’’ which is the most important journal for scientific papers on animal welfare. The journal ‘‘Animal Welfare’’ has also been of major importance in more recent years.

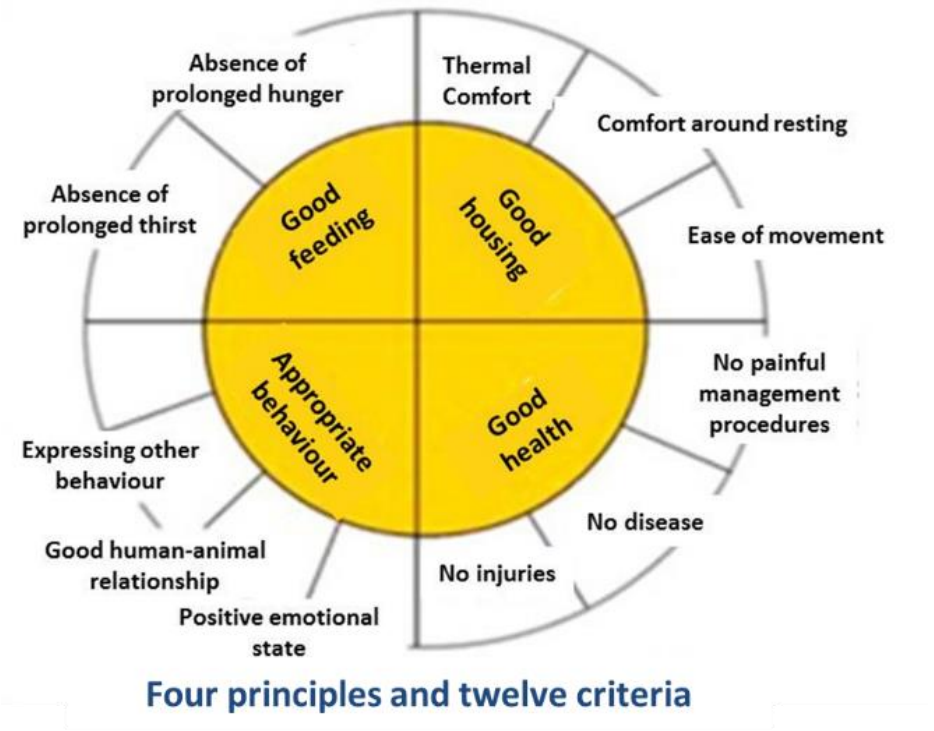


Figure 1.1. The principles and criteria of cow welfare.

1.2. Five Freedom of Farm Animal Welfare

The welfare of an animal includes its physical and mental state and we consider that good animal welfare implies both fitness and a sense of well-being. Any animal kept by man must at least be protected from unnecessary suffering. The animal's welfare, whether on farm, in transit, at market or at a place of slaughter should be considered in terms of five freedoms, as sourced in Farm Animal Welfare Council. (http://www.fawc.org.uk/index.htm). These freedoms define ideal states rather than standards for acceptable welfare. They form a logical and comprehensive framework for analysis of welfare within any system together with the steps and compromises necessary to safeguard and improve welfare within the proper constraints of an effective livestock industry

1. Freedom from hunger and thirst – by ready access to fresh water and a diet to maintain full health and vigour.
2. Freedom from discomfort – by providing an appropriate environment including shelter and a comfortable resting area.
3. Freedom from pain, injury or disease – by prevention or rapid diagnosis and treatment.
4. Freedom to express normal behaviour – by providing sufficient space, proper facilities and company of the animal's own kind.
5. Freedom from fear and distress – by ensuring conditions and treatment that avoid mental suffering.

The word stress should be used for that part of poor welfare that involves failure to cope. If the control systems regulating body state and responding to dangers are not able to prevent displacement of state outside the tolerable range, a situation of different biological importance is reached. The use of the term stress should be restricted to the common public use

of the word to refer to a deleterious effect on an individual.[7] A definition of stress as just a stimulation or an event that elicits adrenal cortex activity is of no scientific or practical value. A precise criterion for what is adverse for an animal is difficult to find but one indicator is whether there is, or is likely to be, an effect on biological fitness. Stress is an environmental effect on an individual that over-taxes its control systems and results in adverse consequences, eventually reduced fitness.[7-9] Using this definition, the relationship between stress and welfare is very clear. Firstly, whilst welfare refers to a range in the state of the animal from very good to very poor, whenever there is stress, welfare is poor. Secondly, stress refers only to situations where there is failure to cope but poor welfare refers to the state of the animal both when there is failure to cope and when the individual is having difficulty in coping. It is very important that this latter kind of poor welfare, as well as the occasions when an animal is stressed, is included as part of poor welfare. For instance, if a person is severely depressed or if an individual has a debilitating disease but there is complete recovery with no long term effects on fitness then it would still be appropriate to say that the welfare of the individuals was poor at the time of the depression or disease. In the circumstances in which people have referred to some stress being good, the effect is not stress but is stimulation that is useful experience in the maintenance or development of individuals. There is no stress that is good and the terms "eustress" and "dystress" are redundant. If an experience is difficult to cope with but beneficial in the long-term, the welfare of the individual is poor at the time of coping difficulty but no stress occurs.

1.3. Thermal Comfort and Environmental Stress in Holstein Dairy Cows

The more attention needs to be placed on the principles of The Welfare Quality Assessment protocol to enhance the performance and productivity of farm animals. Especially, Freedom #2, in which, thermal comfort is one of the most important factors in the management of dairy cow welfare,[5] because cows must make metabolic adjustments to maintain homeothermy.[6] Various stressors could affect on the animal production and welfare, physically as environmental climate, moisture, noise, transport, nutritional or pathologically as biological agent of disease and vaccines. The major factors responsible for Holstein cows were i) it became known worldwide that Holsteins gave higher milk yields than most breeds; ii) dairy farmers' breeding objectives worldwide became increasingly focused on income from sale of milk; iii) technology existed to import Holsteins from the USA into other countries. However, low productive efficiency caused by heat stress among the Holstein breed is a significant issue faced by the main dairy-producing countries worldwide, for instance, the annual production loss of the US dairy herd was reported to be more than $900 million/year. Stressful environmental stimuli are one of the main causes of health issues and deficient immune responses in dairy cows,[10] therefore, heat stress represents a major constraint on animal growth rates, milk yield, and metabolic immune balance.[11] In contrast, dairy cows are relatively resistant to cold stress in winter due to the presence of subcutaneous fat.[12] The ultimate aim of every dairy farm is to produce the highest milk yield; therefore, an efficient management strategy to maintain the thermal comfort of dairy cows is of growing importance. Heat stress occurs when the core body temperature of a given species surpasses the range specified for normal activity due to the total heat load as well as internal production and the environment exceeding the capacity for heat dissipation through sweating and panting. Therefore, heat stress has negative impacts on the health condition of as well as milk production by dairy cows and is associated with serious economic challenges.[13]

1.4. Temperature−Humidity Index (THI)

An index combining temperature and humidity, the temperature−humidity index (THI), has been used in assessments of heat stress in cattle to describe categories of heat stress associated with hot weather for livestock exposed to extreme conditions, even with recognized limitations related to wind speed and radiation heat loads.[14] Previous studies suggested that the general level of thermal comfort in dairy cows estimated by the THI appeared to be <72,[15] with milk production beginning to decrease when the THI exceeds 72.[16] The THI value of dairy cows producing >35 kg/day of milk was found to be 68.[17] Therefore, the sensitivity of dairy cows to heat stress already appears to be enhanced when milk production increases.[18] Because heat stress gradually increases in dairy cows, precise estimations of the heat load must include a measure of the intensity of the heat load and the length of time over which animals are exposed to this stress (for example, THI 78 for a period of >4 h).[19] Moreover, convective and radiant heat exchanges are other factors that contribute to air movement and solar radiation.[20] The presence of sunshine may add several points to the THI, whereas wind may lower it by a few points because it brings cooler air to animals and carries away excess heat. It is important to note that the THI value cannot be used as a feasible indicator to alert farmers to any alterations in an animal's metabolic/homeostatic immune balance perturbed by "complicated heat stress", whereas the THI may be an important parameter for simply showing the temperature and humidity of the feeding environment. Farmers have not been able to fully recognize the signs of cows being physically affected by slight heat stress unless their condition becomes critical. Moreover, traditional physiological approaches are also insufficient for elucidating a direct correlation between hormone secretions with adverse effects on animal well-being due to the stress adaptability of the animal; therefore, the best indicator for identifying animals under stress is the development of a prepathological state, that is, a stress-related change in biological functions with suppression of the immune system.[21]

Therefore, the advent of novel and sensitive serum biomarkers to indicate not only the productivity but also significant changes in the condition of dairy cows caused by unmeasurable stresses may contribute greatly to improving their thermal comfort, performance, and high-quality milk productivity.

1.5. Glycosylation

Glycosylation is considered one of the most prevalent post-transitional modifications of the protein in all eukaryotes. Glycoconjugates are formed when mono-, oligo-, or poly-saccharides (glycans) are attached to protein or lipid. Those glycans attached to proteins either through a nitrogen atom of an asparagine (Asn) residue within the consensus sequence Asn-X-Ser/Thr, (X ≠ Pro) named *N*-glycan.[22] Each glycosylated site may contain many different glycan structures, oligomannose, in which only mannose residues are attached to the core (Manα1-6(Manα1-3)Manβ1)-4GlcNAcβ1 -4GlcNAcβ1-Asn-X-Ser/Thr); complex in which "antenna" initiated by *N*-acetylglucosaminetransferases (GlcNAcTs) are attached to the core; and hybrid, in which only mannose residues are attached to the Manα1-6 arm of the core and one or two antenna are on the Manα1-3 arm.[23,24]

Glycomics can be performed at the whole organism or an individual cell or tissue type. In Chapter 4, I discuss about *N*-glycan analysis of Holstein dairy cow serum. Briefly, the pretreatment of cows' serum glycoproteins was performed to release whole *N*-glycans using PNGase F. Glycans were selectively captured by glycoblotting method using BlotGlycoH beads; methyl esterification of sialic acid residues and trans-iminization reaction to tag *N*-glycans with benzyloxyamine (BOA) were carried out on the beads. BOA-tagged *N*-glycans were subjected to MALDI-TOF/MS analysis. More details on the pretreatment, glycoblotting, mass spectrometry.

***N*-Glycosylation**

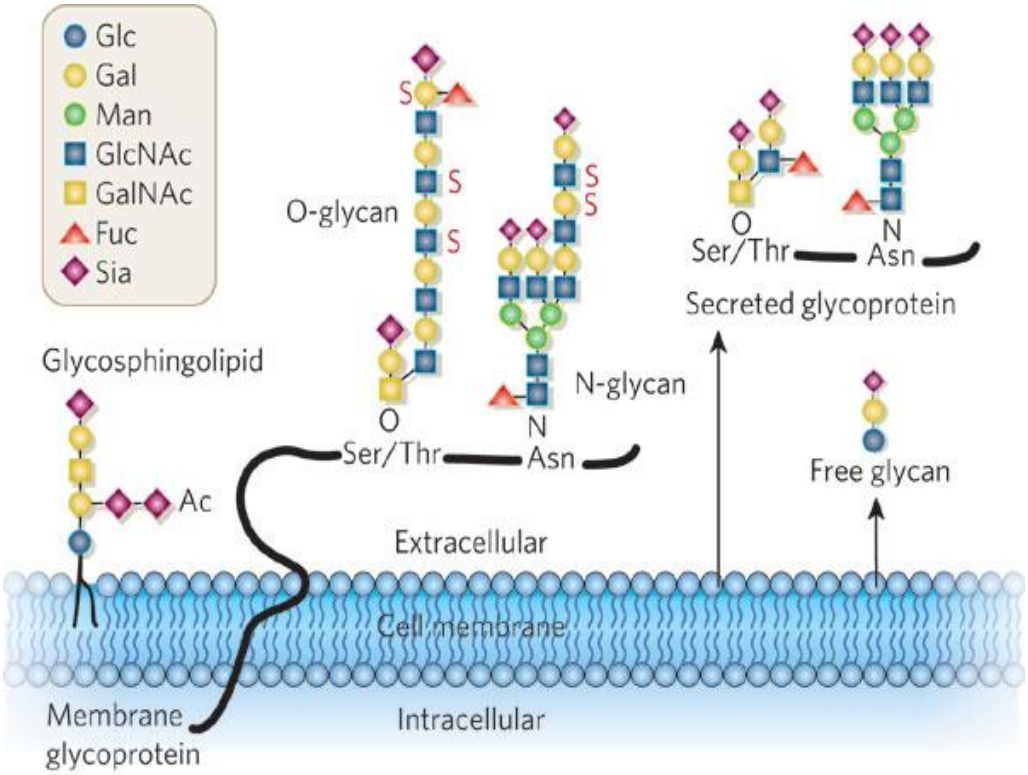


Figure 1.2. Basic structure of glycans.

Post-translational protein glycosylation, (Figure 1.2) is a basic principle for controlling the structures and functions of most glycoproteins in relation to cellular differentiation, adhesion, immunity, signal transduction, growth control, and even malignant alterations.[25–27] Cell surface glycoproteins produced in the same mammalian cells/tissues often share similar glycoforms and glycan profiles, and their expression levels appear to be markedly influenced by environmental stimuli during cell cultivation (exogenous factors) and the genetic background (endogenous factors).[28–31] Protein glycosylation is either *N*-linked (GlcNAcβ1-Asn linkage with a minimal amino acid sequence of Asn-X-Ser/Thr, where X ≠ Pro) or *O*-linked (where GalNAc is added to Ser or Thr), and GSLs are synthesized by a sequential transfer of sugar residues to a ceramide lipid anchor (Figure 1.3). Previous studies demonstrated that the *N*-glycosylation patterns of major serum glycoproteins (extracellular secretary glycoproteins) were significantly

different in patients with various diseases from those in normal human controls.[32–36] These structural alterations in the post-translational glycosylation of major serum proteins may provide highly sensitive biomarkers to indicate changes in general metabolism and the homeostatic immune balance directly/indirectly induced by various diseases without any direct biopsy of the tissues/organs affected. *N*-glycan structure contains high mannose, hybrid and complex. Interestingly, the alteration of *N*-glycan structure/amount is regarded as signals for cancer, rheumatoid arthritis, inflammations and aberrant physiological, pathological conditions in human.[37] The alteration of the glycan structure in biological species had been acquired to adapt themselves to various environmental conditions, for example to defend against infection. However, there is no specific comprehensive *N*-glycomics focusing on livestock towards the discovery of environmental stress biomarkers.

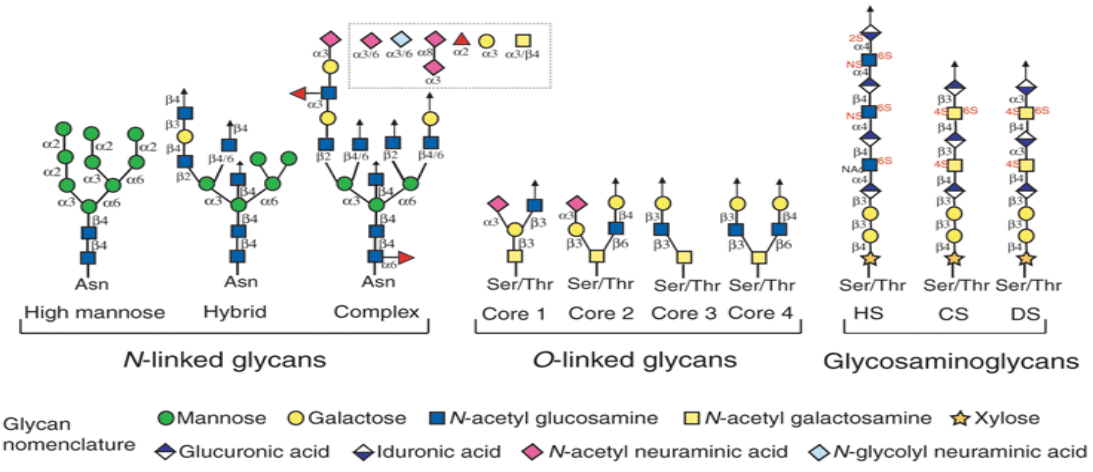


Figure 1.3. Basic structure of *N*-glycans, *O*-glycan and Glycosaminoglycans.

1.6. Sialic Acid and Livestock Immunity.

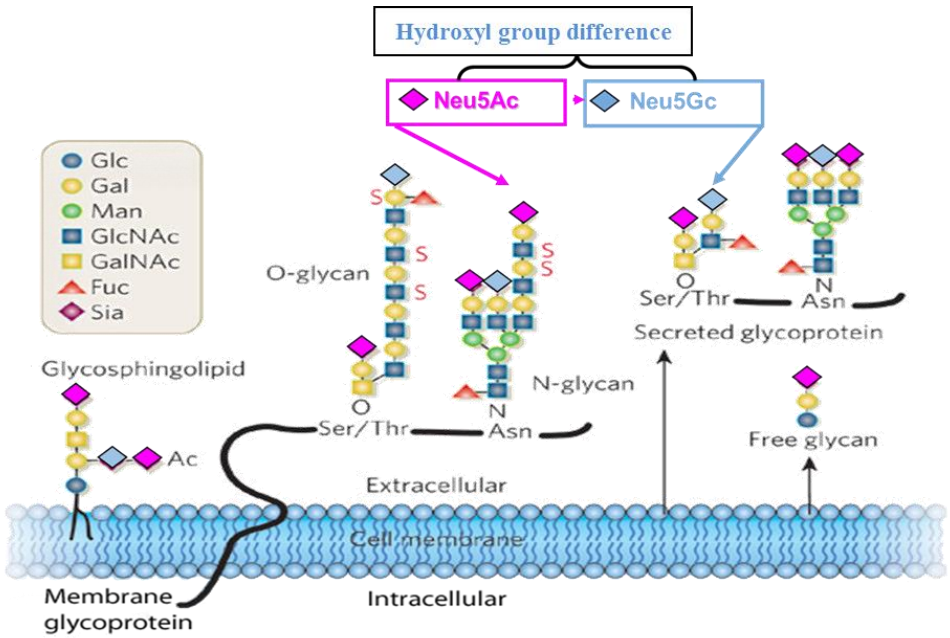
Sialic acids are a family of nine-carbon sugars that are typically found at the terminal end of glycan chains on the cell surface and secreted molecules in the deuterostome lineage of animals.[38-40] (Figure 1.4a). Sialic acids play an important role in various biological processes, including cell differentiation, the immune response, and oncogenesis.[41-43] The two most

common forms of sialic acid are *N*-acetylneuraminic acid (Neu5Ac) which is predominant in livestock to prevent pathogens and *N*-glycolylneuraminic acid (Neu5Gc) provokes the immune response of bovine species and naturally occurs as endogenous sialic acid,[44] and Neu5Gc is enzymatically produced from Neu5Ac by adding a hydroxyl group. A third sialic acid, 2-keto-3 deoxynonulosonic acid (KDN), is known but is less common in mammals, (Figure 1.4b) while avian glycomics revealed the existence of *N*-glycans containing this sialic acid residue in the egg white of Galloanserae.[45] The amino group can also be rarely remained unmodified (Neu) and is regarded as a fourth sialic acid. These four forms of sialic acids can be further modified in various ways, and this diversity is increased by attachments to the underlying sugar chains by different linkages (α2-3, α2-6, and α2-8).[38,46-47] This diversity as presented on the cell surface and secreted molecules has been termed the "sialome".[48]

Sialic acids are synthesized from certain precursor molecules or recycled from glycoconjugates located on the cell surface (Figure 1.4a,b). They can also be taken up from external sources and metabolically incorporated. The activation of free sialic acid to nucleotide donors (CMP-Neu5Ac, CMP-Neu5Gc, and CMP-KDN) occurs in the nucleus, and nucleotide donors are then returned to the cytosol for transport into the lumen of the Golgi apparatus. In the Golgi, sialic acid residues are transferred from the CMP donors to newly synthesized glycoconjugates. Finally, sialic acid- containing glycoconjugates are transported to the cell surface, or secreted. Sialic acids on cell surfaces are often recognized by intrinsic and extrinsic molecules and play an important role in cell–cell communication and host–pathogen interaction. The intrinsic recognition is widely involved in many processes including the immune and nervous systems.[38] Elimination of sialic acid production results in embryonic lethality.[49] On the other hand, several pathogens use sialic acid recognition to initiate their infection.[38] More than 55 vertebrate genes are involved in these biochemical and biological processes.[50]

A surprisingly large number of these have undergone genomic sequence, expression, or function changes uniquely in the human lineage.

A.



B.

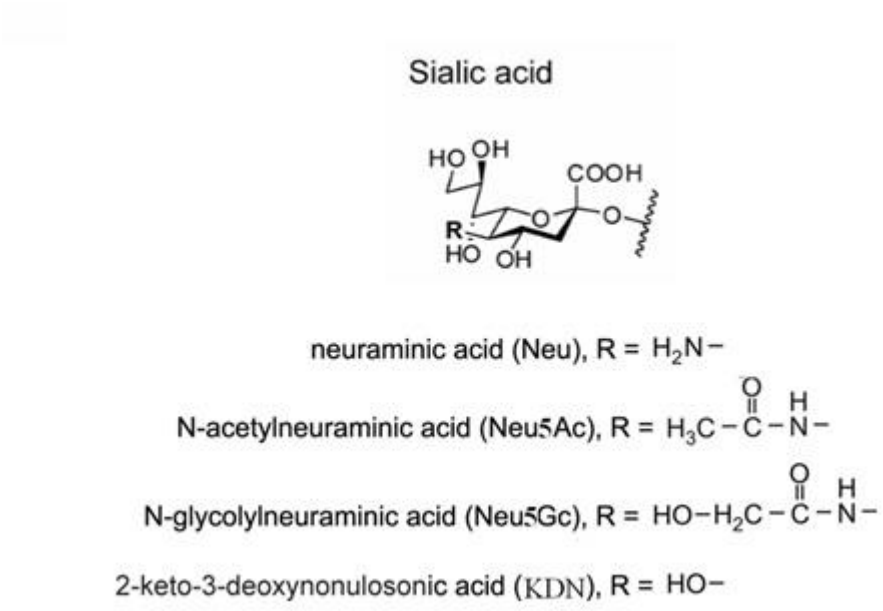


Figure 1.4. (A) Sialic acids on cell-surface and secreated molecules, which are typically found at the terminal position of glycan chains on the cell surface and secreted molecules. (B) The forms of sialic acids Neu, Neu5Ac, Neu5Gc, and KDN.

1.7. Glycoblotting Method

The glycoblotting-based systematic procedure for serum, cell, tissue, etc. is as follow ***c****hemoselective ligation* of whole *N*-glycans of serum glycoproteins by "BlotGlyco" beads; *washing*; *on-bead methylation* of sialic acids; *trans-iminization* by benzyloxiamine to afford BOA-tagged *N*-glycans to be analyzed by MALDI-TOFMS (Figure 1.5).

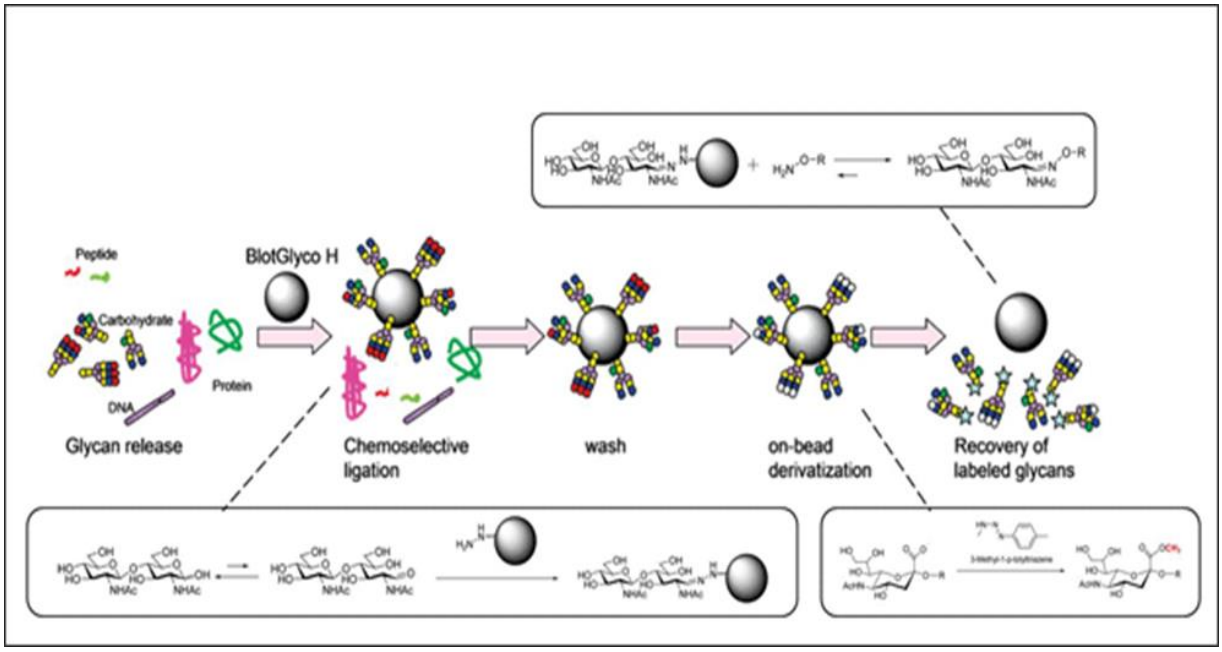


Figure 1.5. Glycoblotting-based systematic procedure for the *N*-glycomics.

The development of a key universal technology for a glycan specific enrichment protocol based on the simple nucleophilic addition reactions between hydrazide/aminooxy- and ketone/aldehyde functional groups, namely, a "glycoblotting method",[37] has allowed, for the first time, for high throughput and quantitative glycomics of various biological samples including serum, cultured cells, and tissues. The researchers at Nishimura laboratory demonstrated that glycoblotting accomplished on the designated bead-platform is the only method applicable to large-scale clinical glycomics of human whole serum glycoproteins (96

samples/plate). This protocol required very small amounts of samples (<100 μL/patient) and, when combined with the automated system "SweetBlot" and common MALDI-TOFMS,[51] took only 24 h to complete from glycoblotting to the glycoform profiling of 96 human serum samples. Recent studies revealed that dynamic alterations in the human serum glycoform profile represent promising diagnostic biomarkers not only in some cancers such as hepatocellular carcinoma,[52-54] pancreatic cancer,[55] renal cell carcinoma,[56] and prostate cancer,[57] but also in various diseases such as osteoarthritis,[58,59] ulcerative colitis,[60] nonalcoholic steatohepatitis,[61] and diabetic retinopathy.[62-63] By employing this approach, we succeeded in obtaining a large avio-*N*-glycome database (88 egg white samples of *Galloanserae*) to investigate the relationship between the function of glycan diversity and evolutionary lineage in some avian species.[45]

Merit of Glycoblotting

Universal platform for glycan sample preparation for MALDI-TOF/MS and DMB-HPLC; High throughput, fast, and quantitative glycomics of various biological samples including serum, cultured cells, tissues, and egg white; Easy to perfectly purified and labeled glycans; Glycans captured by BlotGlyco® beads endure harsh wash. Thereby, impurities, even peptides and surfactants, are easily removed.

In the present study, I performed, for the first time, large-scale glycomics of dairy Holstein cow serum samples collected for 9 months in Hokkaido, Japan (between February and October in 2012), and detected dynamic changes in the expression levels and profiles of the *N*-glycan structures of serum glycoproteins as highly sensitive and efficient serum biomarkers to monitor the heat/environmental stresses influencing the metabolic/homeostatic immune balance, performance, and milk productivity of dairy cows.

Chapter 2

Aim of The Thesis

Despite the field of glycomics have been developed by many scientists, it is still time consuming, in accurate purification process of glycan, and there is no record about large scale glycomics analysis to detect the environmental stress monthly using climate condition in livestock in order to improve their productivity, and to detect the mechanism of action of sialic acid(s) in as a mediator of pathogen infection in neonatal stage of livestock. All these challenges have also leads to interesting study for almost scientists. In this thesis, I aimed to use glycoblotting assisted sample preparation combined MALDI-TOF/MS and DMB-HPLC analysis to estimate the composition and quantify the expression level of the *N*-glycans from the serum of dairy cow. This is a novel and sensitive technique to elucidate purified and labeled glycan easily.

Due to the importance of animal industry and production reported in global survey, it should be considered worldwide, to reduce the risk of animal exposure to the stress, thus in chapter 4, I will discuss in a large-scale analysis about the presumptive composition and expression levels of *N*-glycans of Holstein cow serum samples to detect the infleunce of environmental stress monthly based on climate condition and cow production data, to detect the dynamic changes in the expression levels of *N*-glycan structures monthly. In order to, discovering novel glyco-biomarkers for an easy diagnosis and future biomedical indicator of environmental stress of Holstein cow, which leads to improve cow welfare and to enhance the milk production.

Chapter 3

Materials and Methods

3.1. Animal Management

Animal experiments were performed under the approval of the Committee on Animal Research and Welfare of Hokkaido University. In this study, 336 serum samples were originally collected at the experimental dairy barn to perform other research programs designed by the Field Science Center for Northern Biosphere, Hokkaido University, and they were used in a research agreement to analyze *N*-glycomics in dairy cow serum samples employing the glycoblotting method and establish a bovine *N*-glycome database. Totally 336 serum samples were collected from lactating Holstein cows (n = 21). The experimental cow serum (n = 12) were used or might be more whose obtained by using replicate, over 9 months in 2012 between February and October) to cover the four seasons (spring, summer, autumn, and winter). Among those 336 serum samples 4% (n = 12) were from primiparous and 96% (n = 324) from the multiparous cows, and 46% (n = 155) were collected from pregnant cows. The serum samples were taken outdoor, around Agriculture Faculty inside the campus where cows were exposed to all climatic stress for grazing behaviour (May to October), but being in a shed while temperature was not conditioned in winter and spring (April) during the experiment. All lactating Holstein cows were identified prior to the experiment and mostly housed in an experimental barn in individual tie stalls between October and April, but grazed on a pasture of perennial ryegrass/white clover (4 ha), except when being milked (2:30−4:00 p.m., 8:00−10:00 a.m.). Cows were maintained under the same conditions during the experiments. Rations were frequently changed during the course of the study as part of routine seasonal herd management. Cows were milked in two milking sessions per day. Parity, body weight, and milk yields were recorded per cow per milking (morning and evening). Moreover, composited samples were made from 0.2% of the milk yield to estimate the percentages of fat, protein, and lactose using MilkoScanS54A. Holstein cows were selected randomly to calculate daily milk production

during the experimental period. No specific animal management protocol was necessarily used in this study because only routinely collected field data were used.

3.2. Chemicals and General Methods

3-Methyl-1-*p*-tolyltriazene (MTT), ammonium bicarbonate 99% (ABC), 1,2-diamino-4,5-methylenedioxy-benzene (DMB), sodium 2-hydroxy-3-tetradecanamidopropane-1-sulfonate [1-propanesulfonic acid, 2-hydroxyl-3-myristamido, sodium salt (1:1); PHM], disialyloctasaccharide, and *O*-benzylhydroxylamine hydrochloride (BOA) were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). BlotGlycoH beads were purchased from Sumitomo Bakelite, Co., Ltd. (Tokyo, Japan). Peptide *N*-glycosidase F (PNGase F) was purchased from New England Biolabs Inc. (Ipswich, MA, USA). Trypsin, dithiothreitol (DTT), and iodoacetamide (IAA) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). 5-*N*-Acetylneuraminic acid (Neu5Ac) was purchased from Japan Food & Liquor Alliance Inc., Food & Bio Research Center (Kyoto, Japan). 5-*N*-Glycolylneuraminic acid (Neu5Gc) and fetal bovine serum were from Sigma-Aldrich, Inc. MilkoScanS54A was purchased from Foss Electric Co., Inc. (Tokyo, Japan). SweetBlot was obtained from Systems Instruments Co., Inc. (Hachioji, Japan). MultiScreen Solvinert filter plates (0.45 μm low binding hydrophilic PTFE, MSRLN0410) were purchased from Millipore Co., Inc. (Tokyo, Japan). Mass measurements were performed using MALDI-TOF/MS (Ultraflex III, Bruker Daltonics, Germany). HPLC was performed on a Hitachi D-7000, Hitachi High Technologies Co., Ltd. (Tokyo, Japan), and IMB-SPSS software 22.0 was obtained from IBM Co., Inc. (Armonk, NY, USA).

3.3. Cow Serum Preparation.

After being restrained, at least 12 blood samples were collected from each cow each month. Blood samples were collected into evacuated centrifuge tubes with a capacity of 12 mL. Approximately 1.5 mL of blood was collected from the jugular vein over the inner surface of the tube using syringes. All serum samples were then separated by the centrifugation of clotted blood at 804.96g force for 20 min. Serum samples were clear and free of hemolysis and were then carefully transferred to new Eppendorf tubes and stored at −80 °C until the *N*-glycome analysis.

3.4. Determination of Mean Monthly Climate

The variables (monthly means) of air temperature (°C), maximum temperature (°C), minimum temperature (°C), mean wind speed (m/s), relative humidity (%), and global solar radiation (MJ/m^2) were recorded in 2012 on the basis of a weather station in Sapporo by the Japan Meteorological Agency (JMA) server, which provides an online database (http://www.data.jma.go.jp/obd/stats/data/en/smp/index.html). THI is one of the key parameters defined by measurements of heat intensity, which has been determined to equal [0.8T_a + (RH/100)(T_a − 14.3) + 46.4], where T_a means air temperature (°C) and RH defines relative humidity (%).[64]

3.5. Glycoblotting-based Serum Glycomics

(A) Pretreatment: The premix composition [0.33 M ABC (2.52 mL), 0.4% PHM in 10 mM ABC (1.26 mL), 120 mM DTT (0.84 mL), and made up to 8.4 mL with water] was initially prepared, and 50 μL was then freshly added to the serum of each cow (10 μL). The internal standard (disialyl-octasaccharide) was added carefully (final concentration, 40 μM) and the

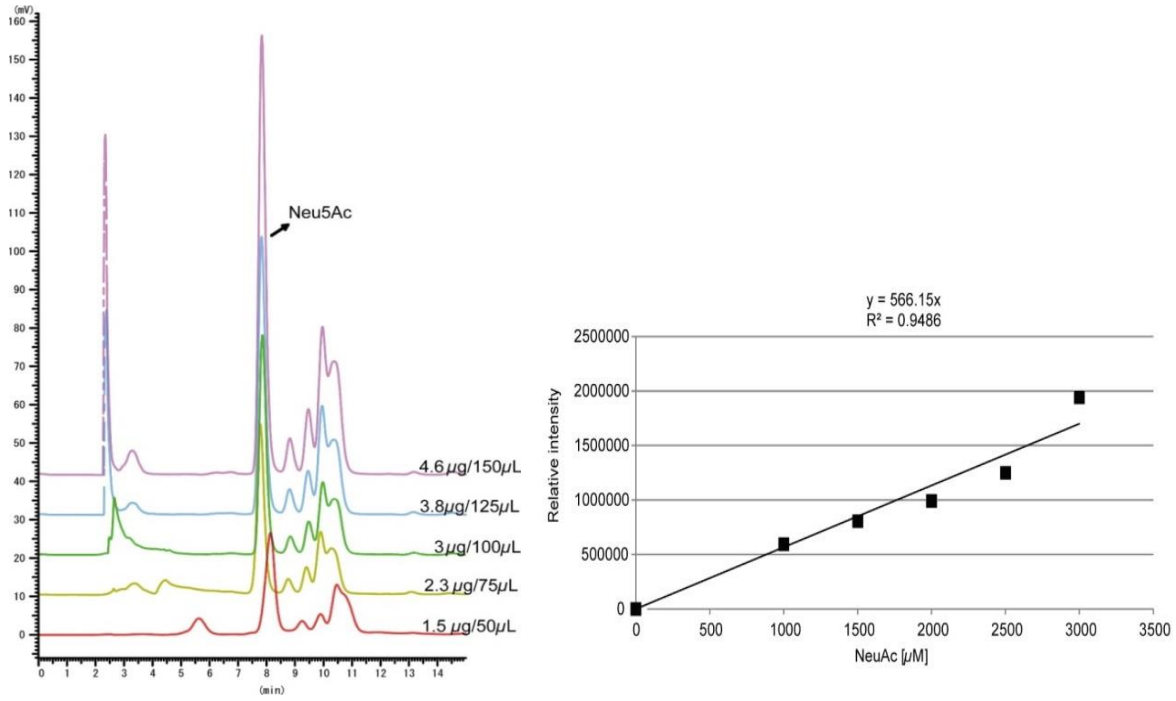
mixture was incubated at 60°C for 30 min. Ten microliters of freshly prepared 123 mM IAA was added to the mixture, which was then incubated at 25°C for 60 min in the dark to allow the alkylation process. The mixture was subsequently treated with trypsin (5 μL, 40 U/μL in 1 mM HCl) at 37°C for 3 hours, followed by heat inactivation of the enzyme at 90°C for 10 min. After being cooled to room temperature, *N*-glycans were enzymatically released from trypsin-digested glycopeptides by an incubation with 65 units of PNGase F at 37°C for 2 hours. Twenty microliters of the digested mixture containing *N*-glycans was directly applied to glycoblotting technology. *(B) Glycoblotting:* Five hundred microliters of BlotGlyco®H beads in a 10 mg/mL suspension with water was initially placed into the wells of a MultiScreen Solvinert®96-well filter plate, and water was then removed by a vacuum. The digested mixtures of cow serum (20 μL) containing released *N*-glycan were applied to each well together with 180 μL of 2% AcOH/CH_3CN. The plate was incubated at 80°C for 45 min to dryness in a thermostat in order to capture the glycans in the sample mixtures onto the beads via stable hydrazone bonds, and this was followed by successive washing using 200 μL each of 2 M guanidine-HCl in 16.6 mM ABC and water and 1% triethylamine in MeOH twice each. The acetyl capping of unreactive hydrazide functional groups was performed using 10% acetic anhydride in MeOH at 25°C for 30 min. The remaining acetic anhydride was removed by a vacuum. In bead methyl esterification, each well was washed twice with 10 mM HCl, MeOH, and dioxane, consecutively, and 150 mM MTT was then added, and the plate was incubated at 60°C for 90 min. The beads were serially washed using 200 μL of dioxane, water, methanol, and water. In order to obtain a perfect transamination reaction, 20 μL of 50 mM BOA was added, followed by a treatment with 180 μL of 2% AcOH in CH_3CN, and an incubation at 80°C for 45 min. Labeled *N*-glycans were finally eluted with 100 μL water. The above pretreatment and glycoblotting of 336 serum samples were performed using SweetBlot™, an automated glycan processing machine. *(C) Mass spectrometry:* The recovered *N*-glycans labeled with BOA were

finally eluted with 100 μL water, analyzed after being spotted on MTP 384 target plates, and then crystallized by drying under a vacuum with an equivalent volume of the liquid matrix solution, 100 mM α-cyano-4-hydroxycinnamic acid diethylammonium salt (CHCA-DEA) dissolved in buffer solution (MeOH : H_2O : DMSO : 10 mM NaOH = 50 : 39 : 10 : 1). MALDI-TOF/MS data were then displayed on an Ultraflex III, equipped with a reflector and controlled by the FlexControl 3.0 software package according to the general protocol. All spectral conditions were obtained using the reflector mode, ions generated by Smartbeam (pulsed UV solid laser, λ_{ex}= 355 nm, 50 Hz) with 25 kV as the acceleration voltage, 26.3 kV as the reflector voltage, 160 ns as pulsed ion extraction in the positive mode, and typically totaling 1,000 shots of each spot. The intensity of the isotopic peak of each glycan was normalized using 40 μM of the internal standard (disialyloctasaccharide) for each status, and their concentrations were calculated from a calibration curve using human serum standards. *(D) Data analysis:* MS data were analyzed with the FlexAnalysis 3.0 software (Bruker Daltonik GmbH, Bremen, Germany, S/N=6, and quality factor threshold of 30). The GlycoMod Tool predicted the possible *N*-glycan compositions of the experimental masses, which were mainly reported on GlycoSuiteDB, (http://web.expassy.org/glycomod/), and non-reported compositions were later checked using Consortium Functional Glycomics Gateway, (CFG), (http://www.functionalglycomics.org), to identify and categorize monthly alterations in *N*-glycan structures of cow serum. *N*-glycan profiling was primarily based on sources from the serum of *Bos Taurus* and other mammals, especially sources from human serum, cells, and tissue.

3.6. Sialic Acid Analysis

Crude mixtures (20 μL each) containing whole *N*-glycans released from cow serum glycoproteins were subjected to glycoblotting with BlotGlycoH beads, and the following steps were performed in a similar manner to that described in procedure B (in Figure 2.6). The beads capturing whole serum *N*-glycans in each well were directly treated with 100 μL of 25 mM HCl at 80 °C for 1 h to hydrolyze *N*-glycans, without further procedures such as acetyl capping and methyl esterification described in procedure B. The filtered hydrolysate (100 μL) containing sialic acids and other monosaccharides was reacted with 100 μL of DMB reagent (7 mM DMB−2 HCl and 1 M 2-mercaptoethanol to stabilize DMB during the reaction and 18 mM $Na_2S_2O_4$ to stabilize the fluorescent products generated from Neu5Ac and Neu5Gc) and then heated at 60 °C for 2.5 h in the dark to develop fluorescence from the sialic acid derivatives. The reaction mixture was cooled in ice water for 15 min to stop the reaction, a 10 μL aliquot of the solution (n = 5) was injected into the HPLC system on a reversed-phase column (Inertsil ODS- 3 μm, ⌀ 4.6 × 250 mm), and the intensity of fluorescence was then measured (λ_{ex} = 373 nm, and λ_{em} = 448 nm). The HPLC settings were as follows:[45] The flow rate was 1 mL/min (ca. 100 kg/cm^2) with a MeOH/ACN/H_2O mixture (3:1:10, v/v). The column temperature was ambient (ca. 40 °C). The quantitation of DMB-tagged sialic acids was performed using the HPLC system equipped with an L-7485 fluorescence detector. The concentrations of sialic acid derivatives were determined on the basis of calibration curves made using unlabeled Neu5Ac and Neu5Gc (5, 7.5, 10, 12.5, and 15 pmol/each) as standards (see Figure 3.1).

A.



B.

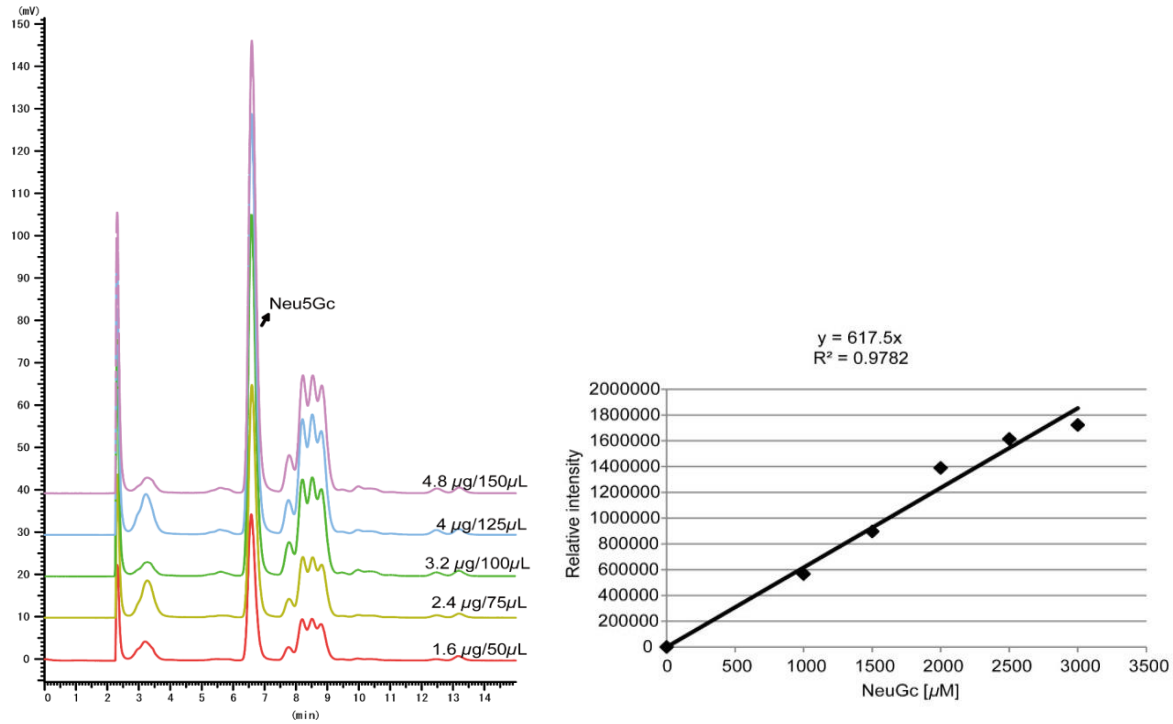


Figure 3.1. Quantitation of Neu5Ac and Neu5Gc by DMB-HPLC method. (A) DMB-HPLC monitored by fluorescence spectroscopy and the calibration curve of DMB-Neu5Ac. (B) DMB-HPLC monitored by fluorescence spectroscopy and the calibration curve of DMB-Neu5Gc.

3.7. Statistical Analysis

The expression levels of *N*-glycans in cow serum are represented as micromolar (mean ± SE). A multivariate analysis was conducted using IMB-SPSS software 22.0, and a one-way ANOVA was performed. Relationships between variables were analyzed by general linear model (GLM) procedures to reduce the possibility of random factors introducing variability into the data and also to allow for the intervention approach. The dependent variable was the average serum concentration (μM) of *N*-glycans with different molecular masses (*m/z*) released from the serum glycoproteins of cows. Fixed factors included the month of the year. Covariates appearing in the model included first treatments (parity, days in milk, body weight, and milk yield) and second treatments (percentages of fat, protein, and lactose in milk). Appropriate interactions were then examined. The significance of differences between two groups was also determined using Student's *t*-test. All reported *P*-values <0.05 for all mean effects were considered significant.

Chapter 4

Results and Discussion

4.1. Correlation of Holstein Cows Data and Climatic Change

As shown in Table 4.1, on the basis of some climatic data of Sapporo, Japan, recorded in 2012, heat stress commonly assessed by the THI was estimated to be maximal in August (THI = 72.2), during which the effects of solar radiation (14.8 MJ/m^2) and rather than low air velocity (3.3 m/s) may have influenced the performance of cows at an air temperature of 23.4 °C in this month (Figures 4.1−3).[65] On the basis of THI values (62.3−70.5), dairy cows were expected to show higher milk productivity in June, July, and September than in other months because the THI of dairy Holstein cows producing >35 kg/day of milk was previously reported to be 68.[17] Furthermore, milk production in August was expected to be minimal because the THI value exceeded 72.[15]

Table 4.1. Monthly mean climatic data in Sapporo, Japan.

Monthly Mean	Jan	Feb	March	April	May	June	July	Aug	Sept	Oct	Nov	Dec
Wind speed	3.2	3.2	3.4	4.2	4	3.3	3.3	3.4	3.2	3.5	3.4	3.3
Global solar radiation	6.8	9.7	12.2	15.6	19.1	20.7	19.3	14.8	13.2	9.7	5.1	4.5
Air temperature	-4.5	-4.4	0.1	7	13	17.1	21.8	23.4	22.4	13	5.5	-2.3
Relative humidity	67	64	66	66	71	72	74	76	74	69	73	69
THI	30.404	31.112	37.308	47.382	56.077	62.29	69.5	**72.23**	70.514	56.10	44.576	33.306

These data were recorded in 2012, using a weather station in Sapporo by the Japan Metrological Agency (JMA) server (http://www.data.jma.go.jp/obd/stats/data/en/smp/index.html). The temperature humidity index (THI) was determined to equal [0.8Ta + (RH/100) (Ta − 14.3) + 46.4], where air temperature (Ta, °C) and humidity (RH, %) peaked in August, 72.236 (cow suffers from heat stress).

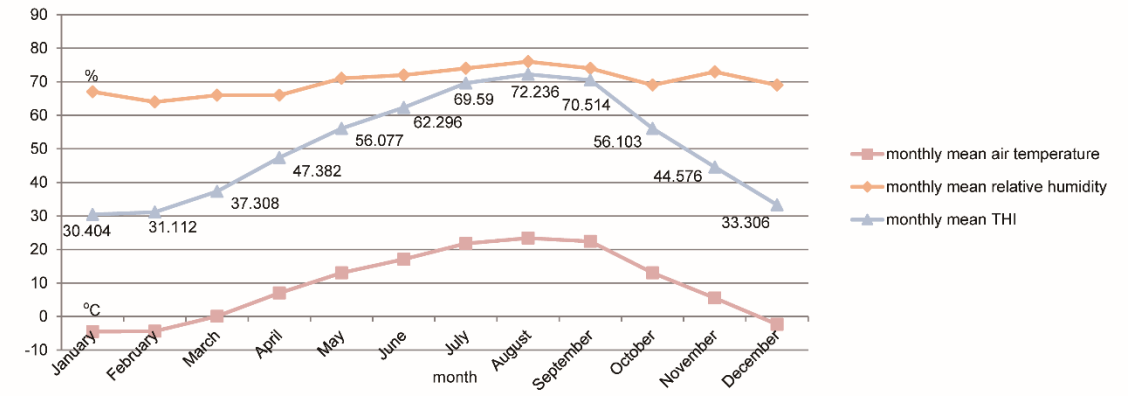


Figure 4.1. Temperature humidity index (THI), monthly mean air temperature, and relative humidity.

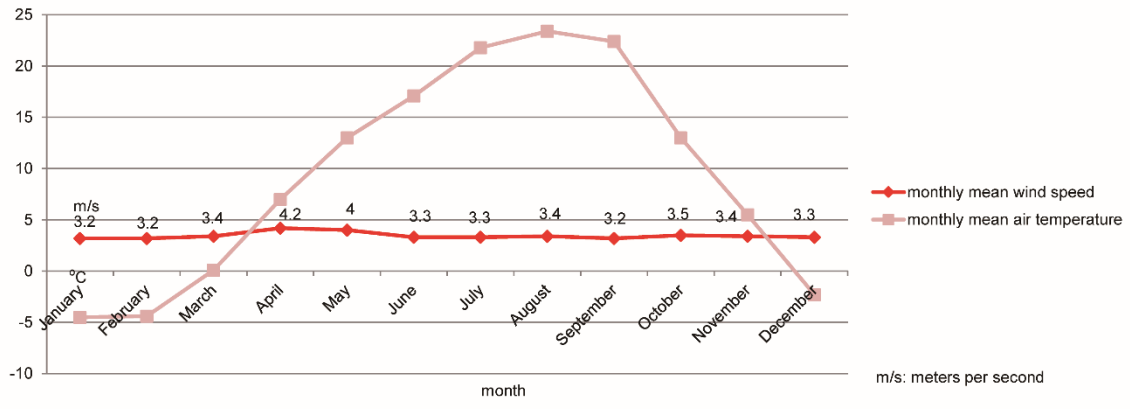


Figure 4.2. Monthly mean air temperature and wind speed.

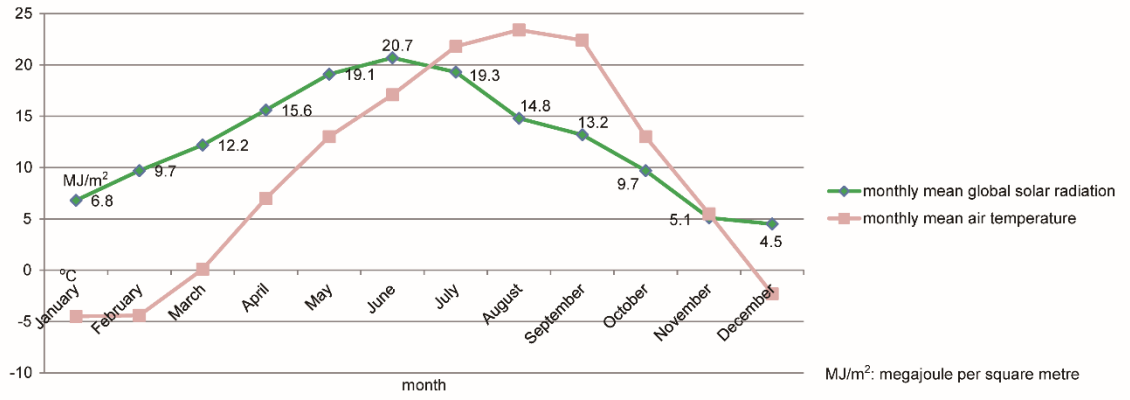
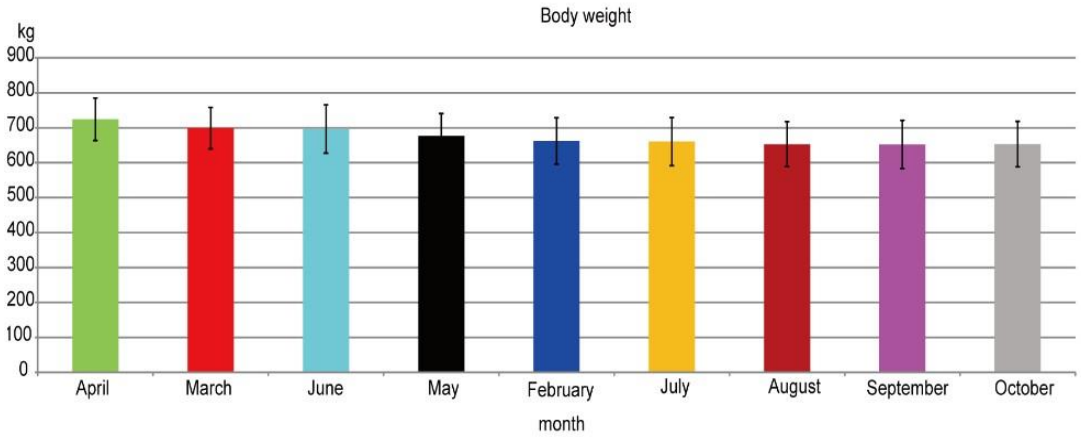


Figure 4.3. Monthly mean air temperature and global solar radiation.

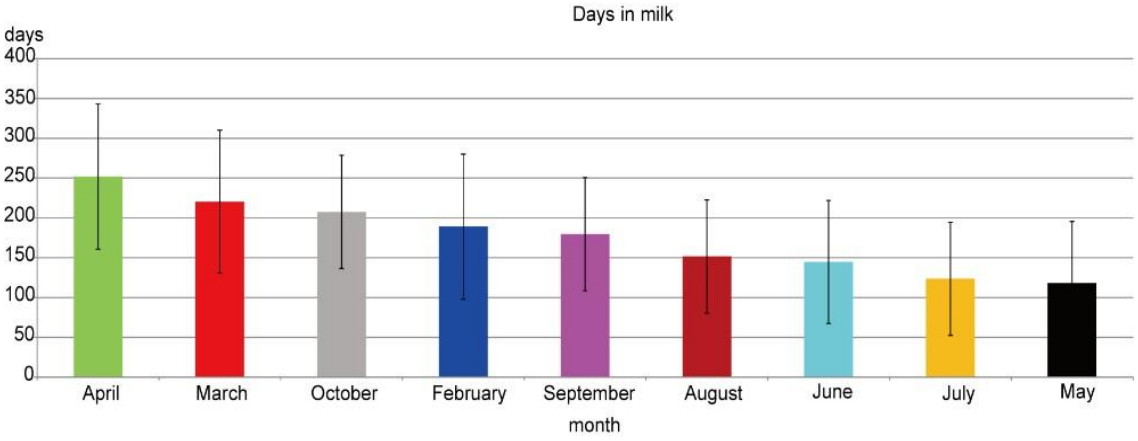
Table 4.2 shows the statistical interpretation of dairy Holstein cows data collected during this study (see also Figure 4.4). Moreover, Table 4.3 and Figure 4.5 show the data of representative cow. These results indicated that animal performance was significantly influenced by seasonal variations. Monthly changes in milk yield and its composition correlated with climatological variables and management. Milk yield increased gradually from February (25.4 kg/day) to spring and reached a maximum in May (36.7 kg/day), see also data of a representative cow (28.4 and 32.6 kg/day in February and May, respectively). As anticipated, milk yield in August was lower (24.8 kg/day) than the average, whereas that in October was the lowest (21.6 kg/day), see also data of a representative cow (22.1, 22 kg/day in August and October, respectively). The THI value in October was identical to that in May (THI =56.1). Milk productivity may have decreased gradually from June to October. Furthermore, the

contents of protein and fat in milk produced in summer and autumn appeared to be lower than those in spring and winter, whereas no significant change was observed in the lactose content (4.36−4.48%), see also data of a representative cow (4.38 and 4.42%). However, days in milk; the days elapsed from calving during which cows are milked every day were obviously varied among cows due to different schedules of artificial insemination and calvig date accordingly. For instance, the days of milk of representative cow #8 supposed to be in February/2012 (97 days in milk), which means this cow had a newly born calf 3 months ago, in November/2011 (calf birth), however, in February/2012 (cow was in maternity with calf of 3 month old) (see Table. 4.4). These seasonal profiles for milk yield and its constituents may strongly depend on marked changes in the diet and subsequent metabolic effects on normal microbial protein synthesis.[66] However, difficulties are associated with directly monitoring changes in the profiles of highly complex metabolites and their effects on the intestinal microbiota. THI values may contribute to the development of a management system for short-term heat stress and environmental assessment guidelines to improve animal comfort. However, the relationship between long-term damage caused by the integration of many environmental stresses and the performance/milk productivity of lactating Holstein dairy cows currently remains unclear due to the lack of sensitive biomarkers to indicate alterations in the general metabolism and homeostatic immune balance of Holstein dairy cows.

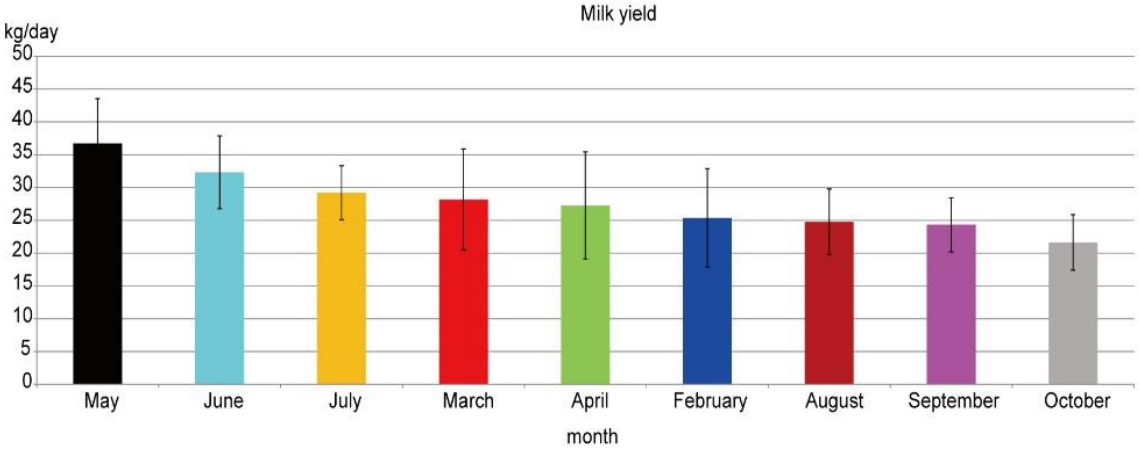
A.



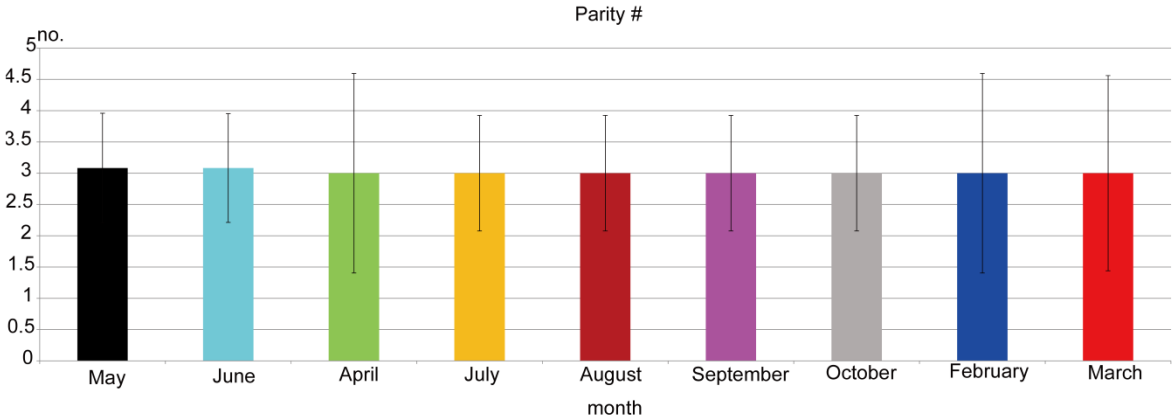
B


Days in milk
days
400
350
300
250
200
150
100
50
0
April
March
October
February
September
August
June
July
May
month

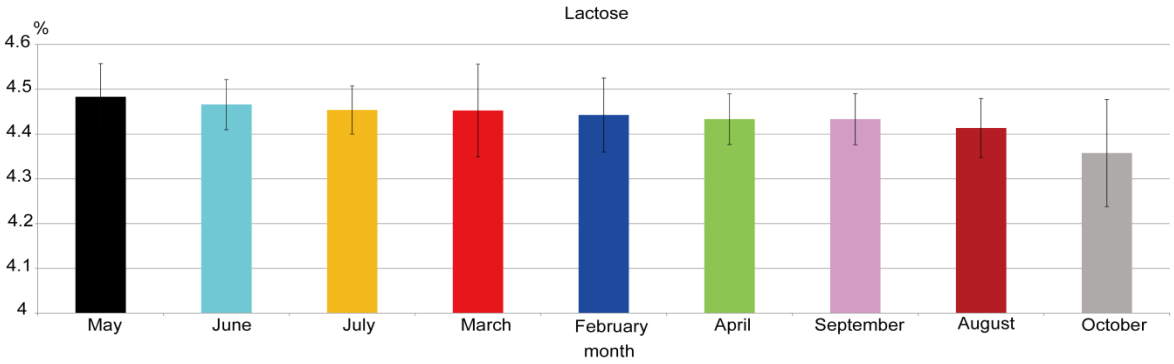
C.


Milk yield
kg/day
50
45
40
35
30
25
20
15
10
5
0
May
June
July
March
April
February
August
September
October
month

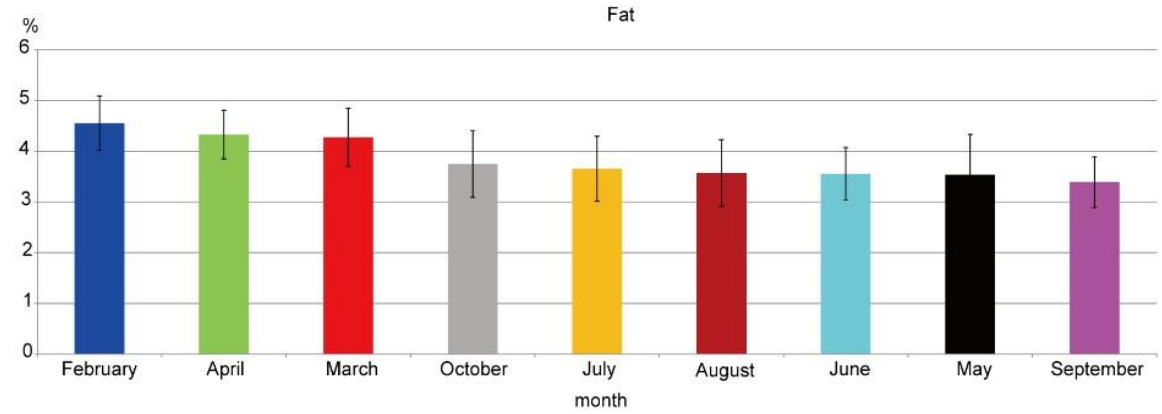
D.


Parity #
no.
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
May
June
April
July
August
September
October
February
March
month

E.



F.



G

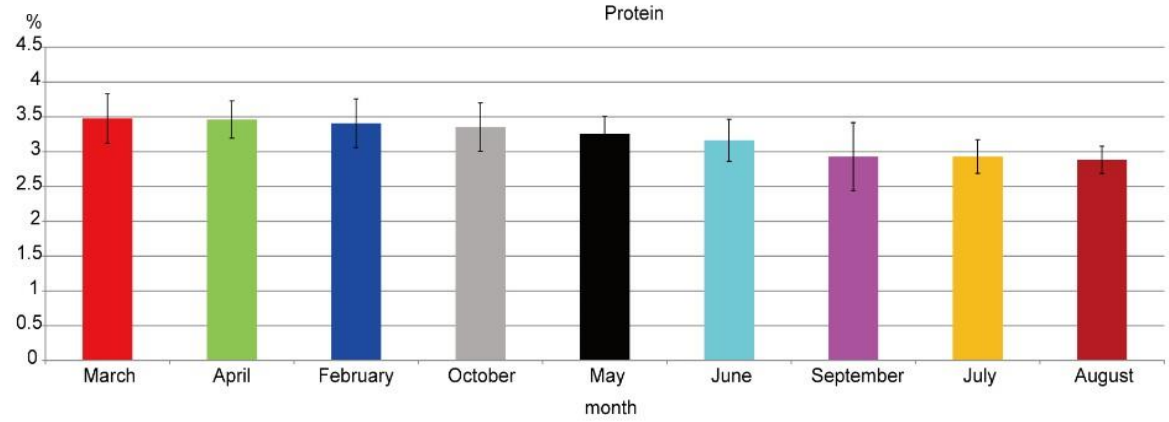


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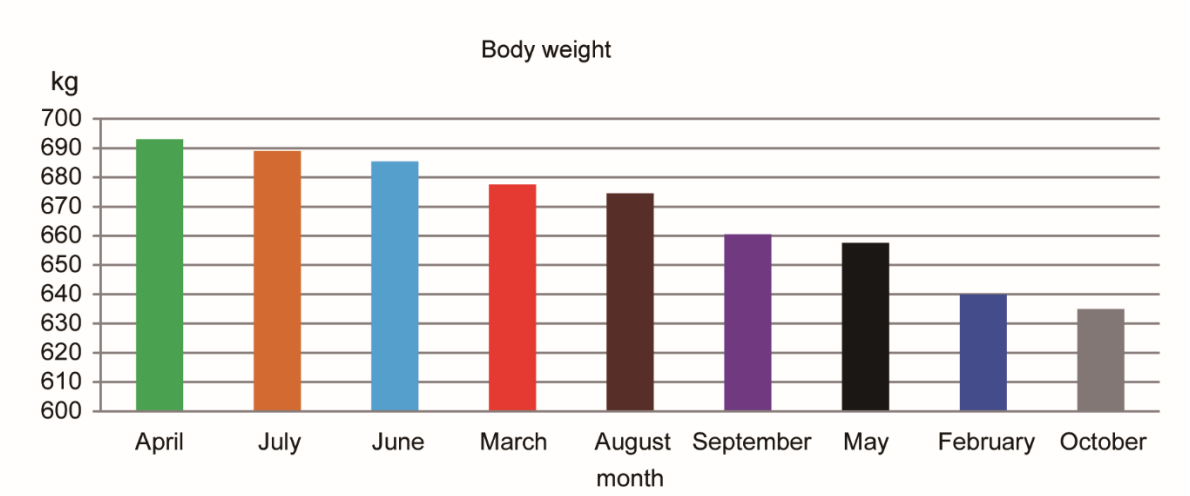
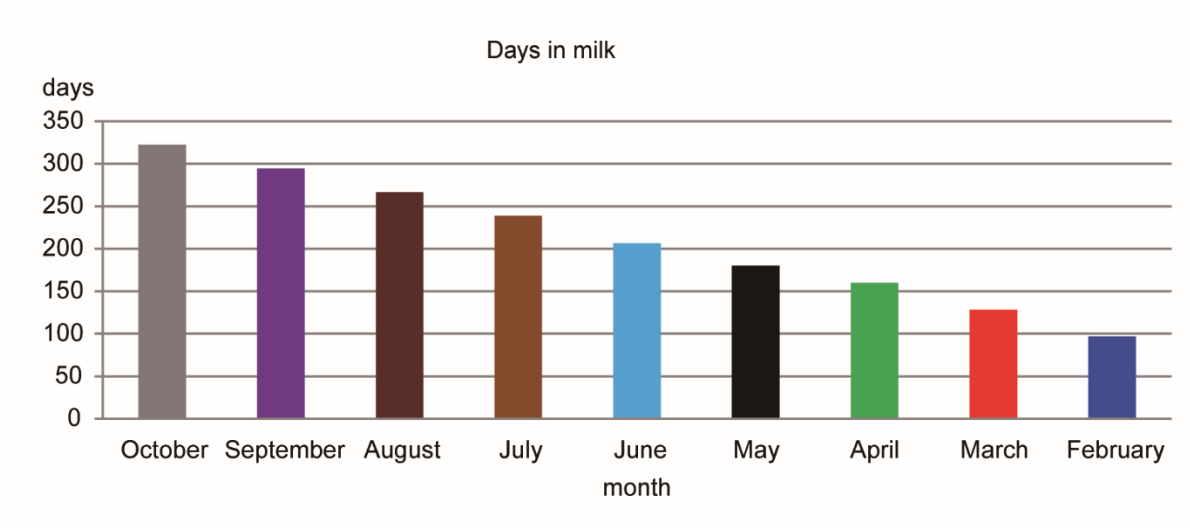
Figure 4.4. (A) Monthly body weight (mean±SD) of Holstein cows (between February and October). (B) Monthly days in milk (mean±SD) of Holstein cows (between February and October). (C) Monthly milk yield (mean±SD) of Holstein cows (between February and October). (D) Monthly parity (mean±SD) of Holstein cows (between February and October). (E) Monthly lactose% in milk (mean±SD) of Holstein cows (between February and October). (F) Monthly fat% in milk (mean±SD) of Holstein cows (between February and October). (G) Monthly protein% in milk (mean±SD) of Holstein cows (between February and October).

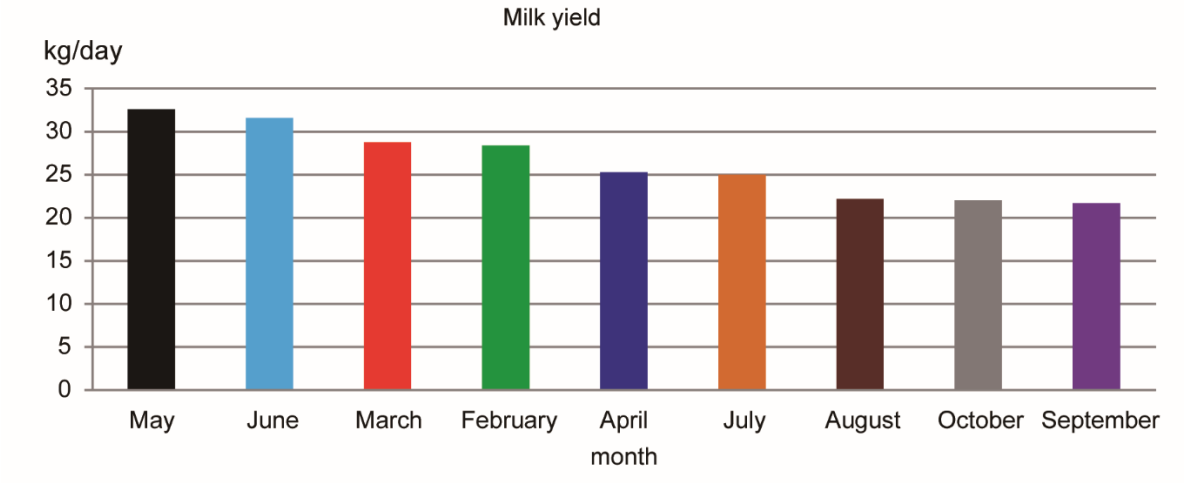
A.


Body weight
kg
700
690
680
670
660
650
640
630
620
610
600
April
July
June
March
August
September
May
February
October
month

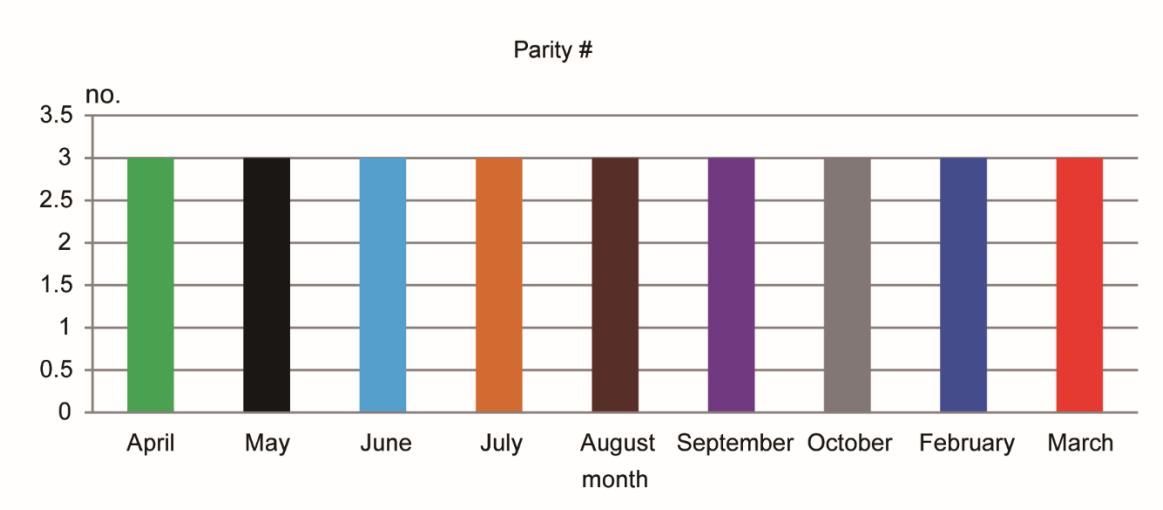
B


Days in milk
days
350
300
250
200
150
100
50
0
October
September
August
July
June
May
April
March
February
month

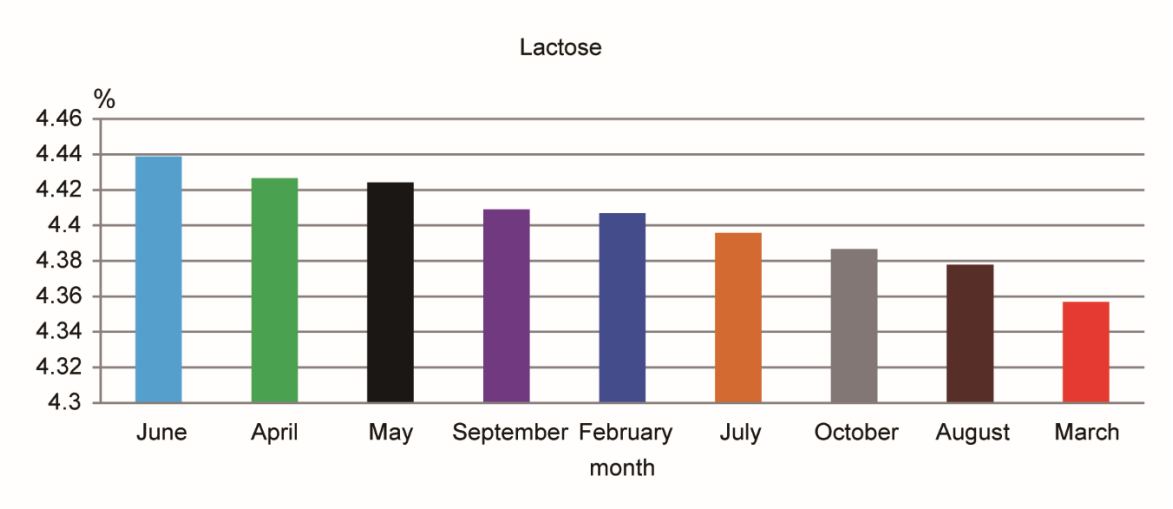
C.


Milk yield
kg/day
35
30
25
20
15
10
5
0
May
June
March
February
April
July
August
October
September
month

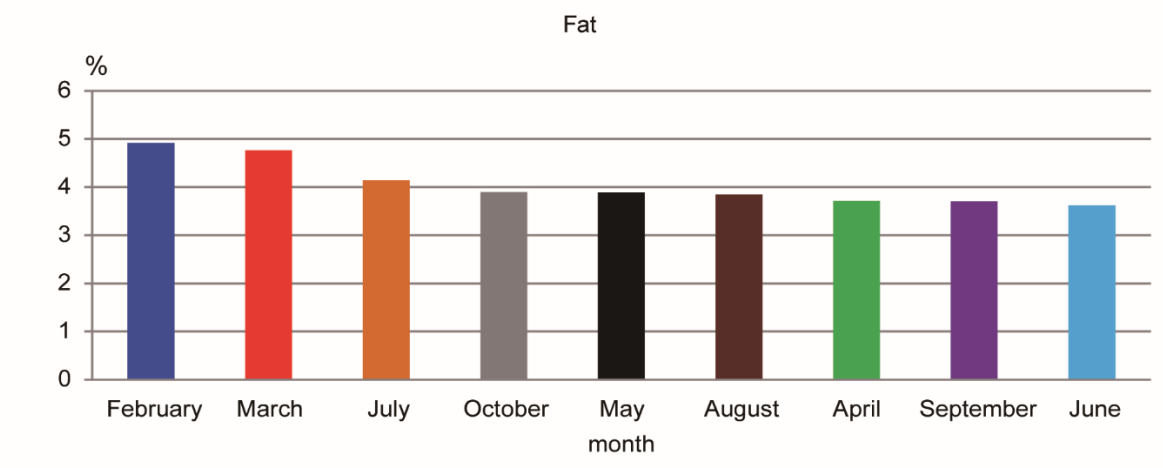
D.


Parity #
no.
3.5
3
2.5
2
1.5
1
0.5
0
April
May
June
July
August
September
October
February
March
month

E.


Lactose
%
4.46
4.44
4.42
4.4
4.38
4.36
4.34
4.32
4.3
June
April
May
September
February
July
October
August
March
month

F.


Fat
%
6
5
4
3
2
1
0
February
March
July
October
May
August
April
September
June
month

G.

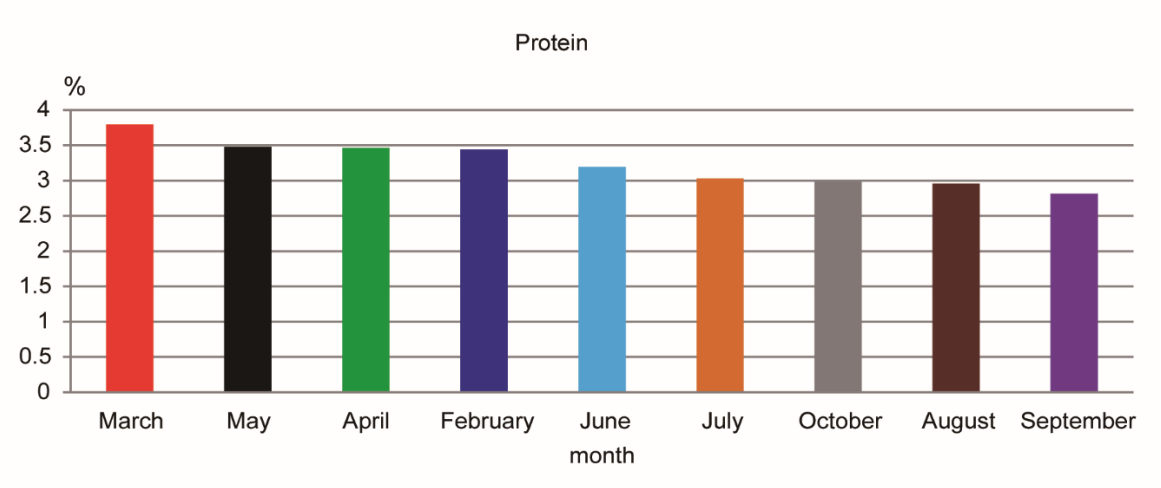


Figure 4.5. (A) Representative monthly body weight of Holstein cow #8 (between February and October). (B) Representative monthly days in milk of Holstein cow (between February and October). (C) Representative monthly milk yield of Holstein cow (between February and October). (D) Representative monthly parity of Holstein cow (between February and October). (E) Representative monthly lactose% in milk of Holstein cow (between February and October). (F) Representative monthly fat% in milk of Holstein cow (between February and October). (G) Representative monthly protein% in milk of Holstein cow (between February and October).

Table 4.2. Statistical interpretation (mean±SD) of some of Holstein cow data during the study.

Performance data	Feb	March	April	May	June	July	Aug	Sept	Oct
Body weight (kg)	662.25±66.559	698.92±59.336	724.33±60.937*	676.78±64.045	696.43±69.0002	660.42±68.830	653.17±64.279	652.38±68.879	653.21±64.784
Parity #	3.00±1.595	3.00±1.560	3.00±1.595	3.0833±0.874	3.0833±0.8692	3.00±0.922	3.00±0.922	3.00±0.922	3.00±0.922
Days in milk (day)	188.92±91.193	220.42±89.831	251.92±91.193	118.25±77.270	144.65±77.2679	123.58±71.184	151.58±71.184	179.58±71.184	207.58±71.184
Milk yield (kg/day)	25.3667±7.489	28.175±7.686	27.2667±8.163	36.7167±6.816***	32.3183±5.5505	29.1996±4.117	24.7792±4.989	24.3121±4.122	21.6429±4.238
Lactose% in milk	4.4425±0.082	4.4525±0.103	4.4333±0.056	4.4833±0.074	4.4658±0.0559	4.4537±0.053	4.4133±0.065	4.4332±0.057	4.3575±0.119
Protein% in milk	3.4058±0.349	3.4767±0.355***	3.4608±0.268	3.2583±0.248	3.1622±0.3024	2.9296±0.240	2.8821±0.196	2.9287±0.487	3.3527±0.348
Fat% in milk	4.5558±0.534***	4.2733±0.573	4.3292±0.479	3.5372±0.796	3.5565±0.5181	3.6573±0.640	3.5696±0.657	3.3200±0.696	3.7496±0.656

- A multivariate analysis was conducted using SPSS software with a one-way ANOVA, (asterisks indicate significance level: ***, $P \leq 0.001$; **, $P \leq 0.01$; ***, $P \leq 0.001$; and *, $P \leq 0.05$), SD; standard deviation.

Table 4.3. Some data of representative Holstein cow #8 during the study.

Performance data	Feb	March	April	May	June	July	Aug	Sept	Oct
Body weight (kg)	640	677.5	693	657.7	685.4	689	674.5	660.5	635
Parity #	3	3	3	3	3	3	3	3	3
Days in milk (day)	97	128.5	160	180	206.4	238.5	266.5	294.5	322.5
Milk yield (kg/day)	28.4	28.75	25.3	32.6	31.6	24.995	22.175	21.7	22.025
Lactose% in milk	4.41	4.36	4.43	4.42	4.44	4.40	4.38	4.41	4.39
Protein% in milk	3.44	3.79	3.46	3.48	3.19	3.03	2.96	2.81	2.98
Fat% in milk	4.92	4.76	3.72	3.89	3.62	4.14	3.84	3.71	3.89

- The cow was randomly selected to show the performance data during the study.

Table 4.4. Etimated maternity state of Holstein cows during the study.

Cow #	Cummulative Days in Milk/Month	Calving Month	Month									Next Calving Date
			Feb	March	April	May	June	July	Aug	Sept	Oct	
			Winter		Spring			Summer			Autumn	
1	203 days/Feb	7/2011	-	-	-	-	-	-	-	-	-	
2	278 days/Feb	5/2011	-	-	-	1st Month	2nd Month	3rd Month	4th Month	5th Month	6th Month	18/02/2013
3	216 days/Feb	07/2011	-	-	-	-	-	-	-	-	-	
4	69 days/Feb	12/2011	maternal care	-	-	1st Month	2nd Month	3rd Month	4th Month	5th Month	6th Month	02/02/2013
5	229 days/Feb	05/2011	-	-	-	-	-	-	-	-	-	
6	234 days/Feb	06/2011	-	-	-	-	-	-	-	-	-	
7	277 days/Feb 26.5 days/July	05/2011 06/2012	6th Month	7th Month	8th Month	9th Month	calf birth	maternal care	maternal care	maternal care	-	
8	97 days/Feb	11/2011	maternal care	-	-	1st Month	2nd Month	3rd Month	4th Month	5th Month	6th Month	11/02/2013
9	27 days/Feb	1/2012	maternal care	-	-	-	-	-	1st Month	2nd Month	3rd Month	23/05/2013
10	73.5 days/May	3/2012	9th Month	calf birth	maternal care	maternal care	1st Month	2nd Month	3rd Month	4th Month	5th Month	07/03/2013
11	52 days/May	3/2012	9th Month	calf birth	maternal care	maternal care	1st Month	2nd Month	3rd Month	4th Month	5th Month	15/03/2013
12	47 days/May	4/2012	8th Month	9th Month	calf birth	maternal care	maternal care	maternal care	1st Month	2nd Month	3rd Month	17/05/2013
13	85 days/May	2/2012	calf birth	maternal care	maternal care	maternal care	-	-	-	-	-	04/08/2013
14	44 days/May	3/2012	9th Month	calf birth	maternal care	maternal care	maternal care	-	-	-	1st Month	03/07/2013
15	103 days/Feb	11/2011	maternal care	-	-	-	-	-	-	-	-	30/11/2013
16	82 days/May	2/2012	calf birth	maternal care	maternal care	maternal care	-	1st Month	2nd Month	3rd Month	4th Month	28/04/2013
17	33.5 days/July	6/2012	6th Month	7th Month	8th Month	9th Month	calf birth	maternal care	maternal care	1st Month	2nd Month	22/06/2013
18	303 days/Feb 21.3 days/July	4/2011 6/2012	6th Month	7th Month	8th Month	9th Month	calf birth	maternal care	maternal care	maternal care	-	06/10/2013
19	231 days/Feb 82.5 days/Sept	6/2011 6/2012	6th Month	7th Month	8th Month	9th Month	calf birth	maternal care	maternal care	maternal care	1st Month	02/07/2013
20	86 days/May	2/2012	calf birth	maternal care	maternal care	1st Month	2nd Month	3rd Month	4th Month	5th Month	6th Month	27/02/2013
21	114 days/July	3/2012	9th Month	calf birth	maternal care	maternal care	1st Month	2nd Month	3rd Month	4th Month	5th Month	13/03/2013

- Based on the days of milk, the estimated monthly calving of cows was conducted during the experiment, ("-") was not pregnant cow. However, there is no accurate interval time after calving for making an artificial insemination unless the cow and her calf confirmed to be in a healthy condition. The cow gestation period is 9 months, and the weaning time of calf is 3 month old.

4.2. Large-Scale Serum *N*-glycomics of Holstein Cows

Figure 4.6 shows a schematic procedure for Holstein cow serum *N*-glycomics based on the glycoblotting method. In the present study, whole serum *N*-glycans captured on the beads were divided into two portions to concurrently perform common *N*-glycan profiling (steps A−D) and its quantitative sialic acid analysis (step E) by modifying an original protocol established for the glycomics of avian egg white.[45] The expression levels of two major sialic acids, Neu5Ac and Neu5Gc, in the glycoproteins/glycosphingolipids synthesized by common mammalian cells, except for human cells, may be critical to indicate marked changes in the metabolic immune balance as well as seasonal alterations in total serum *N*-glycan profiles.

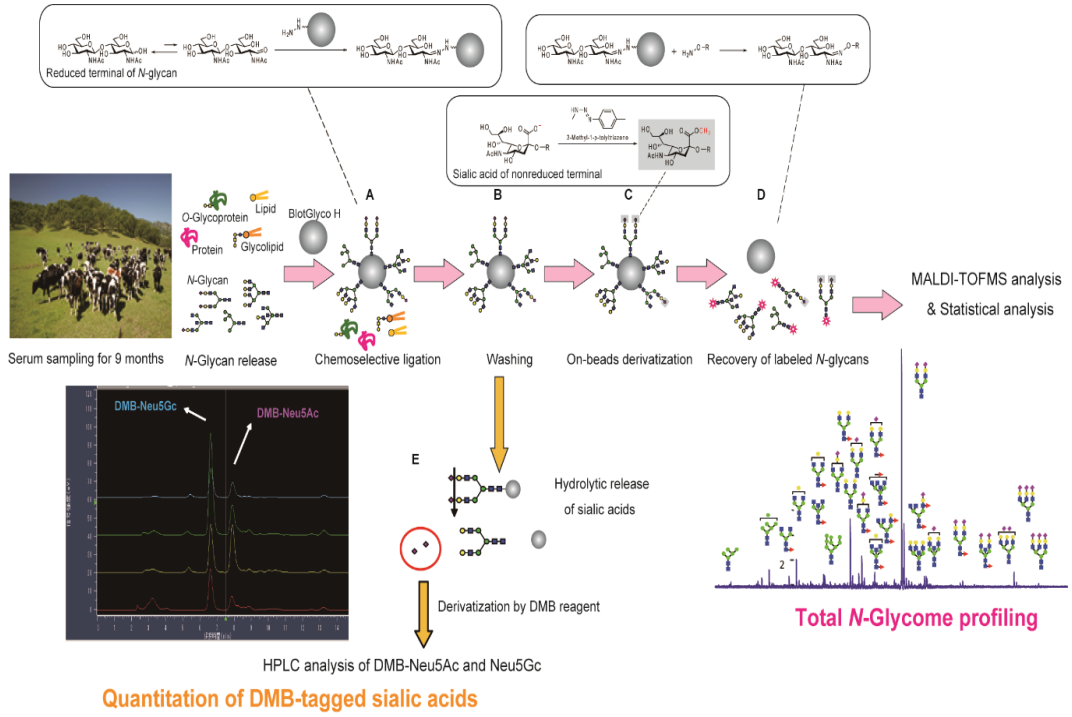
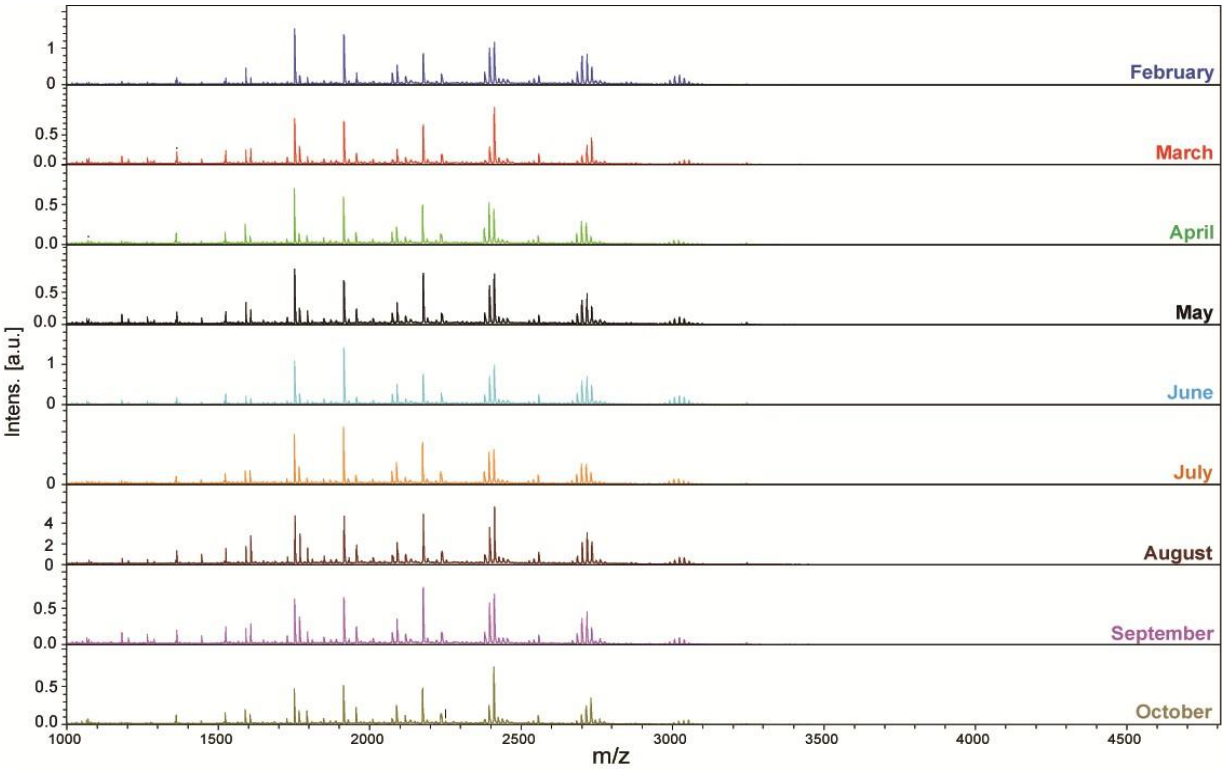


Figure 4.6. Glycoblotting-based systematic procedure for the large-scale glycomics of Holstein dairy cow serum samples. (A) chemoselective ligation of whole *N*-glycans of serum glycoproteins by "BlotGlyco" beads; (B) washing; (C) on-bead methylation of sialic acids; (D) trans iminization by benzyloxiamine to afford BOA-tagged *N*-glycans to be analyzed by MALDI-TOFMS; (E) selective DMB-labeling of the terminal sialic acids of total serum *N*-glycans to be employed for the quantitative HPLC analysis of serum sialic acids derived from *N*-glycans of total glycoproteins.

In the present study, I selected 36 key *N*-glycans in Figure 4.7A and monitored their expression levels because these ion peaks (*m/z*) were detected in ≥80% of the cow serum samples tested herein. Moreover, Figure 4.8A showed the *N*-glycan peaks of representative cow. Although 32 peaks (88.8%) were found in GlycosuitDB and the rest were reported in the database of CFG, only 28 of the *N*-glycan structures were sourced from the serum of *Bos taurus* species, whereas the others were sourced from other species. Unidentified mass peaks with significant intensities may have been due to free reducing oligosaccharides captured by the glycoblotting procedure; however, their metabolic pathways and biological functions have not yet been elucidated in detail. The molecular masses (*m/z*) and estimated sugar compositions of *N*-glycans derived from Holstein cow serum glycoproteins are summarized in Table 4.5. Holstein cow serum *N*-glycans have been categorized into 5 high-mannose types (13.9%), 8 hybrid types (22.2%), and 23 complex types (63.9%), shown in Figure 4.9. Changes in the monthly serum expression levels of these 36 *N*-glycans represented as micromolar (normalized on the basis of 100 μg of serum proteins) between February and October (see Figure 4.7B and Table 4.7). More information of representative cow, see Figure 4.8B and Table 4.9.

A.


February
March
April
May
June
July
August
September
October
Intens. [a.u.]
m/z
1000
1500
2000
2500
3000
3500
4000
4500

B.

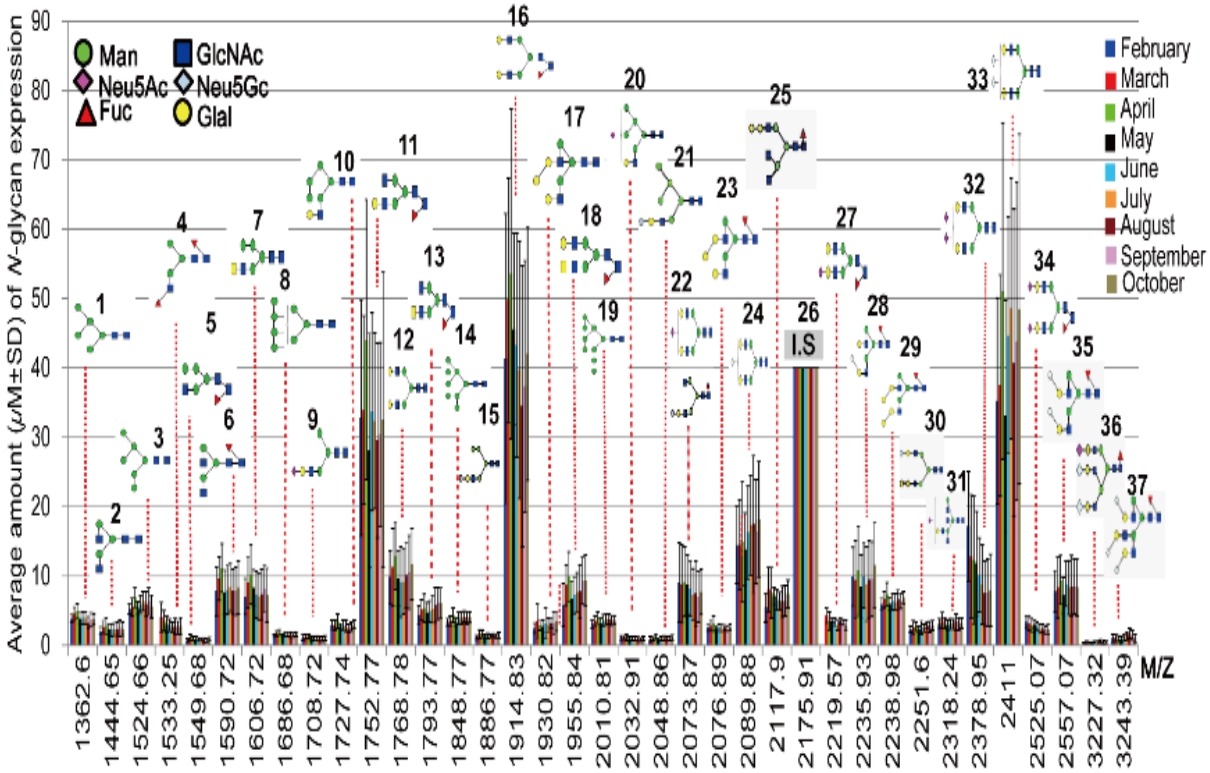
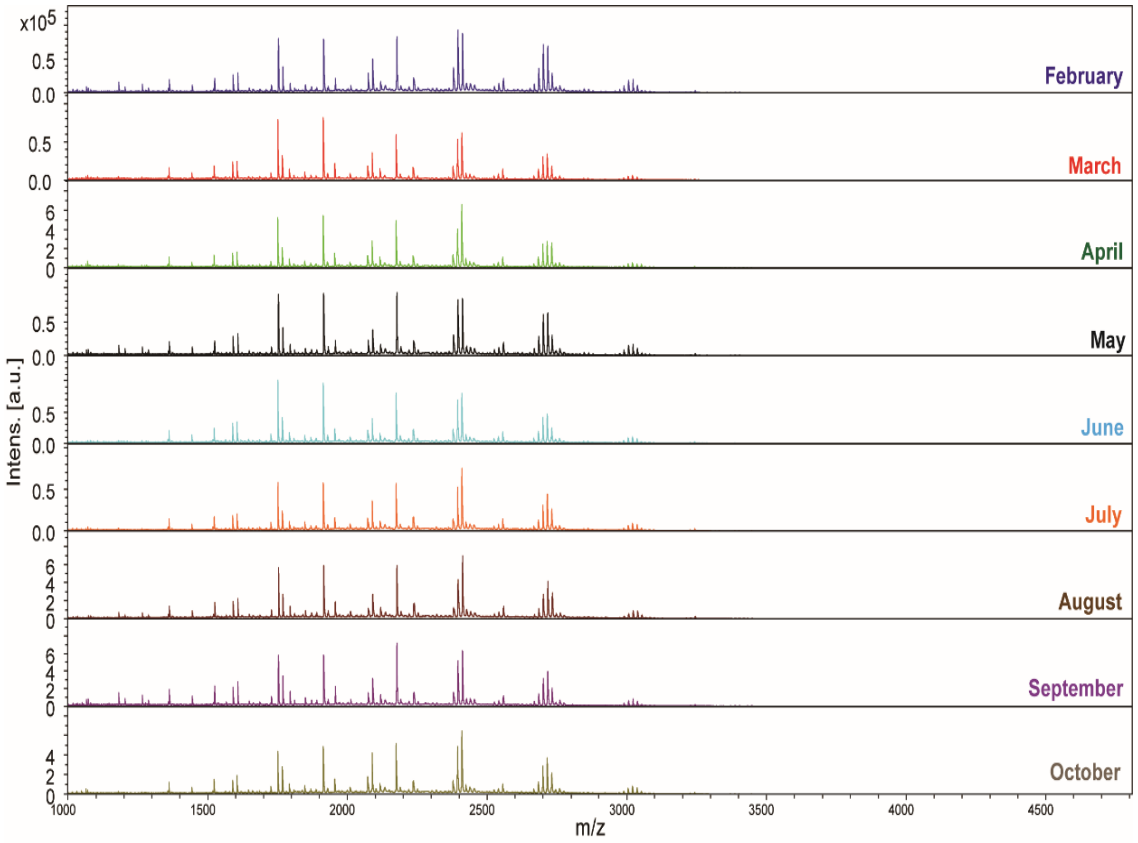


Figure 4.7. Large-scale *N*-glycan profiling of Holstein dairy cows serum glycoproteins by using a standard protocol combining the glycoblotting method and MALDI-TOF/MS, as represented in Figure 4.6: (A) MALDI-TOF/MS spectra showing typical *N*-glycan profiles of Holstein dairy cow serum collected each month; (B) monthly changes in the expression levels of the 36 major *N*-glycans selected from Holstein cow serum glycoproteins. Peak 26 detected at *m/z* 2175 means internal standard (IS) spiked (normalized as 40 μM).

A.


x10^5
0.5
0.0
February
March
6
4
2
0
April
May
June
July
August
September
October
Intens. [a.u.]
1000
1500
2000
2500
3000
3500
4000
4500
m/z

B.

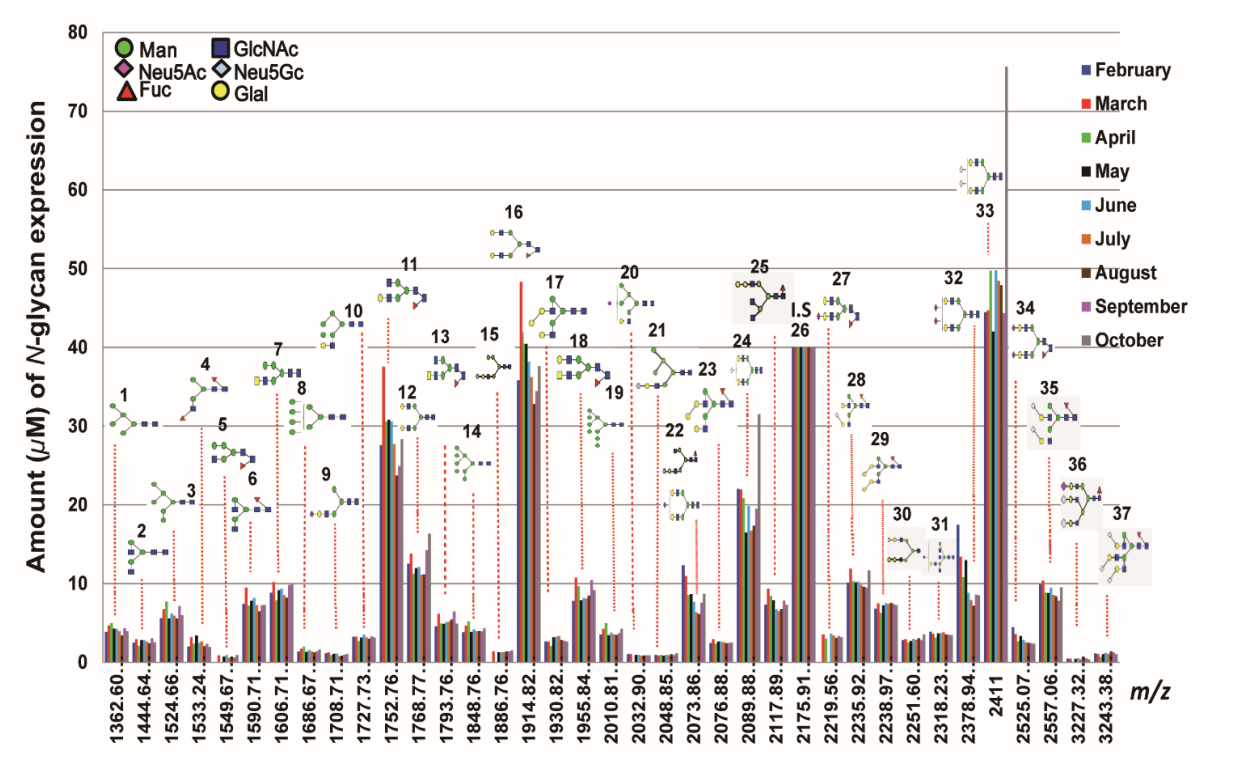


Figure 4.8. Representative *N*-glycan profiling of Holstein dairy cow serum glycoproteins of cow #8 by using a standard protocol combining the glycoblotting method and MALDI-TOF/MS, as represented in Figure 4.6: (A) MALDI-TOF/MS spectra showing typical *N*-glycan profiles of Holstein dairy cow serum collected each month; (B) monthly changes in the expression levels of the 36 major *N*-glycans selected from Holstein cow serum glycoproteins. Peak 26 detected at *m/z* 2175 means internal standard (IS) spiked (normalized as 40 μM).

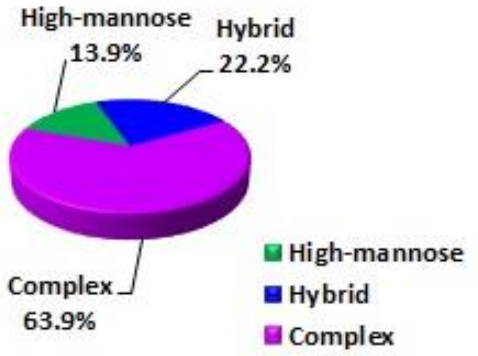
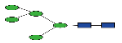
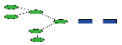
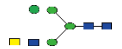
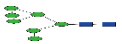
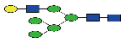
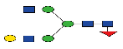
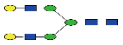
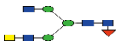
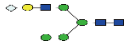
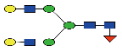
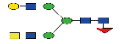
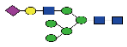
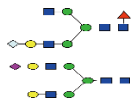
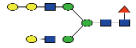
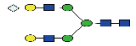
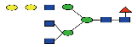
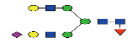
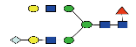
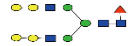
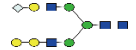
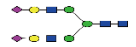
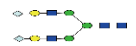
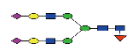


Figure 4.9. Serum *N*-glycan % of Holstein cow.

Table 4.5. Estimated compositions of 36 major *N*-glycans of Holstein cow serum glycoproteins selected.

Common *N*-glycans from serum of Holstein cow						
Peak #	*m/z*	ExPasy MW	Composition	Estimated structure	Type	Link [(source)]
1	1362.6	1216.42	(Hex)2 + (Man)3 (GlcNAc)2		High Mannose	UniCarbKB [(a)]
2	1444.65	1298.48	(HexNAc)2 + (Man)3 (GlcNAc)2		Complex	UniCarbKB [(a)]
3	1524.66	1378.48	(Hex)3 + (Man)3 (GlcNAc)2		High Mannose	UniCarbKB [(a)]
4	1533.25	1387.51	(HexNAc)1 (Deoxyhexose)2 + (Man)3 (GlcNAc)2		Hybrid	UniCarbKB [(b)]
5	1549.68	1403.51	(Hex)1 (HexNAc)1 (Deoxyhexose)1 + (Man)3 (GlcNAc)2		Hybrid	UniCarbKB [(a)]
6	1590.72	1444.53	(HexNAc)2 (Deoxyhexose)1 + (Man)3 (GlcNAc)2		Complex	UniCarbKB [(a)]
7	1606.72	1460.53	(Hex)1 (HexNAc)2 + (Man)3 (GlcNAc)2		Hybrid	UniCarbKB [(a)]
8	1686.68	1540.53	(Hex)4 + (Man)3 (GlcNAc)2		High Mannose	UniCarbKB [(a)]
9	1708.72	1548.54	(Hex)1 (HexNAc)1 (NeuAc)1 + (Man)3 (GlcNAc)2		Hybrid	UniCarbKB [(b)]
10	1727.74	1581.56	(Hex)3 (HexNAc)1 + (Man)3 (GlcNAc)2		Hybrid	UniCarbKB [(a)]
11	1752.77	1606.59	(Hex)1 (HexNAc)2 (Deoxyhexose)1 + (Man)3 (GlcNAc)2		Complex	UniCarbKB [(a)]
12	1768.78	1622.58	(Hex)2 (HexNAc)2 + (Man)3 (GlcNAc)2		Complex	UniCarbKB [(a)]
13	1793.77	1647.61	(HexNAc)3 (Deoxyhexose)1 + (Man)3 (GlcNAc)2		Complex	UniCarbKB [(a)]
14	1848.77	1702.58	(Hex)5 + (Man)3 (GlcNAc)2		High Mannose	UniCarbKB [(a)]
15	1886.77	1726.59	(Hex)2 (HexNAc)1 (NeuGc)1 + (Man)3 (GlcNAc)2		Hybrid	CFG [(c)]
16	1914.83	1768.64	(Hex)2 (HexNAc)2 (Deoxyhexose)1 + (Man)3 (GlcNAc)2		Complex	UniCarbKB [(a)]
17	1930.82	1784.63	(Hex)3 (HexNAc)2 + (Man)3 (GlcNAc)2		Complex	UniCarbKB [(a)]
18	1955.84	1809.67	(Hex)1 (HexNAc)3 (Deoxyhexose)1 + (Man)3 (GlcNAc)2		Complex	UniCarbKB [(a)]
19	2010.81	1864.63	(Hex)6 + (Man)3 (GlcNAc)2		High Mannose	UniCarbKB [(a)]
20	2032.91	1872.65	(Hex)3 (HexNAc)1 (NeuAc)1 + (Man)3 (GlcNAc)2		Hybrid	UniCarbKB [(b)]

21	2048.86	1888.65	(Hex)3 (HexNAc)1 (NeuGc)1 + (Man)3 (GlcNAc)2		Hybrid	CFG [d]
22	2073.87	1913.68	(Hex)1 (HexNAc)2 (Deoxyhexose)1 (NeuGc)1 + (Man)3 (GlcNAc)2 (Hex)2 (HexNAc)2 (NeuAc)1 + (Man)3 (GlcNAc)2		Complex	CFG [f] UniCarbKB [a]
23	2076.89	1930.69	(Hex)3 (HexNAc)2 (Deoxyhexose)1 + (Man)3 (GlcNAc)2		Complex	UniCarbKB [a]
24	2089.88	1929.67	(Hex)2 (HexNAc)2 (NeuGc)1 + (Man)3 (GlcNAc)2		Complex	UniCarbKB [a]
25	2117.9	1971.72	(Hex)2 (HexNAc)3 (Deoxyhexose)1 + (Man)3 (GlcNAc)2		Complex	UniCarbKB [a]
26	2175.91	2001.69	IS: (Hex)2 (HexNAc)2 (NeuAc)2 + (Man)3 (GlcNAc)1			
27	2219.57	2059.74	(Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuAc)1 + (Man)3 (GlcNAc)2		Complex	UniCarbKB [a]
28	2235.93	2075.73	(Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuGc)1 + (Man)3 (GlcNAc)2		Complex	UniCarbKB [a]
29	2238.98	2092.75	(Hex)4 (HexNAc)2 (Deoxyhexose)1 + (Man)3 (GlcNAc)2		Complex	UniCarbKB [a]
30	2251.6	2091.72	(Hex)3 (HexNAc)2 (NeuGc)1 + (Man)3 (GlcNAc)2		Complex	CFG [d]
31	2318.24	2157.78	(Hex)1 (HexNAc)4 (NeuAc)1 + (Man)3 (GlcNAc)2		Complex	UniCarbKB [c]
32	2378.95	2204.77	(Hex)2 (HexNAc)2 (NeuAc)2 + (Man)3 (GlcNAc)2		Complex	UniCarbKB [a]
33	2411	2236.76	(Hex)2 (HexNAc)2 (NeuGc)2 + (Man)3 (GlcNAc)2		Complex	UniCarbKB [a]
34	2525.07	2350.83	(Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuAc)2 + (Man)3 (GlcNAc)2		Complex	UniCarbKB [a]
35	2557.07	2382.82	(Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuGc)2 + (Man)3 (GlcNAc)2		Complex	UniCarbKB [a]
36	3227.32	3039.05	(Hex)3 (HexNAc)3 (Deoxyhexose)1 (NeuAc)1 (NeuGc)2 + (Man)3 (GlcNAc)2		Complex	CFG [e]
37	3243.39	3055.04	(Hex)3 (HexNAc)3 (Deoxyhexose)1 (NeuGc)3 + (Man)3 (GlcNAc)2		Complex	UniCarbKB [e]

[a] Glycosuit and/or CFG database of cow *N*-glycan structures, [b] not identified in cow (reported in human), [c] "no source", [d] "not classified species", [e] not identified structure in cow (reported in pig), and [f] not identified structure in cow (reported in mouse). Peak #26 is a spiked internal standard (quantification). Compositional annotation and estimated structures by GlycoMod (ExPASy proteomics server, Swiss Institute of Bioinformatics (http://www.expasy.ch/tools/glycomod/). However, the non-reported structures from the CFG database. Higher *m/z* values observed in the experimental results than those of Expasy (M/W) are due to the BOA labeling (+123 *m/z*) in addition to (+23 *m/z*) Na adduct under laser irradiation in MALDI. The esterification of sialic acid residues also increases the mass of *N*-glycans (+14 *m/z* per sialic acid residue). *N*-glycan cartoons were conducted using the standard symbol nomenclature as follow, HexNAc, *N*-acetylhexosamine (GlcNAc, *N*-acety-D-lglucosamine [blue square] or GalNAc, *N*-acetylgalactosamine [yellow square] depends on the description); Deoxyhexose, fucose [red triangle]; Hex, hexose (Mannose [green circle], and galactose [yellow circle] depend on the description); Neu5Ac, 5-*N*-acetylneuraminic acid [purple diamond]; and Neu5Gc, 5-*N*-glycolylneuraminic acid [white diamond].

These results showed that the serum expression levels of some characteristic *N*-glycan structures were altered in a seasondependent manner and appeared to peak in some months. A multivariate analysis clearly indicated that monthly expression levels of these *N*-glycoforms (μM ± SE, $P < 0.001$) fit the general linear model and reached the highest level in four different months as follows: April (peaks 1, 3, 8, 14, and 19 at *m/z* 1363, 1525, 1687, 1849, and 2011 corresponding to high-mannose type *N*-glycans assigned as M5−M9) (4.9364 ± 0.3080, 6.9217 ± 0.3997, 1.8851 ± 0.1235, 4.39 ± 0.29874, and 4.0085 ± 0.2378); August (peaks 36 and 37 at *m/z* 3227 and 3243 corresponding to complex type *N*-glycans carrying three sialic acid residues, 3 × Neu5Gc) (0.5919 ± 0.0400, 1.7468 ± 0.1005); October (peaks 24 and 28 at *m/z* 2090 and 2236 corresponding to complex type *N*-glycans bearing one sialic acid residue, 1 × Neu5Gc) (18.2121 ± 1.197, 11.5736 ± 0.877); and February (peaks 32 and 34 at *m/z* 2379 and 2525 corresponding to complex type *N*-glycans bearing two sialic acid residues, 2 × Neu5Ac) (17.182 ± 2.27498, 3.3818 ± 0.22556) (Figure 4.10 and Table 4.6; see also Table 4.7). Interestingly, the result of the serum expression levels of these characteristic *N*-glycan structures of representative cow clearly confirmed the result of multivariate analysis. For instance: April (peaks 1, 3, 8, 14, and 19 corresponding to high-mannose type *N*-glycans) (4.99, 7.701, 1.99, 5.194, and 4.983); August (peaks 36 and 37 corresponding to complex type *N*-glycans carrying three sialic acid residues, 3 × Neu5Gc) (0.478, 1.393); October (peaks 24 and 28) (31.498, 11.664 corresponding to complex type *N*-glycans bearing one sialic acid residue, 1 × Neu5Gc); and February (peaks 32 and 34) (17.475, 4.451 corresponding to complex type *N*-glycans bearing two sialic acid residues, 2 × Neu5Ac) (Figure 4.11 and Table 4.9).

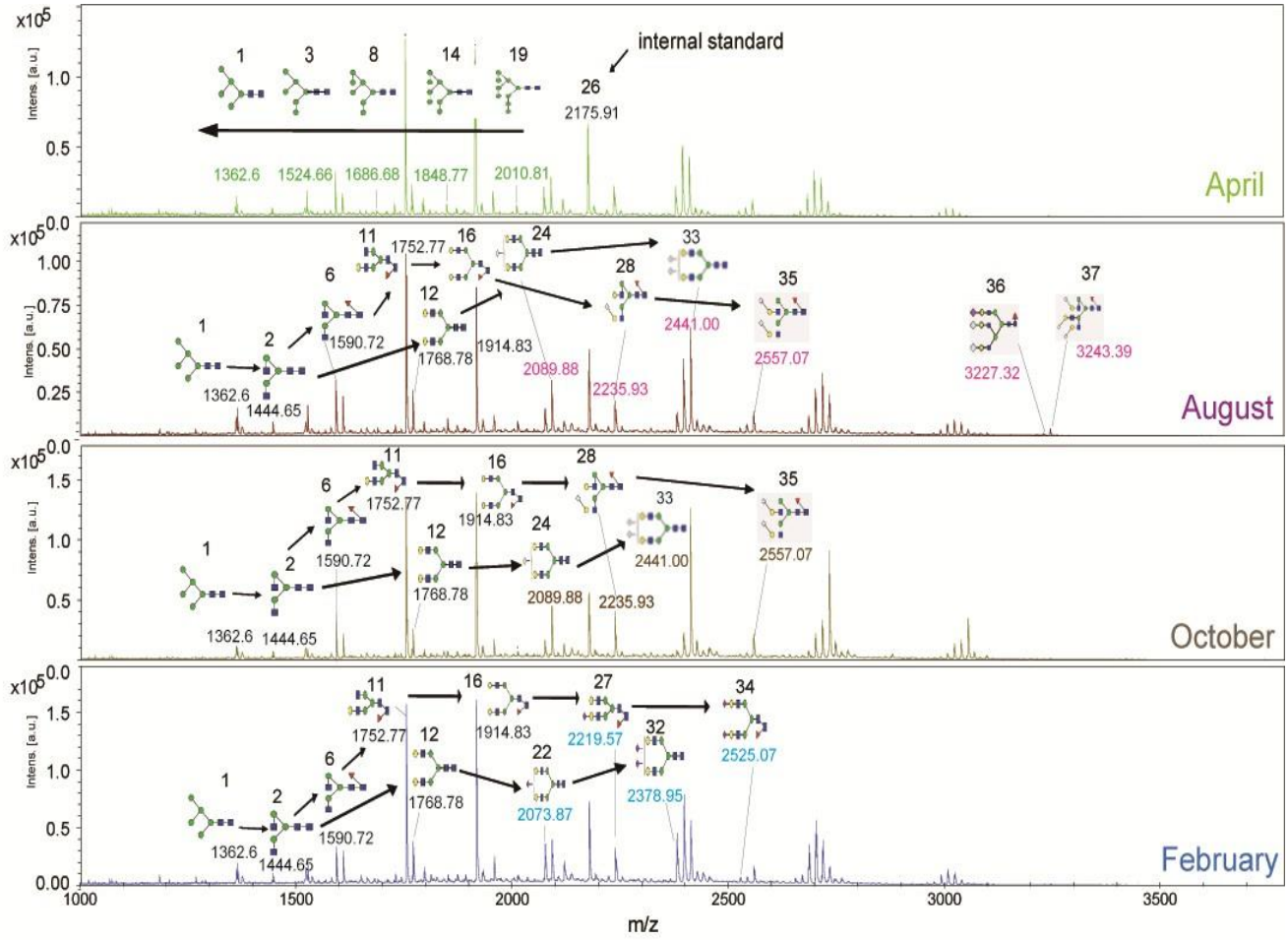


Figure 4.10. Some seasonally relevant serum *N*-glycoforms of Holstein dairy cows given by a statistical analysis of expression levels and plausible biosynthetic pathways (see also Tables 4.7 and 4.7). Peak 26 at 2175.91 indicates an internal standard spiked, [(Hex)2 (HexNAc)2 (Neu5Ac)2 + (Man)3 (GlcNAc)1], and the final concentration was adjusted to 40 μM.

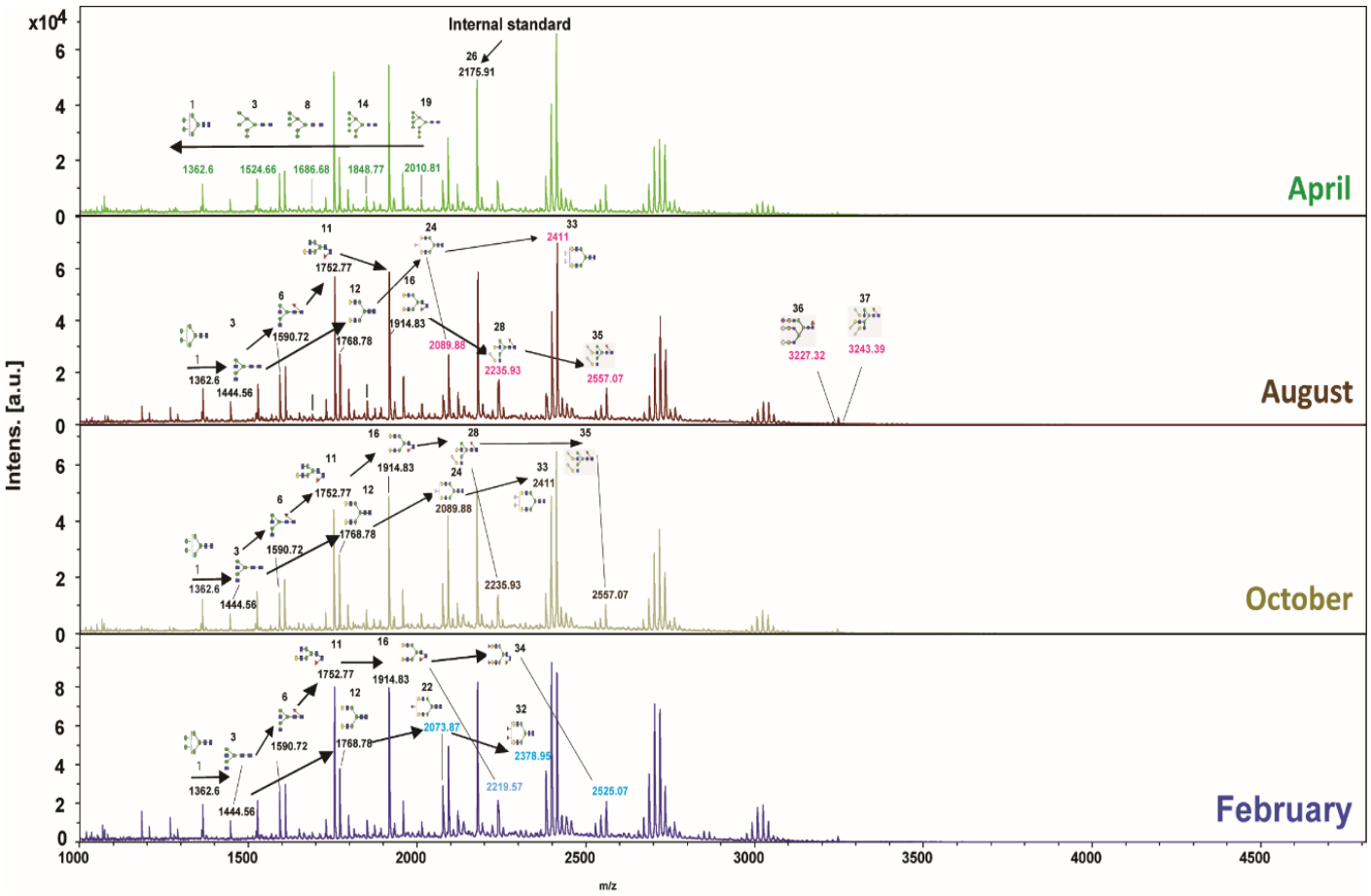
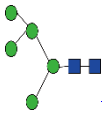
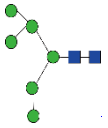
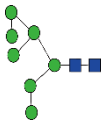
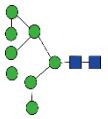
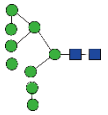
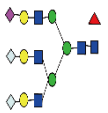
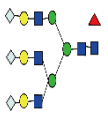
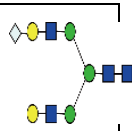
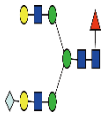
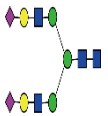
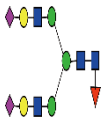


Figure 4.11. Representative MALDI-TOF/MS spectra of some seasonally relevant serum *N*-glycoforms of Holstein dairy cow given by the high expression levels and plausible biosynthetic pathways (see also Tables 4.9). Peak 26 at 2175.91 indicates an internal standard spiked, [(Hex)2 (HexNAc)2 (Neu5Ac)2 + (Man)3 (GlcNAc)1], and the final concentration was adjusted to 40 μM.

Table 4. 6. Specific serum *N*-glycans as biomarkers showing correlations with some characteristic data of dairy Holstein cows

Specific peaks of *N*-glycans from serum of Holstein cow												
Peak #	*m/z*	Treatment	Month									
			February	March	April	May	June	July	August	September	October	Estimated structure
1	1362.61	(a)	3.895a±0.25	4.609a±0.18	5.006a±0.26***	4.011a±0.169	3.995a±0.12	3.945a±0.13	3.233a±0.13	3.642a±0.133	3.701a±0.14	
		(b)	3.782a±0.27	4.345a±0.20	4.781a±0.26**	3.718a±0.157	3.859a±0.11	4.045a±0.13	3.489a±0.14	3.761a±0.136	3.818a±0.14	
3	1524.66	(a)	5.316a±0.44	5.567a±0.31	6.986a±0.45	5.400a±0.289	5.963a±0.21	6.357a±0.22	5.939a±0.23	6.356a±0.227	5.651a±0.24	
		(b)	5.108a±0.47	5.332a±0.35	6.765a±0.46	5.306a±0.272	5.942a±0.20	6.426a±0.23	6.084a±0.25	6.407a±0.234	5.689a±0.24	
8	1686.68	(a)	1.372a±0.1	1.658a±0.07	1.863a±0.10**	1.365a±0.066	1.593a±0.04	1.620a±0.05	1.555a±0.05	1.494a±0.052	1.649a±0.05	
		(b)	1.397a±0.10	1.703a±0.08	1.913a±0.10***	1.380a±0.062	1.609a±0.04	1.607a±0.05	1.526a±0.05	1.463a±0.054	1.639a±0.05	
14	1848.77	(a)	3.341a±0.23	3.615a±0.17	4.407a±0.245*	3.422a±0.157	3.931a±0.11	3.939a±0.12	4.086a±0.12	3.935a±0.124	4.033a±0.13	
		(b)	3.180a±0.25	3.424a±0.19	4.252a±0.25*	3.257a±0.147	3.893a±0.10	3.981a±0.12	4.229a±0.13	3.981a±0.126	4.109a±0.13	
19	2010.81	(a)	3.132a±0.20	3.576a±0.15	4.015a±0.21	3.376a±0.137	3.654a±0.1	3.718a±0.10	3.766a±0.11	3.614a±0.108	3.598a±0.11	
		(b)	3.010a±0.22	3.432a±0.16	3.896a±0.21	3.246a±0.129	3.615a±0.09	3.747a±0.11	3.877a±0.12	3.676a±0.111	3.661a±0.11	
36	3227.32	(a)	0.365a±0.07	0.442a±0.05	0.408a±0.07	0.403a±0.046	0.462a±0.03	0.469a±0.032	0.586a±0.03	0.525a±0.034	0.448a±0.04	
		(b)	0.305a±0.076	0.382a±0.06	0.360a±0.076	0.373a±0.042	0.451a±0.028	0.479a±0.033	0.623a±0.037**	0.545a±0.034	0.460a±0.043	
37	3243.39	(a)	1.043a±0.16	1.034a±0.12	0.933a±0.17	0.980a±0.109	1.171a±0.07	1.415a±0.08	1.745a±0.08***	1.490a±0.086	1.036a±0.09	
		(b)	0.964a±0.179	0.970a±0.13	0.906a±0.175	0.877a±0.102	1.134a±0.07	1.403a±0.08	1.805a±0.09***	1.541a±0.088	1.105a±0.09	

24	2089.88	(a)	14.424a±2.1	14.349a±1.5	14.567a±2.2	14.104a±1.44	16.300a±1.0	17.519a±1.1	17.512a±1.1	16.370a±1.13	18.091a±1.20	
		(b)	14.201a±2.3	13.885a±1.7	14.446a±2.2	13.272a±1.34	16.053a±0.9	17.877a±1.1	18.231a±1.2	16.952a±1.15	17.988a±1.21	
28	2235.93	(a)	9.944a±1.54	9.960a±1.12	11.421a±1.5	8.792a±1.016	10.125a±0.7	8.597a±0.78	9.175a±0.80	9.501a±0.799	11.596a±0.84	
		(b)	10.809a±1.6	10.345a±1.2	11.537a±1.6	8.928a±0.957	10.236a±0.7	8.860a±0.81	9.018a±0.88	9.131a±0.825	11.123a±0.86	
32	2378.95	(a)	17.554a±1.7***	12.836a±1.2	12.255a±1.7	11.194a±1.15	9.536a±0.83	8.583a±0.89	7.754a±0.91	8.046a±0.906	8.723a±0.96	
		(b)	15.997a±1.8*	12.034a±1.4	11.563a±1.8	11.872a±1.08	10.192a±0.8	8.625a±0.92	7.738a±1.00	7.994a±0.933	8.441a±0.97	
34	2525.07	(a)	3.386a±0.19***	3.178a±0.14	2.931a±0.2	3.055a±0.128	2.756a±0.09	2.414a±0.09	2.496a±0.10	2.235a±0.101	2.337a±0.107	
		(b)	3.280a±0.2***	3.099a±0.15	2.843a±0.20	3.071a±0.119	2.781a±0.08	2.439a±0.10	2.527a±0.11	2.271a±0.103	2.294a±0.107	

- Multivariate analyses were performed to evaluate correlations with (a) covariates I "body weight, parity, milk yield, and days in milk" and (b) covariates II "fat, protein, and lactose % in milk", respectively. Dependent variables were the average serum concentrations (μM) of *N*-glycans at each mass peak (*m/z*), and fixed factors included the month of the year appearing in the general linear model, SPSS software. *N*-glycan structures are represented using the standard symbol nomenclature as follows: HexNAc, *N*-acetylhexosamine (GlcNAc, *N*-acetylglucosamine (blue square) or GalNAc, *N*-acetylgalactosamine (yellow square) depending on the description); deoxyhexose, fucose (red triangle); Hex, hexose (mannose (green circle) and galactose (yellow circle) depending on the description); Neu5Ac, 5-*N*-acetylneuraminic acid (purple diamond); and Neu5Gc, 5-*N*-glycolylneuraminic acid (white diamond). A multivariate analysis was conducted by SPSS software with a one-way ANOVA, in which"red results" was not detected. Asterisks show significance level: ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$.

Table 4.7. *N*-glycome expression levels (μM ± SE) of the serum glycoproteins of Holstein cow between February and October.

Peak #	*m/z*	February	March	April	May	June	July	August	September	October
1	1362.61	3.9199±0.14413	4.549±0.20705	4.9364±0.308***	3.8772±0.1411	3.9289±0.11307	3.959±0.13157	3.3053±0.11796	3.6982±0.142	3.7866±0.1192
2	1444.65	2.071±0.16391	2.6376±0.1810	2.8045±0.32055	2.3375±0.136	2.2483±0.11307	2.3284±0.13079	2.3925±0.14091	2.4956±0.165	2.414±0.13966
3	1524.66	5.2963±0.22606	5.5126±0.2657	6.9217±0.399	5.3811±0.1580	5.9725±0.16445	6.4005±0.20298	5.974±0.26452	6.351±0.2828	5.6291±0.2386
4	1533.25	-	4.0702±0.2070	3.5529±0.30809	3.0566±0.1411	2.9198±0.11307	2.6169±0.13157	2.7518±0.11796	2.5762±0.142	2.7801±0.1192
5	1549.67	0.7421±0.06974	1.1757±0.0967	1.0418±0.10342	0.9522±0.0652	0.8676±0.03843	0.8378±0.03893	0.8261±0.05439	0.7289±0.040	0.9026±0.0532
6	1590.72	7.8325±1.01491	9.6377±0.6417	11.1585±1.0127	7.6569±0.5343	7.9923±0.45233	8.374±0.49254	7.8203±0.46348	7.8409±0.500	8.3374±0.5518
7	1606.71	7.0512±0.75789	9.1319±0.7613	10.295±1.2304	8.3254±0.4257	7.3236±0.43513	7.1027±0.48733	7.4032±0.52593	8.1025±0.489	7.262±0.56044
8	1686.68	1.3689±0.05749	1.6703±0.0820	1.8851±0.123***	1.3618±0.0344	1.6016±0.04595	1.617±0.05116	1.5501±0.05984	1.4869±0.046	1.6438±0.0488
9	1708.71	1.124±0.12215	1.3214±0.0916	1.2828±0.11048	1.1156±0.0468	0.9877±0.04557	1.0212±0.06102	0.9972±0.05319	0.9335±0.054	1.0945±0.0573
10	1727.74	2.9935±0.19	2.9445±0.1495	3.5356±0.29498	2.7583±0.0691	2.9549±0.08918	2.8139±0.11826	2.4807±0.08806	2.72±0.09474	3.0187±0.0863
11	1752.77	32.8607±4.9099	33.9478±2.778	44.0812±5.8344	28.1268±2.803	33.7016±1.8399	32.3857±1.83085	29.5508±2.0218	30.3988±1.91	32.5758±3.066
12	1768.78	9.8193±1.11027	11.3113±1.133	12.8438±1.3938	9.6932±0.6676	9.1719±0.65638	9.1788±0.67472	10.1649±0.6829	10.549±0.763	11.7021±0.740
13	1793.77	4.4345±0.37823	5.1747±0.3008	5.3804±0.57866	4.7537±0.2363	4.8811±0.20339	5.2589±0.28992	5.7962±0.36787	6.0867±0.338	6.0735±0.3282
14	1848.77	3.346±0.14784	3.5779±0.1454	4.39±0.29874**	3.3082±0.1034	3.9109±0.11261	3.9563±0.13173	4.1387±0.12137	3.9585±0.131	4.0747±0.1151
15	1886.77	1.1627±0.10177	1.6414±0.2160	1.6885±0.16591	1.3221±0.0685	1.3643±0.05225	1.3671±0.03978	1.4758±0.03638	1.3876±0.049	1.4859±0.0651
16	1914.83	41.3903±6.0247	49.8067±3.589	53.5951±6.8697	45.6033±2.306	43.3027±2.0896	39.7395±2.68272	34.5167±2.9262	37.3794±2.61	42.1001±2.642
17	1930.83	2.4841±0.21117	3.5461±0.4936	2.8897±0.1892	2.7518±0.1535	3.2415±0.27693	2.9783±0.14169	3.2813±0.1116	3.1995±0.245	3.4129±0.1941
18	1955.84	5.8502±0.89391	8.6881±0.5845	9.9325±1.0258	6.7123±0.4363	7.2796±0.34094	7.724±0.40748	7.7944±0.55463	9.301±0.4887	9.4072±0.5256
19	2010.81	3.1431±0.17005	3.5559±0.1429	4.0085±0.237	3.2862±0.1034	3.628±0.10175	3.723±0.112	3.8033±0.10175	3.6388±0.106	3.6405±0.0940
20	2032.91	1.1182±0.08837	1.1528±0.0816	1.1911±0.14363	1.0194±0.0498	1.0044±0.03668	0.9834±0.04986	0.9595±0.04603	0.8618±0.043	0.9851±0.0476
21	2048.86	0.9198±0.11582	0.9673±0.0717	1.2935±0.11986	0.8957±0.0515	1.0038±0.04406	1.0821±0.05385	1.0718±0.0402	1.0241±0.053	1.1571±0.0542
22	2073.87	9.1094±1.65309	8.8677±1.1455	9.1719±1.45003	8.8058±0.7042	8.1498±0.51109	7.3482±0.54233	7.5075±0.68405	7.0002±0.530	7.7308±0.4715
23	2076.89	2.6104±0.15961	2.8211±0.1455	3.1662±0.32045	2.5138±0.0626	2.5503±0.06636	2.4944±0.06792	2.5362±0.05797	2.4654±0.059	2.6669±0.0563
24	2089.88	14.4745±1.6102	14.5556±1.323	14.9618±2.5238	13.8732±0.87	16.3035±0.8622	17.3736±1.0397	17.4766±1.4241	16.3838±1.08	18.2121±1.19
25	2117.89	5.4668±0.60491	8.0593±0.6517	7.5889±1.0466	7.1273±0.2146	6.585±0.19994	6.3829±0.18665	6.3104±0.32023	6.4554±0.319	7.4375±0.2823
27	2219.57	-	4.3049±0.3557	3.5553±0.40754	3.6295±0.0947	3.4224±0.09824	2.9172±0.12969	3.4537±0.0976	3.3549±0.114	2.9606±0.1297
28	2235.93	9.8593±1.23784	9.4806±1.1494	10.803±1.85661	8.5567±0.7553	10.0714±0.6021	8.8955±0.77775	9.502±0.81124	9.5565±0.780	11.5736±0.87
29	2238.98	5.995±0.22094	6.6253±0.2366	7.4279±0.4786	5.9999±0.1632	6.3261±0.15655	6.261±0.1761	6.5919±0.19926	6.3662±0.153	6.8794±0.1216
30	2251.6	2.3392±0.12549	2.6347±0.1947	2.7086±0.20195	2.3957±0.0698	2.5727±0.12625	2.5335±0.08485	2.9193±0.07758	2.7024±0.117	2.8882±0.1041
31	2318.24	3.0872±0.22258	3.6544±0.2731	3.2048±0.30231	2.9727±0.1398	3.0661±0.13657	3.0633±0.15146	3.3921±0.23175	3.0699±0.231	3.2562±0.1708
32	2378.95	17.182±2.274***	12.8175±1.817	12.3291±2.6503	11.8628±1.248	10.1646±0.6904	8.8066±0.82594	7.5391±0.67546	7.5779±0.701	7.9739±0.7498
33	2411	35.2825±4.2887	37.52±3.24869	51.1407±6.9979	33.092±2.7784	44.7274±2.2048	48.6048±2.71908	40.7311±3.2009	43.8459±3.30	48.5359±3.637
34	2525.07	3.3818±0.225***	3.1722±0.1998	2.9267±0.26987	3.0485±0.0921	2.7602±0.08421	2.4204±0.0932	2.5013±0.0871	2.2327±0.079	2.3324±0.0961
35	2557.07	7.7943±0.87022	8.3507±0.8878	9.4348±1.02103	7.2503±0.4328	8.8882±0.42608	8.3294±0.56529	8.5823±0.63158	8.4875±0.573	8.3548±0.6035
36	3227.32	0.366±0.02846	0.4373±0.0398	0.4026±0.04436	0.3913±0.0229	0.4582±0.02258	0.4703±0.02787	0.5919±0.04**	0.528±0.0453	0.4551±0.035
37	3243.39	1.0681±0.15023	1.0676±0.0999	0.9946±0.12251	0.9086±0.0756	1.144±0.06812	1.3838±0.085	1.7468±0.10***	1.5131±0.095	1.0896±0.0822

- Based on protein concentration 100 μg proteins for *N*-glycans and 10 μL of serum glycoproteins of each sample. A multivariate statistical analysis was conducted by SPSS software, one-way ANOVA, ("-") was not detected, the asterisk"***" showed a significance level, $P \leq 0.001$,"**" showed a significance level, $P \leq 0.01$.

Table 4.8. Relationship between *N*-glycome expression levels (μM ± SE) of the serum glycoproteins of Holstein cow and basic data between February and October.

A. In correlation with covariates I "body weight, parity, milk yield, and days in milk".

Peak #	*m/z*	February	March	April	May	June	July	August	September	October
1	1362.61	3.895a±0.257	4.609a±0.186	5.006a±0.26***	4.011a±0.169	3.995a±0.123	3.945a±0.131	3.233a±0.135	3.642a±0.133	3.701a±0.143
3	1524.66	5.316a±0.44	5.567a±0.319	6.986a±0.451	5.400a±0.289	5.963a±0.21	6.357a±0.224	5.939a±0.231	6.356a±0.227	5.651a±0.242
5	1549.67	0.734a±0.096	1.198a±0.072	1.092a±0.113	0.978a±0.063	0.891a±0.044	0.828a±0.049	0.796a±0.053	0.717a±0.054	0.870a±0.06
8	1686.68	1.372a±0.1	1.658a±0.074	1.863a±0.103**	1.365a±0.066	1.593a±0.048	1.620a±0.051	1.555a±0.053	1.494a±0.052	1.649a±0.055
9	1708.71	1.124a±0.102	1.297a±0.085	1.262a±0.114	1.085a±0.073	0.979a±0.051	1.034a±0.057	1.017a±0.055	0.942a±0.056	1.105a±0.067
10	1727.74	2.978a±0.196	2.951a±0.142	3.531a±0.201	2.833a±0.129	2.984a±0.094	2.819a±0.1	2.455a±0.103	2.695a±0.101	2.974a±0.108
14	1848.77	3.341a±0.239	3.615a±0.173	4.407a±0.245*	3.422a±0.157	3.931a±0.114	3.939a±0.122	4.086a±0.127	3.935a±0.124	4.033a±0.132
16	1914.83	41.396a±5.23	50.786a±3.79	55.111a±5.37	45.948a±3.44	43.734a±2.50	39.168a±2.66	33.868a±2.72	37.081a±2.70	41.950a±2.87
18	1955.84	6.783a±0.889	8.427a±0.513	8.854a±0.631	6.804a±0.497	7.457a±0.357	7.584a±0.518	7.987a±0.535	9.116a±0.46	9.289a±0.447
19	2010.81	3.132a±0.209	3.576a±0.151	4.015a±0.214	3.376a±0.137	3.654a±0.1	3.718a±0.106	3.766a±0.11	3.614a±0.108	3.598a±0.115
23	2076.89	2.601a±0.146	2.813a±0.106	3.142a±0.149	2.564a±0.097	2.562a±0.07	2.502a±0.074	2.526a±0.077	2.455a±0.076	2.642a±0.08
24	2089.88	14.424a±2.19	14.349a±1.59	14.567a±2.25	14.104a±1.44	16.300a±1.05	17.519a±1.12	17.512a±1.14	16.370a±1.13	18.091a±1.20
27	2219.57	-	4.232a±0.236	3.507a±0.224	3.588a±0.242	3.378a±0.15	2.919a±0.135	3.495a±0.15	3.384a±0.198	3.008a±0.151
28	2235.93	9.944a±1.546	9.960a±1.121	11.421a±1.58	8.792a±1.016	10.125a±0.74	8.597a±0.787	9.175a±0.805	9.501a±0.799	11.596a±0.84
29	2238.98	5.964a±0.334	6.665a±0.242	7.468a±0.343	6.137a±0.22	6.395a±0.16	6.256a±0.17	6.528a±0.175	6.310a±0.173	6.790a±0.186
30	2251.6	2.317a±0.204	2.573a±0.152	2.600a±0.22	2.479a±0.142	2.588a±0.101	2.559a±0.109	2.930a±0.109	2.692a±0.108	2.844a±0.12
32	2378.95	17.554a±1.7***	12.836a±1.26	12.255a±1.79	11.194a±1.15	9.536a±0.838	8.583a±0.891	7.754a±0.919	8.046a±0.906	8.723a±0.963
33	2411	37.973a±5.59	37.882a±3.23	49.925a±3.97	35.982a±3.12	44.429a±2.25	47.398a±3.26	38.576a±3.36	41.632a±2.89	50.191a±2.81
34	2525.07	3.386a±0.19***	3.178a±0.141	2.931a±0.2	3.055a±0.128	2.756a±0.093	2.414a±0.099	2.496a±0.103	2.235a±0.101	2.337a±0.107
36	3227.32	0.365a±0.07	0.442a±0.055	0.408a±0.076	0.403a±0.046	0.462a±0.03	0.469a±0.032	0.586a±0.033	0.525a±0.034	0.448a±0.043
37	3243.39	1.043a±0.166	1.034a±0.12	0.933a±0.171	0.980a±0.109	1.171a±0.079	1.415a±0.085	1.745a±0.088***	1.490a±0.086	1.036a±0.091

- Based on protein concentration 100 μg proteins for *N*-glycans and 10 μL of serum glycoproteins of each samples. Moreover, dependent variable was the average amounts (μM) of *N*–glycans *m/z* released from the serum glycoprotein of cow, and fixed factor included month of the year appearing in the general linear model, SPSS software, ("-") was not detected, the asterisk "***" showed a significance level, $P \leq 0.001$, "**" showed a significance level, $P \leq 0.01$,"*" showed a significance level, $P \leq 0.05$).

B. In correlation with covariates II “fat%, protein%, lactose% in milk”.

Peak #	*m/z*	February	March	April	May	June	July	August	September	October
1	1362.61	3.782a±0.275	4.345a±0.206	4.781a±0.269**	3.718a±0.157	3.859a±0.116	4.045a±0.134	3.489a±0.147	3.761a±0.136	3.818a±0.142
3	1524.66	5.108a±0.473	5.332a±0.354	6.765a±0.463	5.306a±0.272	5.942a±0.201	6.426a±0.231	6.084a±0.252	6.407a±0.234	5.689a±0.244
5	1549.67	0.746a±0.101	1.157a±0.079	1.030a±0.112	0.927a±0.058	0.856a±0.042	0.843a±0.051	0.850a±0.058	0.738a±0.054	0.920a±0.059
8	1686.68	1.397a±0.107	1.703a±0.082	1.913a±0.105***	1.380a±0.062	1.609a±0.046	1.607a±0.052	1.526a±0.058	1.463a±0.054	1.639a±0.055
9	1708.71	0.984a±0.109	1.186a±0.091	1.146a±0.117	1.066a±0.066	0.968a±0.048	1.056a±0.058	1.083a±0.059	0.992a±0.056	1.107a±0.067
10	1727.74	2.905a±0.21	2.830a±0.157	3.445a±0.206	2.679a±0.12	2.920a±0.089	2.854a±0.103	2.575a±0.112	2.759a±0.104	3.044a±0.108
14	1848.77	3.180a±0.256	3.424a±0.191	4.252a±0.25*	3.257a±0.147	3.893a±0.109	3.981a±0.125	4.229a±0.138	3.981a±0.126	4.109a±0.132
16	1914.83	43.214a±5.654	50.942a±4.22	54.860a±5.536	45.202a±3.245	42.999a±2.40	39.808a±2.761	34.396a±2.996	36.834a±2.795	42.071a±2.918
18	1955.84	6.502a±0.712	8.696a±0.536	9.390a±0.728	6.643a±0.509	7.331a±0.41	7.763a±0.478	7.692a±0.511	9.677a±0.525	9.218a±0.427
19	2010.81	3.010a±0.224	3.432a±0.167	3.896a±0.219	3.246a±0.129	3.615a±0.095	3.747a±0.11	3.877a±0.121	3.676a±0.111	3.661a±0.116
23	2076.89	2.506a±0.156	2.710a±0.116	3.064a±0.152	2.479a±0.09	2.541a±0.066	2.538a±0.076	2.613a±0.084	2.512a±0.078	2.647a±0.08
24	2089.88	14.201a±2.344	13.885a±1.75	14.446a±2.296	13.272a±1.346	16.053a±0.99	17.877a±1.145	18.231a±1.242	16.952a±1.159	17.988a±1.21
25	2117.89	5.756a±0.458	7.705a±0.345	7.247a±0.468	7.115a±0.331	6.623a±0.264	6.438a±0.308	6.208a±0.328	6.467a±0.338	7.320a±0.274
27	2219.57	-	4.258a±0.258	3.544a±0.243	3.617a±0.243	3.404a±0.137	2.873a±0.141	3.438a±0.171	3.347a±0.208	3.057a±0.149
28	2235.93	10.809a±1.668	10.345a±1.24	11.537a±1.633	8.928a±0.957	10.236a±0.71	8.860a±0.814	9.018a±0.884	9.131a±0.825	11.123a±0.861
29	2238.98	5.923a±0.36	6.509a±0.269	7.337a±0.352	5.911a±0.206	6.289a±0.153	6.323a±0.175	6.702a±0.193	6.386a±0.178	6.873a±0.186
30	2251.6	2.354a±0.223	2.647a±0.171	2.705a±0.226	2.430a±0.133	2.593a±0.097	2.558a±0.115	2.915a±0.121	2.672a±0.112	2.809a±0.122
32	2378.95	15.997a±1.88*	12.034a±1.41	11.563a±1.849	11.872a±1.083	10.192a±0.80	8.625a±0.923	7.738a±1.007	7.994a±0.933	8.441a±0.975
34	2525.07	3.280a±0.2***	3.099a±0.155	2.843a±0.203	3.071a±0.119	2.781a±0.088	2.439a±0.101	2.527a±0.112	2.271a±0.103	2.294a±0.107
36	3227.32	0.305a±0.076	0.382a±0.06	0.360a±0.076	0.373a±0.042	0.451a±0.028	0.479a±0.033	0.623a±0.037**	0.545a±0.034	0.460a±0.043
37	3243.39	0.964a±0.179	0.970a±0.133	0.906a±0.175	0.877a±0.102	1.134a±0.076	1.403a±0.087	1.805a±0.09***	1.541a±0.088	1.105a±0.092

- Based on protein concentration 100 μg proteins for *N*-glycans and 10 μL of serum glycoproteins of each samples. Moreover, dependent variable was the average amounts (μM) of *N*–glycans *m/z* released from the serum glycoprotein of cow, and fixed factor included month of the year appearing in the general linear model, SPSS software, (”-”) was not detected, the asterisk ”***” showed a significance level, $P \leq 0.001$, ”**” showed a significance level, $P \leq 0.01$,”*” showed a significance level, $P \leq 0.05$).

Table 4.9. *N*-glycome expression levels of the serum glycoproteins of a representative Holstein cow #8 between February and October.

Peak #	*m/z*	February	March	April	May	June	July	August	September	October
1	1362.61	3.853	4.655	4.990	4.271	4.216	3.958	3.423	4.324	3.946
2	1444.65	2.497	2.915	2.085	2.814	2.813	2.611	2.412	3.035	2.545
3	1524.66	5.593	6.757	7.701	5.547	6.199	5.910	5.562	7.144	5.999
4	1533.25	1.995	3.190	2.385	3.419	2.491	2.657	2.031	2.335	1.949
5	1549.67	-	0.882	-	0.752	0.929	0.568	0.720	0.612	0.908
6	1590.72	7.434	9.482	7.199	7.816	8.172	7.250	6.457	7.271	7.276
7	1606.71	8.837	10.188	7.867	9.169	9.337	8.521	8.191	9.799	9.914
8	1686.68	1.351	1.710	1.990	1.321	1.547	1.394	1.264	1.390	1.646
9	1708.71	1.155	1.254	0.934	1.086	1.110	0.787	0.826	0.962	1.063
10	1727.74	3.244	3.238	2.705	3.116	3.510	3.166	2.966	3.289	3.147
11	1752.77	27.574	37.551	30.559	30.818	30.568	27.706	23.707	24.917	28.360
12	1768.78	12.528	13.814	11.248	11.972	12.077	11.131	11.166	14.229	16.326
13	1793.77	4.577	6.148	4.941	4.876	5.120	5.172	5.433	6.445	4.915
14	1848.77	3.806	4.665	5.194	3.843	4.166	3.957	3.972	3.918	4.316
15	1886.77	-	1.356	-	1.285	1.306	1.303	1.366	1.363	1.510
16	1914.83	35.811	48.309	41.913	40.827	38.187	36.209	32.792	34.440	37.610
17	1930.83	2.665	2.603	2.061	3.187	3.241	3.340	2.859	2.741	2.647
18	1955.84	7.824	10.746	9.650	7.854	8.168	8.105	8.486	10.431	9.184
19	2010.81	3.547	4.243	4.983	3.434	3.778	3.547	3.461	3.700	4.274
20	2032.91	1.045	1.020	-	0.922	0.963	0.828	0.872	0.891	0.885
21	2048.86	0.942	0.851	0.939	0.829	0.949	0.900	1.011	0.924	1.147
22	2073.87	12.327	10.969	8.536	8.681	7.672	6.346	6.133	7.586	8.686
23	2076.89	2.458	2.929	2.356	2.632	2.653	2.536	2.416	2.460	2.523
24	2089.88	22.029	21.942	20.850	16.459	19.833	16.708	17.330	19.491	31.498
25	2117.89	7.313	9.357	8.409	7.863	6.740	6.494	6.764	7.837	7.303
27	2219.57	-	3.557	2.999	-	3.635	3.413	3.088	3.340	3.194
28	2235.93	10.116	11.884	10.293	10.143	10.258	9.836	9.597	9.466	11.664
29	2238.98	6.788	7.471	6.279	7.236	7.485	7.447	7.534	7.395	7.256
30	2251.6	2.820	2.914	2.515	2.688	2.975	2.864	3.058	2.800	3.555
31	2318.24	3.889	3.632	3.153	3.650	3.712	3.798	3.549	3.488	3.448
32	2378.95	17.475	13.422	10.812	12.960	8.839	7.880	7.175	8.569	8.516
33	2411	44.463	44.674	49.733	41.992	49.769	48.459	47.901	44.359	75.632
34	2525.07	4.451	3.591	2.675	3.344	2.872	2.542	2.474	2.405	2.349
35	2557.07	9.912	10.380	8.852	8.830	9.458	8.549	8.380	7.831	9.515
36	3227.32	0.448	0.443	-	0.426	0.471	0.403	0.478	0.465	0.384
37	3243.39	1.117	1.030	0.671	1.017	1.190	1.065	1.393	1.267	1.013

- Based on protein concentration 100 μg proteins for *N*-glycans and 10 μL of serum glycoproteins of each sample, ("-") was not detected, and the cow was randomly selected to show the expression levels of the serum glycoproteins during the study.

Furthermore, statistical analyses revealed that the expression levels of these *N*-glycoforms correlated with covariate I (parity, days in milk, body weight, and milk yield) and covariate II (fat %, protein %, and lactose % in milk constituents), as shown in Table 4.8. These results clearly showed the significant relationship between the seasonal metabolic/homeostatic immune balance and changes in serum *N*-glycan expression profiles. A biosynthetic pathway analysis toward some season-relevant *N*-glycans revealed that *N*-glycans detected at *m/z* 2411 (peak 33) and *m/z* 2557 (peak 35) markedly increased in August and October and were assigned as counterparts of the *N*-glycans observed at *m/z* 2379 (peak 32) and *m/z* 2525 (peak 34), notably characteristic biomarkers that correlated well with the results of the multivariate analysis of Holstein cow data in February (Table 4.8). As anticipated, serum expression levels of their precursors detected at *m/z* 2090 (peak 24) and *m/z* 2236 (peak 28) also appeared to be upregulated in accordance with the increase observed in serum *N*-glycans at *m/z* 2411 (peak 33) and *m/z* 2557 (peak 35) in August and October. Similarly, *N*-glycans detected at *m/z* 2074 (peak 22) and *m/z* 2220 (peak 27), the precursors of the *N*-glycans observed at *m/z* 2379 (peak 32) and *m/z* 2525 (peak 34), were dominantly synthesized in February, as shown in Figure 4.10 and Table 4.7. In April, serum levels of a series of high-mannose type *N*-glycans appeared to be higher than other matured glycoforms such as hybrid and complex type *N*-glycans, suggesting the down-regulation of exo-mannosidases, key enzymes responsible for trimming the multiple mannose residues of highmannose type *N*-glycans, leading to the universal intermediate structure observed at *m/z* 1445 (peak 2), an indispensable substrate for further synthetic processes of all complex type *N*-glycans. These results suggested that the accumulation of such seasonally characteristic *N*-glycoforms was caused by changes in the expression levels of *exo*-mannosidases/glycosyltransferases and intracellular concentrations of various sugar nucleotides in relation to their biosynthetic pathways, as shown by the arrows in Figure 4.10. This biosynthetic pathways has been confirmed in representative cow #8, see Figure 4.11 and Table

4.9. It is important to emphasize that the dynamic interconversion between Neu5Ac and Neu5Gc in serum *N*-glycans provides highly sensitive biomarkers. Seasonal alterations in the expression levels of two biantennary *N*-glycans carrying Neu5Ac or Neu5Gc, notably peak 32 at *m/z* 2379 and peak 33 at *m/z* 2411, represent promising serum biomarkers for monitoring unmeasurable damage or the disruption of the homeostatic immune balance of lactating Holstein dairy cows under integrated heat/environmental stresses.

I then focused on the significance of the sialylation of highly abundant serum IgG Fc *N*-glycans and regulatory mechanism of immunity in dairy cows, as explained in schematic Figure 4.12, because serum IgG Fc *N*-glycans were needed for optimal Fcγ receptor binding through modulations of the quaternary structure and thermodynamic stability of the Fc region.[67,68] The addition of sialic acids to the ends of Fc *N*-glycans was previously shown to convert human IgG from a pro-inflammatory to an anti-inflammatory agent.[69,70] The interaction between sialylated IgG Fc *N*-glycans and dendritic-cell-specific ICAM-3 grabbing non-integrin (DCSIGN in humans and SIGN-R1 in the mouse) may be an essential step to suppress inflammation by providing an intrinsic mechanism to regulate the T_H2 pathway for maintaining immune homeostasis.[71]

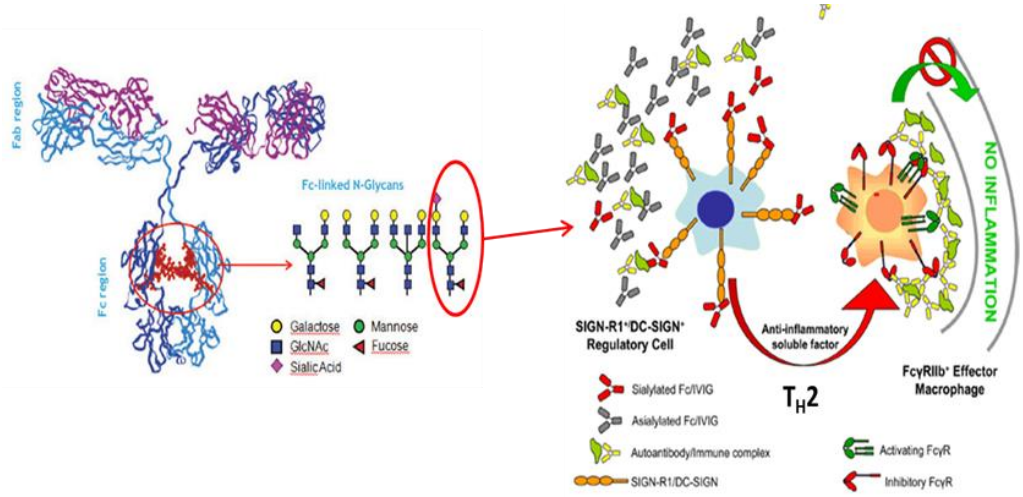


Figure 4.12. Schematic cartoon for the explanation of the addition of sialic acids to the ends of Fc *N*-glycans can convert human IgG from a pro-inflammatory anti-inflammatory, and The interaction between sialylated IgG Fc *N*-glycans and (DCSIGN, SIGN-R1 might suppress inflammation by regulate the T_H2 pathway.

Moreover, IgG in bovine/buffalo colostrum obtained 1 day after calving was found to be more heavily terminated by Neu5Ac and Neu5Gc than that in milk obtained 1−4 weeks postpartum.[72,73] Therefore, the sialylation of IgG and other serum glycoproteins was considered to putatively modulate immune cell functions in infectious diseases, inflammation, neurodegeneration, autoimmune diseases, and cancer through interactions with Fcγ/FcRn receptors and/or various pattern recognition receptors (PRRs) including sialic acid-binding immunoglobulin- like receptors (Siglec) on dendritic cells and macrophages.[74]

I hypothesized that the sialylation profile of serum IgG Fc *N*-glycans as well as those of major serum glycoproteins in fetal bovine serum may differ markedly from those observed in the serum samples of mature Holstein dairy cows. As shown in Figure 4.13, the *N*-glycan profiles of fetal bovine serum glycoproteins were different from those in Holstein cow serum. Fetal bovine serum glycoproteins carried only 8 major *N*-glycoforms, and the expression levels of high-mannose type *N*-glycans were markedly lower than those in mature bovine serum. Three major peaks detected at *m/z* 2379, 3049, and 3354 were fully sialylated complex type *N*-glycans

that were mostly modified with Neu5Ac residues. The triantennary *N*-glycan detected at *m/z* 3354 was modified with four Neu5Ac residues, suggesting that this glycoform involved the typical glycoside unit Neu5Acα2,8Neu5Ac widely found in the embryonic stage.[42] However, it is important to note that peaks due to *N*-glycans having the Neu5Gc residue were not detected in fetal serum glycoproteins. Highly sialylated multivalent *N*-glycans may be released from some fetal serum-relevant glycoproteins such as α-fetoprotein (AFP) and carcinoembryonic antigen (CEA) in addition to serum IgG.[75,76] The results also revealed that the peak (3 in Figure 4.13) detected at *m/z* 2379, assigned as biantennary *N*-glycan having two terminal Neu5Ac residues, was identical to peak 32 listed in Table 4.6, and the serum expression level of which in Holstein dairy cows was highest in February among the 10 months examined. Given the facts that approximately 50% of the present serum samples were collected from pregnant cows during this study and the expression levels of this *N*-glycan (peak 32) and its monofucosylated glycoform (peak 34 detected at *m/z* 2525) in February strongly correlated with parity data (Table 4.10 and 4.11), in addition it seems to be related to cows in pregnancy or presence of calves (Table 4.4, see also Figure 4.14), in addition to *N*-glycan terminated with two groups Neu5Ac was highly expressed in pregnant cow than non pregnant one (Figure 4.15 a,b). I considered that these biantennary *N*-glycan structures bearing two Neu5Ac residues have potential as specific serum biomarkers closely related to maternal immune defenses.[77,78] However, I have to notice that further glycomics analysis should be done to easily confirm the maternal relationship.

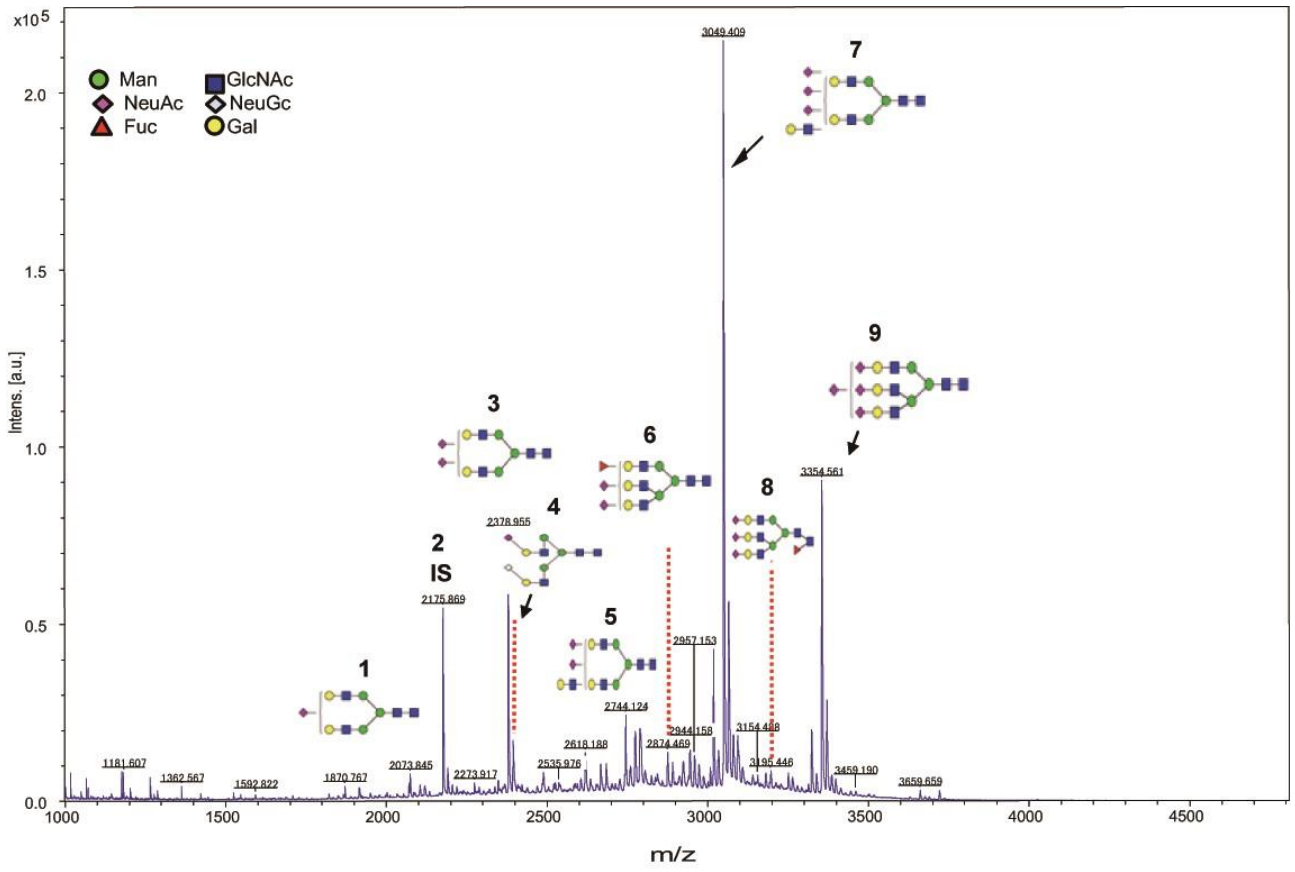
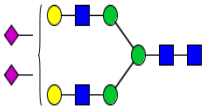


Figure 4.13. MALDI-TOF/MS of *N*-glycans of fetal serum glycoproteins. Peak 2 detected at *m/z* 2175 indicates an internal standard (IS) spiked [(hex)2(HexNAc)2(Neu5Ac)2 + Man)3(GlcNAc)1] during the glycoblotting protocol, and the final concentration was adjusted to 40 μM.

It was reported that proteomic analysis of bovine pregnancy-specific serum proteins by two dimensional-fluorescence difference gel electrophoresis, used as comparative proteomics, for the development of pregnancy-diagnostic markers in early pregnancy bovine serum. The differentially expressed proteins were identified by MALDI-TOF/MS.[79] Moreover, proteomic approach based on 2-DE combined with MS revealed the ovine serum protein pattern and identified 42 medium-high-abundance proteins that could potentially serve as biomarkers in early diagnoses and monitoring of ovine physiology and metabolism would be useful for recognising subclinical and pathological conditions, certifying welfare and the quality/safety of ovine products.[80] On the other hand, saliva with its hundreds of proteins, peptides, hormones, electrolytes and other chemical compounds might be a good source of biomarkers which helps

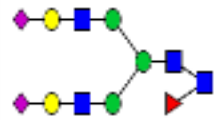
in assessing stress factors such as malnutrition as well as diseases or ongoing viral infections in farm animals. Also the difference in the proteome of tears in various animal species could explain their adaptation to their climatic conditions.[81] Thus this study is unique to get a biomarker in case of time independent in cow serum correlated with such environmental stressors.

Table 4.10. The statistical analysis of *N*-glycome concentration (μM) of the serum glycoproteins from primi-parous and multi-parous-6 Holstein cow in February, particularly in (2378.95 *m/z*).

t-Test: Two-Samples			
Holstein cow serum	*N*-glycome amount (μM), 2378.95 *m/z*		Estimated structure
	Primi-parous	Multi-parous-6	
Mean	12.9398448	26.62677067	
Variance	57.11289771	8.396654138	
Observations	8	3	
Hypothesized Mean Difference	0		
Degrees of Freedom	5		
t-Stat	-4.064886542		
P(T<=t) one-tail	0.004841242**		
t-Critical one-tail	2.015048373		
P(T<=t) two-tail	0.009682483**		
t-Critical two-tail	2.570581836		

-The corresponding statistical significance was calculated using the student *t*-test, ("primi-parous" is a cow who has given birth once, "multi-parous-6" is a cow has given birth 6 times. The asterisk "**" showed a significance level, $P \leq 0.01$).

Table 4.11. The statistical analysis of *N*-glycome concentartion (μM) of the serum glycoproteins from primi-parous and multi-parous-3 Holstein cow in February, particularly in (2525.07 *m/z*).

t-Test: Two-Samples			
Holstein cow serum	*N*-glycome amount (μM), 2525.07 *m/z*		Estimated structure
	Primi-parous	Multi-parous-3	
Mean	2.939932525	3.711270609	
Variance	0.713717055	0.501408827	
Observations	8	11	
Hypothesized Mean Difference	0		
Degrees of Freedom	14		
t-Stat	-2.100894552		
P(T<=t) one-tail	0.027121648*		
t-Critical one-tail	1.761310136		
P(T<=t) two-tail	0.054243295		
t-Critical two-tail	2.144786688		

-The corresponding statistical significance was calculated using the student *t*-test, ("primi-parous" is a cow who has given birth once, "multi-parous-3" is a cow has given birth 3 times. The asterisk "*" showed a significance level, $P \leq 0.05$).

In contrast, in July, August, September, and October, these *N*-glycans (peak 32 at *m/z* 2379 and peak 34 detected at *m/z* 2525) markedly decreased and a new peak, 33, at *m/z* 2411, corresponding to the biantennary *N*-glycan having two Neu5Gc residues, was observed with high intensity (Figures 4.7A and 4.9 and Table 4.7). Because the *N*-glycans of bovine colostrum IgG were modified with Neu5Gc and Neu5Ac residues, the immunity of newborn calves may be specifically controlled by the Neu5Gc residues of IgG *N*-glycans because, as shown in Figure 4.13, fetal bovine serum glycoproteins were not modified with the Neu5Gc residue. In other words, Neu5Gc appeared to be an unusual sialic acid for newborn calves and may be required epigenetically to adapt to the dynamic changes associated with early environmental stresses. As a result, IgG *N*-glycans,[72,73] and free oligosaccharides involving Neu5Gc residues in colostrum were provided by cows to newborn calves through lactation. This step was definitely essential for newborn calves to construct a beneficial intestinal

microbiota and shape early immunity, as has been reported for the functions of human milk oligosaccharides in the formation of infant gastrointestinal microbiota.[82–84] However, there is no example to investigate th relation between milk glycan composition and THI/diet. I also felt that such approach is very interesting and important because changes in the milk glycan composition influences significantly human gastrointestinal microbiota. The results clearly suggest that the quantitation of serum expression levels of whole Neu5Ac and Neu5Gc residues provides highly sensitive biomarkers for matured Holstein cows because the expression levels (ratio) of these two sialic acids (Neu5Ac/Neu5Gc) differed significantly between some of the major *N*-glycoforms observed in February, April, August, and October (Figure 4.10 and Table 4.6). The upregulation of serum total Neu5Gc expression levels may directly indicate an increase in damage caused by various environmental stresses in Holstein dairy cows. The quantitation of serum Neu5Gc expression levels may facilitate the monitoring of damage and disorders in Holstein dairy cows by directly reflecting their general immune responses and milk productivity.

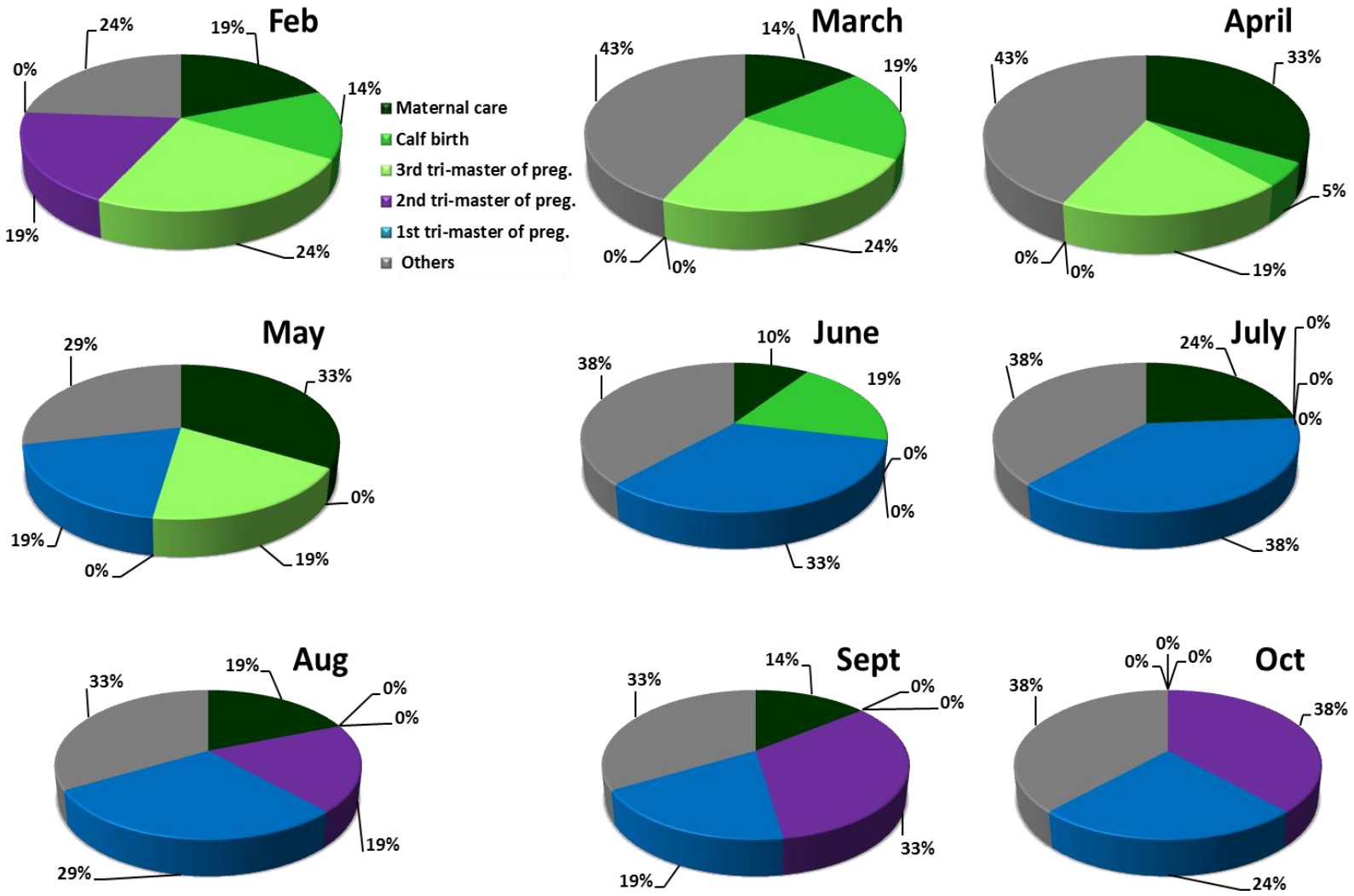
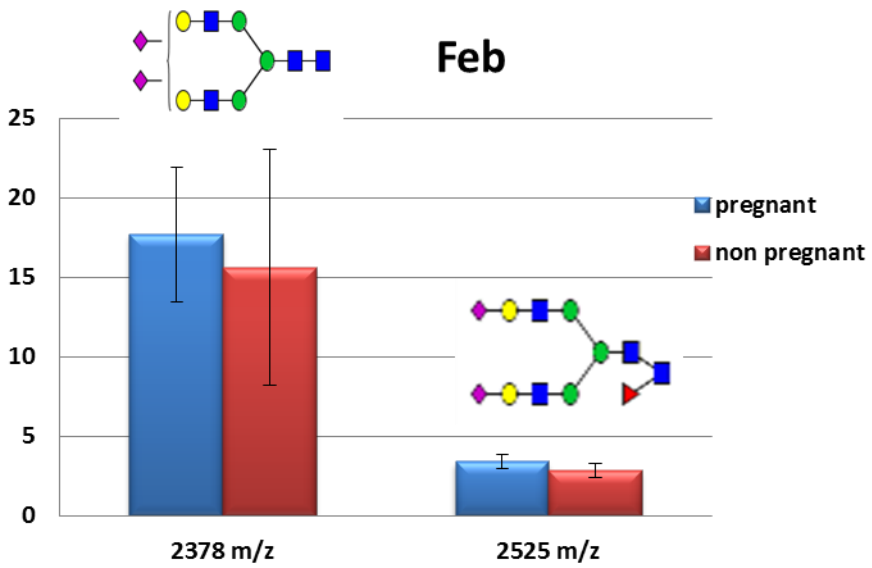


Figure 4.14. Monthly description of Holstein cow state during the study.

A.



B.

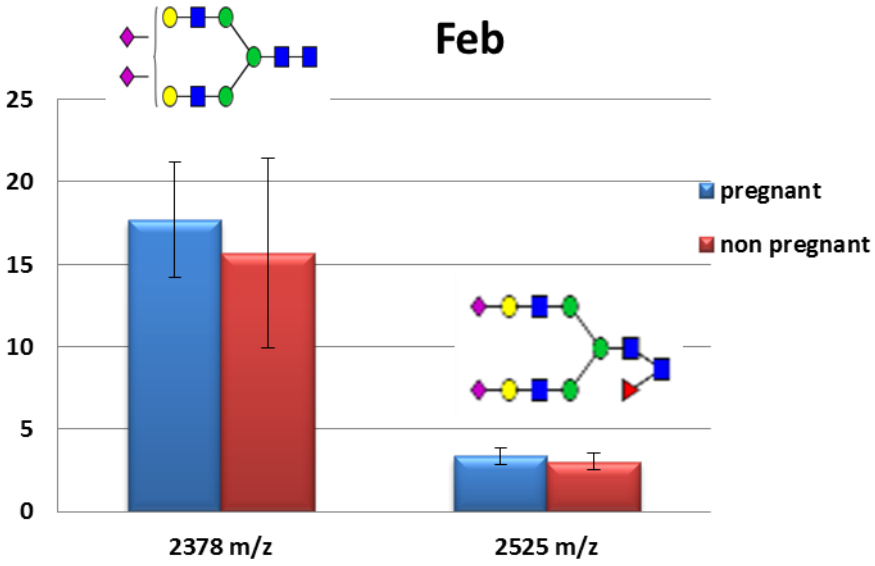


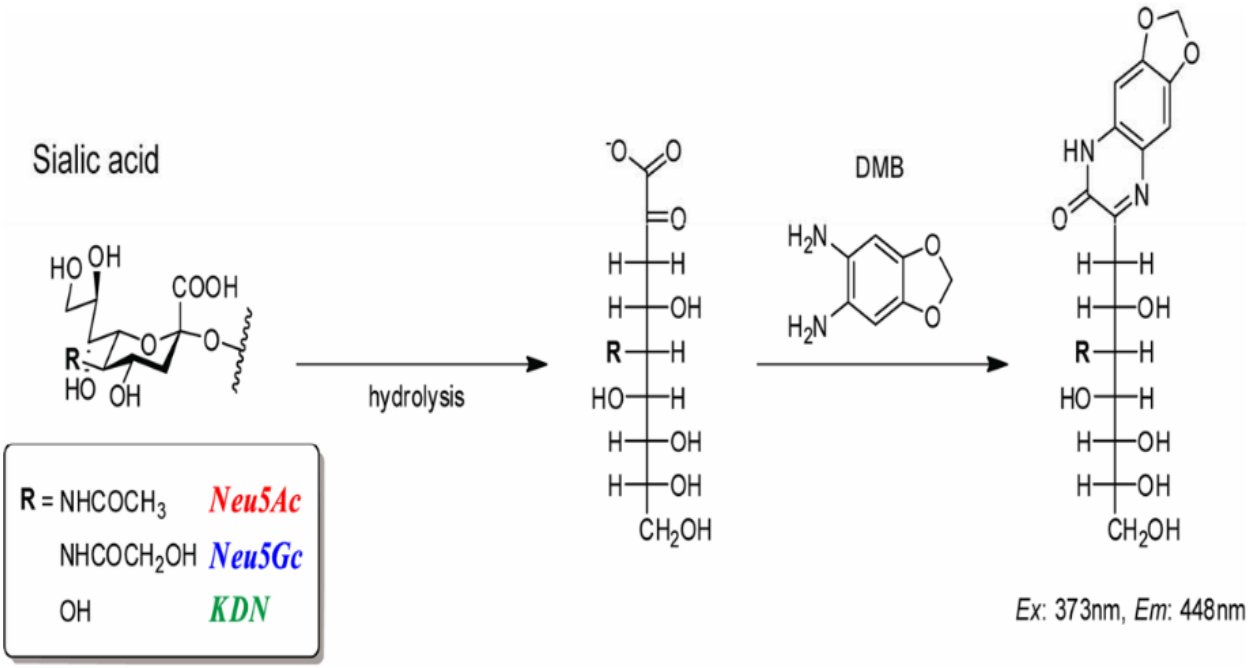
Figure 4.15. A statistical analysis of the average amounts (μM±SE) of *N*–glycans (2378, 2525 *m/z*) released from the serum glycoprotein of preganant and non pregnant Holstein cows was conducted in February during the study. (A) in correlation with covariate I (body weight, milk yield, days in milk, parity). (B) in correlation with covariate II (fat%, protein%, lactose% in milk).

4.3. Quantitative Analysis of Dynamic Sialylation in Serum Glycoproteins.

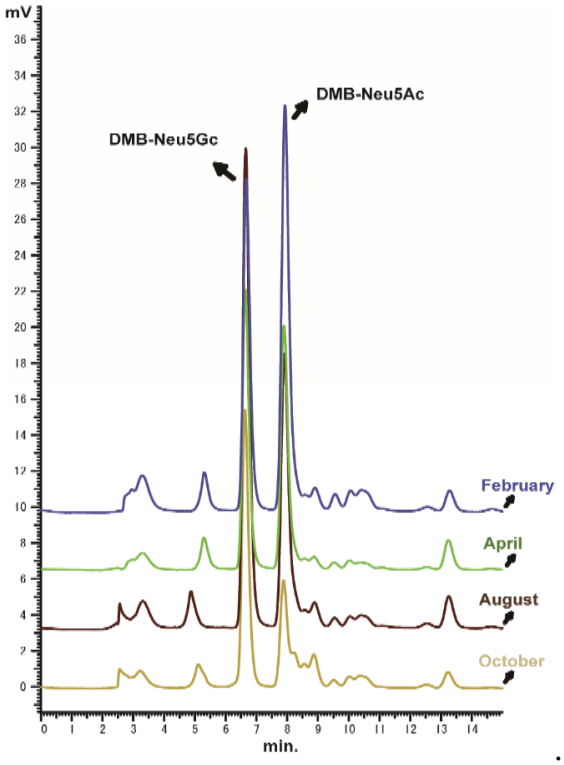
To assess the feasibility of simple biomarkers based on the quantitation of whole sialic acids released from serum glycoproteins, I established an easy and efficient protocol for the quantitation of Neu5Ac and Neu5Gc concentrations in Holstein cow serum samples by modifying a general glycoblotting platform (Figure 4.6).[45] This standardized protocol facilitated a quantitative analysis of Neu5Ac and Neu5Gc derived directly from total serum *N*-glycans captured by the common glycoblotting procedure. Thus, acid hydrolysis followed by the derivatization of whole sialic acids with DMB,[85] selectively afforded DMB−Neu5Ac and DMB−Neu5Gc without any side reaction in other monosaccharides because the reagent reacted specifically with the α-keto acid moieties of sialic acids (Figure 4.16A). Figure 4.16B shows typical HPLC profiles of DMB−Neu5Ac and DMB−Neu5Gc derived from the serum samples of Holstein dairy cows in four different months. The quantitation of DMB−Neu5Ac and DMB−Neu5Gc (represented as μM in serum, n = 5) was performed using calibration curves with replicate analysis (R^2 = 0.9503 for DMB−Neu5Ac and R^2 = 0.9783 for DMB−Neu5Gc; see also Figure 3.1 in materials and methods). The results obtained demonstrated that the Neu5Ac content in whole serum *N*-glycans was markedly higher in winter (February) than in the other seasons (April, August, and October), as shown in Figure 4.16C. On the other hand, serum *N*-glycans in August and October were dominantly terminated by the Neu5Gc residue, suggesting that serum Neu5Gc concentrations are a promising alternative biomarker to the *N*-glycans identified by the present large-scale glycomics study. The markedly higher expression ratio of the Neu5Gc against Neu5Ac in October than in August correlated with the lowest milk productivity (21.6 kg/day) of this month. The THI value of October (56.1) was identical to that of May, which had the highest milk yield (36.7 kg/day) in the year; therefore, the impact of heat/environmental stress in August (THI = 72.2) was beyond my conception and caused long-term damage and disorders in the homeostatic immune balance of dairy cows during summer

and autumn. The present results demonstrated that the Neu5Gc/ Neu5Ac ratio of whole serum glycoproteins was a highly sensitive and efficient biomarker for indicating the general performance and milk productivity of Holstein dairy cows. However, it is important to note that the present protocol covered the quantitation of sialic acids derived selectively from the *N*-glycans of whole serum glycoproteins (Figure 4.6). In addition, it seems likely that an HPLC-based assay is not suited for large-scale analysis needed in the case for serum glycomics study. The influence of sialic acids in free oligosaccharides, *O*-glycans, or glycosphingolipids in serum remains unclear; however, their serum expression levels appear to be very low.[86–88]

A.



B.


mV
DMB-Neu5Ac
DMB-Neu5Gc
February
April
August
October
min.

C.

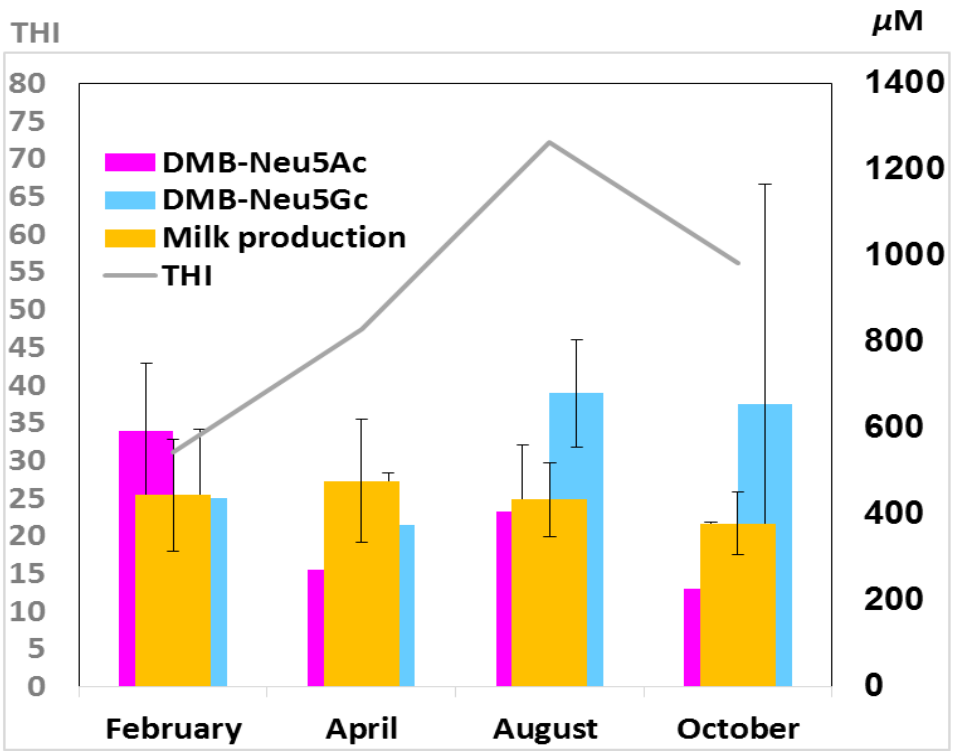

THI
μM
DMB-Neu5Ac
DMB-Neu5Gc
Milk production
THI
February
April
August
October

Figure 4.16. Quantitation of nonreducing terminal sialic acids of total serum glycoproteins. (A) Sialic acid-selective fluorescent labeling by means of DMB reagent. Nonreducing terminal sialic acids of *N*-glycans captured by the glycoblotting method were digested predominantly from BlotGlyco beads and allowed to react specifically with DMB reagent at the α-keto acid moiety to afford fluorescence-tagged sialic acid derivatives showing the characteristic HPLC profiles based on the structure at the C5 position of Neu5Ac, Neu5Gc, and KDN. (B) HPLC profiles monitored by fluorescence spectrometry of total DMB−Neu5Ac and DMB-Neu5Gc derived from serum glycoproteins captured by the glycoblotting method. In the present study, DMB−KDN was not detected, whereas avian glycomics revealed the existence of *N*-glycans containing this sialic acid residue in the egg white of Galloanserae. (C) Seasonal alterations in the THI value, milk production, and the expression levels of total sialic acids as DMB−Neu5Ac and DMB−Neu5Ac in the *N*-glycans of serum glycoproteins.

Because the conversion from CMP−Neu5Ac into CMP−Neu5Gc was conducted by cytidine monophosphate *N*-acetylneuraminic acid hydroxylase (CMAH), the long-term damage caused by heat/environmental stress appeared to influence the up-regulation of this enzyme and, thus, this step may be essential for the activation of immune responses in Holstein dairy cows in these months. Furthermore, Neu5Gc residues used for the biosynthesis of *N*-glycans may be taken up into intracellular recycling systems through the endocytic incorporation of glycoproteins and lysosomal sequestration, as described in the schematic carton Figure 4.17. However, the effects of heat stress on these recycling systems of Neu5Gc have not yet been examined in detail. Although there is currently no evidence to demonstrate that the enzymatic activity of such a pathway in human cells results in the loss of Neu5Gc due to genetic mutations, the unusual incorporation of Neu5Gc residues from the diet into some human glycoconjugates has been implicated in various human diseases such as cancer, atherosclerosis, diabetes, and heart attacks.[43,89]

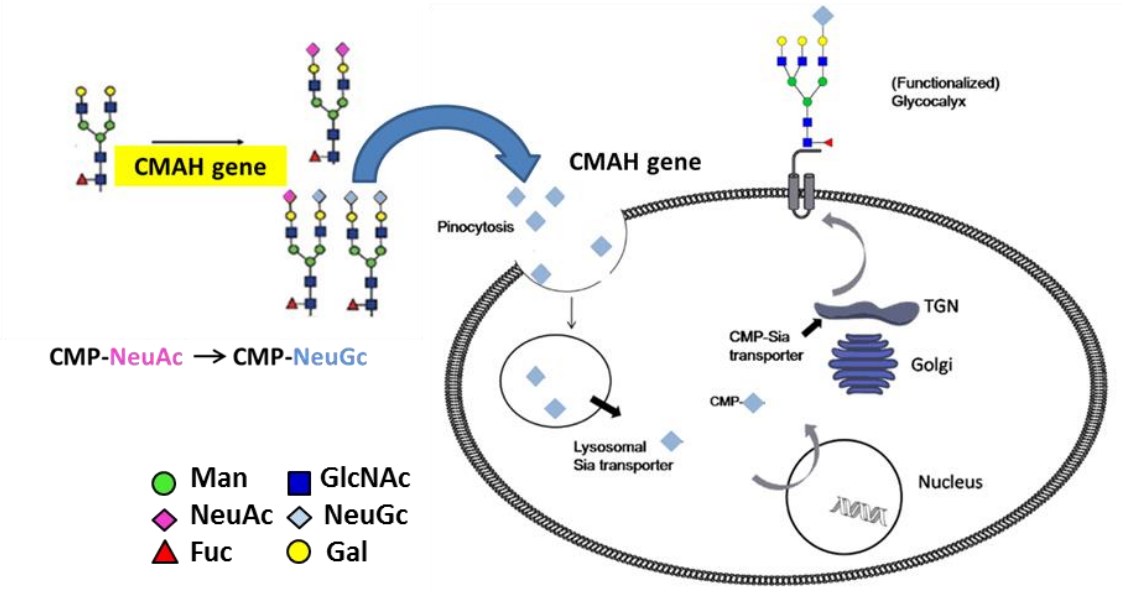


Figure 4.17. Schematic cartoon of the biosynthetic pathway for sialoglycoprotein (Neu5Gc) in Holstein cows.

As for the effects of diet of the cows on the milk production and glycosylation of serum proteins, I thought that: (a) Given the accumulating evidences in the previous studies done by our lab members on human and avian glycome that diet and medication as well as life styles influence significantly glycosylation profiles of serum glycoproteins and glycosphingolipids, it is not surprising that seasonal changes in the quality of diet can have influence serum glycan composition found in the present study. However, (b) It is important to note that the seasonal glycan alteration observed in the serum can not directly predict the milk productivity of the cows while this is strongly related to changes in the homeostatic immune balance caused both by heat stress and quality of the diet. (c) To explain molecular mechanisms explaining the present results, it is clear that further experiments are needed by focusing on the functional role of serum proteins modified with key glycan biomarkers revealed in this study.

Chapter 5

Summary

Because various stresses strongly influence the food productivity of livestock, biomarkers to indicate unmeasurable environmental stress in domestic farm animals are of increasing importance. Thermal comfort is one of the basic principles of dairy cow welfare that enhances productivity, so that the more attention needs to be placed on the principles of The Welfare Quality® Assessment protocol. THI value cannot be used as a feasible indicator to alert farmers to any alterations in an animal's metabolic/homeostatic immune balance perturbed by "complicated heat stress", whereas the THI may be an important parameter for simply showing the temperature and humidity of the feeding environment. Farmers have not been able to fully recognize the signs of cows being physically affected by slight heat stress unless their condition becomes critical. Moreover, traditional physiological approaches are also insufficient for elucidating a direct correlation between hormone secretions with adverse effects on animal well-being due to the stress adaptability of the animal; therefore, the best indicator for identifying animals under stress is the development of a prepathological state, that is, a stress-related change in biological functions with suppression of the immune system. Post-translational protein glycosylation is a basic principle for controlling the structures and functions of most glycoproteins in relation to cellular differentiation, adhesion, immunity, signal transduction, growth control, and even malignant alterations. These structural alterations in the post-translational glycosylation of major serum proteins may provide highly sensitive biomarkers to indicate changes in general metabolism and the homeostatic immune balance directly/indirectly induced by various diseases without any direct biopsy of the tissues/organs affected. According to some climatic data of Sapporo, Japan, recorded in 2012, heat stress commonly assessed by the THI was estimated to be maximal in August. These seasonal profiles for milk yield and its constituents may strongly depend on marked changes in the diet and subsequent metabolic effects on normal microbial protein synthesis. However, difficulties are associated with directly monitoring changes in the profiles of highly complex metabolites

and their effects on the intestinal microbiota. THI values may contribute to the development of a management system for short-term heat stress and environmental assessment guidelines to improve animal comfort. However, the relationship between long-term damage caused by the integration of many environmental stresses and the performance/milk productivity of lactating Holstein dairy cows currently remains unclear due to the lack of sensitive biomarkers to indicate alterations in the general metabolism and homeostatic immune balance of Holstein dairy cows. Thus, for the first time using glycomics technological techniques to determine the concentration and amount of dedicated animal serum proteins (dairy cows) that reveal the relationship between these proteins and combinations of climate change and seasonal productivity. Large-scale glycomics of dairy Holstein cow serum samples (n=336) collected for 9 months from the farm, Faculty of Agriculture, Hokkaido University, Japan (between February and October in 2012). Samples were analyzed using glycoblotting combined with MALDI-TOF/MS and DMB/HPLC to detect the dynamic changes in the expression levels and profiles of the *N*-glycan structures of serum glycoproteins as highly sensitive and efficient serum biomarkers to monitor the heat/environmental stresses influencing the metabolic/homeostatic immune balance, performance, and milk productivity of dairy cows. The analysis demonstrated a correlation between the levels of *N*-glycan and productivity, where there was major serum glycoproteins in summer and autumn mainly terminated with Neu5Gc residues, whereas glycoproteins in winter used Neu5Ac. This was caused by changes in the expression levels of enzymes and intracellular concentrations of various sugar nucleotides in relation to their biosynthetic pathways. The dynamic interconversion between Neu5Ac and Neu5Gc in serum *N*-glycans provides highly sensitive biomarkers due to the change in the functions of immune cells across the infectious diseases, inflammation, neurological diseases, and autoimmune diseases, and cancer through interactions with receptors of these sugars. Also, serum *N*-glycans profiles derived from bovine serum embryo (containing sugars Neu5Ac),

which differ markedly from serum samples of mature Holsteins cows which means an immune defense from mothers to fetuses through the placenta and blood circulation. However, vaccinating newly born calves specifically through the of Neu5Gc residues in colostrum to adapt to the dynamic changes associated with early environmental stresses in order to construct a beneficial intestinal microbiota and shape early immunity, because it does not have this kind of the protein bovine serum to transfer from mother to child or through the placenta. After the quantitative analysis of the dynamic in *N*-glycans in the serum of Holstein cows, Neu5Ac quantifications ratio is a promising alternative embryonic biomarker to the individual *N*-glycans of serum in cows, while in summer and autumn dominated the existence of the Neu5Gc residue to indicate the environmental stress resulting from climate change and the results showed that the serum ratio Neu5Gc/Neu5Ac indicated the heat/environmental stress and milk productivity/performance of Holstein dairy cows.

Chapter 6

Conclusion and Recommendation

In this thesis, I have discussed about the a glycoblotting-based systematic strategy that concurrently allowed for a large-scale serum *N*-glycome analysis and the quantitation of nonreducing terminal sialic acids was established in the present study. By applying the present protocol, I discovered highly sensitive serum *N*-glycan biomarkers indicating an environmental/heat stress affecting the general health condition, especially homeostatic immune balance, of Holstein dairy cows. The results obtained clearly showed the seasonal alterations in serum *N*-glycan expression levels and their structural characteristics. The *N*-glycans of major serum glycoproteins in summer and autumn mainly terminated with Neu5Gc residues, whereas glycoproteins in winter used Neu5Ac as the main sialic acid instead of Neu5Gc residues. A multivariate analysis revealed a correlation between productivity and the expression levels of season-specific glycoforms. Dynamic alterations in modifications to the nonreducing terminals of di/triantennary *N*-glycans provide highly sensitive serum biomarkers indicating an environmental stress/thermal comfort in dairy cows that affects their immune responses, performance, and milk productivity. Consequently, serum Neu5Gc levels are a promising alternative biomarker to the individual *N*-glycans. The merit of this biomarker is evident because the serum ratio Neu5Gc/Neu5Ac correlated well with the performance and milk productivity of Holstein dairy cows, whereas the THI value was not capable of predicting such long-term damage by a heat/environmental stress in October (THI = 56), which ultimately resulted in the lowest milk yield (21.6 kg). By applying DMB-HPLC analysis, I can quantify the nonreducing terminal sialic acids, in which the significance of the interconversion between Neu5Ac and Neu5Gc of serum glycoproteins in the regulatory mechanism of immune responses may be determined in terms of the interactions between glycoform-engineered IgG and Fcγ/FcRn receptors or key animal lectins/PRRs on dendritic cells/macrophages.

Herein, I have confirmed the accuracy and effectiveness of using glycoblotting for a large-scale serum *N*-glycome analysis and DMB-HPLC analysis in order to quantify of non-reducing terminal sialic acids. Moreover, the serum ratio Neu5Gc/Neu5Ac indicated the heat/environmental stress and milk productivity/performance of Holstein dairy cows. However, Neu5Ac quantifications ratio is a promising alternative embryonic biomarker to the individual *N*-glycans of serum in cows.

Finally, I strongly recommend that glycomics profiling is one of the non-invasive approaches to investigate the immune balance and productivities in livestock (e.g., Holstein cow) management under integrated heat/environmental stresses.

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