Machine Vision Diagnosis of Eyes for Vitamin A Conditions in Japanese Black Cattle

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Chapter 1

Introduction

1.1. Motivation

1.1.1. Vitamin A and beef quality

Beef is the third most widely consumed meat in the world following pork and poultry. In Japan, the most desired beef is genetically predisposed to intense marbling—intramuscular fat and to produce a high percentage of oleaginous unsaturated fat. The amount of marbling is the most influential factor to determine the beef quality grade in the USA, Japan and Australia (Strong 2004). Usually, the beef with higher marbling becomes more tender and juicy than beef with lower marbling after cooking. In Japan, Beef Marbling Standard (BMS) is established by Japanese Meat Grading Association (1988). Silicone resin models used for the evaluation of beef marbling are shown in Fig. 1.1. There are 12 models and the NO. 12 model represents the highest marbling standard.

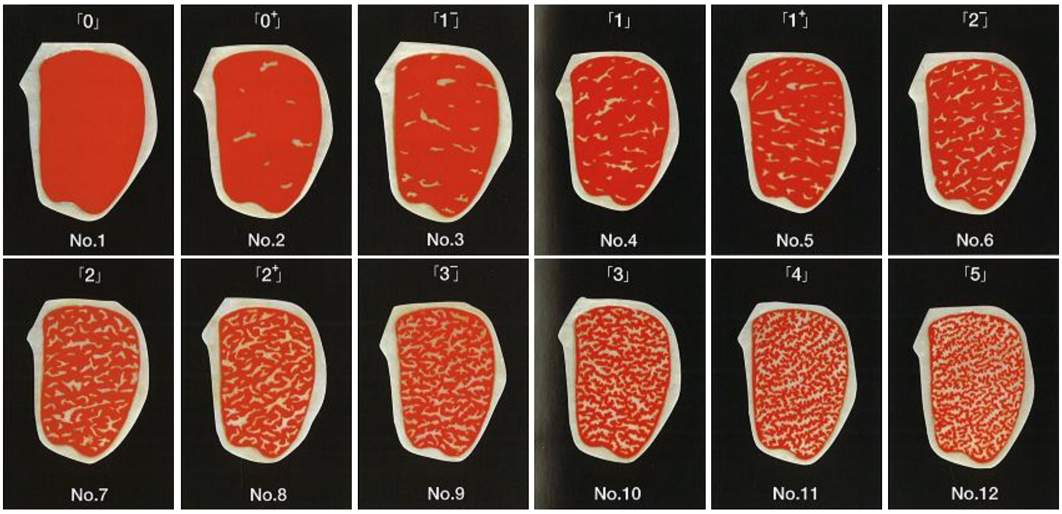

Figure 1.1 Silicone resin models used for the evaluation of beef marbling developed by the Japanese National Institute of Animal Industry (JMGA 1988).

Some researches have been conducted on the relation between vitamin A and beef marbling. Because retinoic acid, a derivative of vitamin A, regulates cell differentiation and proliferation, and prevents the process of adipogenic differentiation (Kuri Harcuch 1982; Pairault *et al.*, 1988), it is possible that vitamin A influences the differentiation of adipocytes and hence marbling. Oka *et al.* (1998) found that the mean marbling score of the low vitamin A group was significantly higher than that of the group supplemented with high vitamin A. Similarly, Adachi *et al.* (1999) investigated the biochemical parameters related to marbling. They found that high marbling scored group showed significant lower level in serum vitamin A at 10 to 14 months old than low marbling scored group. Not only in Japan, another research conducted in United States stated that regardless of the corn type used, feeding low vitamin A diets for 145 day to Angus-cross steers increase marbling and quality grade without affecting yield grade, animal health, or performance (Gorocica-Buenfil *et al.*, 2007). In Austrilia, Kruk *et al.* (2008) concluded that the reduced vitamin A consumption, leading to increases intramuscular fat. Siebert *et al.* (2006) found that feeding vitamin A was associated with less intramuscular fat. All these researches confirmed that manipulating vitamin A at a lower level during fattening stage is effective to increase beef marbling.

Currently, most Japanese black cattle farmers actively manipulate the vitamin A levels in fattening cattle to get beef with higher beef marbling standard. Ideal serum vitamin A level in cattle during the fattening stage in Hyogo Prefectural Hokubu Agricultural Institute is shown in Fig. 1.2. However, it is not easy to maintain the minimum desired serum vitamin A level (30 IU/dL). Vitamin A deficiency may increase disease susceptibility, such as night blindness, xerophthalmia, diarrhea, or even death (Moore 1941; O'donoghue 1955; Millemann *et al.*, 2007; Issi *et al.*, 2010). Yano *et al.* (2009) evaluated the relationship between the serum vitamin A level and immune function in seventy non-diseased Japanese black cattle during their fattening stages. The result showed that immune function imbalance was present in Japanese black cattle with low serum vitamin A levels during the fattening stage. Thus, monitoring the vitamin A levels in Japanese black cattle during their vitamin A manipulated period is extremely important. Blood assay is the conventional way to measure serum vitamin A levels. However, it is expensive (about 2000 Japanese yen per measurement) and cannot be afforded by most farmers for frequent measurements. Besides that, it takes about two weeks to get the result of measurements. Thus, an alternative system which can indicate the vitamin A status of cattle would contribute to feeding management during vitamin A manipulated period. Early clinical signs of vitamin A deficiency (VAD) usually related to some ocular changes like papilledema, mottling of the tapetum, pallor of the nontapetum, and xerophthalmia (Maggs *et al.*, 2008). The ocular changes may be utilized to diagnosis the vitamin A deficient status.

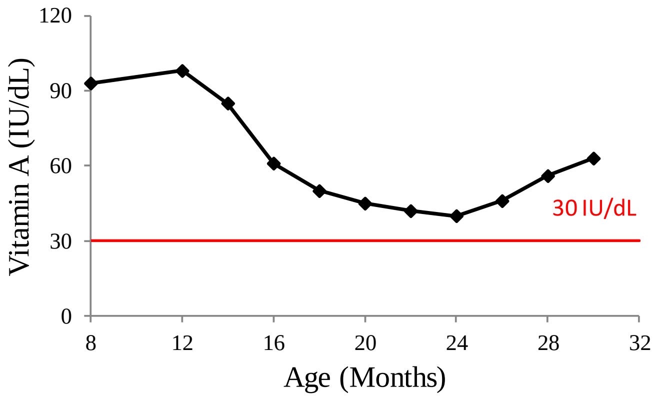

Figure 1.2 Ideal serum vitamin A level in cattle during the fattening stage. The red line indicates the minimum desired level (30 IU/dL) of vitamin A in Japanese black cattle. For vitamin A, 1 IU is the biological equivalent of 0.3 μg retinol, or of 0.6 μg β-carotene. (Provided by Hyogo Prefectural Hokubu Agricultural Institute).

1.1.2. Previous Studies

To estimate serum vitamin A levels based on the reflectance of tapetum, many efforts have been made in our research group.

To determine the optimum wavelength for measuring pupil reflection, a multispectral camera was used in capturing slaughtered and live cattle eye images. A linear relationship between pupil reflection and serum vitamin A level was shown, the highest coefficient of determination (0.76) occurring at 500 nm in slaughtered cattle. The result is shown in Fig. 1.3.

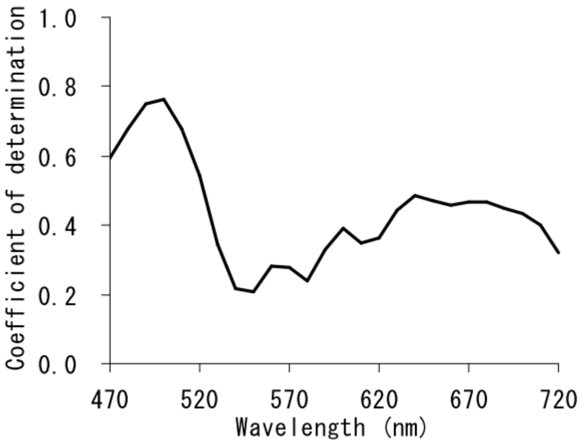

Figure 1.3 Coefficient of determination between pupil reflection and serum vitamin A level in slaughtered cattle at different wavelengths (Takahashi *et al.*, 2010).

However, for live cattle, no significant correlation between pupil reflection and serum vitamin A level was observed at the 500 nm wavelength (Takahashi *et al.*, 2010).

For the visual pigment rhodopsin located in the retina has absorption peaks at about 500 nm (Bownds 1967) and would shift from 500 nm to 380 nm after exposuring to light (Sakamoto *et al.*, 1995). Because 380 nm belongs to ultraviolet (UV) region, Takahashi *et al.* (2011) investigated the relationship between serum vitamin A levels and the tapetum reflection using UV imaging. Negative relationships were found between serum vitamin A level and reflection of tapetum lucidum, tapetum nigrum, and the optic disc. These results confirmed Moore's observation in vitamin A deficient cattle that bleaching of tapetum lucidum, whitely mottling on the tapetum nigrum, and pinker and paler optic disc were presented (Moore 1941).

UV imaging was also used to investigate the relationship between the pupil reflection and serum vitamin A level in live cattle. However, a positive correlation between the pupil reflection and serum vitamin A level was acquired, which was opposite from the result of tapetum. Yoshida *et al.* (2010) concluded that UV light could not reach the tapetum in live cattle and it was difficult to get the tapetum information in pupil images using UV light.

Because of the unsatisfied result of multispectral and UV imaging, the pupil images in the visible region would be investigated in this study.

1.1.3. Computer aided diagnosis system

Computer-aided diagnoses are procedures in medicine that assist physicians in the interpretation of medical images. It combines artificial intelligence and digital image processing. It has been applied in the early detection of lung nodules in thoracic computed tomography (Armato Iii *et al.*, 2004), microaneurysm and glaucoma in color fundus images (Walter *et al.*, 2007; Bock *et al.*, 2010), breast cancer in ultrasound images (Cheng *et al.*, 2010) and referable diabetic retinopathy in retinal images (Abràmoff *et al.*, 2013). Computer aided diagnosis system holds the potential to detect of VAD related ocular changes in the early stage.

1.2. Research Objective

The overall objective of this research is to develop a machine vision diagnosis system of eyes or a computer-aided diagnosis (CAD) system that can help farmers make the correct diagnosis of vitamin A deficient levels of Japanese black cattle during their vitamin A manipulated period. This system includes hardware and software. The hardware is a customized machine vision system that can capture high quality eye images and record pupillary light reflex videos, which would be used as the input of the software. The software should include image processing algorithms and classification algorithm. The image processing algorithm is supposed to extract many ocular features of VAD, which can be used for the final classification. The classification algorithm should be able to classify cattle into different VAD levels, like mild, moderate or severe.

To achieve the final goal, three studies were included in this thesis:

(1) to measure the color changes of pupil area in eye images (pupil color) during the vitamin A manipulated period and to build an estimation model of serum vitamin A based on the color changes.

(2) to develop a robust image segmentation algorithm to calculate pupil area for pupillary light reflex analysis and to investigate the feasibility of using one second PLR analysis to identify vitamin A deficient cattle.

(3) to determine VAD levels based on the ocular changes caused by VAD in Japanese black cattle.

1.3. Thesis Structure

The thesis structure can be shown in Fig. 1.4.

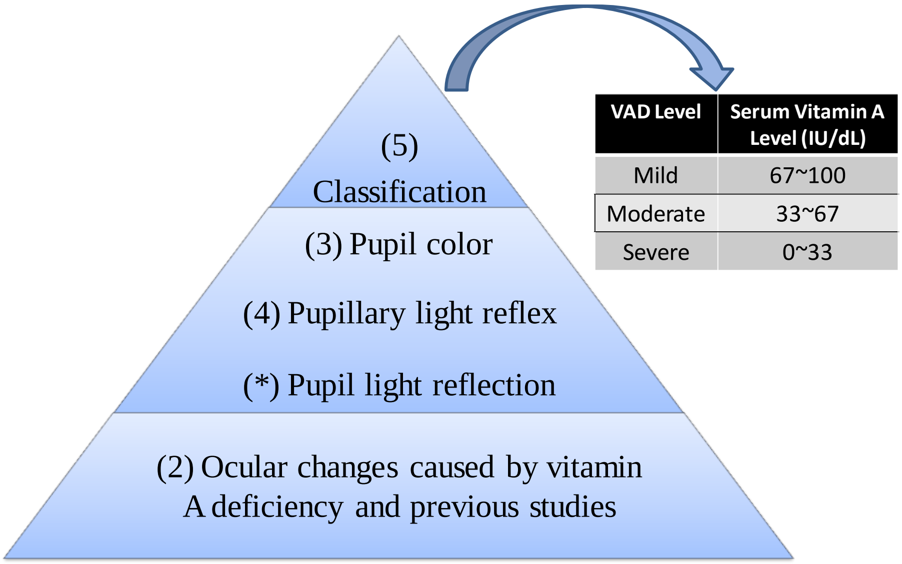

Figure 1.4 Thesis structure. The number in parenthesis indicates the chapter number. * means this research was conducted by our group members.

Chapter 2 briefly summarized the ocular changes caused by VAD reported in literature and some sample images of eye diseases were shown for a better understanding of ocular changes. Besides that, what we learned from previous investigation was introduced and the optical properties of lens and vitreous humor were shown.

Based on the knowledge of Chapter 2, the relation between serum vitamin A level and three aspects (pupil color, pupillary light reflex and light reflection) were investigated individually. These three aspects were selected because they were easy to be extracted from the images acquired by a customized machine vision system.

In Chapter 3, we studied color changes of pupil area in Red, Green, Blue (RGB) and Hue, Saturation, Intensity (HSI) color models from July to November in 2010 and from May to December in 2011 to estimate the serum vitamin A level of Japanese black cattle during their vitamin A controlled stage. A serum vitamin A estimation model based on color changes was achieved with an estimation error of 10 IU/dL.

Chapter 4 described an image processing algorithm that was used to segment pupil from background. The algorithm was proved to be effective in pupillary light reflex analysis. Two parameters, constriction amplitude and initial pupil roundness were introduced and used to evaluate pupillary light reflex changes. Based on the data of 43 cattle in 9 months, the potential and limitation of pupillay light reflex analysis were discussed.

In Chapter 5, five parameters extracted from pupil color, pupillary light reflex and light reflection analysis, were used for classification. The performance of two multivariate classification methods, soft independent modelling of class analogies (SIMCA) and partial least squares discriminant analysis (PLS-DA) were evaluated. Finally, a vitamin A deficient index (VADI) was proposed.

Chapter 6 summarized the main contributions of the works, and explained current limitations and future works.

1.4. Research Contributions

The major contributions of the author in this thesis are as follows:

- A negative correlation between the red component ratio of cattle pupil images and serum vitamin A levels, a positive correlation between the saturation value of fattening cattle pupil images and serum vitamin A levels were found in 2010, and confirmed in 2011.
- A serum vitamin A levels estimation model based on color changes in pupil area was built.
- An image segmentation method to calculate pupil area of cattle was proposed.
- The proposed pupillary light reflex analysis method was proved to able to detect fattening cattle with weak pupillary light reflex.
- Cattle were found to possess smaller resting pupils ($p<0.05$) in a dark environment during vitamin A deficient stage.
- A VAD index (VADI) was proposed and proved to be effective in representing VAD status of cattle.

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Chapter 2

Literature Review and Anatomy Investigation

Abstract

Ocular changes caused by VAD including papilledema, bleached tapetum lucidum, mottled taptetum nigrum, atrophy of retina, xerophthalmia, dilated pupil, beautiful blindness, loss of pupillary light reflex and exophthalmos were briefly summarized in this chapter. To estimate serum vitamin A levels from reflection of tapetum, multispectral and ultraviolet (UV) imaging was used in previous studies. However, the result was not satisfied by using UV imaging, because most UV light was absorbed by lens. In anatomy investigation, transmittance of lens and vitreous humor were measured.

2.1. Vitamin A and its function

Vitamin A is a group of fat-soluble retinoids that includes retinol, retinal, retinoic acid, and several provitamin A carotenoids. Vitamin A is important for growth and development, for the maintenance of the immune system and good vision (Tanumihardjo 2011). Vitamin A is needed by the retina of the eye in the form of retinal, which combines with protein opsin to form rhodopsin the light-absorbing molecule (Wolf 2001), that is necessary for both low-light (scotopic vision) and color vision. The structure of all-*trans* retinol is shown in Fig. 2.1.

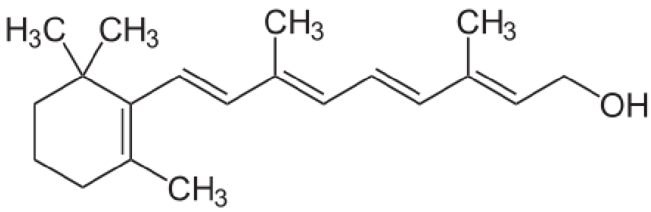


Figure 2.1 Structure of all-*trans* retinol (Tanumihardjo 2011).

2.2. Ocular Changes Caused by Vitamin A Deficiency

Deprivation of the vitamin A produces effects largely attributable to disturbance of dim-light vision, normal bone growth and maintenance of normal epithelial tissues. The major pathophysiological effects of VAD include night vision, cerebrospinal fluid pressure (CSF), bone growth, epithelial tissue, embryological development and immune mechanisms. Cattle eye diagram is shown in Fig. 2.2. Ocular changes caused by VAD are summarized below.

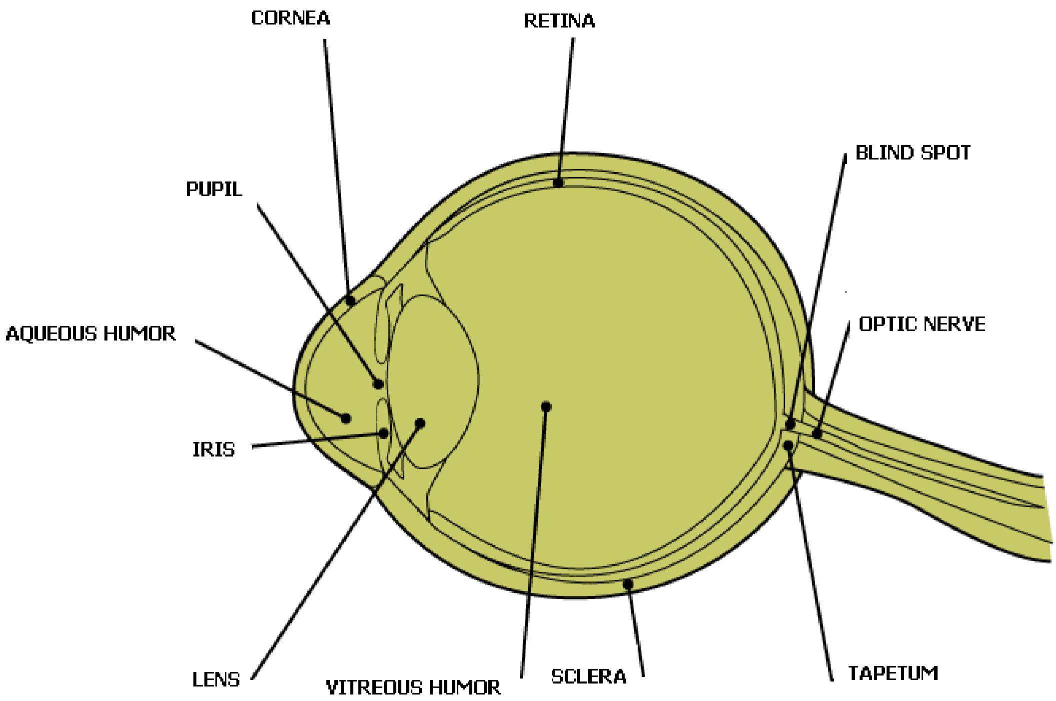
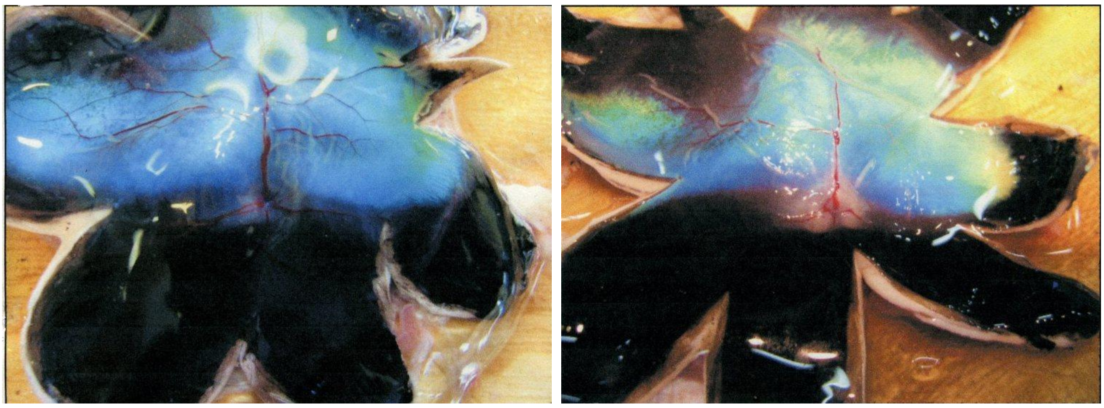


Figure 2.2 Cattle eye diagram.

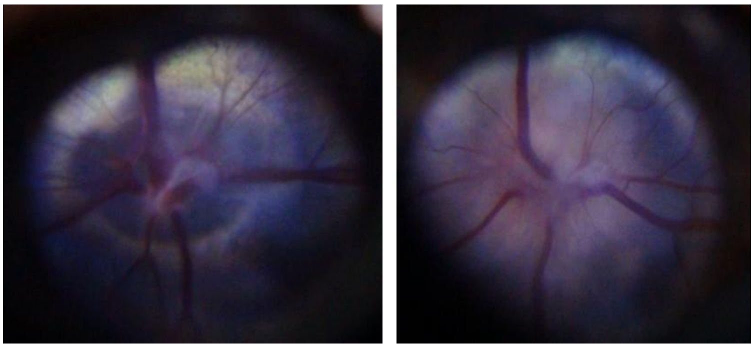
2.2.1. Optic nerve

Optic nerve is the bundle of nerve fibers that carries information from the retina to the brain as shown in Fig. 2.2. It was believed that papilledema ("choked disc"), edema of optic nerve, is the first objective sign of optic disc changes caused by VAD and it is reversible in the early stage (Barnett et al., 1970; He et al., 2012). If treatments of vitamin A are not given, papilledema worsens, the optic disc is swollen, elevates above the surrounding retina and becomes pink and pale, as shown in Fig. 2.3. Ophthalmoscopic examination shows the disc has a "watery pink" appearance and indistinct edges, as shown in Fig. 2.4.



(a) (b)

Figure 2.3 Cut eyeball of normal cattle (a) and the eyeball from cattle suffered from VAD (b).



(a) (b)

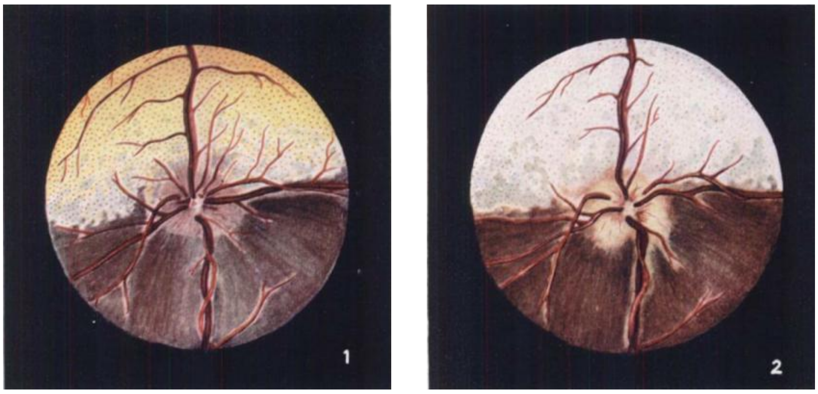
Figure 2.4 Normal optic disc (a) and optic disc showed papilledema (b).

The constriction of optic nerve is associated with a narrowing of optic canal (Vandonkersgoed et al., 1988) in calves. Although papilledema does not cause primary loss of vision, chronic papilledema may lead to progressive visual deficits due to optic nerve atrophy. Mature cattle develop papilledema on vitamin A deficient rations due to malabsorption of cerebrospinal fluid but never show the permanent type of blindness. The reason they do not acquire the permanent type of blindness is because the bony canal has fully developed and calcified and therefore no abnormal growth anomaly results (Moore 1939).

2.2.2. Tapetum

The tapetum is a reflective layer located in the choroid, as shown in Fig. 2.2. It acts as a mirror that reflects the light back toward the photoreceptor layer, thus increasing the probability that the light will be absorbed by the photopigment and contribute to visual sensation in dim light. The photon that is not absorbed by rods and cones is reflected in the tapetal fundus, thus the photon has the second chance to be absorbed by rods and cones which would increase the sensitivity to low levels of light. In the nontapetal fundus, the photon would not have the second chance to be absorbed and wasted.

Moore (1939) investigated ocular changes of calves when feeding them with a low carotene grain ration. The tapetum lucidum became bleached and clinical signs of papilledema were shown after a low carotene grain ration, as shown in Fig. 2.5. Finally, he concluded that under proper conditions of observation, bleaching of the tapetum lucidum of the retina was a good guide for the diagnosis of VAD in the bovine.



(a) (b)

Figure 2.5 Fundus images of a healthy calf (a) and a calf suffered from VAD (b) (Moore 1939).

In 1941, Moore investigated the ocular changes in mature cows fed a ration deficient in vitamin A. The tapetum nigrum and lucidum developed a mottled appearance. Usually the changes were more easily observed and more marked in the nigrum than in the lucidum as shown in Fig. 2.6. Another two mottled tapetum nigrum example caused by vitmamin A deficiency were given by Vandonkersgoed *et al.* (1988) and Gelatt (2001).

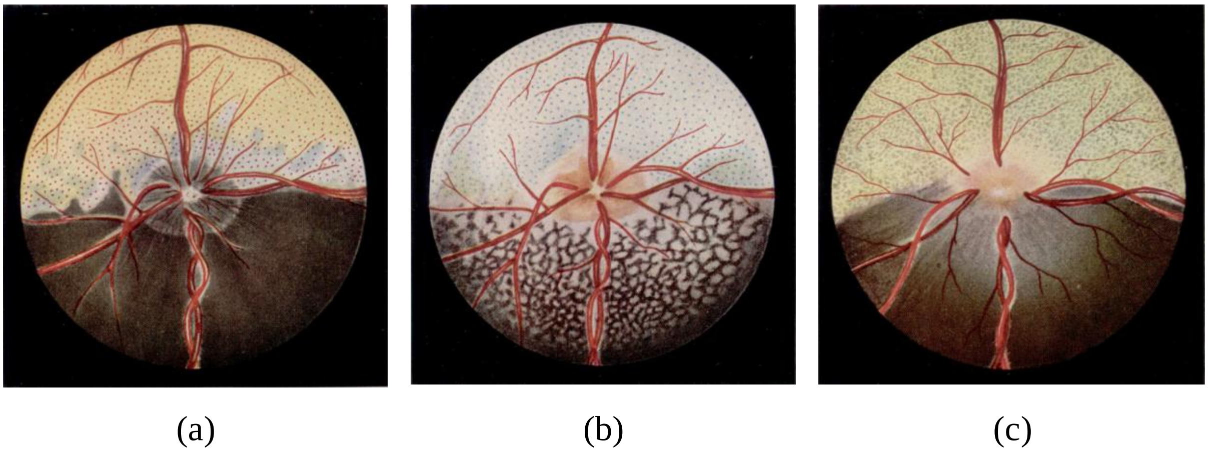


Figure 2.6 (a) Normal bovine fundus, (b) Fundus showing papilledema, bleached tapetum lucidum and a mottled tapetum nigrum, (c) Fundus showing papilledema and a mottled tapetum lucidum (Moore 1941).

2.2.3. Retina

Vitamin A deficiency can cause the atrophy of the photoreceptor layer of the retina. If the tapetum and transparent retina are compared to a mirror (tapetum) and curtain (retina), when the curtain is removed (retinal atrophy), reflectivity is increased, as shown in Fig. 2.7 (Maggs *et al.*, 2008). The increased reflectivity is visible ophthalmoscopically (Maggs *et al.*, 2008).

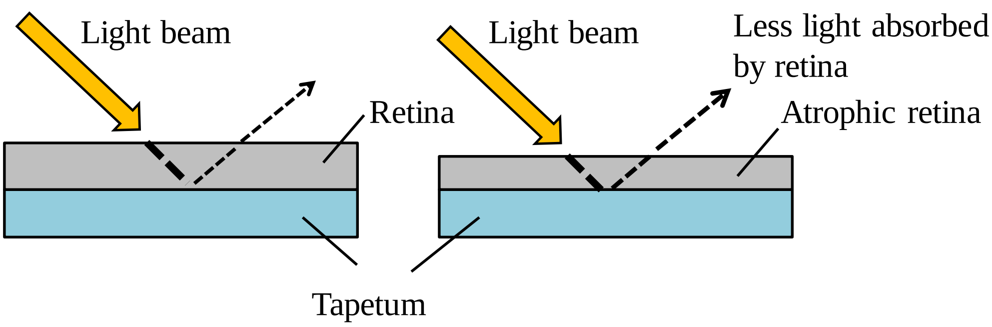


Figure 2.7 Pathogenesis of increased tapetal reflectivity in cases of retinal atrophy. A thinner (atrophied) retina absorbs less light; hence more light reaches the tapetum and is reflected toward the observer.

2.2.4. Cornea

Xerophthalmia (dry eye) is a medical condition in which eyes fails to produce tears. It may be caused by VAD. If untreated, the cornea would become rough and finally damaged which result in blindness. However, xerophthalmia are not always present in cattle experiments, especially when animals were brought to the clinic at an early stage (Issi *et al.,* 2010). Another reason for the few occurrences of xerophthalmia might be the eyes of the animals during the deficiency period were probably not subjected to the presence of large amounts of abrasive dust particles and possibly the proper type of bacteria (Moore 1941).

2.2.5. Pupil and pupillary light reflex

Dilated pupil, "beautiful blindness" and loss of react to light stimulation were believed to be another clinical sign of VAD (Moore 1939; Vandonkersgoed *et al.,* 1988; Mason *et al.,* 2003; Millemann *et al.,* 2007; Issi *et al.,* 2010; He *et al.,* 2012). As Fig. 2.8 shows, the affected cattle showed dilated pupil in day time and loss of pupillary light reflex. The fattening cattle were fed on a ration free of vitamin A for 9 months. After 6 months of low vitamin A diets, serum vitamin A levels of this cattle arrived a minimum desired level 30 IU/dL, to prevent serious disease occurred, oral administrations was given, however, the serum vitamin A level was not increased as expected. In the 7th month of low vitamin A diets, edema of optic disk was found by ophthalmoscopic examination. In the 8th month of low vitamin A diets, papilledema was found. Finally, in the 9th month, loss of pupillary and palpepral light reflex, dilated pupil and beautiful blindness were shown as same as the clinical signs described by other reports (Moore 1939; Vandonkersgoed *et al.,* 1988; Mason *et al.,* 2003; Millemann *et al.,* 2007; Issi *et al.,* 2010; He *et al.,* 2012).

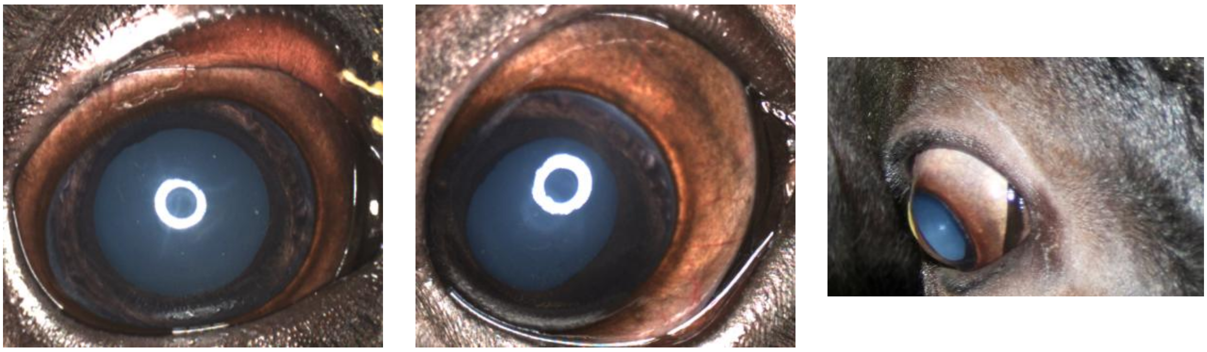
(a)

(b)

Figure 2.8 Normal face image of a steer, notice the horizontal and oval shape of resting pupil in day time (a), face image of a steer suffered from VAD, notice the dilated pupil and beautiful color of the pupil area (b).

2.2.6. Eyeball

Exophthalmos is a bulging of the eye anteriorly out of the orbit. It has been found in many VAD cases (O'Donghue 1955; Vandonkersgoed *et al.*, 1988; Millemann *et al.*, 2007; Issi *et al.*, 2010). An example is shown in Fig. 2.9. The eye of this steer slightly bulged, in the 7th month of low vitamin A diets. Edema of optic disk was found in the 8th month of low vitamin A diets. The exophthalmos became obvious and got noticed after 9 months of low vitamin A diets.



(a) (b) (c)

Figure 2.9 Eye image of a steers in 3rd (a) and 9th (b) months of low vitamin A diets, and a front image in 9th month shows the exophthalmos (c).

Even though many ocular changes caused by VAD have been summarized, the identification of vitamin A deficient cattle based on these features is still difficult, because the reproduction of these symptoms is not easy. First, cattle have different tolerance of VAD. Second, how soon clinical signs appear on low vitamin A diets will depend the extent of liver stores of vitamin A (Vandonkersgoed *et al.*, 1988).

2.3. Optical Properties of Lens and Vitreous Humor

When taking an image of the pupil, the light goes through lens and vitreous humor, arrives in the retina, and part of light would be absorbed by rods and cones in the retina. The left light is reflected by the tapetum and has a second chance to be absorbed by rods and cones, as shown in Fig. 2.10. Then the reflected light passes through the vitreous humor and lens again. To understand the difference between the images of tapetum and pupil, it is important to understand the optical property of lens and vitreous humor.

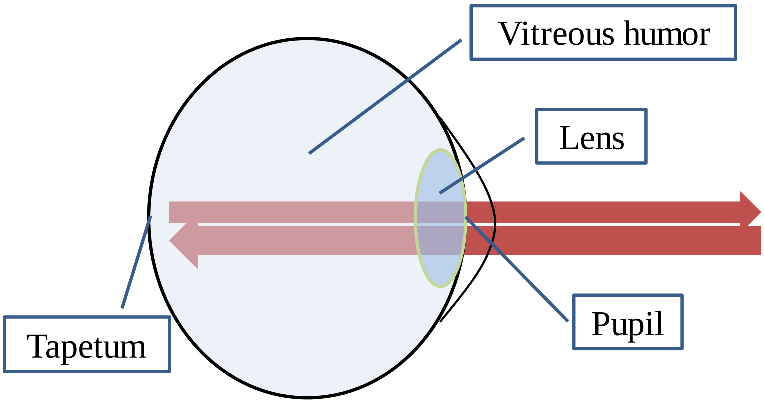


Figure 2.10 Light path when taking a pupil image.

The experiment was conducted at Hyogo Prefecture Meat Inspection Center (Kakogawa, Japan). Lens were dissected from eyeballs and installed in a holder and compressed to 5 mm thickness to reduce the refraction of light caused by lens when measuring transmittance by using UV2500 Spectrometer (Shimadzu, Japan). The lens holder is shown in Fig. 2.11. Vitreous humor was filled into a cell to measure the transmittance.

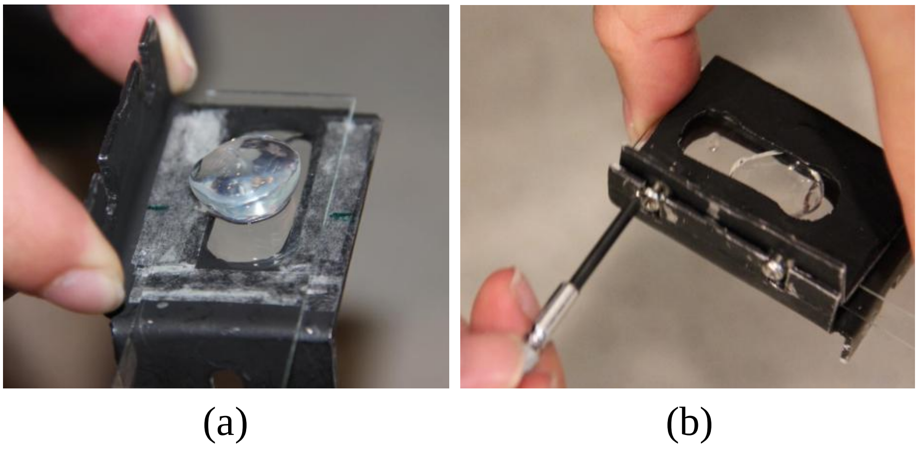


Figure 2.11 Lens on the holder (a), compressed lens with the thickness of 5 mm for transmittance measurement (b).

The result is shown in Fig. 2.12. The transmittance of lens dropped abruptly below 400 nm, which indicated that most UV light was absorbed by the lens. This result is consistent with Yoshida *et al.*'s conclusion (2010). Vitreous humor has a high transmittance over the measuring region, which can be regarded as transparent.

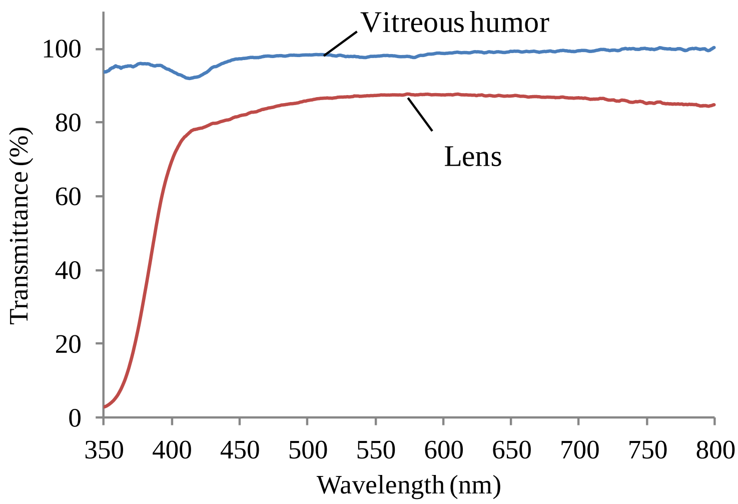


Figure 2.12 Transmittance of lens and vitreous humor in a steer.

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Chapter 3

Estimation of Serum Vitamin A Level by Color Change of Pupil in Japanese Black Cattle

Abstract

Color change of pupil area was investigated in Red, Green, Blue (RGB) and Hue, Saturation, Intensity (HSI) color models from July to November in 2010 and from May to December in 2011 to estimate the serum vitamin A level of Japanese black fattening cattle during their vitamin A manipulated stage. A 2CCD camera was used to acquire the eye images. The results showed cattle with lower serum vitamin A level usually accompanied with higher red component value, lower saturation in their eye images. An estimation model was built based on red and green component ratio. The estimation error is about 10 IU/dL. The reasonable result shows the feasibility to estimate the serum vitamin A level by color change of pupil area in Japanese black cattle.

3.1. Introduction

To meet the demand in the Japanese market for beef with high marbling scores, Japanese black cattle farmers, generally, control the cattle's vitamin A level. Studies have shown that low levels of vitamin A in cattle can result in a high degree of marbling (Oka *et al.*, 1998; Adachi *et al.*, 1999; Gorocica-Buenfil *et al.*, 2007). Recently, Japanese farmers have begun to active manipulate their cattle's serum vitamin A level (30-40 IU/dL) during the middle fattening stage: 16 to 24 months (see Fig. 1.2). Thus, it is important for farmers to monitor the vitamin A level during this middle fattening stage.

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In addition, maintaining a minimum vitamin A level is critical, since a VAD below this level increases disease susceptibility, such as nyctalopia, xerophthalmia, papilledema, or diarrhea (Moore 1941; O'Donghue 1955; Divers *et al.*, 1986; Mason *et al.*, 2003). The conventional way of measuring serum vitamin A levels in cattle is by blood assay. This test is invasive and stressful to the cattle. Besides that, complicated procedures and expensive devices, such as high-performance liquid chromatography (HPLC) system are needed. Thus, an alternative that can estimate vitamin A levels quickly and non-invasively is desirable.

Low vitamin A levels in cattle are known to cause retinal degeneration that can lead to increased tapetal reflection and loss of pigmentation of the nontapetal fundus (Moore 1941; Vandonkersgoed and Clark 1988; Gelatt 2001). As VAD progresses, the disease papilledema can occur, where the optic discs enlarge and become pink and pale. Maggs *et al.*, (2008) showed that mottling of the tapetum and pallor of the nontapetum also appear with VAD.

Our laboratory (Takahashi *et al.*, 2011) found a negative relationship between vitamin A levels and the reflection of tapetum lucidum, tapetum nigrum and the optic disc using ultraviolet imaging, suggesting hyper-reflectivity in cattle pupils at lower vitamin A levels. To our knowledge, this is the first research to estimate serum vitamin A levels using color change of pupil area. The aim of the current study is to (1) measure changes in pupil color, and then (2) estimate the serum vitamin A levels in Japanese black cattle using the relationship with pupil color during the middle fattening stage.

3.2. Materials

This experiment was conducted from July to November in 2010 and from May to December in 2011 with two different groups of fattening cattle at Hyogo Prefectural Hokubu Agricultural Institute, Japan.

In 2010, images of the left eye of 42 Japanese black cattle were taken at 6 different times. The fattening cattle were made up of 22 Yoshihisa-Yoshinaka-Shigeyasu, 15 Nobufu-Teruharu, and 5 locally bred cattle. The measurements were conducted on July 23rd, September 15th, October 14th, October 27th, November 10th and November 24th. The cattle’s ages ranged from 15–18 months and at the beginning of the experiment serum vitamin A levels ranged from 17 to 90 IU/dL (mean ± SD 46 ± 20 IU/dL).

In 2011, images of the right eye of 42 Japanese black fattening cattle were taken once a month. The cattle were made up of 6 inbred lines (Table 3.2) and divided into two groups by date of arrival. Group A consisted of 24 cattle: “Hiroiwadoi”, “Yoshigakidoi” and “Hiromi-Yoshigaki” inbred lines. They arrived at the feedlot in December 2010, their ages ranged from 14–16 months and at the beginning of the experiment their serum vitamin A levels ranged from 35 to 80 IU/dL (mean ± SD 59 ± 11 IU/dL). Group B consisted of 18 cattle: “Miyagikujyo”, “Kikuyu-Hiromasa -Miyagikujyo” and “Kikuyu- Hiromasa” inbred lines. They arrived at the feedlot in February 2011. Their ages ranged from 12–14 months, and at the beginning of the experiment their serum vitamin A levels ranged from 35 to 80 IU/dL. From the beginning of April 2011, the cattle were subjected to low vitamin A diets. All the cattle used in this study were clinically healthy during the experimental period.

3.3. Devices and Methods

3.3.1. Experimental devices

A 2CCD multi-spectral camera AD-080CL (JAI) was used to acquire both color and Near Infrared (NIR) images. The camera’s spectral sensitivity is shown in Fig. 3.1. Light entering the lens was separated into two light beams by a beam splitter; one was focused on a color image sensor and the other was focused on a monochrome image sensor. The color image sensor was a single-chip image sensor with Bayer filters. The monochrome image sensor was equipped with a filter to capture a NIR image in the wavelength range 750–900nm. The image was 1024 pixels in width and 768 pixels in height. The lens was a TF15D-8 (Focus Length 15 mm, F 2.2, FUJINON) in 2010 and NT63-240 (Focus Length 12 mm, F 1.8, EDMUND) in 2011. The camera was combined with two ring-shaped light-emitting diode (LED) lights; a MDRL-CW50 (MORITEX) white LED light and a MDRL-CIR31 (MORITEX) NIR LED light which has a central wavelength of 850 nm. Two polarizing (PL) filters were installed to reduce the specular reflection from the surface of the eye. One of them was installed in front of the LED, the other in front of the camera, perpendicular to the former PL filter. A plastic tube was installed in front of the LED lights to exclude ambient light. The sketch of the device is shown in Fig. 3.2. Fig. 3.3 shows the appearance of the device.

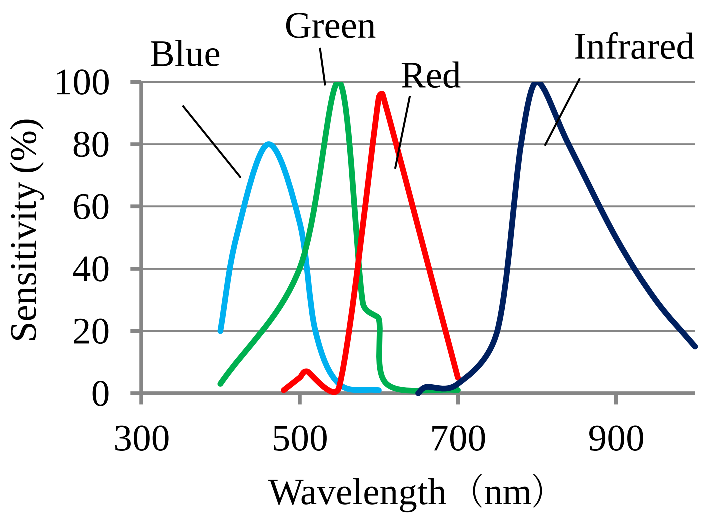

Figure 3.1 Total spectral sensitivity of camera including prism and sensor.

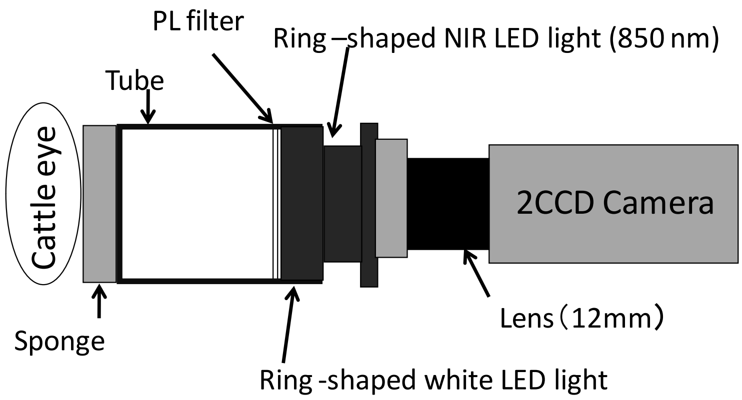

Figure 3.2 Sketch of experimental device.

Figure 3.3 Appearance of experimental device.

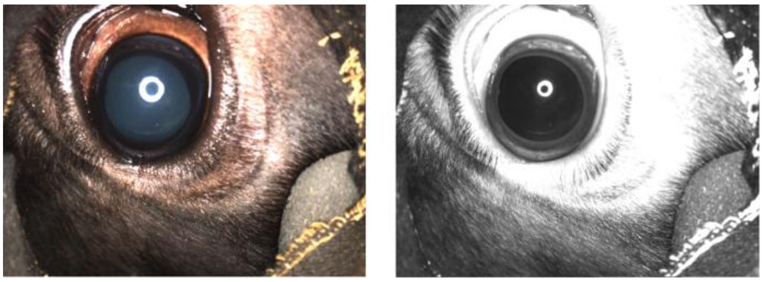

Figure 3.4 Color and NIR images of cattle's right eye captured without PL filter.

3.3.2. Method

Color calibration was performed prior to each experiment to ensure high quality images and minimize operation error. System setting and color calibration was carried out according to the procedure shown in Table 3.1.

Table 3.1 Machine vision system setting procedure.

NO.	Setting
1	Set the brightness at the end of tube to 1700 lx
2	Set the focus to 8 mm from the end of tube
3	Set F value to 2.2 in 2010 and 1.8 in 2011
4	Set shutter speed to 1/30 s
5	Set master gain to 336
6	Check white balance and calibrate camera by adjusting red and blue gain
7	Take images of Color Checker (color calibration board)

After calibration, the cattle's eyes were covered for two minutes to adapt to a dark environment. This black cloth cover was kept on until the eye images were captured. Then, we adjusted the aim of the camera to the cattle's eye using NIR-LEDs lighting. After a focused image of the pupil was obtained on the NIR video monitor, the white LEDs were turned on and the eye images were captured simultaneously. Fig. 3.4 shows sample images.

Blood assays were conducted one to two days before image acquisition. Serum vitamin A levels in the blood were determined by HPLC.

The images were processed by software developed in Microsoft Visual Studio 2008 to obtain the color information. The yellow color in Fig. 3.5 shows the region of interest (pupil area). The region of interest was determined by the hue value of the color image and the brightness of the NIR image. As shown in Fig. 3.4, very little NIR light was reflected from the tapetum, hence the NIR images were not used in any subsequent analysis.

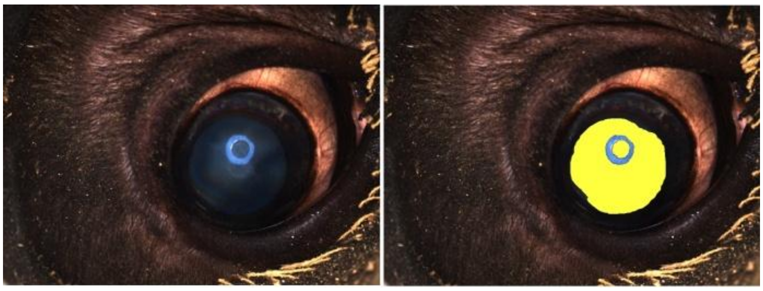

Figure 3.5 Original image (Left), Region of Interest shown in yellow color (Right).

A comparison of the color change in RGB color model and HSI color model based on Gonzalez *et al*. (1992) was conducted. Red (*r*), green (*g*), and blue (*b*) component ratios were calculated by the formulas below:

$$r = R/(R+G+B) \tag{3.1}$$

$$g = G/\,(R+G+B) \tag{3.2}$$

$$b = B/\,(R+G+B) \tag{3.3}$$

where *R*, *G* and *B* represent red, green and blue, respectively. They have been normalized and are in the range [0, 1]. The HSI color space is claimed to be the closest approximation to a human interpretation of colors. There are many different models for HSI color space. Here, we use the triangular pyramid model. The calculation method is shown below.

$$H = \begin{cases} \theta & if\ B \leq G \\ 360 - \theta & if\ B > G \end{cases} \text{ with } \theta = \cos^{-1}\left\{\frac{\frac{1}{2}[(R-G)+(R-B)]}{[(R-G)^2+(R-B)(G-B)]^{\frac{1}{2}}}\right\} \tag{3.4}$$

$$S = 1 - \frac{3}{(R+G+B)}[\min\,(R, G, B)] \tag{3.5}$$

$$I = \frac{1}{3}[R + G + B] \tag{3.6}$$

To make "Saturation" and "Intensity" easy to interpret, they are multiplied by 100, and 255, respectively. So they are in the range of [0,100] and [0,255], respectively.

3.3.3. Statistics

Data were expressed as means ± SD, and were statistically analyzed using Student's *t*-test. Statistical significance was set at $p < 0.01$.

3.4. Results

3.4.1. Relationship between individual color factors and serum vitamin A in 2010

In 2010, the serum vitamin A level in all the cattle declined and then stayed at a low level around 20–30 IU/dL. By the end of the experiment, the cattle's serum vitamin A levels ranged from 7 to 35 IU/dL (mean ± SD 19 ± 7 IU/dL).

Fig. 3.6 shows changes in serum vitamin A (mean ± SD) and *r* (mean ± SD) over time. This indicates that when vitamin A drops, *r* would increase.

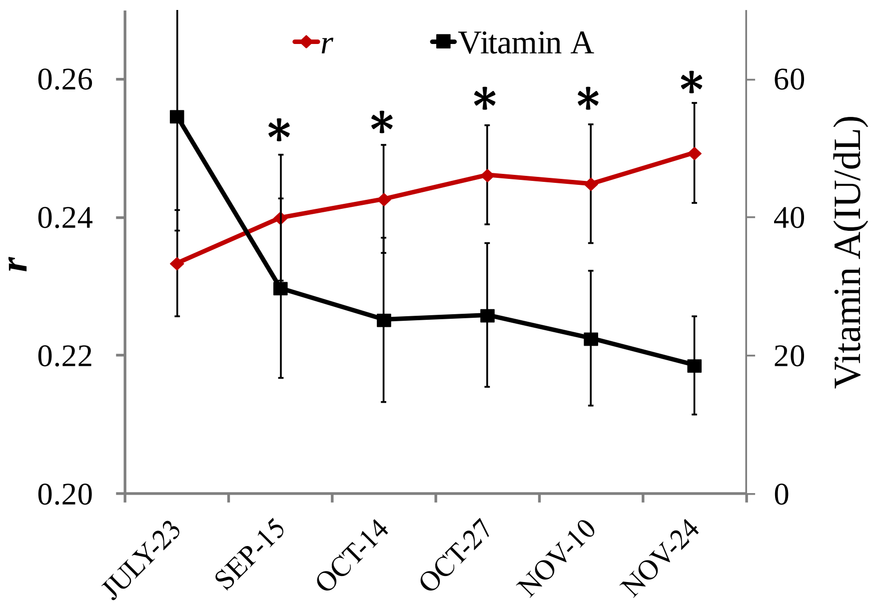

Figure 3.6 Changes in serum vitamin A and red component ratio from July to November 2010. Error bars indicate the standard deviation of all the cattle (N = 42). *Significant difference from values in July 23rd ($p < 0.01$).

In 2010, only average serum vitamin A levels on July 23rd (mean ± SD 46 ± 20 IU/dL) were significantly above 30 IU/dL. In subsequent months, levels were around or below 30

IU/dL. A comparison between data on July 23rd and November 24th was conducted. This represents the highest and lowest levels of serum vitamin A, respectively. The results are shown in Fig. 3.7. The *r* on July 23rd were significant lower than that on November 24th ($p<0.001$). This indicates there is a negative correlation between *r* and serum vitamin A. Saturation was also investigated in the same way. The results show that vitamin A deficient in fattening cattle is usually accompanied by a lower saturation value, as shown in Fig. 3.8. The significant color change in the pupil area indicates that changes in the tapetum and retina induced by low serum vitamin A levels can be detected by measuring the reflected light from the back of the pupil.

To confirm these findings, a similar experiment was conducted again in 2011 with another group of fattening cattle. This time the experimental period was extended from the 5 months to 8 months in order to cover a wider range of serum vitamin A levels. In addition, a new lens with a smaller F value (1.8) was used in 2011 to acquire brighter eye images, and thus make it easier to detect color changes.

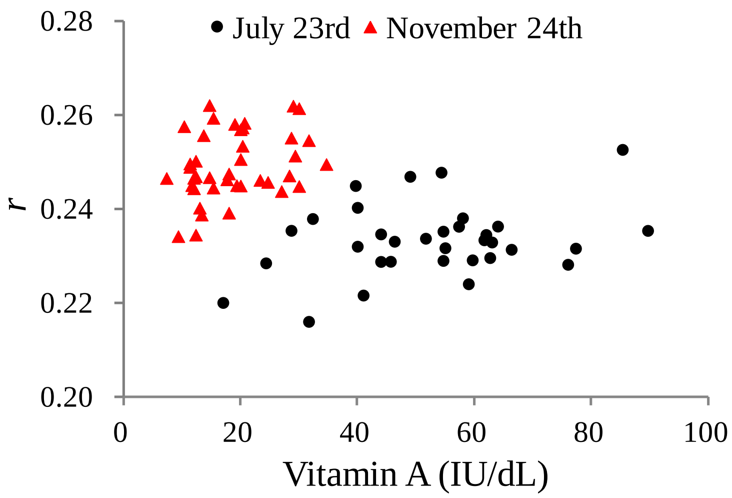

Figure 3.7 Relationship between red component ratio and serum vitamin A level in July 23rd and November 24th 2010.

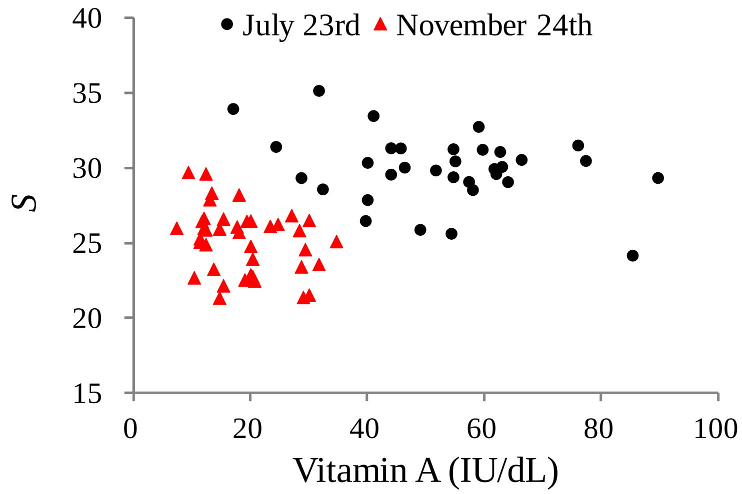

Figure 3.8 Relationship between saturation and serum vitamin A level in July 23rd and November 24th 2010.

3.4.2. Relationship between individual color factors and serum vitamin A in 2011

Serum vitamin A levels in all the fattening cattle gradually declined during the experimental period in 2011. By the end of the experiment, the experiment cattle's serum vitamin A levels ranged from 11 to 41 IU/dL (mean ± SD 28 ± 7 IU/dL).

The relationship between serum vitamin A level and *r*, *g*, *b*, hue, saturation and intensity were investigated. Fig. 3.9 shows the correlation between *r* and serum vitamin A level from May (12–16 months old) to December (19–23 months old) 2011. The correlation is weak. Similar to the results in 2010, lower serum vitamin A levels tend to be associated with a higher red component. As the serum vitamin A level dropped, the red color component value increased. The changes in serum vitamin A level (mean ± SD) and *r* (mean ± SD) over time are shown in Fig. 3.10. This data also show that as serum vitamin A levels decline, *r* rises.

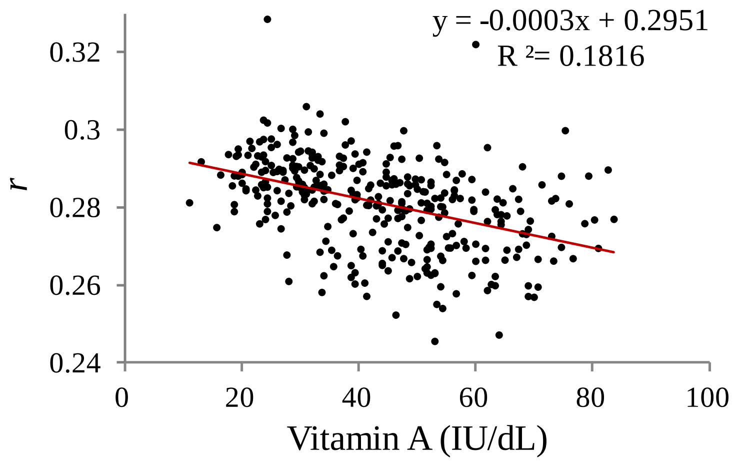

Figure 3.9 Relationship between red component ratio and serum vitamin A level.

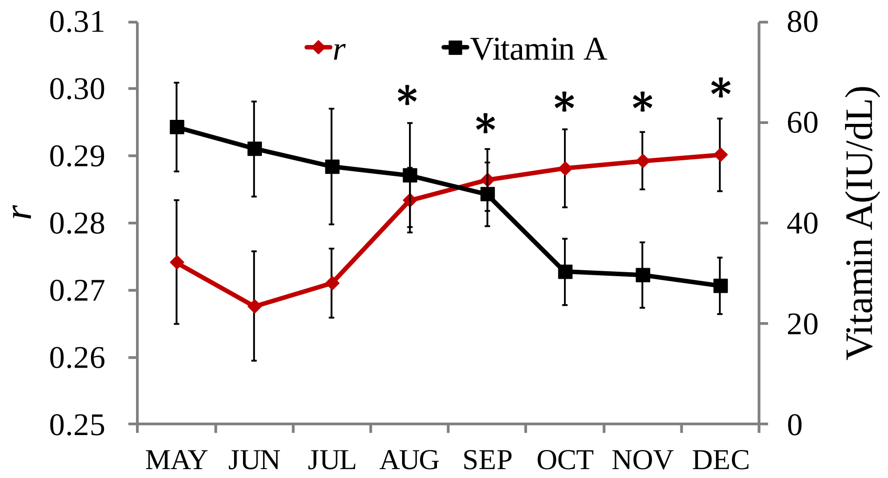

Figure 3.10 Changes in serum vitamin A and red component ratio from May to December 2011. Error bars indicate the standard deviation of all the cattle (N = 42). *Significant difference from values in May ($p < 0.01$).

According to past veterinary experience, at the beginning of the fattening stage serum vitamin A levels of cattle will be around 80 IU/dl. After 3 months of low vitamin A diets, serum vitamin A levels will be around the critical 30 IU/dl value. The symptoms of VAD may not show for another 3 months. Based on this information, we choose the data in May, August and December; corresponding to the beginning, middle and end of middle fattening stage, for correlation analysis. The correlation between color factors (*H*, *S*, *I*, *r*, *g*, *b*) and serum vitamin A levels in the selected three months were investigated. Table 3.2 shows the correlation coefficients between color factors and serum vitamin A levels. In Group A, the correlation coefficients of *r*, *S*, *H* were higher than 0.6, indicating a strong correlation. Generally, cattle with lower serum vitamin A levels had higher *r*, *H*, *I* and lower *b*, *S*, as Table 3.2 shows. This result for *r* and *S* was consistent with the result obtained in 2010. This higher correlation was obtained using the HSI color model.

Group B showed a weak correlation between color factors and serum vitamin A levels, where all the correlation coefficients were lower than 0.5. Compared to Group A, cattle in Group B had significantly higher serum vitamin A levels, especially in October, November and December ($p<0.01$), as shown in Table 3.3. It is possible that Group B had more vitamin A storage in their body, so the time taken to deplete vitamin A stores took longer. Which in turn means the detection of color changes will take longer.

Table 3.2 Correlation coefficients between color factors and serum vitamin A levels.

	Inbred lines	Number of cattle	*r*	*g*	*b*	*H*	*S*	*I*
A	Hiroiwadoi	8	0.52^-	0.76^+	0.23^+	0.86^-	0.56^+	0.68^-
	Yoshigakidoi	4	0.75^-	0.50^+	0.65^+	0.82^-	0.75^+	0.58^-
	Hiromi-Yoshigaki	12	0.68^-	0.29^-	0.56^+	0.70^-	0.68^+	0.57^-
	Group A	24	0.60^-	0.51^+	0.42^+	0.77^-	0.62^+	0.55^-
B	Miyagikujyo	4	0.27^-	0.09^-	0.29^+	0.05^-	0.28^+	0.65^-
	Kikuyu-Hiromasa-Miyagikujyo	5	0.39^-	0.08^+	0.30^+	0.21^-	0.39^+	0.24^-
	Kikuyu-Hiromasa	9	0.17^-	0.44^+	0.01^+	0.45^-	0.17^+	0.65^-
	Group B	18	0.21^-	0.28^+	0.09^+	0.33^-	0.21^+	0.55^-
	Overall	42	0.42^-	0.38^+	0.27^+	0.55^-	0.42^+	0.55^-

Note: "+" indicates positive correlation; "-" indicates negative correlation.

Table 3.3 Serum vitamin A levels from August to December.

	Inbred lines	NO.	Aug*	Sep*	Oct*	Nov*	Dec*
A	Hiroiwadoi	8	44±5	45±6	25±3	26±3	26±5
	Yoshigakidoi	4	44±4	45±6	28±3	21±2	25±4
	Hiromi-Yoshigaki	12	39±8	39±5	26±5	24±4	24±4
	Group A	24	42±9	42±7	26±5	24±4	25±6
B	Miyagikujyo	4	57±6	47±5	39±5	37±4	31±3
	Kikuyu-Hiromasa-Miyagikujyo	5	58±5	54±8	39±6	39±7	36±4
	Kikuyu-Hiromasa	9	62±10	50±7	33±7	35±8	28±6
	Group B	18	60±10	51±9	36±9	37±8	31±7
	Overall	42	49±13	46±9	30±9	30±9	28±7

Unit: IU/dL. Serum vitamin A levels were shown in means ± SD. * Serum vitamin A levels in group A were significant lower than that of group B May ($p < 0.01$).

3.4.3. Result of Multiple Linear Regression (MLR) Analysis

To estimate serum vitamin A levels, a MLR model of serum vitamin A levels and color factors was developed in Excel 2007. To build the calibration models, the data at three sampled dates (May, August and December) for sixteen Group A cattle were used, and the data at three sampled dates (May, August and December) for 8 Group A cattle were used for validation. Because *H*, *S* and *I* were calculated from *R*, *G* and *B*, they were interrelated. *r* and *g* were used to build the estimation model. The results are shown in Table 3.4.

Table 3.4 Multiple Linear Regression Models.

Color Factors	MLR Model	R^2
r, g	$y = -915x_r + 2372x_g - 465$	0.67*

Note: * $p < 0.05$, where x_r, x_g, is the value of r, g respectively; y is the estimated serum vitamin A level.

The "*r*, *g*" model was used to estimate the serum vitamin A values of the other 8 cattle. The performance of the "*r*, *g*" model was evaluated in terms of root mean square error of calibration (RMSEC) and root mean square error of prediction (RMSEP), and the coefficient of determination (R^2). The results are shown in Table 3.5. Scatter plots of the "*r*, *g*" models for calibration and validation are shown in Fig. 3.11. The data are evenly distributed around the regression line with a 45 °slope, which indicates estimated and measured values are very close to each other.

Table 3.5 Result of "*r*, *g*" estimation model.

Model	RMSEC(IU/dL)	R^2(Calibration)	RMSEP(IU/dL)	R^2(Validation)
r, *g*	9.3	0.67	10.3	0.62

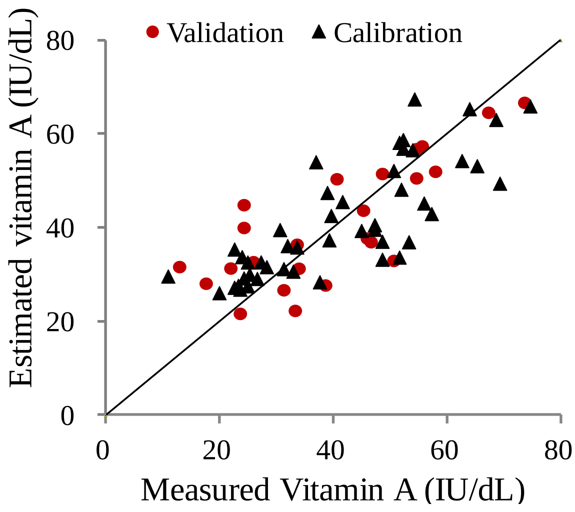

Figure 3.11 Measured and estimated serum vitamin A by "*r*, *g*" model.

3.5. Discussion

The result shows that fattening cattle with a lower serum vitamin A level usually have a lower pupil saturation and lower blue component value. The decreased saturation, a measure of the degree to which pure color is diluted by white light (Gonzalez *et al.*, 1992), may indicate more white LED light is reflected by the tapetum when there is VAD. As the tapetum is blue in color, a low blue component value for cattle with a low serum vitamin A level may indicate the mottled appearance of the non-tapetal area because of retina degeneration (Maggs *et al.*, 2008). However, this result is based on the subjective notion that the main factor impacting color changes in the eyes of Japanese black cattle is associated with serum vitamin A levels. Further research, with a vitamin A supplement group, is need to confirm pupil color changes are caused by serum vitamin A levels, and not other factors, such as age.

The RMSEP (about 10 IU/dL) may be affected by the individual differences between cattle. To decrease the effect of individual differences on the estimation error, it may be effective to conduct a blood assay of serum vitamin A levels at the beginning of vitamin A control stage. Thus, the estimation error could be reduced based by taking into account color changes and initial serum vitamin A levels. According to the Japan Livestock Technology Association, three cattle blood assays are needed to identify VAD during the middle fattening stage. With the suggested sampling regime, blood assays could be reduced to 1. Issi *et Al.* (2010) reported that the loss of pupillary light reflexes is also a clinical sign of VAD. To make the estimation procedure more robust and accurate, other factors such as pupillary light reflex and surface reflection, which may be affected by low serum vitamin A levels, should also be investigated in the future.

3.6. Conclusions

A negative correlation between the red component ratio of cattle pupils and serum vitamin A levels, a positive correlation between the saturation value of cattle pupils and serum vitamin A levels were found in 2010, and confirmed in 2011. For 24 cattle, with a wider range of serum vitamin A levels than that in 2010, a multiple linear regression analysis was conducted, and a model y= -915x_r+2372x_b-465 based on the red and green component ratio was selected. The coefficient of determination for calibration and validation was 0.67 and 0.62, respectively. RMSEC was 9.3 (IU/dL) and RMSEP was 10.3 (IU/dL). This model may not be applicable to other cattle inbred lines, but the results show the feasibility of estimating serum vitamin A levels using the color signatures of Japanese black cattle during the middle fattening stage, when their serum vitamin A levels are depleted. This information derived from pupil color changes will be a valuable aid to farm management.

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Chapter 4

A Feasibility Study of Using Pupillary Light Reflex Analysis to Identify Vitamin A Deficient Japanese Black Cattle

Abstract

To produce beef with higher marbling, Japanese black cattle farmers usually actively manipulate the serum vitamin A in fattening cattle at a minimum desired low level (about 30 IU/dL). Identification of vitamin A deficient fattening cattle in the early stage is important to prevent fattening cattle from getting serious diseases. In this study, we investigated the feasibility of using Pupillary Light Reflex (PLR) analysis to identify vitamin A deficient fattening cattle. PLRs of 43 cattle were monthly recorded from June 2012 to February 2013 by using a 2CCD camera based handy machine vision system. A new image processing algorithm to segment pupil from background was presented. Compared with manually selected results, the root mean square error of constriction amplitude (*CA*) acquired by image processing was 2.3%, which indicates the effectiveness of this algorithm. In 2011, cattle in low serum vitamin A level period had larger *CA* in 1 second ($p<0.01$) and smaller resting pupil ($p<0.01$). In 2012, no significant difference were found between results of *CA* from fattening cattle in high (>60 IU/dL) and low (<30 IU/dL) vitamin A period. However, two fattening cattle were identified as severe vitamin A deficient cattle, because of their weak PLR ($CA<10\%$). Initial pupil roundness (*IPR*) results showed cattle possessed less-dilated pupil in a dark environment during vitamin A deficient period ($p<0.05$). These results showed the potential and also limitation of this method. Due to the variation of PLR in healthy cattle, monthly measurements are not enough to make accurate identification. To realize early identification of severe vitamin A deficient fattening cattle, more frequent PLR analyses are necessary.

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4.1. Introduction

Marbling—intramuscular fat, is the intermingling or dispersion of fat within the lean. The amount of marbling is the most influential factor to determine the beef quality grade in the USA, Japan and Australia (Strong 2004), because it indicates the expected eating characteristics (tenderness, juiciness and flavor) of the cooked product. Beef Marbling Standard (BMS) established by Japanese Meat Grading Association are applied in beef grading in Japan. Silicon resin models, as shown in Fig. 1.1, are used to standardize the range of the degree of marbling. The model of No. 12 indicates the highest marbling standard.

Oka *et al.* (1998) found maintaining low serum vitamin A levels of Japanese black cattle during the fattening age, from 15 to 23 months old, could produce beef with significant higher BMS. Similar experiments conducted by other researches (Siebert *et al.*, 2006; Gorocica-Buenfil *et al.*, 2007; Kruk *et al.*, 2008) also confirmed this finding. Currently, most Japanese black cattle farmers actively manipulate the serum vitamin A levels in fattening cattle to get beef with high BMS. Ideal serum vitamin A level in cattle during the fattening stage in Hyogo Prefectural Hokubu Agricultural Institute is shown in Fig. 1.2. However, maintaining a minimum vitamin A level is difficult. Besides that, a VAD below 30 IU/dL would increase disease susceptibility, such as night blindness, xerophthalmia, or diarrhea, even death (Moore 1941; O'Donghue 1955; Millemann *et al.*, 2007; Issi *et al.*, 2010). In a word, it is important to monitor changes of serum vitamin A levels to assure all the cattle in healthy condition during vitamin A controlled period. Blood assay is the conventional way to measure serum vitamin A levels. However, it is invasive and stressful to the cattle. Furthermore, this measurement is inconvenient and expensive which cannot be afforded by most farmers. Thus, an alternative system which can identify cattle with low serum vitamin A levels below 30 IU/dL would contribute to feeding management during vitamin A controlled period.

Early clinical signs of VAD usually related to some ocular changes like papilledema, mottling of the tapetum, pallor of the nontapetum, and xerophthalmia (Maggs *et al.*, 2008). Previous studies from our group showed there were some correlations between serum vitamin A levels and tapetum reflection (Takahashi *et al.*, 2011) pupil color (Takao *et al.*, 2011; Han *et al.*, 2013) and pupil light reflection (Tanigawa *et al.*, 2011). Among these factors, pupil color showed the highest correlation with serum vitamin A levels. A serum vitamin A estimation model with the estimation error about 10 IU/dL was built based on color (Han *et al.*, 2013). To make the identification of vitamin A deficient cattle more reliable, considerations of multiple factors are necessary. In this study, the relation between Pupillary Light Reflex (PLR) and serum vitamin A levels would be investigated.

O'Donghue (1955) reported some cattle which were vitamin A deficient showed dilated pupil and no response to light. Another report reassured the loss of PLR in cattle is one clinical sign of VAD (Issi *et al.*, 2010). PLR analysis is an indirect measure of neural network function. Some features extracted from PLR such as initial pupil diameter, constriction latency, constriction amplitude, constriction velocity and pupil diameter at max constriction have been studied for aging effects (Bitsios *et al.* 1996; Daluwatte *et al.*, 2012), time-of-day effects (Yu *et al.*, 2007) and functions of melanopsin-containing retinal ganglion cells (Ishikawa *et al.*, 2012) in human. It is a reliable test and has the potential to be used to assist the diagnosis of eye related diseases.

With the notion of VAD may affect PLR in cattle, Matsuda *et al.* (1999) investigated the relation between constriction duration and serum vitamin A levels. The result showed cattle with lower serum vitamin A levels tended to need longer time to stop constriction. These findings indicate that it is worthy to test the feasibility of using PLR analysis to identify vitamin A deficient cattle. The experiment Matsuda *et al.* conducted took more than 10 seconds to record the full PLR of cattle. A long time illumination might make cattle stressful. For the considerations of animal welfare and instant identification, one second PLR was used in this experiment.

In previous works, a new machine vision system based on 2CCD camera was introduced by us (Han *et al.*, 2011). That handy machine vision system is capable of capturing images in a full PLR process. We investigated the relation between PLR and serum vitamin A levels from five aspects pupil area, normalized pupil area, starting shrinking time, slope of pupil contraction regression line and ratio of max length and breadth of pupil before shrinking. The linear correlations between these factors and serum vitamin A levels were not found. However, in that preliminary experiment, the PLR analysis was not carried out accurately. Pupil area calculation was based on binarization by hue value. The results were incorrect when boundaries of pupil were overlapped with a bright image of LED ring that was formed by a specular reflection. The image of LED ring is shown in Fig. 4.2. Especially, after the constriction, the pupil was usually small and easy to be overlapped by the image of LED ring. Therefore, the objective of present study is to develop a robust image segmentation algorithm to calculate pupil area and to investigate the feasibility of using one second PLR analysis to identify vitamin A deficient cattle during vitamin A manipulated fattening stage.

4.2. Materials and Methods

4.2.1. Cattle

This experiment was conducted from May 2011 to December 2011 and from June 2012 to February 2013 with two different groups of cattle at Hyogo Prefectural Hokubu Agricultural Institute, Japan.

In 2011, PLRs of right eyes of 40 Japanese black cattle were recorded once a month. At the beginning of the experiment, ages of the cattle ranged from 14 to 16 months and serum vitamin A levels ranged from 34 to 80 IU/dL (mean $\pm$ SD 59 $\pm$ 11 IU/dL). From April 2011, the cattle were subjected to low vitamin A diets.

In 2012 and 2013, PLRs of right eyes of 43 Japanese black cattle were recorded once a month. At the beginning of the experiment, ages of the cattle ranged from 13 to 16 months and serum vitamin A levels ranged from 38 to 151 IU/dL (mean $\pm$ SD 76 $\pm$ 18 IU/dL). From April 2012, the cattle were subjected to low vitamin A diets.

4.2.2. Device

A 2CCD multi-spectral camera AD-080CL (JAI) installed NT63-240 (Focus Length 12 mm, F 1.8, EDMUND) lens was used to acquire both color and Near Infrared (NIR) images. The camera was combined with two ring-shaped LED lights; a MDRL-CW50 (MORITEX) white LED light and a MDRL-CIR31 (MORITEX) NIR LED light which has central wavelength of 850 nm. A plastic tube was installed in front of the LED lights to block the ambient light. The sketch of the device is shown in Fig. 4.1. The captured image is 1024 pixels in width and 768 pixels in height.

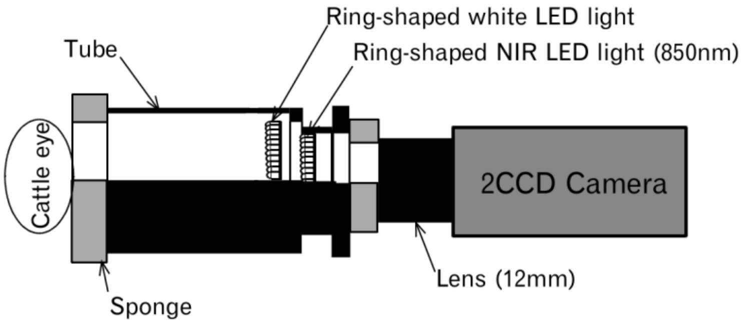

Figure 4.1 Sketch of experimental device. This device is the same as the device as shown in Fig. 3.3. However, PL filter was not used in this experiment.

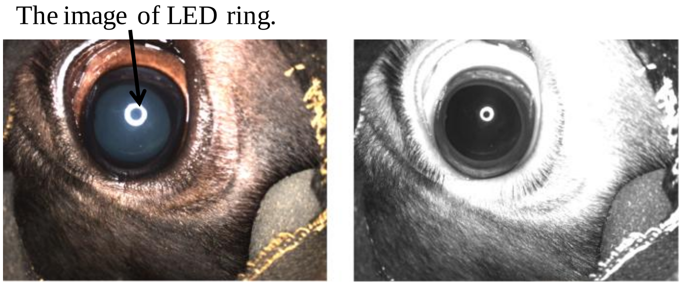

Figure 4.2 Color image without PL filter and NIR images of cattle's right eye.

4.2.3. Experiment Procedures

Eyes of the cattle were covered about two minutes to adapt a dark environment. This black cloth cover was kept on during recording. At the beginning of the measurement, the light intensity at the end of the plastic tube was set to 1700 lx and the same lighting condition was kept in all measurements. The aim of the camera was adjusted to the cattle's eye with NIR LED lighting. After a focused image of the pupil was obtained on the NIR video monitor, the white LEDs were turned on and the eye images were obtained simultaneously. Sample images are shown in Fig. 4.2. Blood was sampled one to two days before image acquisition. Serum vitamin A levels in the blood were determined by using High-Performance Liquid Chromatography (HPLC) device.

4.2.4. Image Processing

Due to the reflection of white LED light from tapetum lucidum of cattle, unique blue color was shown in pupil area. It is easier to segment pupil from background in color images than

NIR images, as shown in Fig. 4.2. Color images were processed by program developed in Matlab 2007 (Mathworks). Procedures of pupil area acquisition are shown in Fig. 4.3.

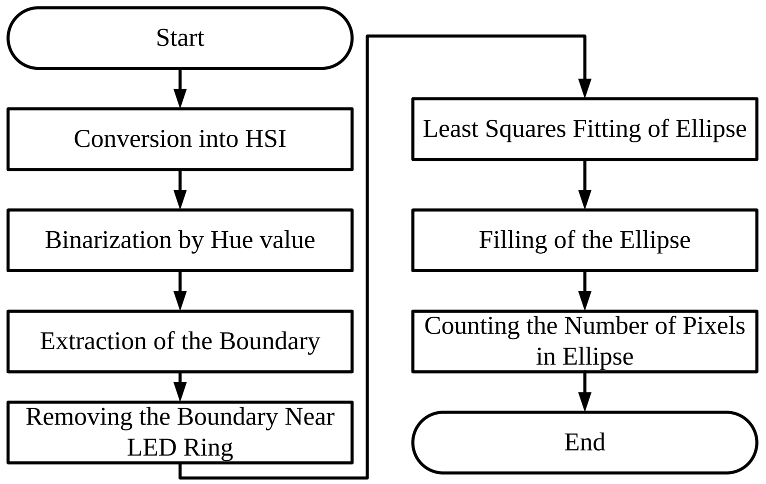

Figure 4.3 Pupil area acquisition procedures.

The color image was converted into the HSI color space. The hue image is shown in Fig. 4.4 (b). After binarization based on hue value, as shown in Fig. 4.4 (c), the boundary of the largest region of interest (ROI) was selected, as shown in Fig. 4.4 (d). Because the pupil was partially overlapped by the image of LED ring, it was difficult to select the correct pupil area by thresholding, as shown in Fig. 4.4 (c). Because of the oval shape of the pupil of cattle, an ellipse is good for estimation of pupil area. The boundary of the largest ROI after removing near LEDs' reflection part, as shown in Fig. 4.4 (e) was used to conduct ellipse fitting based on least squares method introduced by Rosin (Rosin 1993). The fitted result is shown in Fig. 4.4 (f). The ellipse shown in Fig. 4.4 (g) was used to represent the pupil area for subsequent PLR analysis.

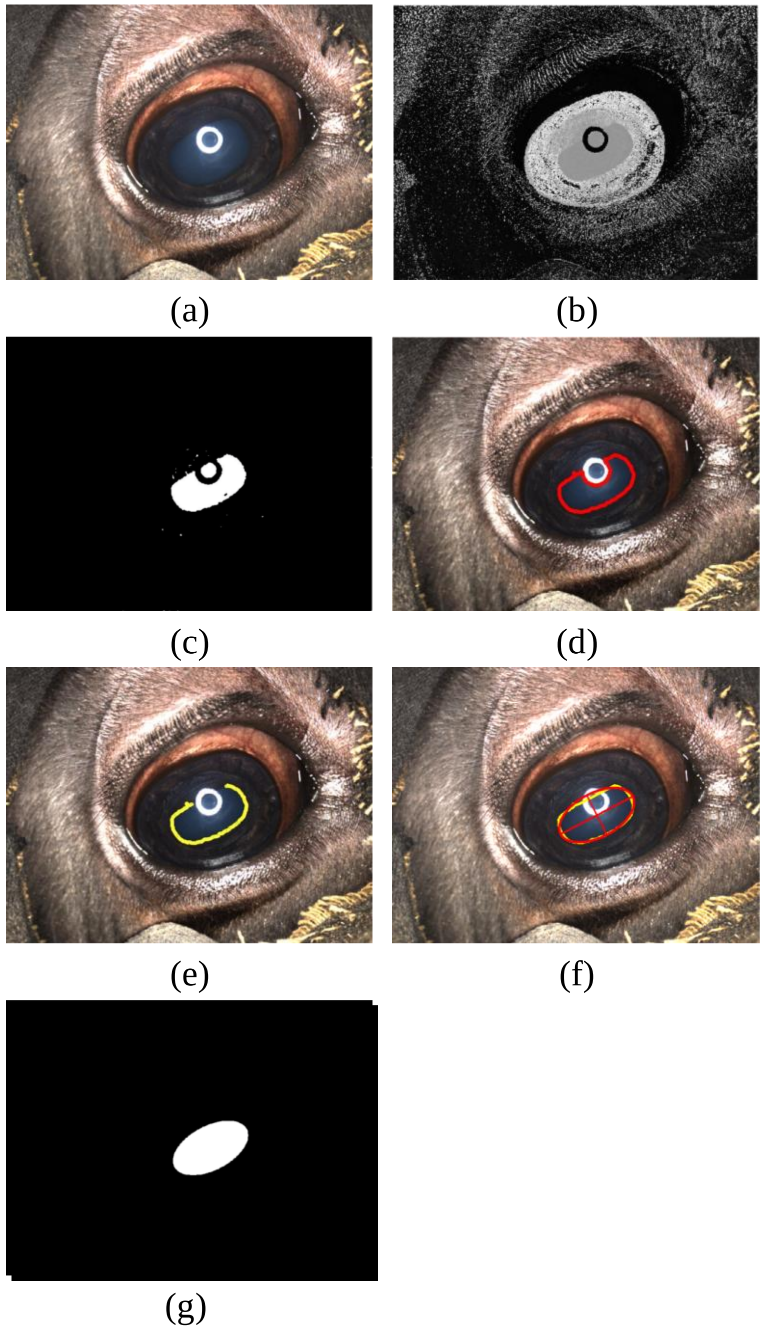

Figure 4.4 (a) Original color image, (b) Hue image, (c) Binarized image by hue value,(d) Color image with boundary of largest region of interest shown in red, (e) Color image with boundary of pupil selected for fitting shown in yellow, (f) Fitted ellipse based on least squares method with its long and short axis shown in red, (g) Fitted ellipse used to represent pupil area.

4.2.5. PLR Analysis

In order to correct for differences in the pupil area among different cattle and different measurements, all pupil data were normalized by regarding resting pupil area, the largest pupil area before onset of white LED light, as baseline. Once all normalized pupil area was calculated from 30 images taken in 1 second, a pupilogram curve was constructed after smoothing by using Savitzky–Golay filter (Savitzky *et al.*, 1964), as shown in Fig. 4.5. The following parameters were used for subsequent analysis, Constriction Amplitude in 1 second (*CA*), and Initial Pupil Roundness (*IPR*).

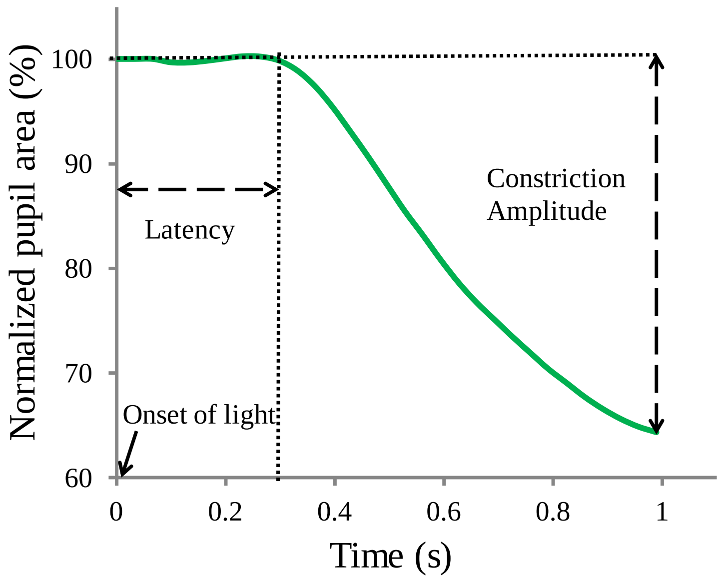

Figure 4.5 An example of pupilogram from cattle's right eye in response to white LED light stimulation with light intensity of 1700 lx.

After the onset of white LED lighting, there is latency time about 0.3 second, as shown in Fig. 4.5. *CA* in 1 second was calculated by using the following equation (1).

$$CA = \frac{S_0 - S_1}{S_0} \tag{4.1}$$

Where s_0 is the resting pupil area and s_1 is the pupil area at the end of 1 second constriction.

IPR was used as the indicator of resting pupil shape. The *IPR* was calculated using the following equation (2).

$$IPR = \frac{4\pi S}{P^2} \tag{4.2}$$

Where *S* is the area of pupil and *P* is the perimeter of the pupil.

4.2.6. Data Analysis

The performance of image processing results were evaluated by root mean square error (RMSE) regarding manually selected pupil area as the true value. To study the effect of VAD on PLR, data were investigated by unpaired *t*-tests and paired *t*-tests to examine differences between the means of low (< 30 IU/dL) and high (> 60 IU/dL) serum vitamin A level groups for PLR parameters, *CA* and *IPR*.

4.3. Results and Discussion

4.3.1. Evaluation of image processing

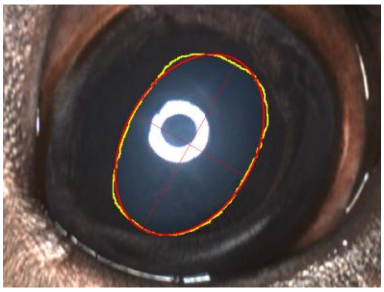

Figure 4.6 A comparison of ellipse fitted (in red) and manually selected (in yellow) pupil.

Sixty PLR measurements were randomly selected from nine months experiments conducted from June 2012 to February 2013 with 43 Japanese black cattle. First and thirtieth images from each measurement were used for comparisons. Pupil area results acquired by image processing were compared with manually collected results by using ImageJ (Abràmoff *et al.*, 2004), as shown in Fig. 4.6. The correlation coefficient between these methods is 0.99 and 0.95 in pupil area and *CA*, respectively. Root mean square error (RMSE) of pupil area and *CA* is 930 pixels and 2.3%, respectively. As shown in Fig. 4.7, the results of pupil area and *CA* were normally distributed around the line of equality, which means the results acquired by these two methods were close to each other. Paired *t*-test showed there was no significant difference between means of these two results (p>0.05). It indicates the proposed image processing method is effective in estimating pupil area and *CA*.

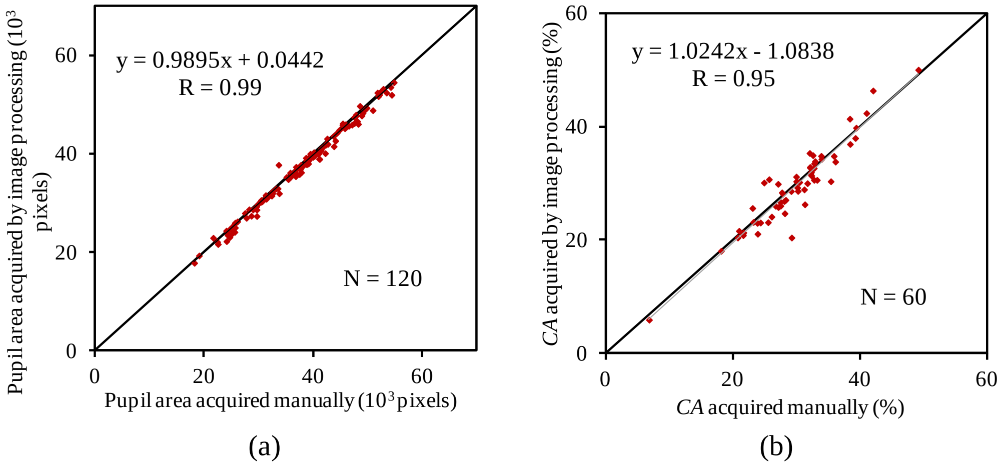

Figure 4.7 Pupil area (a) and *CA* (b) acquired by manually and image processing.

4.3.2. PLR of low and high serum vitamin A group in 2011

All the cattle's vitamin A level gradually declined during the experiment period, as shown in Fig. 4.8. The average vitamin A levels of October (mean ± SD 24 ± 4 IU/dL), November (mean ± SD 23 ± 4 IU/dL) and December (mean ± SD 22 ± 4 IU/dL) were lower than the critical level 30 IU/dL. PLR parameters' average values in these 3 months were considered to represent PLR features of low vitamin A cattle. On the contrary, the average values in May, June and July were used to represent PLR features of high vitamin A cattle. The age effects on PLR were not considered.

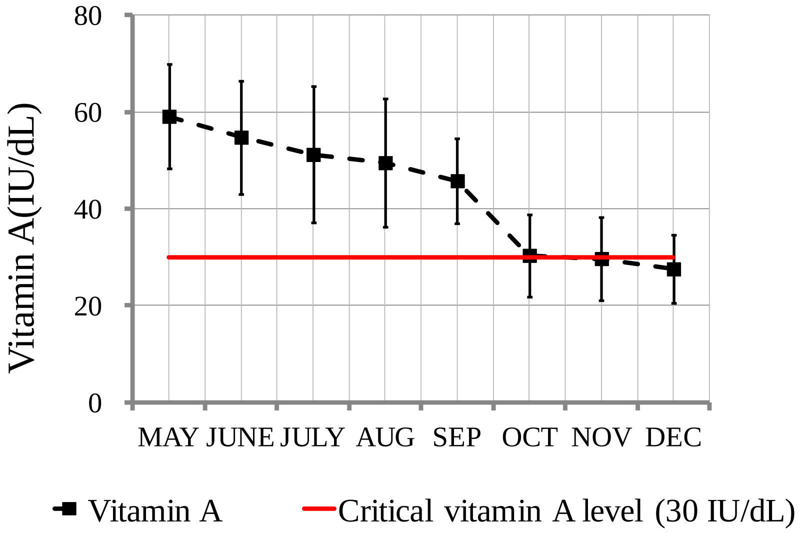

Figure 4.8 Change in serum vitamin A levels from May to December 2011. Error bars indicate the standard deviation of all the cattle (N = 40).

Paired *t*-tests showed that cattle in low serum vitamin A period showed significant larger *CA* ($p < 0.01$) and smaller *IPR* ($p < 0.01$) as shown in Fig. 4.9. Larger *CA* in low vitamin A cattle can be caused by shorter constriction latency, as reported by (Yoshida et al. 2011). Compared with the cattle with high vitamin A levels, there were many cattle with lower vitamin A levels showed smaller *IPR*, which means thinner resting pupil. The thinner resting pupil may be caused by the change of retina sensitivity after vitamin A deficiency.

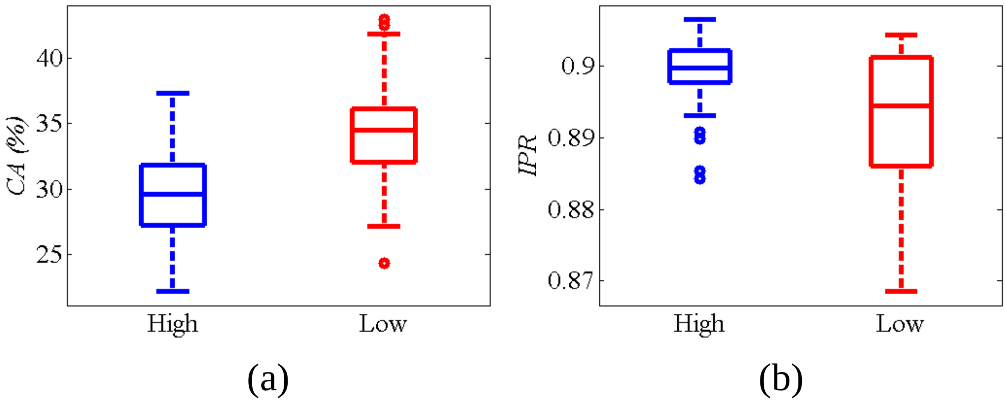

Figure 4.9 Comparison of *CA* and *IPR* between low (in red) and high (in blue) serum vitamin A level cattle (N = 40). The horizontal lines in the boxes are medians, the bottom and top of the boxes are the first and third quartiles, the 'whiskers' are extreme values in each group and the dots are outliers.

4.3.3. PLR of low and high serum vitamin A group in 2012 and 2013

The serum vitamin A levels in all the cattle gradually declined during the experiment period, as shown in Fig. 4.10. Once the serum vitamin A levels in cattle measured by blood assay were lower than 30 IU/dL, oral supplementation with vitamin A was conducted. However, the restoration of serum vitamin A levels usually took long period. There were many cattle possessed serum vitamin A levels lower than 30 IU/dL at the end of the experiment.

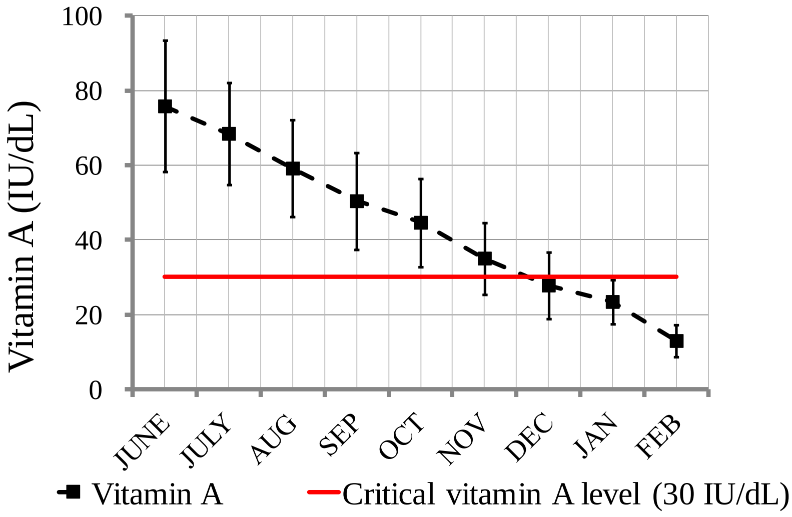

Figure 4.10 Change in serum vitamin A levels from June 2012 to February 2013. Error bars indicate the standard deviation of all the cattle (N = 43).

4.3.3.1. *CA of low and high vitamin A group*

Because initial pupil shape would have effect on the *CA*, to avoid complicated discussion about this effect, only the *CA* with *IPR* larger than 0.89, indicating the fully dilated resting pupil, were used for subsequent comparisons. Seventy measurements with the serum vitamin A levels in cattle higher than 60 IU/dL were considered as high vitamin A group. On the contrary, seventy seven measurements with serum vitamin A levels under the minimum desired serum vitamin A level (30 IU/dL) were considered as low vitamin A group. Their histogram is shown in Fig. 4.11. Unpaired 2-tailed *t*-test showed there are no significant differences between means of high (28.8 ± 5.5%) and low (28 ± 7.4%) vitamin A group (p=0.44). However, the low group showed larger deviation, which may be caused by VAD. To exclude the effect of individual difference, paired *t*-test was carried out on 30 fattening cattle which appeared both in high and low vitamin A group. Their histogram is shown in Fig. 4.12. Paired 2-tailed *t*-test also showed there are no significant difference between means of high (28.3 ± 6.6%) and low (28.8 ± 8.2%) vitamin A group (p=0.78). During our experiment two cattle showed loss of PLR and weak PLR, which was similar as the report of O'Donghue, Issi *et al.* (O'Donghue 1955; Issi *et al.*, 2010). *CA*s of these two cattle were less than 10%, as shown in Fig. 4.13. The possible reasons that the significant difference of mean of *CA* between high and low vitamin A group could not be found might be attributed to lack of measurements, individual differences between cattle on tolerance of VAD, variation of

PLR in healthy cattle and the effect of unavoidable physical stimulation on PLR during recording.

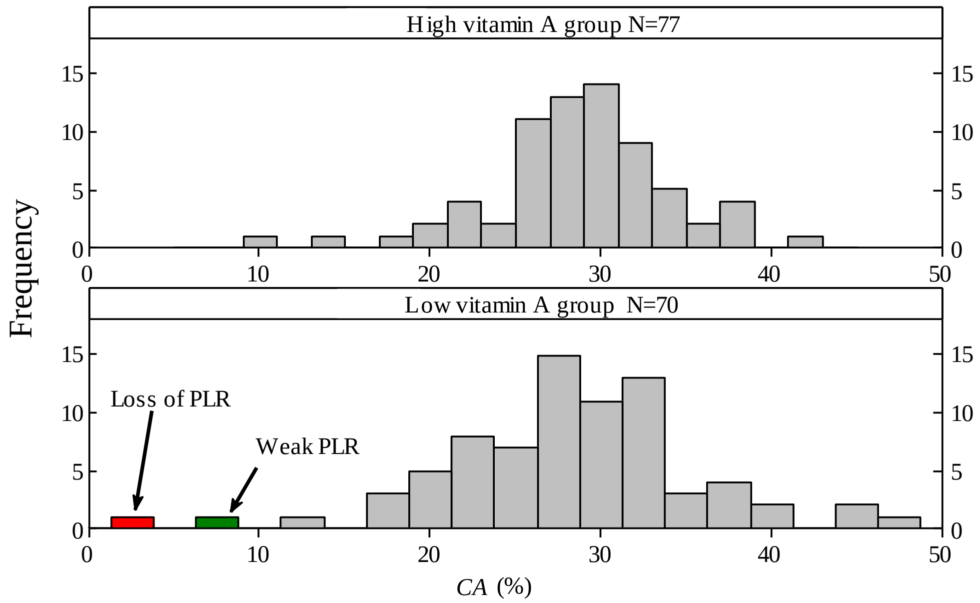

Figure 4.11 Histogram of *CA* from high (>60 IU/dL) and low (<30 IU/dL) vitamin A group cattle. Two cattle with *CA* less than 10% were recognized as loss of PLR and weak PLR, respectively.

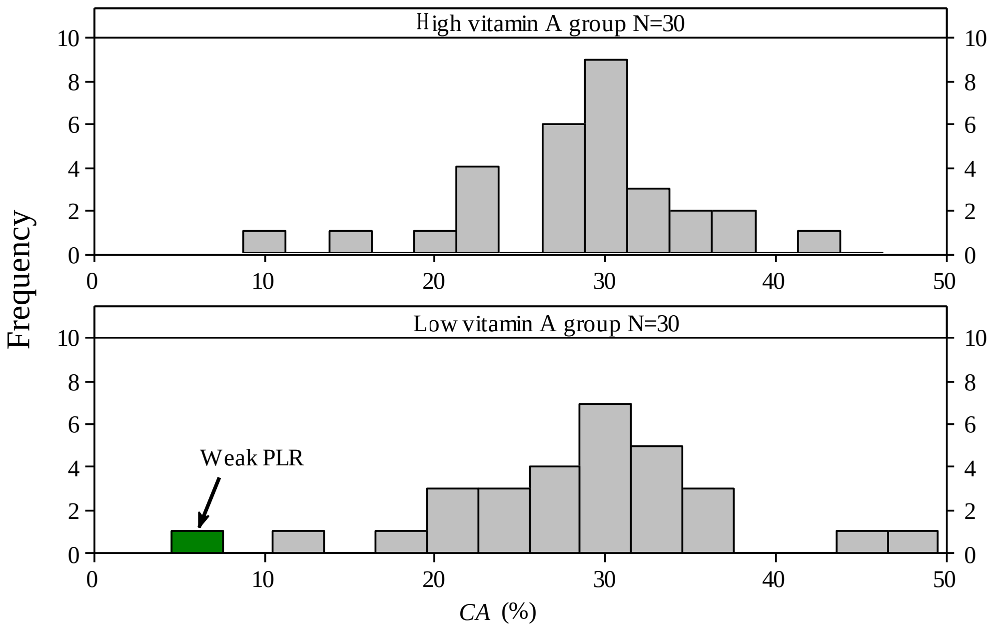

Figure 4.12 Histogram of *CA* from high (>60 IU/dL) and low (<30 IU/dL) vitamin A group cattle in pair. One cattle showed weak PLR.

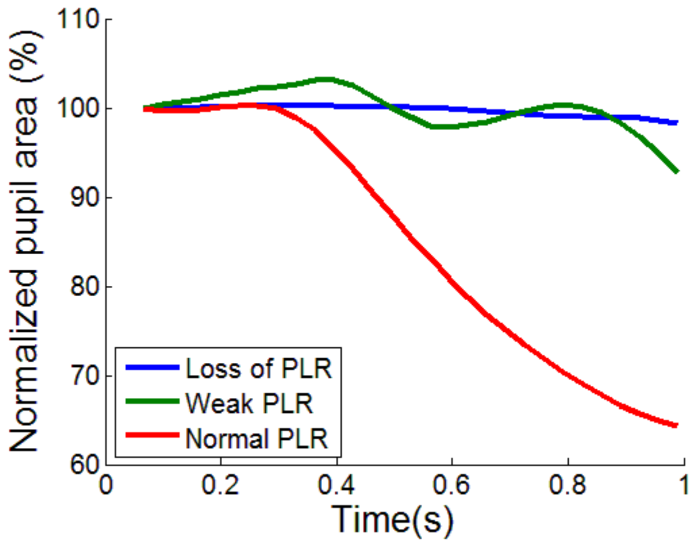

Figure 4.13 Examples of Loss of PLR, Weak PLR and Normal PLR.

Currently, because of the complicated procedures and cost of blood test, we can only afford doing PLR analysis monthly. More frequently measurements are necessary to make a clear judgment and the early detection of VAD. Besides that, pupil was affected by physical stimulation, as shown in Fig. 4.14. Six cattle were selected to record their PLRs during continuous patting their back. The results showed clearly that *CA* became smaller and pupil became larger, as Fig. 4.15 shows. This is because the emotional status of the cattle (e.g., fear, anger, or excitement), which influences the sympathetic system and cause pupillary dilation (Maggs *et al.*, 2008). Even though we tried to keep the cattle calm during recording, to take clear and focused image, it was necessary to hold the upper and lower eyelids of cattle to prevent blinking. This action affected the PLR results randomly. However, two cattle showed weak PLR. This identification, which may not be noticed by farmers during daily feeding, can aid feeding management. To realize early identification and free of stress, an automatic capturing system installed at the watering place that does not need to fix the cattle is one of the solutions.

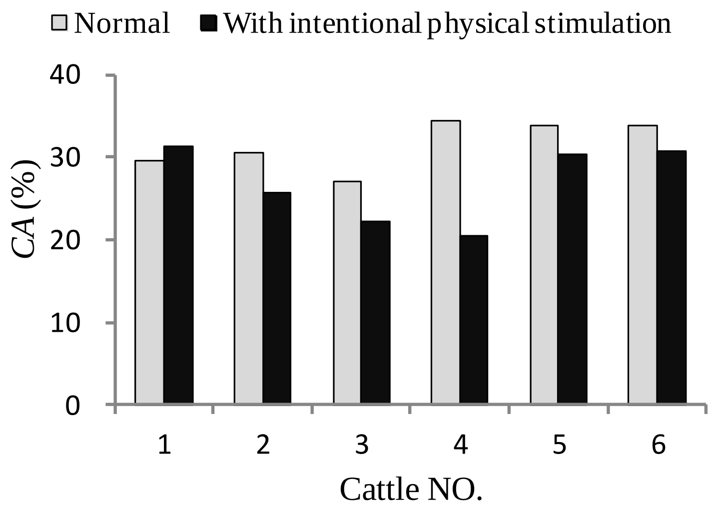

Figure 4.14 *CA*s from six cattle's PLRs recorded normally and with intentional physical stimulation.

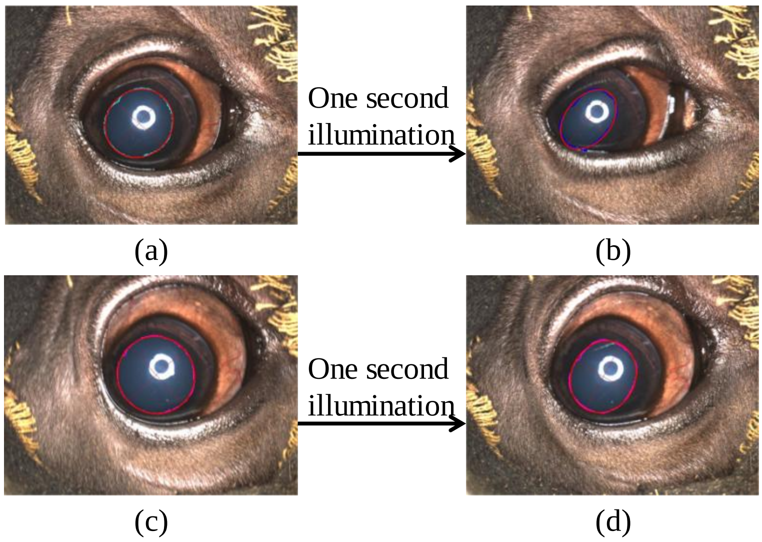

Figure 4.15 First and last images from 1 second PLR of cattle NO.4. Physical stimulation can dilate the resting pupil as showed in (c), compared with (a). Besides that, it can attenuate pupil constriction, as shown in (d), compared with (b).

4.3.3.2. *IPR of low and high vitamin A group*

The histogram of *IPR* of low and high vitamin A group are shown in Fig. 4.16. Unpaired 2-tailed *t*-test showed that *IPR* of low vitamin A group were significant smaller than high vitamin A group (p=0.007). To avoid the effect of individual difference, paired *t*-test was carried out between *IPR* of 34 cattle from high and low vitamin A period. The same result was shown (p=0.013). Resting pupil size may be related to the dark adaption ability. It is possible that when VAD occurred, the dark adaption process become sluggish in cattle, as in human (Kemp *et al.*, 1988). That might explain that there were more less-dilated pupils during vitamin A deficient stage, as shown in Fig. 4.17. This result indicates *IPR* can also be used as an indicator of VAD.

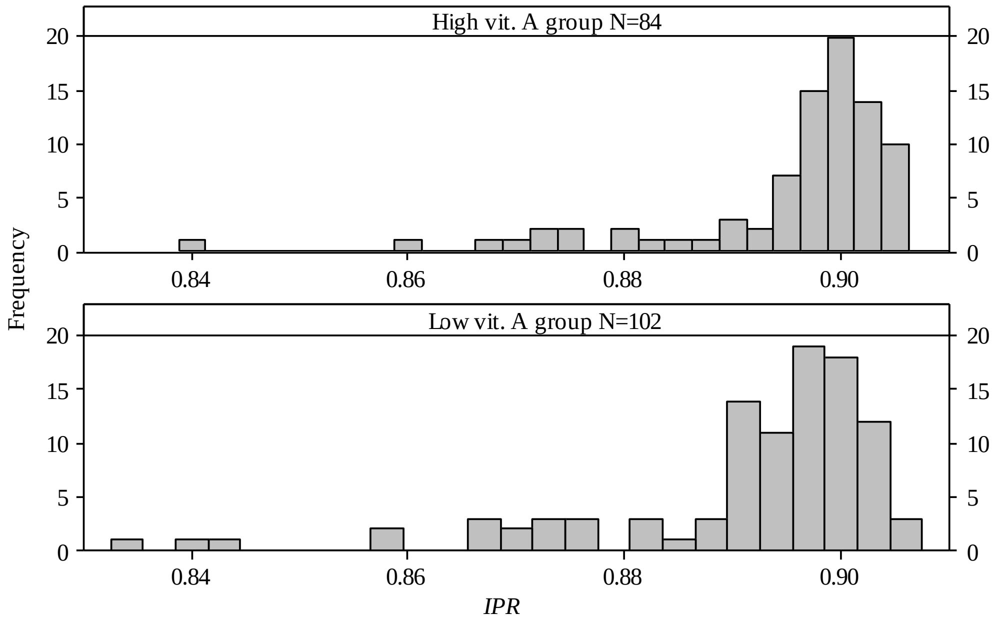

Figure 4.16 Histogram of *IPR* from high (>60 IU/dL) and low (<30 IU/dL) vitamin A group cattle.

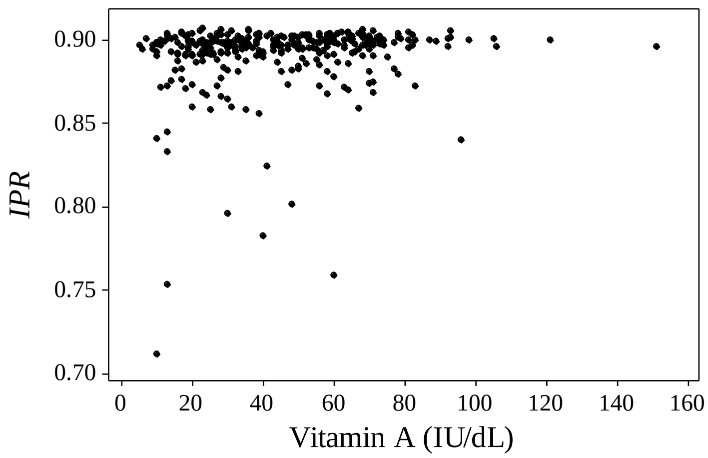

Figure 4.17 Relationship between IPRs and serum vitamin A levels.

4.4. Conclusions and Future Work

An image processing algorithm based on least squares ellipse fitting was successfully applied on pupil area calculation from images of cattle eye. The root mean square error of *CA* was 2.3% regarding manually selected results as the true values. In 2011, Japanese black cattle in low vitamin A level period showed significant larger *CA* in 1 second and significant smaller *IPR* value. In 2012, two sample *t*-test and paired *t*-test showed there were no significant difference (p>0.05) in *CA* between low and high vitamin A group cattle. However, two cattle were identified weak PLR (*CA*<10%) which was similar as the cases reported by O'Donghue, Issi *et al.* (O'Donghue 1955; Issi *et al.*, 2010). It indicates the potential of PLR analysis can be used to identify severe vitamin A deficient cattle but also shows the limitation of this method, which cannot detect the difference in the cattle which had serum vitamin A levels lower than 30 IU/dL by currently monthly measurement. More frequent measurements are necessary to detect the early change of PLR caused by VAD. On the other hand, compared with cattle in high vitamin A stage, there were more cattle possessed smaller resting pupils (p<0.05) in a dark environment during vitamin A deficient stage. *IPR* may also be used as an indicator of VAD. To realize frequently measuring, an automatic image capturing system installed at the watering place is one of the solutions. *CA* and *IPR* can be analyzed every time when the cattle are drinking water. Besides that, water use of individual cattle can also be recorded. Because feed and water are usually close related (Kashiha *et al.*, 2013), reduced drinking activity may indicate anorexia, which is another early clinical sign of VAD (Issi *et al.*, 2010).

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Chapter 5

Determination of Vitamin A Deficiency Levels by Ocular Changes in Japanese Black Cattle

Abstract

To help Japanese black cattle farmers make the diagnoses of VAD levels, ocular features of 40 fattening cattle were investigated monthly from May to December in 2011 during their vitamin A manipulated period. Multivariate classification methods (SIMCA and PLS-DA) were used to classify fattening cattle into the mild, moderate and severe VAD groups. Five variables (*r*, *I*, *CA*, *IPR*, *I_RFL*) were used for classification. The mild and severe VAD group could be classified with over 85% correct classification rate. However, the moderate VAD group could not be discriminated. A VAD index was proposed and proved to be effective in representing VAD status. The results showed the potential that ocular changes can be utilized to aid farmers.

5.1. Introduction

Beef marbling standard is an important indicator of beef quality. Several studies have shown that beef marbling standard can be increased by keeping cattle at low serum vitamin A levels during middle fattening stage (Oka *et al.*, 1998; Adachi *et al.*, 1999; Siebert *et al.*, 2006; Gorocica-Buenfil *et al.*, 2007; Kruk *et al.*, 2008). Currently, Japanese farmers actively manipulate the cattle's serum vitamin A at a minimum desired level to produce beef with higher marbling standards. However, if the serum vitamin A levels were lower than the minimum desired level 30 IU/dL, cattle become susceptible to nyctalopia, xerophthalmia, papilledema, diarrhea, etc. (Moore 1941; O'Donghue 1955; Divers *et al.*, 1986; Mason *et al.*,

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2003). Thus, knowing the vitamin A status is the key for precision feeding management. Blood assay is the conventional way to measure serum vitamin A levels. However, it is expensive and the procedure to acquire blood may bring stress to the cattle. Thus, some alternatives that can estimate serum vitamin A levels or VAD levels are desirable.

VAD are known to cause ocular changes (Barnett *et al.*, 1970), including corneal ulcers and xerophthalmia, ocular globe modifications, "beautiful blindness" and loss of palpebral and pupillary reflexes and etc. (Issi *et al.*, 2010). The relationships between serum vitamin A levels and ocular features, including color in pupil area, pupillary light reflex (PLR) and light reflection on pupil have been studied in previous investigations. A negative correlation between the red component ratio of pupil color images and serum vitamin A levels was found in 2010, and confirmed in 2011 (Han *et al.*, 2013b). The estimation error of serum vitamin A levels based on color changes in pupil area was about 10 IU/dL. However, the relative large estimation error restricted the field application of this method. For the PLR analysis, *CA* in 1 second and *IPR* were found to increase ($p < 0.01$) and decrease ($p < 0.05$), respectively, during the vitamin A manipulated stage. It indicated that parameters (*CA* and *IPR*) extracted from PLR could also contribute to the diagnosis of VAD status.

Due to the large estimation error of serum vitamin A levels, diagnosing VAD levels instead of directly predicting serum vitamin A levels seems to be more reliable and easier to be accepted by farmers. VAD can be classified into three levels, mild (67-100 IU/dL), moderate (33-67 IU/dL) and severe (0-33 IU/dL) VAD. Early detection of fattening cattle with severe VAD is the key for vitamin A manipulation, and it is essential to prevent fattening cattle from getting serious diseases. Therefore, the objective of this study is to determine VAD levels based on the ocular changes caused by VAD in Japanese black cattle during the vitamin A manipulated stage. To our knowledge, this is the first attempt to develop a method to aid farmers make correct veterinary ophthalmology diagnoses and ultimately contribute to feeding management.

5.2. Materials and Methods

5.2.1. Cattle

The experiment was conducted from May to December in 2011 at Hyogo Prefectural Hokubu Agricultural Institute, Japan. Forty fattening cattle were included in the experiment.

The eye images were recorded once a month. At the beginning of the experiment, ages of the cattle ranged from 14 to 16 months and serum vitamin A levels ranged from 34 to 80 IU/dL (mean $\pm$ SD 59 $\pm$ 11 IU/dL). From the beginning of April 2011, the cattle were subjected to low vitamin A diets.

5.2.2. Devices

A 2CCD multi-spectral camera AD-080CL (JAI) installed with lens of NT63-240 (Focus Length 12 mm, F 1.8, EDMUND) was used to acquire both color and Near Infrared (NIR) images. The camera was combined with two ring-shaped LED lights; a white LED light and a NIR LED light. To focus on the light reflected from tapetum, two polarizing (PL) filters were installed to reduce the specular reflection from the surface of the eye. One of them was installed in front of the LED, the other in front of the camera, perpendicular to the former PL filter. The details of experiment devices, procedures and image processing algorithms were introduced in Chapter 3 and Chapter 4 (Han *et al.*, 2013a; Han *et al.*, 2013b).

5.2.3. Ocular features

Ocular features were extracted from captured images. The parameters used for multivariate classification were briefly introduced as below.

5.2.3.1. *Pupil color*

A sample image is shown in Fig. 5.1. The captured images were processed by software developed in Microsoft Visual Studio 2008 to obtain the color information.

Figure 5.1 Original image with PL filter (Left), Region of Interest shown in yellow color (Right).

The yellow color in Fig. 5.1 shows the region of interest (ROI). The ROI was defined as the whole pupil area after removing a bright image of LED ring that was formed by a specular reflection. Red component ratio (r) and intensity (I) were calculated by the following equations.

$$r = R/(R+G+B) \tag{5.1}$$

$$I = (R + G + B)/3 \tag{5.2}$$

where R, G and B represent red, green and blue, respectively.

5.2.3.2. *Pupillary light reflex*

An example of pupilogram is shown in Fig. 4.5. After the onset of white LED lights, there is latency time about 0.3 second, as shown in Fig. 4.5. CA in 1 second was calculated by using the following equation (5.3).

$$CA = \frac{S_0 - S_1}{S_0} \tag{5.3}$$

Where s_0 is the resting pupil area and s_1 is the pupil area at the end of 1 second constriction.

IPR was used as an indicator of resting pupil shape. The IPR was calculated using the following equation (5.4).

$$IPR = \frac{4\pi S}{P^2} \tag{5.4}$$

Where S is the area of pupil and P is the perimeter of the pupil.

5.2.3.3. *Light reflection*

Different from pupil color, to focus on the light scattered on the cornea, a small region in pupil area was selected as the ROI. A circumscribed rectangle (shown in red in Fig. 5.2) of the LED ring image formed by a specular reflection was drawn. The area inside this rectangle after removing the saturated part was regarded as ROI, as shown in Fig. 5.2. Light reflection intensity (I_RFL) was calculated by the following equation (5.5).

$$I\_RFL = (R + G + B)/3 \tag{5.5}$$

where R, G and B represent red, green and blue, respectively.

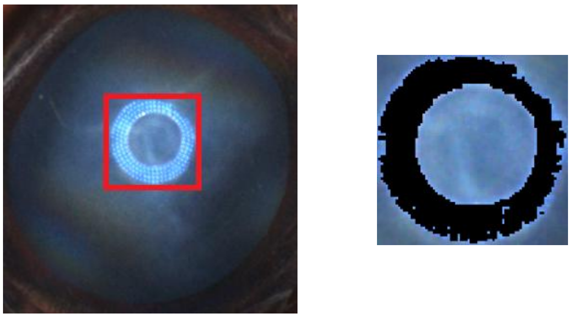

Figure 5.2 Original image (Left), the ROI for light reflection analysis (Right). All the parameters used for subsequent analyses are summarized in Table 5.1.

Table 5.1 Parameters extracted from ocular features.

Pupil Color		Pupillary Light Reflex		Light Reflection
r	*I*	*CA*	*IPR*	*I_RFL*

5.2.4. Multivariate classification

5.2.4.1. *Principal component analysis (PCA)*

PCA is one unsupervised approach used mainly for preliminary detection of sample clustering or outlier detection. The scores and loadings plot of PCA can be used to interpret which variables describe the differences between clusters and which variables contribute most to an observed difference. In this study, PCA was used to obtain an overview and insight into the data and the scores plot gives information about patterns within the samples.

5.2.4.2. *Soft independent modelling of class analogies (SIMCA)*

SIMCA is a class-modeling technique proposed by Wold (1976). It makes use of principal components to model a class of material on the basis of samples in a training set. Samples in a prediction set are then compared to different models and assigned to classes according to their proximity to the training samples. Different from PCA, SIMCA is known as a supervised pattern recognition method as the individual PCA models define classification rules. In this study, the five variables were used for classification analyses. Performance of classification models (95% confidence levels) was evaluated using three parameters; overall correct classification rate, sensitivity (percentage of samples in the external prediction set known to belong to the modeled category which were accepted by the model) and specificity (percentage of samples in the external prediction set not belonging to the modeled category which were correctly rejected by the model) (Osorio *et al.*, 2013).

5.2.4.3. *Partial least squares discriminant analysis (PLS-DA)*

PLS-DA consists in a classical PLS regression where the response variable is a categorical one expressing the class membership of the statistical units. A set of dummy variables is used to describe the categories. In this study, -1, 0 and 1 were used to denote the severe, moderate and mild VAD cattle, respectively. The dummy variable was regarded as a VAD index (VADI). If VADI is close to 1, it indicates the sample is likely to be mild VAD. On the other hand, if VADI is close to -1, it indicates the sample is probably severe VAD.

5.2.4.4. *Data Analysis*

After removing samples with blurred images or unsuccessfully recorded PLRs, 286 samples were used in SIMCA and PLS-DA. Half of them were randomly selected as the training set and the remaining samples were used for prediction. The same training set was also used in the PCA analyses. Because different units were used among variables, all the data were scaled. The value of each variable for a given sample was divided by the standard deviation of the values for that variable calculated over all samples. Due to the known less importance of *IPR* and *I_RFL*, they were given smaller weights (0.5) than others (1) subjectively. All the data analyses were performed using THE Unscrambler (version 9.8, CAMO Software AS, Oslo, Norway).

5.3. Results and Discussion

Serum vitamin A levels in all the cattle gradually declined during the experiment period, as shown in Fig. 5.3. By the end of the experiment, the experiment cattle's serum vitamin A levels ranged from 11 to 41 IU/dL (mean ± SD 27 ± 7 IU/dL).

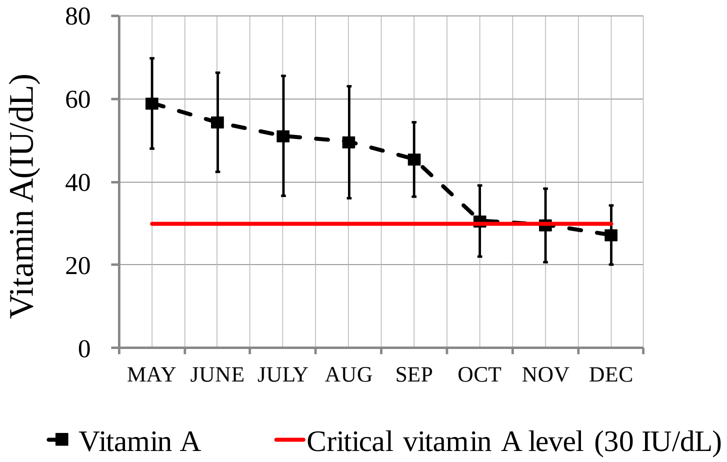

Figure 5.3 Changes in serum vitamin A levels from May to December 2011. Error bars indicate the standard deviation of all the cattle.

5.3.1. Result of PCA analysis

The first and second principal components (PC) accounted for 54% and 24% of the total variance in the data set, as shown in Fig. 5.4. The sum of 78% is high enough for a good representation of the five variables. The severe (shown in red) and mild (shown in green) VAD group could be separated in the first and second PC scores plot. However, the moderate VAD group which had the serum vitamin A levels between the severe and mild vitamin A group was difficult to be discriminated. This may be caused by the individual different tolerance of VAD among cattle. Belonging to the same moderate group, some cattle developed ocular changes and some cattle did not.

Comparing the relative position of groups in the scores plot and the relative position of variables in the loadings plots, it could be learned that the severe VAD group accompanied with higher *I*, *r*, *I_RFL* and *CA* value, while the mild VAD group accompanied with higher *IPR*, as shown in Fig. 5.5. Higher intensity (I) indicated the higher reflectivity of tapetum, caused by degeneration of retina (Maggs *et al.*, 2008). Higher red component ratio (r) indicated the mottled appearance and loss of pigmentation in the nontapetal fundus (Gelatt. 2001; Takahashi *et al.*, 2011). The reason why higher *CA* and smaller *IPR* were accompanied with severe VAD is still not clear, further investigations are necessary.

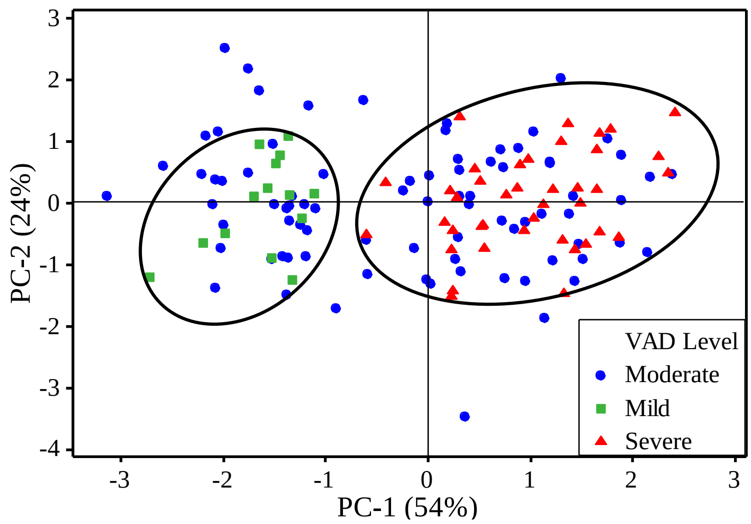

Figure 5.4 Scores plot of the mild, moderate and severe VAD groups in the training set. All variables were used for the calculation of scores.

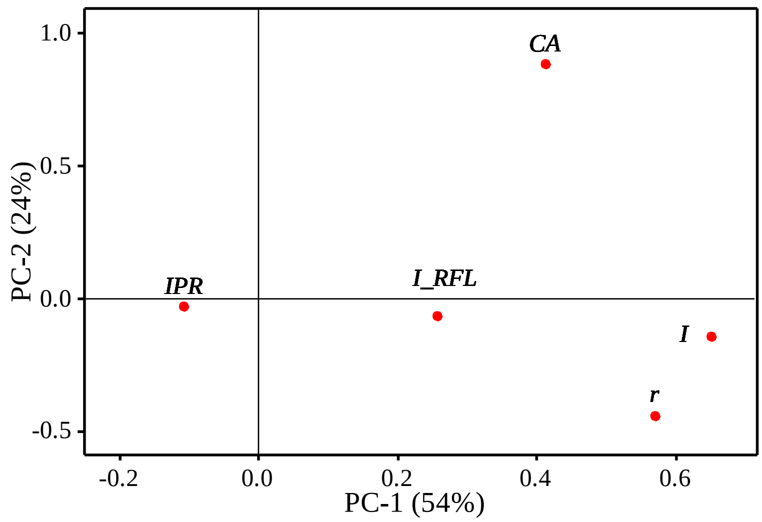

Figure 5.5 Loadings plot of the mild, moderate and severe VAD groups in the training set.

5.3.2. Result of SIMCA analysis

Firstly, only samples belong to the mild and severe VAD group were used for prediction. The result is shown in Table. 5.2. The overall correct classification rates for the mild and severe VAD group were 86.8% and 92.5%, respectively. However, the sensitivity for the mild VAD was 61.5% and the specificity for the severe VAD was 76.9%, which means some cattle with mild VAD were misclassified into the severe VAD group. The result needs further improvement.

Table 5.2 SIMCA classification results of the mild and severe VAD groups.

Target Group	Sample NO.	Sensitivity (%)	Specificity (%)	Overall correct classification rate (%)
Mild	13	61.5	95	86.8
Severe	90	97.5	76.9	92.5

The classification result using all the samples in prediction set is shown in Table 5.3. Except the overall correct classification rate of the mild VAD group was 74.8%, the classification results for the other two groups were poor. The poor performance of SIMCA may be caused by the unbalanced sample number and lack of measurements. There were few samples belong to the mild VAD group (N = 13) and most samples were belong to the moderate VAD group (N = 90). In this study, the experiments were conducted monthly. To build a robust classification model, more frequent measurements are necessary.

Table 5.3 SIMCA classification results of all samples in the prediction set.

Target Group	Sample NO.	Sensitivity (%)	Specificity (%)	Overall correct classification rate (%)
Mild	13	61.5	76.2	74.8
Moderate	90	81.1	15.1	56.6
Severe	40	97.5	48.5	62.2

5.3.3. Result of PLS-DA

The prediction result of PLS-DA is shown in Fig. 5.6. For the mild and severe VAD group, the samples with positive value were regarded as the mild VAD and vice versa. The result is shown in the confusion matrix (Table 5.4). Overall correct classification rate was 94.3%. However, same as the problem of SIMCA, the moderate VAD group could not be discriminated. Different from SIMCA, PLS-DA can generate the VADI which indicates the similarity to a class membership. To confirm the effectiveness of VADI, the samples in the moderate group were divided into positive and negative VADI groups. Their histograms of serum vitamin A levels are shown in Fig. 5.7. It is obvious that the samples with negative VADI possessed lower serum vitamin A levels (p<0.01). It indicates the VADI can be used as an indicator of VAD status.

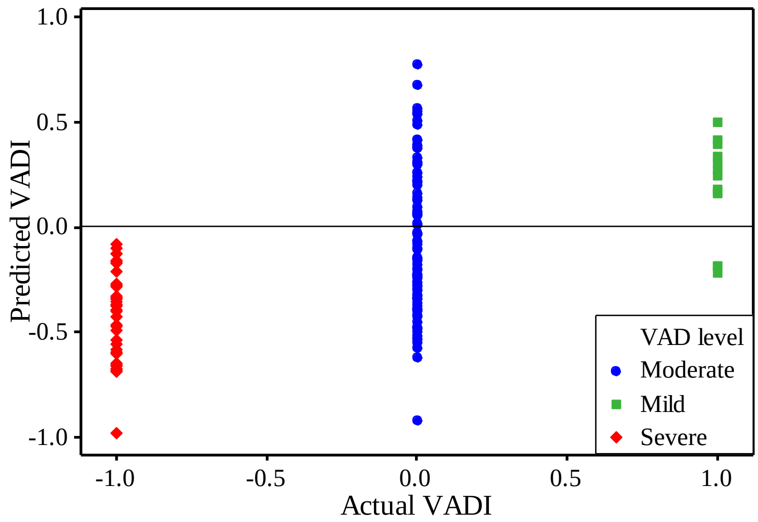

Figure 5.6 Actual and predicted VADI in prediction set.

Table 5.4 The confusion matrix.

		Predicted	
		Mild	Severe
Actual	Mild	10	3
	Severe	0	40

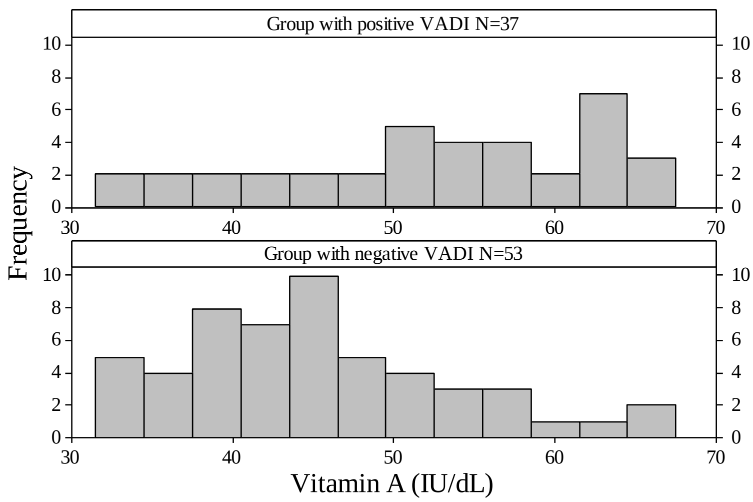

Figure 5.7 Histograms of samples with negative and positive VADI in the moderate VADI group.

5.4. Conclusions

Five variables (*r*, *I*, *CA*, *IPR*, *I_RFL*) from ocular changes were used for VAD levels determination. Both SIMCA and PLS-DA achieved good performance on discriminating the mild and severe VAD group with overall correct classification rate (over 85%). However, the moderate VAD group cannot be discriminated from the other two groups. The cattle with positive VADI, the predicted value by using PLS-DA, showed significant larger actual serum vitamin A levels than the cattle with negative VADI. The results indicated that VADI can be used to aid VAD diagnosis. To make the computer aided VAD levels diagnosis system more reliable, further more frequent investigations are necessary.

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Chapter 6

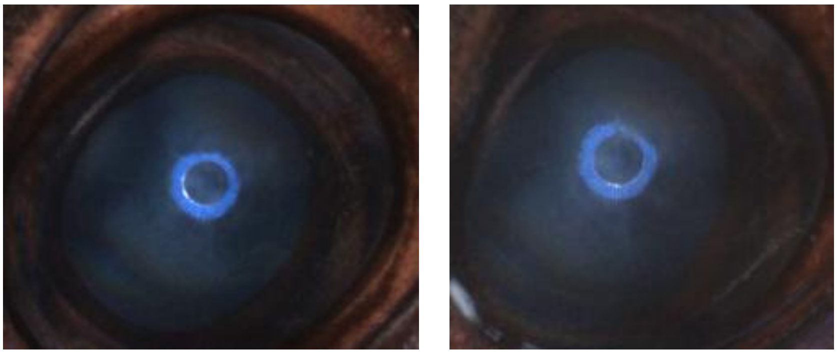
Conclusion and Future Work

To meet the demand for a computer-aided diagnosis system for VAD levels determination in Japanese black cattle, some preliminary works have been done and they are summarized here. Firstly, the feasibility of using pupil color changes to estimate serum vitamin A levels was investigated in 2010 and 2011. The consistent result suggested that pupil color can be used as an indicator of VAD, which is also recommended by Moore (1939). Secondly, the feasibility of using pupillary light reflex changes to estimate serum vitamin A levels was investigated in 2011 and 2012. Constriction amplitude (*CA*) and initial pupil roundness (*IPR*) were selected as two parameters to represent pupillary light reflexes. The result indicates the potential and limitation of the pupillary light reflex analysis in estimating serum vitamin A levels. Finally, the data of pupil color, pupillary light reflexes and light reflection in 2011 were used for classifying fattening cattle into different serum vitamin A deficient levels. The results showed the potential that ocular changes can be utilized to help farmers make the correct diagnosis of vitamin A status. It would contribute to the beef industry.

6.1. **Main contributions**

6.1.1. **A negative correlation between the red component ratio of cattle pupil images and serum vitamin A levels**

In 2010, eye images of 42 cattle were captured biweekly from July to November. The result showed there was a negative tendency between the red component ratios and serum vitamin A levels. In 2011, the same experiment was conducted again to confirm the findings with other 42 cattle from May to December. Eye images were captured monthly. The same tendency was acquired. The relative high red component ratio during the low vitamin A period may indicate the mottled appearance of the non-tapetal area. This knowledge maybe used as a guide for farmers. However, because there was no control group in the experiments, the finding can only be regard as some experience, not knowledge. One example was shown in Fig. 6.1, compared with the left image taken when the serum vitamin A level was higher (54 IU/dL), the right image taken when the serum vitamin A level was lower (23 IU/dL) was brighter and less blue.



(a) (b)

Figure 6.1 Sample images of one cattle taken in high (a) and low (b) vitamin A period captured with installation of PL filter.

6.1.2. **A serum vitamin A levels estimation model based on color changes in pupil area was built.**

In 2011, a multiple linear regression model was built based on the data from 24 cattle with a wider range of serum vitamin A levels. The model had an estimation error about 10 IU/dL. This is the first model that uses pupil color data to predict serum vitamin A levels. Even though the model may not work well with other eye images, it showed the potential of this method.

6.1.3. **An image segmentation method to calculate pupil area was proposed.**

To study the pupillary light reflex, a large amount of images have to be processed. It is impossible to deal with these images manually, a good image processing algorithm is essential for the success of PLR analysis. Due to the reflection of light sources, some part of region of interest in the captured images is covered. Thresholding method cannot be used.

The pupil of human is a circle, while the pupil of cattle is oval. The proposed algorithm utilizes this information and uses an ellipse to fit the pupil. Thanks to the least squares method, part of the pupil boundary is enough for the calculation. Compared with the manually selected result, the root mean square error of pupil area and constriction amplitude is only 930 pixels (The whole image pixel number is 786432) and 2.3%.

6.1.4. **Cattle were found to possess smaller resting pupils (*p*<0.05) in a dark environment during vitamin A deficient stage.**

To our knowledge, this is the first report about changes of resting pupil size during vitamin A deficient period. Further investigation is necessary to understand the reason.

6.1.5. **A Vitamin A deficiency index (VADI) was proposed and proved to be effective in representing VAD status of cattle.**

Estimating actual serum vitamin A levels by ocular changes is difficult and the estimation error is large. Farmer would not like to accept such a system with a large error. On the other hand, the actual serum vitamin A levels are not important for farmers. An indicator to show which cattle are in a dangerous vitamin A deficient level is enough. The proposed VADI can be used as that indicator and proved to be able to distinguish cattle with severe VAD from cattle with mild VAD. It indicates the potential of actual application of VADI.

6.2. **Current Limitations and Future Works**

6.2.1. **Establish a standard for camera calibration.**

The serum vitamin A estimation model developed in Chapter 3 may not be applicable to other inbred line. Even for the same inbred line, the models built based on images captured under difference calibration conditions may vary. Because images can be affected by many factors like: light intensity, the spectral characteristic of light sources, shutter speed, aperture, gain of camera, installation of polarized filter, white balance setting, relative angle between camera and eye and measuring distances. It was found that the image could be affected by the relative rotating angle between camera and eye when polarized filters were used, as shown in Fig 6.2. Notice the dark center in the left image, which appeared after rotating the camera 45 degree clockwise from the position when capturing the left image.

Without a standard for calibration, the knowledge of pupil color cannot be accumulated and contribute to the final classification. If an open standard for calibration can be established, then the developed model based on the images captured following the same calibration standard can be compared and evaluated. The model can be used universally.

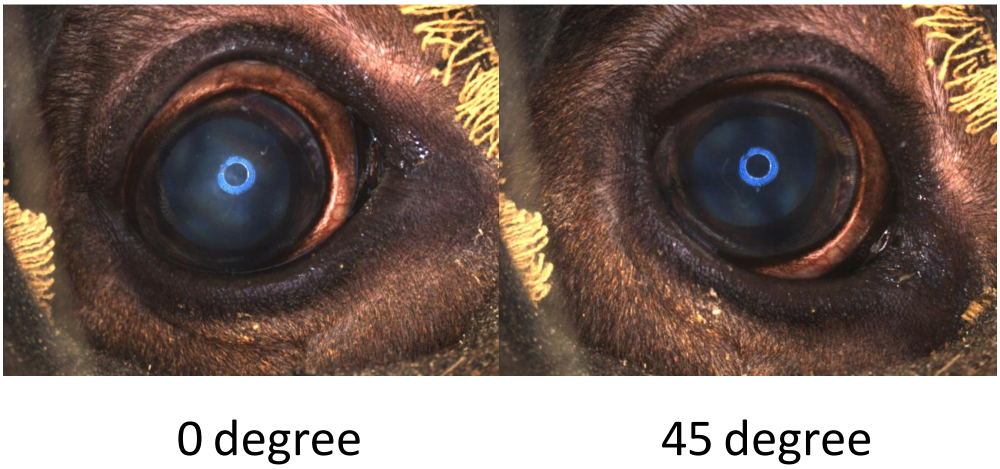


Figure 6.2 The color change of pupil area after rotating the camera on the optic axis.

6.2.2. **Develop an automatic image capturing system.**

Currently, the experiment was conducted monthly, because the experiment is labor intensive. Five labors are necessary: two for fixing the cattle to the bars, one for capturing images, one for operating the software and one for covering the cattle's heads with black cloth. The scenery of image capturing is shown in Fig. 6.3. It is impractical to take images every day.

However, to realize early detection of loss of pupillary light reflex and prevent the affected cattle getting serious diseases, it is necessary to check the pupillary light reflex frequently. Meanwhile, if more data can be acquired and used for the calibration, the classification model would be more robust. An automatic capturing system is desired, as shown in Fig. 6.4, the camera can be installed at the drinking place. With that system, the pupillary light reflexes of cattle can be checked every time when they are drinking water. That would be greatly improve the effectiveness of the system.

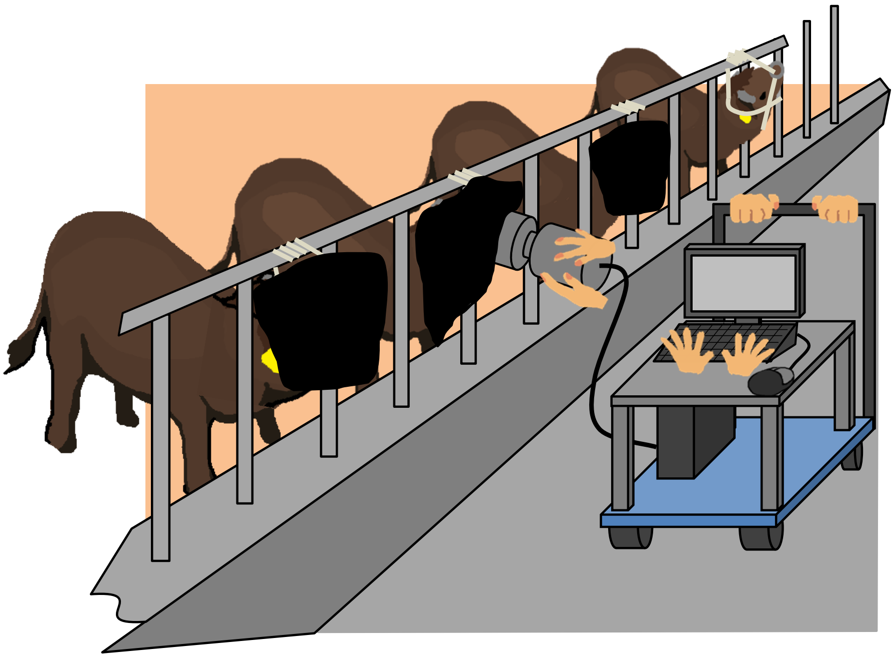


Figure 6.3 Experiment scenery.

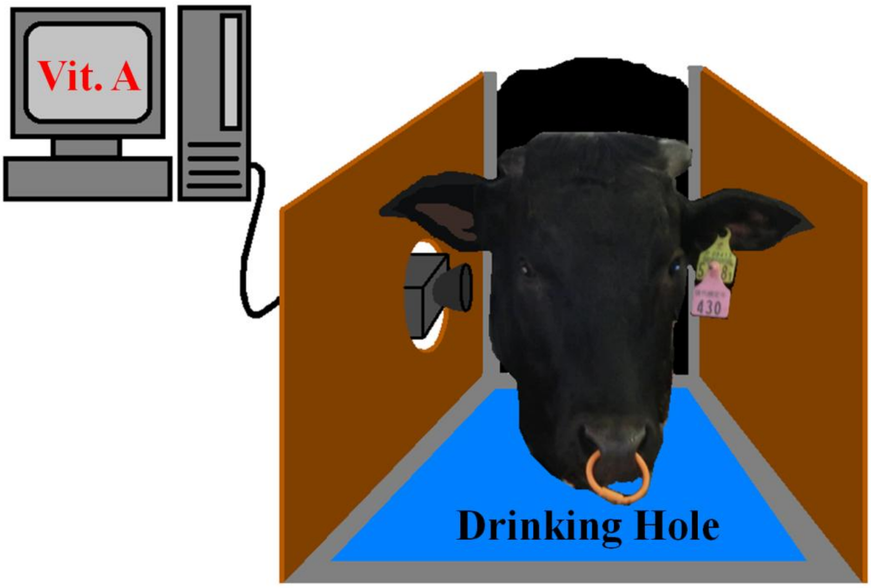


Figure 6.4 The proposed automatic image capturing system.

6.2.3. **Investigate the PLR with longer duration and chromatic PLR analysis**

For the consideration that short measuring period would reduce the stress of cattle during experiments, one second PLR was used in the experiment. However, the result in 2012 showed the difference of constriction amplitude (*CA*) between high and low vitamin A period was not significant (p=0.78). According to Matsuda (1999)'s report, longer time PLR may give us good result. However, if the measuring time is long, the quality of the images would be decreased, because most cattle would try to move or blink because of the stimulation of light. The movement would cause the pupil be covered by the eyelid or unfocused images. All in all, the balance between measuring duration and image qualities should be considered in the future.

There are two kinds of PLRs, rod-cone-mediated PLRs and intrinsic melanopsin-mediated PLRs. To study these two PLRs separately, different lighting (red and blue) should be used. Grozdanic found the PLRs of dogs with sudden acquired retinal degeneration syndrome (SARDS) can be elicited under blue light, but not under red light (Grozdanic *et al.,* 2007). Because vitamin A deficient may only affect the rod-cone-mediated PLRs, to remove the disturbance caused by intrinsic melanopsin-mediated PLRs, it is worthy to try to use red light as a stimuli of PLRs.

6.2.4. **Add more features that can improve the classification performance.**

The classification result in Chapter 5 achieved with correct classification rate of over 85% for mild and severe VAD group. However, what farmers desired most is to separate severe VAD group from moderate VAD group. The current classification result is not satisfied.

Loss of appetite and slow growth were believed to be another two early symptoms of VAD (Issi *et al.,* 2010; He *et al.,* 2012). They should also be included as the inputs for classification. The classification result may be improved.

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List of Publications

Peer Reviewed Journal:

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Han, S., N. Kondo, T. Fujiura, Y. Ogawa, S. Tanigawa, M. Fukushima, O. Watanabe and N. Kohama. 2013. An approach for identification of vitamin A deficient cattle by pupillary light reflex analysis in Japanese black cattle. SPIE SeTBio 2013, Yokohama, Japan, 23-25 April (Oral presetation)

Han, S., N. Kondo, T. Fujiura, Y. Ogawa, S. Tanigawa, M. Fukushima, O. Watanabe and N. Kohama. 2012. Estimation of serum vitamin A level by pupil color and pupillary light reflex analysis in Japanese black cattle. 2012 Summer School of the HeKKSaGOn Consortium, Heidelberg University, Germany, September 17-26, 2012 (Poster and oral presetation)